Emerging Trends in Medicinal and Pharmaceutical Chemistry

Chemistry of BIOLOGICALLY POTENT NATURAL PRODUCTS and SYNTHETIC COMPOUNDS

Edited by
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Preface

The increasing incidences of multidrug resistance toward conventional pharmaceuticals have increased the demand for the discovery and development of new drug candidates with novel action mechanisms. Natural products and their synthetic analogs have shown interesting preclinical and clinical results as pharmaceuticals and have recently gained considerable attention in the modern medicinal chemistry and drug design.

This book is organized into 13 important chapters that focus on the progress made by natural products and their synthetic analogs obtained directly from natural sources, as well as modified by synthesis/semi-synthesis procedures in medicinal chemistry as antiviral, anticancer, antibacterial, and anti-inflammatory agents. The various methods for enhancing the secondary metabolite concentration for efficient drug design are also described. This book is an essential source of information for professors, researchers, teaching staff, postgraduate students, and practitioners working in medicinal chemistry, medical science, pharmacy, biotechnology, and biomedical engineering.

We would like to convey our appreciation to our all experienced contributors for investing their valuable time in writing informative chapters for this book. Last but not the least, the editors would like to thank Mr. Martin Scrivener, President of Scrivener Publishing, USA, who accepted and supported this project.

Shahid-ul-Islam
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Medicinal Importance of Plant Metabolites

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Abstract
Medicinal herbs are plants utilized for remedial purposes or antecedents of another type of supposed chemo pharmaceutical semi-synthetic. The two types of chemical compounds received from plant sources are primary and secondary metabolites. Although secondary substances assume a significant role in plant protection against microscopic organisms, growths, and bugs and enormous creatures, people use them as medications, food added substances, flavors, and recreational medications. These days, most plants utilize secondary metabolites in present day medication. Secondary metabolites have a wide scope of therapeutic functions including shielding the body against free radicals and protection against heart ailments. These therapeutic activities can be attributed to their capability of interacting with the receptors, cell layers, and nucleic acids.

***Keywords*:** Microscopic organisms, free radicals, biotic & abiotic, immobilization, phytoalexines

1.1 Introductory Note

The expression "metabolism" alludes to the whole biochemical procedure that a living entity makes. Substances produced from anabolism and catabolism known as metabolites, typically limited toward tiny living atoms [1]. The plant produces many chemicals, which are classified as basic metabolites, which are essential for plant work and are pervasive. Because of its various applications in logical exploration and commercial exploitation,

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different compounds have been classified as secondary metabolites [2]. The word "secondary" was coined by A. Kossel in 1891. Basic metabolic pathways exist in each living cell capable of dividing. Secondary metabolites appear just accidentally and are not important to organisms. Although secondary metabolites originate from primary metabolic procedures, yet they do not make vital molecular frame of the organism. The absence of this does not legitimately restrain the existence of a living organism, a trait that is dissimilar to basic metabolism, however somewhat the survival of the organism [1].

Secondary metabolites are separately placed within the restricted scientific categorizations of the plant kingdom. As of late, the business/commercial significance of secondary metabolites has pulled in much consideration, most prominently the utilization of plant tissue culture innovation to change bioactive plant metabolism [3].

Secondary metabolites can be depicted as an assorted group of common and natural metabolic products that require the development of the vegetation of the living beings they produce. They are viewed as differential exacerbates that assume positive jobs in ecological collaborations or in defensive compounds, for example, signal atoms, metal transport, advantageous and interaction/symbiosis [4]. Secondary metabolic structures, the low molecular weight containing compounds are typically cell, tissue, and organ-explicit. They provide shield to the plants from biotic (feeding microbes, organisms, nematodes, insects, or creatures) and abiotic (elevated temperature and dampness, concealing, injuries, or heavy metals). Because of its extraordinary monetary worth, secondary metabolites are utilized as medications, flavoring agents, aroma producing compounds, pesticides, and colors. The secondary metabolic structures are classified into various categories (polyketides, terpenoids, and phenylpropanoids) in view of their biosynthesis derivation [5]. Further, secondary metabolites also comprise of alkaloids which are made up of biodegradable nitrogen-containing moieties, i.e., amino acids, namely, arginine, lysine, tyrosine, phenylalanine, and tryptophan [6].

As of late, traditional medication has become a subject of worldwide discussion. Numerous plants distinguished as therapeutic flora have been experimentally assessed for the potential utilization. It is hard to get compounds derived from plants. Phyto-compounds have assumed a significant function in the adjustments of plants to their ambient conditions and as indispensable pharmaceutical assets [7]. Secondary metabolite has gained extensive consideration in the course of recent decades because of its pivotal significance in human nourishment, beauty care products, prescriptions, and plant protection. The emerging importance of secondary metabolites is

not only vital from educational point of view but also from business aspect. Owing to the marketable significance of secondary metabolites as colors, drugs, polymers, waxes, pastes, strands, antimicrobials, herbicides, and pesticides, fascination toward secondary metabolites continues to grow [8, 9].

1.2 Primary and Secondary Metabolites

All plants have primary metabolites that add to sustenance and reproduction and assume significant metabolic jobs [6]. The plant's essential metabolites speak to nucleic acids, proteins, sugars, and lipophilic compounds. They are identified with compositional, functional, and hereditary qualities and represent to a significant job in plant improvement. Conversely, secondary metabolites, as a rule, happen in small compounds at lesser amount. The way toward making Krebs cycle's carboxylic acids is alluded to as primary metabolite. Secondary metabolite is basic forever, yet in addition for species endurance. Secondary metabolic compounds are utilized as markers of plant scientific categorization (chemotaxonomy). Plant secondary metabolic products may be classified in following structurally diverse classes. These are terpenes, phenol-containing, N (nitrogen)–containing, and S (sulfur)–containing compounds [10].

Since there are numerous secondary metabolic mediators, the contrast among primary and secondary metabolites is conceptual [11]. Amino acids, which are viewed as the primary metabolic product, are the secondary metabolites. In spite of the perception, sterols are secondary metabolites in numerous auxiliary structures of cells. The mosaic properties of the middle of the road direct a typical biochemical pathway that shares main and resultant metabolism [12]. Resultant metabolites are used as moderator, whereby extra nitrogen and carbon structure a latent piece of the primary metabolite. At the point when important, put away nitrogen and carbon can rear to primary metabolic substance through the degradation of secondary metabolites. There is a dynamic and fragile harmony between the elements of primary and secondary metabolite, which influence development, segregation of tissues, and external stresses of the body [13].

1.3 Functional Roles of Secondary Metabolites

For some secondary compounds, signaling movement influences the capacities and impacts of different cells and their metabolism and coordinates overall improvement. Materials, for example, bloom colors, help to

speak with pollinators. Secondary metabolites protect plants from nature and take care of plants infectious diseases from contamination [14]. Plants utilize secondary metabolites (volatile essential oils, colored flavonoids, or tetraterphanes) to catch the attention of pollinators or different creatures for kernel circulation; thus, the secondary metabolites used as signal compounds. Organic compounds are classified as terpenoids, alkaloids, and flavonoids and, at present, being utilized as medicines or dietary supplements to fix or forestall different infections [15]. In many plants, 14%–28% is dealt therapeutically and 74% of pharmacologically dynamic plant extracts are found after ethno-clinical utilization of plants [16]. A significant assurance system was given to the plants by the existence of volatile essential oils or mono-terpenes, fundamentally against herbivorous insects and infectious fungi. The above-mentioned terpenoids assume a significant job in plant to plant communications and act as pollinating agents [17]. They represent developmental associations by the way of their practical jobs and act as signaling molecules. Secondary substances which are soluble, for example, cyanogenic glycosides, isoflavonoids, and alkaloids, are additionally harmful to creatures [10].

The fundamental elements of secondary metabolites, as well as antibiotics, are as follows:

- Competitive weapons on microorganisms against creatures, plants, creepy crawlies/insects, and different organisms.
- Metal transport operators.
- Agents for symbiotic associations with different organisms.
- Agents for reproduction.
- Differential impacts.
- As corresponding agents between organisms.

Different exercises incorporate fertilization (not obligatory) and germination directions [4]. Specifically, different biological functions, like antimicrobial, anti parasitic agents, enzyme inhibitors, anti tumor agents, and immunosuppressive agents, utilized secondary metabolites [19].

1.4 Source and Production of Secondary Metabolites

Plant life is the primary/essential source of secondary metabolic substances (80% of secondary metabolites), microscopic organisms, parasites, and numerous marine organisms (wipe, tunicate, coral, and snails) [20]. Plants

are wealthy in an assortment of many substances, including tannins, terpenoids, alkaloids, and flavonoids, with antimicrobial properties *in vitro*. Plants can create aromatic substances, the vast majority of which contain phenol or their oxygen-substituted derivatives. The precursor iso-pentenyl diphosphate (IPP) produced around 25,000 terpenoids known as secondary compound. Altogether, 12,000 identified alkaloids have been distinguished that possess at least one nitrogen atom that can be synthesized biologically from amino acids [22]. The secondary metabolites originate from plant and their functions are indexed in Table 1.1 [23].

Table 1.1 Secondary metabolites with biological function.

S. no.	Secondary metabolites	Biological functions
1.	Pyrethrins	Insecticidal
2.	Nicotine	Insecticidal
3.	Rotenoids	Insecticidal
4.	Azadirachtin	Insecticidal
5.	Phytoecdysones	Insecticidal
6.	Bruceantine	Antineoplastic
7.	Baccharine	Antineoplastic
8.	Gsaline	Antineoplastic
9.	3-Doxycolchicine	Antineoplastic
10.	Ellipticine	Antineoplastic
11.	Fagaronive	Antineoplastic
12.	9-methoxyellipticine	Antineoplastic
13.	Tlarringtovinl	Antineoplastic
14.	Jandicine N-oxide	Antineoplastic
15.	Maytansive	Antineoplastic
16.	Taxol	Antineoplastic
17.	Podophyllotoxin	Antineoplastic
18.	Thalicarpine	Antineoplastic

(*Continued*)

Table 1.1 Secondary metabolites with biological function. (*Continued*)

S. no.	Secondary metabolites	Biological functions
19.	Tripdiolide	Antineoplastic
20.	Vinblastin	Antineoplastic
21.	Quinine	Antimalarial
22.	Digoxin	Cardiac tonic
23.	Diosgunin	Antifertility
24.	Thebaine	Source of codeine
25.	Morphine	Analgesic
26.	Suolpolanine	Anti-hypertension
27.	Codeine	Analgesic
28.	Atropine	Muscle relaxant
29.	Shikonin	Dye, pharmaceutical
30.	Anthroquinones	Dye, laxative

Conventional strategies for secondary metabolite production include immobilization and *in vitro* cell, tissue, and organ culture. The traditional technique for secondary metabolite fabrication relies on metabolite extraction, which depends on vapor, solvent, and supercritical extraction (the phytochemical methods). In the immobilization process, the cell or biocatalysts are constrained by the entanglement, adsorption, or covalent linkage in the network. Notwithstanding the ideal substrate and state on the best possible physicochemical parameters, the necessary secondary metabolites are synthesized. The instability of a suitable bio-reactor framework gives numerous focal points, for example, persistent/continuous process operation [18].

Tissue and cells cultures of plant can be distinguished from clean leaves, stems, roots, and meristem by duplication and characterization of various secondary metabolic compounds. Root, cones, stem, suspension of cells, and hair root culture delivered the ideal/desired metabolite [24]. The treatment of plant cultures with biotic and/or abiotic elixir can improve the measure of secondary metabolites in plant parts [25]. Metabolic production by the hair follicle framework dependent on the infusion of *Agrobacterium rhizogens* has attracted attention. The magnitude and

eminence of secondary metabolic matter of root hair frameworks is comparable to or superior to the synthesis of damaged host root of plant [26].

Current work to change/modify the secondary metabolites production is paying attention on the following points:

- To improve the profitability of target molecules, chemical maneuvring of cell cultures are done during chemical processing and bio-reactor execution [27].
- Target studies signal transduction pathways that represent to various effectors techniques prompting the biosynthesis of desired secondary metabolites [28].
- The production of target secondary metabolites may also be enhanced by genomic management of regulator genes, i.e., transcription factors and its regulatory systems [28].
- Cloning can target metabolic flow so the cloned biosynthetic genes and alteration of key genes prompt compounds, which are used for this purpose [29].
- Path specification metabolic flows and metabolic mediators to comprehend the general pathways and overall guideline of target joint aggregation [30].
- A review/investigation of gene transcripts for plant secondary metabolites to completely comprehend plant secondary metabolite by analyzing global gene expression profiling under various conditions [31].

Advances in tissue culture have opened up additional opportunities for high level production of pharmaceuticals, nutraceutical, and other useful materials when joined with genetic engineering and, in particular, transformation innovations. So, there is still a room in molecular biology, fermentation technology, and enzyme study to make these frameworks a significant viable source of secondary metabolites [32].

1.5 Classification of Secondary Metabolic Substances

Characterization of secondary metabolites can be done by chemical composition (e.g., possessing rings and having sugar), miscibility in a variety of solvents, or by the delivery manner, the structure (whether nitrogen is available), (e.g., tannins producing compound and phenylpropanoid) and normally divided on the basis of their biosynthetic pathways [33]. The British Nutrition Foundation divides secondary metabolic products into four primary classes [34]. These four groups incorporate terpenoids

(sterols, carotenoids, cardiovascular glycosides, and plant instabilities), phenolics (lignans, phenolic acids, tannins, coumarins, lignin, stilbenes, and flavonoids), compounds having nitrogen (e.g., lectins), and compounds having sulfur (GSH, GSL, thionin, lectins, pytoalexins, etc.) [35].

1.5.1 Terpenes

Terpenes are huge and differing organic compounds that produce a wide variety of plants (conifers), and resin is the important ingredient. The name originates from the word turpentine, which is the principle biosynthetic structure block, for example, a derivative of steroids, triterpene squalene. In warm climate, trees release increasingly active terpenes, which are progressively common in cloud seeding, prompting daylight, which permits the forests to keep up its temperature normal. When the rearrangement or oxidative process of carbon framework increases the terpenes, the subsequent products are terpenoids, also called isoprenoids. Basic constituents of essential oils, terpenes and terpenoids, are broadly utilized as natural flavor added substances to foods, as aromatherapy and as an option in contrast to vitamin A and aromatherapy. The aroma and flavor are myrecene, b-pinin, b-caryophyllene, and a-humulin [36].

Terpenes have the largest group of secondary metabolites, independent of the common biosynthetic root of acetyl-CoA or glycolytic intermediates. Terpenes are categorized in monoterpenes, diterpenes, triterpenes, sesquiterpenes, and polyterpenes. The flowers and leaves of chrysanthemum species contain pyrethroid (monoterpenes esters), which have a strong reaction to pesticides and are a well-known constituent in commercial pesticides due to lesser persistence and mammalian toxicity to the environment. Because of their role in plant defense, various sesquiterpenes have been found, for example, castunolides are antiherbivore species, which contain five-membered lactone rings (cyclic esters) that can inhibit huge quantities of herbivores, insects, and vertebrates. Abietic acid (a sesquiterpene), which assumes key administrative roles in initiating and protecting seed and bud inactivation, acts as a transcriptional activator by modifying membrane properties [37]. Abscisic acid and sterols play diverse functional roles as structural components of cellular membrane in tetones phytol, gibberellins, and hormones. Xanthophylls, lycopene, and α- and β-carotenes are red, yellow, and orange lipid soluble pigments, i.e., carotenoids. Many green leafy vegetables are covering up in chlorophyll. The bright color in tomatoes, carrots, zucchini, and potatoes are because of carotenoids. Carotenoids are photo oxidant protector of different pigments as well as precursors of abscisic acids, which modulate improvement and stress reactions [38].

1.5.2 Phenol-Based Compounds

Plant life produces a wide diversity of secondary products, including the phenolic group, i.e., a-OH group attached to an aromatic ring known as phenolic compounds [39]. Phenols have unique properties with higher acidities due to aromatic rings. Phenolic acids are spread all through the plant and are fundamental ingredient for plant development and reproduction, which proceed to a response for plant injury against pathogens. The total content of phenolic acid reaches from 0 to 103 mg/100 g (Table 1.2). Phenolic acids have been seen as high in grapefruit, anthocyanins, catechins, proanthocyanidins, and stilbenes in skin and seeds [40].

In the course of recent decades, medications/formulations based on antioxidant used to cure and prevention of specific disorders, like cardiovascular disease, atherosclerosis, Alzheimer's ailment, diabetes, and

Table 1.2 List of phenolic compounds and sources [2].

S. no.	Compounds	Sources
1.	Butylated Hydroxytoluene	A fat soluble antioxidant & food additive
2.	Capsaicin	Pungent Compound in chillies, Pepper
3.	Cresol	Present in coal tar + creosote
4.	Estradiol	Estrogin - hormones
5.	Eugenol	Essential oil of clove
6.	Gallic Acid	Present in galls
7.	Guaiacol (2-methoxyphenol)	Smokey flavor found in roasted coffee, whisky
8.	Orthophenyl Phenol	Antifungal agent used for waxing citrus fruits
9.	Picric acid	Dangerous substance
10.	Polyphenol	Flavonoids and tannin
11.	Propofol	Anesthetic
12.	Raspberry Ketone	Raspberry taste
13.	Serotonin/Doamine	Neurotransmitter
14.	Thymol	Antiseptic used in mouthwashes

malignancy contains increasing amount of natural phytochemicals, particularly phenolic compounds found in berries, tea, oil-seeds, beans, and fruits [41]. So far, almost 8,000 phenolics have also been established. In view of composition, these are classified into two main classes, flavonoids, and non-flavonoids [42].

Flavones, isoflavones, anthocyanidins, flavan-3-ols, and flavonones are the principle constituents of flavonoids. Flavonoids, for example, coumarins, oranges, chalcones, flavan-3, 4-diols, and dihydrochalcones [42] are accessible in lesser concentration. Flavonoids are present on the external surface of fruit or leaves and serve significant task. They provide colors to plant parts, protection against UV radiation and protection against microorganism [43, 44]. Compounds having phenolic acids, tannins, hydroxy cinnamates, stilbenes, polyphenolics, and their conjugated derivatives are known as non-flavonoids [45].

1.5.3 Nitrogen-Containing Secondary Metabolites

Nitrogen-containing secondary metabolic substance resulted from various amino acids, i.e., tyrosine, tryptophan, lysine, and aspartic acid [46]. In 20% of plants, these are perceived as secondary metabolites and play as herbicides and anti- pathogens [47, 48]. A number of alkaloids which are biologically active are extracted from various plants. The alkaloid's action mode is unpredictable; some may control the nervous system, some may influence synthesis of protein, and some have an effect on enzyme activity and transportation within membrane [49].

1.5.3.1 Alkaloids

Carl F. W. Meissner, a chemist from Germany, gave the name alkaloids in 1819. He used "ine" for the class or generic name like atropine and strychnine, which is extracted from *Atropa belladonna* and Strychnine tree seeds, respectively [50]. Alkaloids are ring containing organic compound having lesser Mw and negative oxidation state nitrogen. It has a defensive reaction to creatures or ads to vegetarianism and has limited distribution among organisms [51]. Various microbes, fungi, plants, and animals are utilized for the alkaloids production. Their crude extract undergoes purification by acid-base extraction and utilized as recreational medication or endogenous methods like local sedative, stimulant cosine, caffeine, nicotine, pain killer morphine, and anti-neoplastic agents, i.e., vincristine, antihypertensive agent reserpine, cholinominary, gelatomine, and spasmolytics agent atropine, and as local anti-infective drugs [52].

Alkaloids are classified into the following major classes [53]:

- Original alkaloids: It includes heterocyclic compounds having nitrogen derived from amino acids like atropine, nicotine, and morphine.
- Proto-alkaloid: It also includes heterocyclic compounds with nitrogen but derived from other amino acids like mescaline, adrenaline, and ephedrine.
- Polyamine alkaloids: It contains derivatives of putrescine, spermidine, and spermine.
- Alkaloids having cyclopeptides and peptides.
- Pseudo alkaloids: Compounds show mimicry with alkaloids but not invented from amino acids like caffeine, theobromine, and theophylline.

There are two pathways for the synthesis of alkaloids: the shikimic acid and pyruvate pathway. Both the pathways consume aromatic and aliphatic amino acids. Pyruvate and shikimate produced by phosphonol pyruvate and erythrax 4-phosphate, respectively. Although, all alkaloids are essentially formed by L-amino acids like tyrosine, lysine, tryptophan, and aspartic acid; yet, there are a few contrasts in the biological synthetic pathways. For instance, xanthoxin, a free pool of purine nucleotides produced purine alkaloids. Pyrolizidine alkaloids are derived from polyamines spermidine and putrescine. Decarboxylation of S-adenosylmethionine leads in the formation of spermicidin [54].

1.5.4 Secondary Metabolites Having Sulfur

These are the result of two different pathways. The first group contains hydrolyzed glucosinolate (GSL) by myosinase enzyme and it is present in broccoli, nasturtium, and cabbage, i.e., the crucifers.

The subsequent group resulted by the hydrolysis of allinin via an enzyme (alliinase) present in Allium genus like Allium *prorrum* (Leeks), A. *cepa* (onion), and A. *sativum* (garlic). Above-mentioned crop bunches are significantly utilized as vegetables, flavors, and immune boosters due to the presence of these two pathways [55].

Glutathione (GSH) is natural sulfur found in the plants and acts as a remedy against reducing plant intensification and also as an antioxidant agent in stress reactions [56]. GSL is a cluster of glycosides with small compounds of sulfur and nitrogen. These are responsible for the resistance against competitors, predators, and parasites in plants because

volatile substances resulted from the breakdown of GSL have harmful, repellent, and detoxifying impacts [57]. GSLs are converted in isothiocyanates via metabolism and absorbed which influences GST (glutathione S-transferase) activity and cell damage against DNA damage and manipulate the antioxidant resistance method and detoxify the xenobiotics [58].

Organic phytoalexins is a family of sesquiterpenoids in Solanaceae, isoflavonoids of Leguminosae and phytoalexins from Brassicae which has a basic structural moiety, i.e., S-atom, indole or related ring structures. The fungal and bacterial assault and associated conditions of plants produced defensins, lectins, and thionines, i.e., S-rich plant proteins. They are responsible for the development or growth of numerous fungi [10]. Sulfur compounds are abundantly present in Cruciferae and Allium genus members. They give a volatile, trademark fragrance, and flavor to these plants. Crucifers are hot and pungent due to the presence of isothiocyanates. There is a danger of attack of vegetarian/vegan and pathogens; GSLs prevents this by imparting bitter taste. These compounds are also utilized as protective source against atherosclerosis and inflammatory diseases [56].

1.6 Bioactivity of Secondary Metabolites

1.6.1 As Antioxidants

There are firm inferences that plants produce antioxidants which are able to destroy free radicals within the human body. Antioxidant-based formulation is utilized to protect and treat infections engaged with the process of oxidative stress. So, the huge interests recently centered around natural foods, their own ability to suppress free radicals. They may give a safety option in contrast to toxic and synthetic antioxidant agents [59]. Secondary metabolites derived from the accompanying plants have antioxidant activity:

- *Limonia acidissimia* [60]
- *Murraya koenigii* [61]
- *Duranta erecta* [62]
- *Petrea volubilis* L. [63]
- *Rumex vesicarius* [64]
- *Sisymbrium irio* [65]
- *Digera muricata* [66]
- *Gomphren acelosioides* Mart. [67]
- *Trichosanthes cucumerina* [68]
- *Melothria maderaspatana* [69]

1.6.2 As Antimicrobials

The breakthrough of antimicrobial medications is a great gift toward the control of infectious disease but whole world is worried about antibiotic resistance. Presently, available drugs are not potent enough toward multi-drug resistant pathogens. So, the variety of solvent extract has been explored to overcome this existing risk of the anti-infective properties of different herbs and spices in roots, leaves, stems, flowers, and fruits [59].

- *Sisymbrium irio* [65]
- *Duranta erecta* [62]
- *Gomphrena celosioides* [70]
- *Petrea volubilis* [63]
- *Melothria maderaspatana* [71]
- *Trichosanthes curcumerina* [71]
- *Limonia acidissimia* [60]
- *Murraya koenigii* [72]
- *Sidaacuta Burm* F. [73]

1.6.3 As Anti-Diabetics Agents

Type I diabetes (dependent upon insulin) is due to insulin inadequacy and lack of actuated β-cells and type II (insulin-independent diabetes) does not react to the enzyme insulin and proper diet, work out and medication can treat this type of hypoglycemia. In our digestive tract, starch is initially hydrolyzed into maltose, and finally into glucose, this process is catalyzed by α-amylase enzyme present in pancreas. The degradation of this starch can lead quick and prominent postprandial hyperglycemia (PPHG). In this way, reducing starch digestion by inhibiting enzymes, for example, α-amylase is important in controlling diabetes. Type 2 diabetes can be treated by various ways like bignonides, sulfonylureas, and thiazolidinones; however, undesirable side effects are also there. Natural concentrates/extracts from traditional herbs can possibly make new and potential anti-diabetes drugs [59].

- *T. cucumerina* [71]
- *Melothria maderaspatana* [71]
- *Aloe vera* L. [74]
- *Azadirachta indica* A. Juss [75]
- *Allium cepa* L. [76]
- *Allium sativum* L. [76]

- *Mangifera indica* L. [77]
- *Andrographis paniculata* Nees [78]
- *Vitex negundo* Linn. [78]

1.7 Conclusion and Future Perspectives

In contrast to basic metabolism (carbohydrates, fats, proteins, vitamins, and minerals), secondary metabolites have no healthy benefit to human, yet have scientifically demonstrated inline properties. Primary metabolic molecule generates secondary metabolites via a variety of biological processes. The biological assay-based separation of bioactive compounds and preparation of their analogs has surfaced as a new effectual move toward designing of derivatives. Secondary metabolic substances are one of the most important tools for protection as well as development of plants and are easily accessible for innovation. Secondary metabolic compounds possess great biological activity; therefore, it is hard to replace them with synthetic medications and other market available significant drugs. From the viewpoint of evolutionary pharmacology, secondary metabolites constitute an attractive collection of bioactive compounds showcasing as range of functions for human cells, viruses, bacteria, fungi, and parasites. Secondary metabolites have significant natural functions in plants. These natural compounds are generated when plants undergo abiotic stresses, for example, saltiness, dry season, ultraviolet radiation, heavy metals and extreme climate. The in-depth study of various secondary metabolic products of plant life has prompted the development of plant meta-bolomics, an innovative field associated with discovery and detection of the biosynthetic pathways of these compounds and identification of their structure and practical uses. These days, analytical assessment of plants is being widely carried out using metabolite finger printing and metabolite profiling. In spite of these advances, there is a room for elevated throughput strategies for prompt investigation of plant samples. Computational devices ought to be developed to rapidly analyze and detect metabolite information.

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2

Advances in Natural Products-Based Antiviral Agents

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Abstract
Long-term use of approved antiviral drugs can lead to drug resistance and side effects. By the other hand, there are currently no vaccines or antiviral drugs available to treat several important viral diseases as well as newly emerging viral infections. Therefore, antiviral drug research has always been the hot research topic in the field of medicinal chemistry. In addition, natural products are a crucial source of antiviral drugs. In this chapter, we review recent progress in the discovery and development of natural products as antiviral compounds, paying special attention to those molecules that provide potential lead compounds for drug development.

***Keywords*:** HIV, hepatitis C, alkaloids, influenza, herpesvirus, dengue, coronavirus

2.1 Introduction

Viral infections are major health threats across the world, causing millions of deaths every year. Although highly effective antiviral therapies are available for some pathogens, several important emerging viral diseases remain without effective treatments. Dengue virus (DENV), Ebola virus (EBOV),

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Zika virus (ZIKV), Chikungunya virus (CHIKV), and several coronaviruses are the most notable examples. Among them, the coronavirus SARS-CoV-2 has become the most threatening since its initial outbreak in Wuhan, Hubei province, central China, in November to December 2019. Its control and further expansion across all the world led to unseen measures of confinement in many countries, while coronavirus infectious disease (Covid-19) is claiming thousands of lives everywhere. However, despite promising results [1] and important efforts of the scientific community, there are only a few therapies available that show some efficacy against Covid-19.

For treatable viral infections [e.g., human immunodeficiency virus (HIV), hepatitis C virus (HCV), or herpes simplex virus (HSV)], the emergence of drug-resistant mutants and adverse effects caused by approved antiviral drugs, particularly after long-term administration constitute major threats for therapy success [2]. In this context, efforts in the discovery of new antiviral agents must be maintained and should be a priority in coming years [3, 4]. In this review, we will center on advances achieved during the last 5 years in medicinal chemistry of antiviral inhibitors, paying attention to those obtained directly from natural sources, as well as modified natural compounds prepared by synthesis/semi-synthesis procedures in organic chemistry.

2.2 Anti-HIV Agents

HIV type 1 (HIV-1) is the etiological pathogen of acquired immunodeficiency syndrome (AIDS), which still remains a serious worldwide public health problem. The viral replicative cycle of HIV-1 offers several drug targets for the treatment of AIDS [5, 6] (Figure 2.1). The most widely

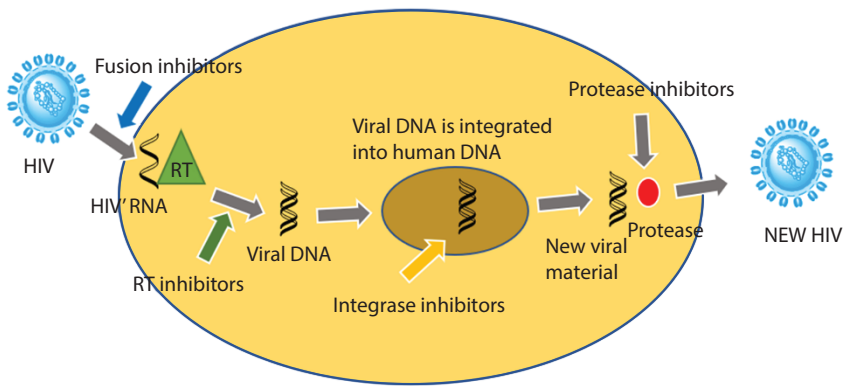

Figure 2.1 The viral replicative cycle of HIV-1 and potential targets of inhibition.

used antiretroviral agents act on viral enzymes, mainly reverse transcriptase (RT), but also protease (PR), and integrase (IN). Apart from other approved drugs targeting viral entry (e.g., virus-receptor recognition and cell membrane-viral envelope fusion), there are additional molecules in preclinical or early clinical development that inhibit other viral processes (e.g., maturation inhibitors and capsid binding compounds) as well as new drugs targeting reverse transcription, virus attachment, or molecules interfering with CD4 or coreceptor binding [7, 8].

Although several natural products have been considered for development as anti-HIV drugs and extensively characterized in preclinical studies and early clinical trials (e.g., bryostatin-1, calanolide A, and betulinic acid derivatives), none of them has been approved. These natural products target various steps of the virus replicative cycle. However, their mechanism of action has not been elucidated. In the following sections, we describe structures and anti-AIDS activities of natural products whose antiviral activities have been reported during the last 5 years.

2.2.1 Terpenes

Ingenane-type diterpenoids (1~4) were isolated from *Euphorbia ebracteolata* (Figure 2.2), guided by bioassay fractionation [9]. They showed potent activities against HIV-1, with IC_{50} values ranging from 0.7 to 2.3 nM and selectivity index (SI) values in the range of 11,307 to 20,263. Structure-activity relationship (SAR) studies indicated that their anti-HIV activity is related to long aliphatic chain substituents at positions C-3, C-5, or C-20. A synthetic ingenane analog (5) that inhibits viral infection (IC_{50} = 1.3 nM) and reactivates latent HIV-1 has been identified as the most promising candidate for further development [10].

In 2017, Zhang *et al*. reported four new oxazole-containing diterpenoids isolated from *Salvia miltiorrhiza* Bunge (*Labiatae*) [11]. In HIV-1 long terminal repeat (LTR) reporter assays, reporter gene expression which was driven by the LTR promoter was suppressed by compound **6** by a dose-dependent manner, both in the absence or presence of Tat. Compound **6** was identified as a new lead for the development of HIV-1 transcription inhibitors and blocked viral transcription at submicromolar concentration.

Several lupane-type triterpenoids with anti-HIV activity have been obtained from *Cassine xylocarpa* and *Maytenus jelskii* extracts. Compound **7** and its derivatives (**8**, **9**) possessed potent anti-HIV-1 activity with IC_{50} values below 5 μM [12]. A preliminary SAR study indicated that a relevant structural pharmacophore of these molecules is the formyl group, although their antiviral activity is also related to their oxidation level.

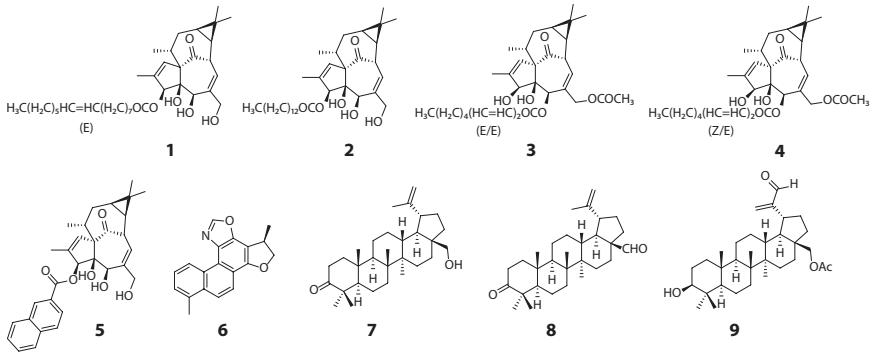

Figure 2.2 Terpenes with anti-HIV activity (**1**–**9**).

2.2.2 Phenylpropanoids

The anti-HIV activity of coumarin derivatives has been reported, and in many cases, attributed to their potential as HIV PR [13] and IN inhibitors [14]. Chromenone derivatives have a coumarin-based scaffold and were developed to inhibit many HIV-1 enzymatic functions, most notably, HIV-1 IN and Ribonuclease H (RNase H) activities. Among them, compound **10** (Figure 2.3) showed IC_{50} values of 6.75 ± 0.51 and 6.45 ± 0.45 μM, in IN strand transfer and RNase H activity assays, respectively [15].

Tetradecyl ferulate (**11**) was isolated from the dichloromethane extract of holy basil (*Ocimum sanctum*) leaves, a showed remarkable RNase H inhibitory activity with an IC_{50} value of 12.4 μM. Derivatives of **11** such as *N*-oleylcaffeamide (**12**) inhibited HIV-1 RT-associated RNA-dependent DNA polymerase and RNase H activity (IC_{50} = 2.3 and 0.68 μM, respectively) [16]. Molecular docking and Yonetani-Theorell studies predicted that compound **12** could bind at two allosteric sites in the viral RT, one located near to the non-nucleoside RT inhibitor (NNRTI) binding pocket, the other near to RNase H catalytic site.

In 2017, Zhang *et al.* reported the antiviral effects of arylnaphthalide lignan (ANL) glycosides with antiviral efficacy. Patentiflorin A (**13**) and justiprocumin B (**14**) were isolated from methanol extracts of stems and bark of *Justicia gendarussa* by bioassay-guided separation [17, 18]. Further modification of these molecules revealed that the quinovopyranosyloxy group was crucial for retaining potent anti-HIV activity in these ANL glycosides. Compound **13** was active against M- and T-tropic HIV-1 isolates, and was more effective than 3′-azido-3′-deoxythymidine (AZT) in inhibiting multiple different HIV-1 clinical isolates, with IC_{50} values ranging from 14 to 32 nM.

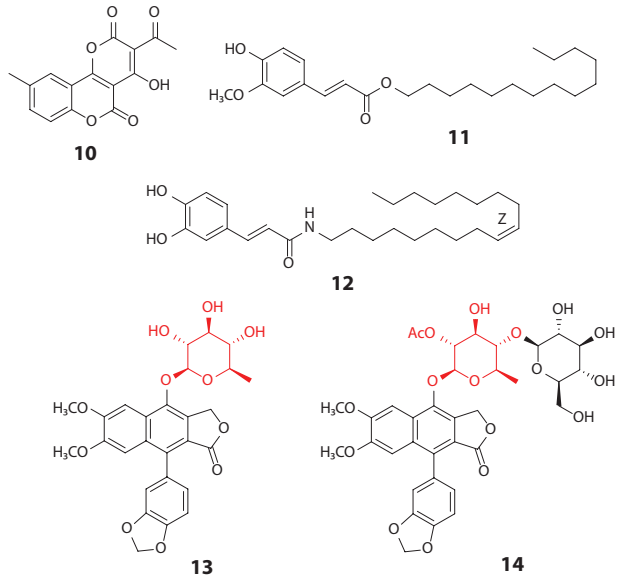

Figure 2.3 Phenylpropanoids with anti-HIV activity (**10**–**14**).

2.2.3 Anthraquinones

Sennoside A (**15**) (Figure 2.4) is an anthraquinone derivative extracted from *Rheum officinale* Baill and *Rheum palmatum* L. that inhibits both HIV-1 DNA polymerase and RNase H activities [19]. Sennoside A showed antiviral activity in cell culture with EC_{50} values in the low micromolar range. Related anthraquinones containing sulfur-bridged pyranonaphthoquinone (PNQ) dimers were isolated from soil-dwelling *Streptomyces* sp. Among them, naquihexcin E (**16**) exhibited inhibitory effects against HIV-1 (EC_{50} = 2.8 μM; TI = 22.5) [20].

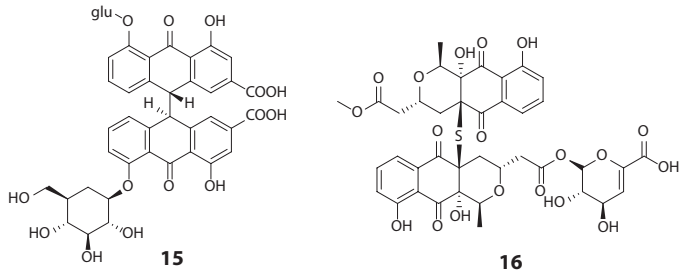

Figure 2.4 Anthraquinones with anti-HIV activity (**15**, **16**).

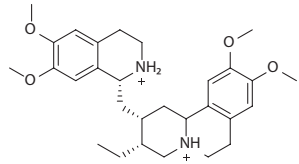

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Figure 2.5 Structural formula of emetine (**17**).

2.2.4 Alkaloids

Various structural alkaloids have been reported to be active against HIV [21]. HIV-1 RT DNA polymerization inhibition by emetine (**17**), which has a monoterpenoid-tetrahydroisoquinoline skeleton (Figure 2.5), has been demonstrated *in vitro* using purified enzyme, and in virions, using an endogenous RT activity assay. Emetine also showed inhibitory activity in cell culture with low cytotoxicity, although total abrogation of HIV replication was not observed [22].

2.3 Natural Alkaloids With Activity Against HBV and HCV Infections

Hepatitis B virus (HBV) and HCV infections are among the most prevalent infectious diseases around the World and remain as major health threats. Based on the World Health Organization, hepatitis B affects more than 250 million people worldwide, while the estimated population infected with HCV is around 71 million (https://www.who.int/). During the last few years, there has been significant progress in controlling those diseases, particularly HCV infection with the introduction and improvement of directly acting antiviral agents and interferon-free therapies that allow eradication of the virus in infected patients [23].

Among natural products recently described as potential inhibitors of HBV or HCV replication, alkaloids represent the most important group. Thus, matrine (**18**) exhibited potent antiviral activity against HBV. Li and Wang's group reported novel alkaloids (**19**~**25**) that possessed a matrine skeleton (Figure 2.6), isolated from the ethanol extract of seeds of *Sophora alopecuroides*. The results of the assay for antiviral activities demonstrated that these natural products have similar potencies in comparison with the positive control lamivudine [24, 25]. Recently, several matrine-type analogs

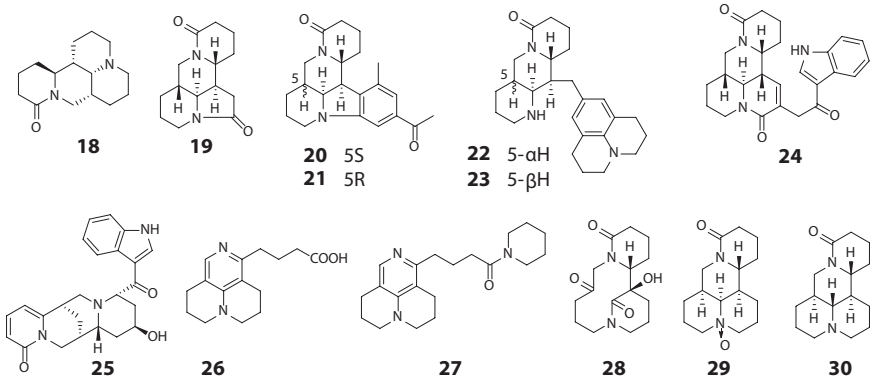


Figure 2.6 Alkaloids with anti-HBV activity (**18–30**).

were isolated from the roots of *Sophora flavescens* ("Kushen" in Chinese). All alkaloids displayed inhibitory effects, based on the levels of hepatitis B surface antigen (HBsAg). Specifically, compounds **26**–**30** inhibited HBsAg secretion by 37.2% to 46.0% at non-cytotoxic concentrations, suggesting that they are more active than compound **18** (34.7% at a concentration of 0.4 mM) [26]. Apparently, these compounds inhibit virus replication by making the heat shock cognate 70 (Hsc70) messenger RNA unstable [27].

The inhibitory activity of compound **31** (12N-4-methoxybenzyl matrinic acid) against HCV was detected after screening a library of specific tricyclic matrinic derivatives [28]. The EC_{50} value obtained for compound **31** was 22.7 ± 0.67 μM. Based on **31** (Figure 2.7), a new class of 12N-substitited matrinic ethanol derivatives has been developed [29]. After evaluation of their activity against HCV *in vitro*, authors determined an EC_{50} value of 3.2 μM for the best molecule in the series (**32**), with an SI of 96.6. These compounds, as Hsc70 down-regulators, blocked HCV propagation at the post transcriptional level.

An alkaloid containing a quinolizidine group (**33**) and extracted from *Sophora alopecuroides* inhibited HCV replication (EC_{50} = 4.32 μM; SI = 54.5), while another derivative in the series (**34**) was found to inhibit both wild-type and HCV variants [30].

New indole diketopiperazine alkaloid (**35**) and new benzodiazepine (**36**) were isolated from the fungus *Penicillium raistrickii*. Compounds **35** and **36** displayed inhibitory activity against HCV (EC_{50} = 5.7 and 7.0 μM, respectively) [31]. Structural modification of these new alkaloids may provide a new series of potential anti-HCV candidates useful in medicinal chemistry studies.

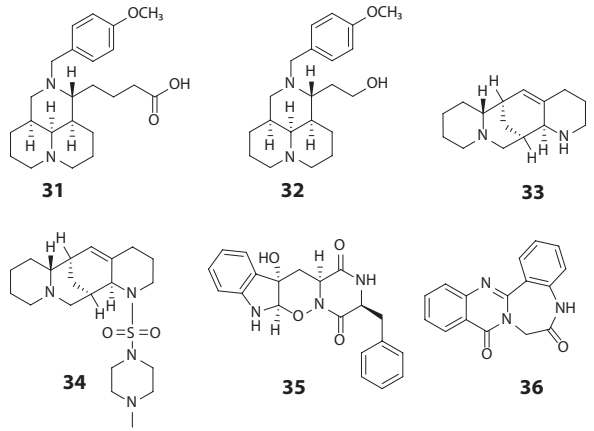


Figure 2.7 Alkaloids with anti-HCV activity (**31–36**).

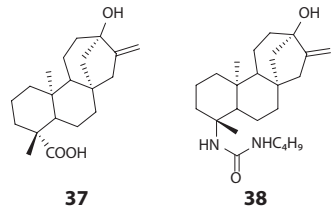


Figure 2.8 Structures with anti-HBV activity (**37, 38**).

Steviol (*ent*-13-hydroxykaur-16-en-19-oic acid) (**37**) (Figure 2.8) extracted from *Stevia rebaudiana* Bertoni was found to inhibit the secretion of HBV antigens, suggesting an antiviral effect against HBV. A series of C-4-substituted steviol derivatives were designed, synthesized, and evaluated for their effects against HBV. Overall, compound **38** was identified as the most active steviol derivative (EC_{50} = 16.9 μM, SI = 57.7) [32]. Studies aimed to unveil their mechanism of action revealed the involvement of NF-κB and MAPK signaling pathways, although the precise mechanism was not determined.

2.4 Anti-Influenza Virus Agents

Influenza virus has a segmented single-stranded RNA genome belonging to the *Orthomyxoviridae* family, which is classified into three types (A, B, and C) based on viral antigenicity. Seasonal flu is caused by influenza A and B viruses. Influenza A viruses are divided into many subtypes based on hemagglutinin (H) and neuraminidase (N) composition. Based on their

mechanism of action, approved drugs blocking influenza virus propagation can be classified in two groups: M2 ion channel inhibitors and neuraminidase inhibitors. Globally, the annual death toll of influenza virus is estimated at 291,000–645,000 [33]. Besides, seasonal flu can occassionally turn into a pandemic, as occurred several times during the last one hundred years. Effective anti-influenza drugs are necessary to combat seasonal infections as well as any eventual future pandemics.

Under these circumstances, some natural products have received attention from the research community. For example, new flavonoid glycosides (**39**) (Figure 2.9) isolated from the rhizomes of *Matteuccia struthiopteris* (L.) Todar, which showed significant anti-influenza virus (H1N1) activity (IC_{50} = 6.8 ± 1.1 μM, SI = 34.4) [34]. Molecular docking analysis showed that compound **39** fits deep into an extended binding pocket, with hydrogen bonds and hydrophobic interactions in the active site.

Phenolic lactones obtained from a deep-sea fungus (*Spiromastix* sp.) exhibited potential inhibitory activity against an influenza A virus WSN strain with low cytotoxicity. Among them, compound **40** was the most potent, inhibiting different influenza A and B virus strains (EC_{50} = 6.0 and 33.45 μM, respectively), as well as clinical isolates resistant to neuraminidase inhibitors [35]. Mechanistic studies showed that compound **40** targets hemagglutinin protein (HA), disrupting the interaction between HA and sialic acid receptor, blocking virus attachment to host cells.

In 2015, Botta *et al.* reported that catechol derivatives exhibit activity against RNA and DNA viruses [36]. Thus, tocopheryl hydroquinone derivatives were shown to be active against influenza A virus by altering cell's internal redox potential [37]. Pyrogallol and other catechol derivatives were synthesized to evaluate the function of the oxidation state of different catechol and pyrogallol derivatives in anti-influenza activity. Among them, pyrogallol derivatives **41** and **42** were the most effective inhibitors against the virus, as determined in hemagglutination assays (EC_{50} = 8.7 and 2.5 μM, respectively) [38].

Bark extracts of wild syringa (*Burkea Africana*), a tropical African tree, were used to isolate triterpene saponins **43** and **44** that showed EC_{50} values ranging from 0.05 to 0.27 μM in influenza virus inhibition assays, without relevant cytotoxicity [39].

Spirostaphylotrichin X (**45**), a new spirocyclic γ-lactam, isolated from the fungus *Cochliobolus lunatus* SCSIO41401 (one of the marine-derived fungus), was active against various influenza virus strains (IC_{50} = 1.2–5.5 μM) [40]. Mechanistic studies revealed that compound **45** inhibited influenza virus polymerase activity through binding the highly conserved binding pocket of polymerase.

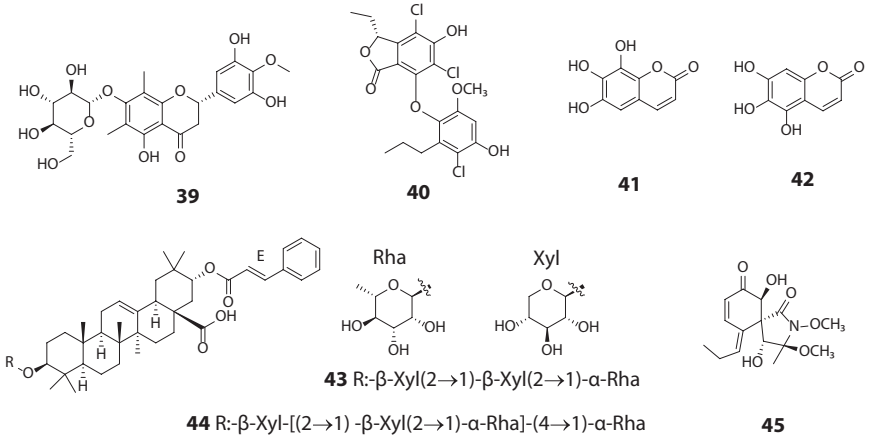


Figure 2.9 Structures with anti-influenza virus activity (**39–45**).

2.5 Natural Products Active Against Herpesviruses

Herpesviruses are enveloped double-stranded DNA viruses that often produce cutaneous lesions, frequently seen as blisters, occurring in flares of herpes simplex 1, herpes simplex 2, and herpes zoster (shingles). There are nine types of viruses in the *Herpesviridae* family that infect humans: herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6A and 6B, human herpesvirus 7, varicella-zoster virus (VZV), and Kaposi's sarcoma-associated herpesvirus (KSHV). Herpesviruses are very rarely responsible for life-threating diseases, but their infections can be serious and can last the whole life.

Houttuynoid A (**46**) and M (**47**) are flavonoids (Figure 2.10) isolated from an herbal medicine *Houttuynia cordata* that showed inhibitory activity against HSV with EC_{50} values of 12.42 and 17.72 μM in a plaque formation assay. Biochemical and cell biology studies showed that houttuynoid A inactivated HSV-1 infectivity by blocking viral membrane fusion [41, 42].

EBV is associated with several kinds of cancer, including Burkitt lymphoma, gastric carcinoma, nasopharyngeal carcinoma, and Hodgkin's disease. The coumarin (+)-rutamarin (**48**), isolated from *Ruta graveolens* L. (*Rutaceae*), was found to be active against EBV in lytic replication assays (EC_{50} = 7.0 μM) [43]. Compound **49** [(−)-chalepin], used as precursor of (−)-rutamarin, was 10 times less potent than **48** in those assays (EC_{50} = 69.9 μM). Rutamarin derivatives **50** and **51** exhibited EC_{50} values of 1.5 and 0.32 μM, respectively, while showing SI values of 801 for compound **50** and 211 for compound **51** [44].

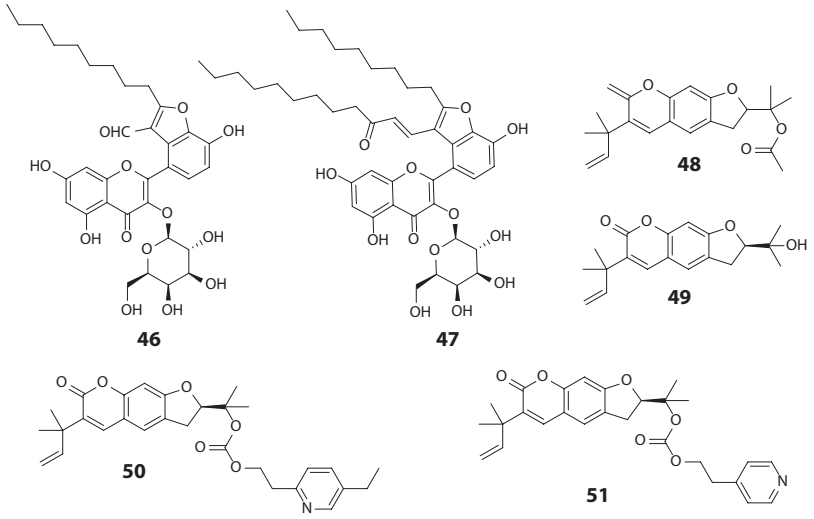


Figure 2.10 Structures with anti-HSV activity (**46–51**).

2.6 Natural Products Against Chikungunya Virus

CHIKV is a mosquito-borne arbovirus that has become a prominent public health threat. It can induce high fever, myalgia, and vomiting in the acute phase. Currently, there are no vaccines or antiviral drugs available for the treatment of CHIKV fever.

A series of 29 diterpene esters were evaluated in cell culture assays against CHIKV and HIV [45]. Most of them showed low to moderate activity against CHIKV, while phorbol-12,13-didecanoate (**52**) emerged as the most promising compound (Figure 2.11), with EC_{50} value of 6.0 ± 0.9 nM and an SI value of 686. After comparing the chemical properties and structures of diterpene esters with those of naturally occurring and chemically modified phorbol and deoxyphorbol esters, authors concluded that compound **52** and related drugs could act as anti-CHIKV agents by interfering with the activity of protein kinase C (PKC).

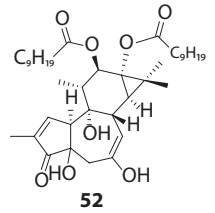
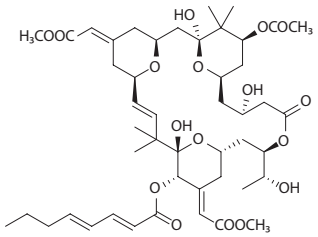


Figure 2.11 Structural formula of compound **52**.



53

Figure 2.12 Structural formula of bryostatin 1.

Interestingly, however, bryostatin 1 (**53**) (Figure 2.12), a promising pan-PKC modulator, is inactive against CHIKV in cell culture assays [46]. However, several bryostatin analogs were found to be selective inhibitors of CHIKV replication. These new findings indicated that the anti-CHIKV activity of bryostatin analogs is not only mediated by PKC modulation, suggesting the involvement of other unidentified targets of CHIKV therapeutic intervention.

2.7 Natural Products Targeting Dengue Virus

DENV is an arbovirus that causes dengue in humans, with symptoms ranging from mild to severe diseases like hemorrhagic fever and shock syndrome. The prevalence of DENV has increased during the last 20 years, and currently, it is responsible for about 400 million infections per year, with half a million causing hemorrhagic fever [47]. Although a vaccine has been recently approved, effective antiviral treatments are still missing.

Several natural products have been recently described as potential antivirals against dengue virus. Cavinafungin (**54**) is a linear lipopeptide (Figure 2.13) isolated from the fungus *Colispora cavincola* with potent inhibitory effect against ZIKV and all four DENV serotypes [48]. By using a large genome-wide profiling strategy involving the use of CRISPR-Cas technologies, researchers have shown that cavinafungin inhibits a signal peptidase associated with the endoplasmic reticulum. This peptidase plays a critical role in flavivirus polyprotein processing. Another interesting natural product is (2*R*,4*R*)-1,2,4-trihydroxyheptadec-16-yne (THHY, **55**). THHY has been extracted from avocado (*Persea americana*) fruits and exhibits inhibitory activity against all DENV serotypes. Mechanistic studies revealed that THHY inhibits DENV replication through impairment of the NF-κB–mediated interferon antiviral response [49].

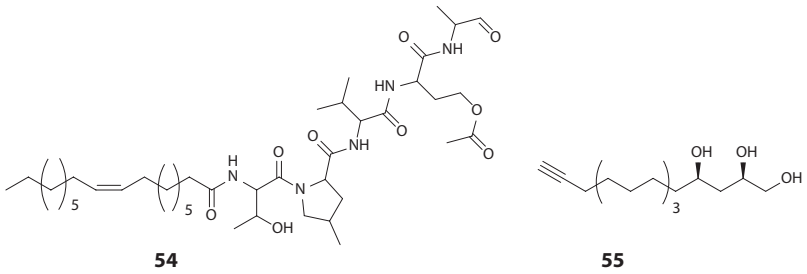


Figure 2.13 Structures with anti-DENV activity (**54, 55**).

2.8 Natural Products Targeting Coronaviruses

Viruses of the family *Coronaviridae* are enveloped, positive-strand RNA viruses, which, in humans, cause respiratory infections that include mild forms (e.g., cold) to serious and life-threatening infections that constitute a major threat to public health. Examples can be found in the last two decades with the emergence and dispersion of coronaviruses (CoVs) such as MERS-CoV, SARS-CoV, and, presently, SARS-CoV-2 which is causing a large pandemic across the World starting at the end of 2019. However, there are only a few approved antiviral agents to treat infections caused by CoVs, and large efforts are being concentrated on the development of urgently needed drugs [1].

In CoVs, the chymotrypsin-like protease 3 ($3CL_{pro}$) enzyme, also determined as the main protease, is responsible for cleavage of the viral precursor polyprotein at eleven sites, resulting in the production of 16 non-structural proteins. Therefore, it is considered as a major target for CoV infection. Inhibitors of SARS-CoV $3CL_{pro}$ were detected in ethanol extracts of the leaves of *Torreya nucifera*. These are bioflavonoids (Figure 2.14), among which biflavone amentoflavone (**56**) was the most effective compound (IC_{50} = 8.3 μM) [50]. Other flavonoids, including helichrysetin (**57**), herbacetin (**58**), isobavachalcone (**59**), and quercetin 3-β-D-glucoside (**60**) were also found to inhibit the enzymatic activity of MERS-CoV $3CL_{pro}$ [51]. Results of experimental and computational studies indicated that flavonol and chalcone are favorite scaffolds for binding to the catalytic site of MERS-CoV $3CL_{pro}$. Therefore, flavonoids with those characteristics are potential templates for development of potent CoV inhibitors.

Iguesterin (**61**), pritimererin (**62**), tingenone (**63**), and quinone-methide triterpenoids celastrol (**64**), obtained from the shrubby yellow vine

Tripterygium regelii, were found to be good SARS-CoV $3CL_{pro}$ inhibitors with IC_{50} values ranging from 2.6 μM in the case of iguesterin to 10.3 μM for celastrol. In contrast, the related semisynthetic analog dihydrocelastrol (**65**) had an IC_{50} of 21.7 μM [52]. A SAR study indicated that the quinone-methide moiety in the A ring and the more hydrophobic E-ring are major contributors for the observed inhibitory activity.

Angiotensin-converting enzyme 2 (ACE2) is the cellular receptor of SARS-CoV and SARS-CoV-2, and a potentially important target to stop SARS-CoV-2 infection [53]. Based on molecular docking studies, Chen and Du (2020) have recently proposed that natural compounds, such as baicalin (**66**), hesperetin (**67**), scutellarin (**68**), glycyrrhizin (**69**), and nicotianamine (**70**) are potential ACE2 ligands (Figure 2.14), and may block SARS-CoV-2 entry [54].

Additional compounds targeting CoVs have been identified in high-throughput screening campaigns. One of them involved a library of 2,000 compounds and allowed researchers to identify 56 potentially active molecules [55]. Several of them showed low-micromolar inhibitory activity against reference strains of human CoVs such as OC43. Emetine, lycorine, monensin sodium, mycophenolic acid, mycophenolate mofetil, pyrvinium pamoate, and phenazopyridine were characterized as broad-spectrum CoVs inhibitors. These molecules showed good inhibitory activity against four different types of CoVs in viral replication assays carried out in cell culture. Among them, emetine exhibited the strongest effect against MERS-CoV (EC_{50} = 0.34 μM; SI = 9.06). Data from pseudovirus entry assays were consistent with an inhibitory effect on viral entry for these compounds. Lycorine (**71**), an alkaloid from the red spider lily (*Lycoris radiate*), showed potent anti-CoV activity, with EC_{50} values in the range of 0.15 to 1.63 μM and an SI value of 29.13. Mycophenolic acid (**72**) and its derivative mycophenolate mofetil (**73**) were both good inhibitors of human CoV OC43 replication, suggesting that the presence of similar core structures in both molecules was determinant for having similar antiviral activities and molecular mechanisms of action.

Lianhuaqingwen (LH) as traditional Chinese medicine formula composed of 13 herbs recommended in the latest guidelines for treatment of CoV infectious disease (Covid-19) (http://www.nhc.gov.cn/). LH has been used to treat influenza and recent studies have demonstrated its anti-CoV activity *in vitro* (IC_{50} = 411.2 μg/ml). The formula inhibited the replication of SARS-CoV-2 in Vero cells, while decreasing the release of pro-inflammatory cytokines in Huh-7 cells [56]. These results suggest a protective effect of LH against Covid-19, although further studies are required to demonstrate its real value.

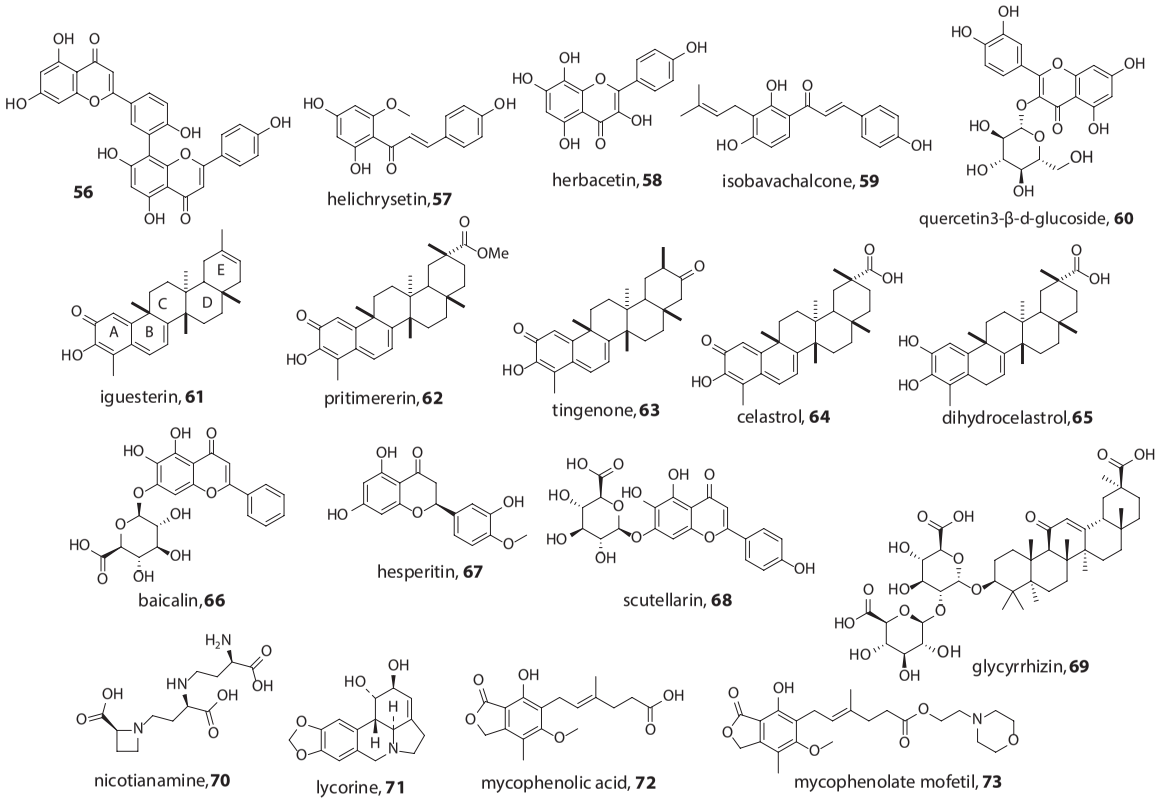


Figure 2.14 Structures with anti-CoV activity (**56**–**73**).

2.9 Natural Products Against Other Viral Infections

Marburg virus (MARV) and EBOV are both extremely contagious pathogens belonging to the family *Filoviridae*. They can both cause lethal hemorrhagic fever disease in human beings and animals. Aloperine (**74**) (Figure 2.15) showed potent antiviral activity against EBOV and pseudotyped MARV (EC_{50} = 12.4 μM and 10.2 μM, respectively) [57]. Compound **75** was active against both viruses *in vitro* and *in vivo* (EC_{50} = 4.8 μM and 7.1 μM, for EBOV and pseudotyped MARV, respectively) and was described as the most promising compound in a series of 23 aloperine derivatives. Mechanistic studies revealed that compound **75** could block a late step in the viral life cycle by inhibiting cathepsin B, a cysteine protease present in the host cell.

Enterovirus 71 (EV71) belongs to the family *Picornaviridae* of non-enveloped single-stranded positive-sense RNA virus. Aqueous extracts of the plant *Schizonepeta tenuifolia* (Benth.) Briq. showed anti-EV71 activity [58]. These extracts seem to have multiple effects on enterovirus replication: (i) interfere with virus attachment to host cells, (ii) suppress cleavage of eukaryotic translation initiation factor 4G, and (iii) hinder cytoplasmic translocation of heterogeneous nuclear ribonucleoprotein A1. Taken together, these findings suggest that the referred plant extracts could serve as candidate antiviral drugs or healthy food for the treatment of EV71.

Human rhinovirus (HRV) is one of the most frequent infectious pathogens that cause mild upper respiratory illness in humans worldwide. Compounds **76** and **77**, isolated from dichloromethane extracts of leaves of *Bupleurum fruticosum* by bioassay-guided fractionation, displayed the most potent inhibitory effect against the replication of HRV39 serotype, with EC_{50} values of 1.8 ± 0.02 μM for compound **76** and 2.4 ± 0.04 μM for compound **77** [59]. Mechanism of action studies indicated that compound **77** binds to the viral capsid, thereby interfering with early events in virus replication, but also seems to inhibit late phases in the virus replicative cycle.

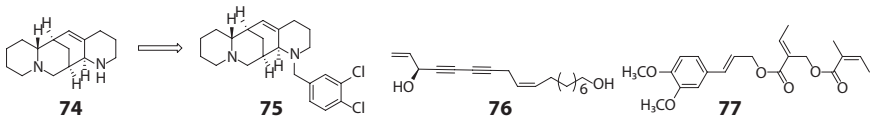


Figure 2.15 Structures of antiviral compounds **74**–**77** targeting filoviruses and enteroviruses.

2.10 Conclusion

Considering the continuous spread of major infectious agents as well as unpredictable outbreaks and spillovers of novel virus pathogens, it seems advisable to accelerate drug discovery. Natural products have been—and would continue to be—one rich source of small molecules. Due to the large structural diversity and complexity, natural product libraries play a crucial role in drug discovery.

In order to build up the knowledge base required to address the urgent need for novel antiviral compounds, researchers have exploited many strategies to obtain diverse natural products and their analogs. The strategies are mostly focused on finding new active compounds from novel organisms and interfering with natural metabolic pathways but also used chemical synthesis and biological synthetic and semisynthetic approaches to obtain better compounds. On the other hand, natural products with low bioactivity could be modified synthetically to ameliorate their pharmacological profiles. It is especially crucial that synthesis of new analogs must be comprehensive used of multiple strategies appropriate biological assays to successfully optimize lead compounds into clinical trial candidates.

Acknowledgements

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3

Bioactive Component of Black Pepper-Piperine: Structure-Activity Relationship and Its Broad-Spectrum Activity—An Overview

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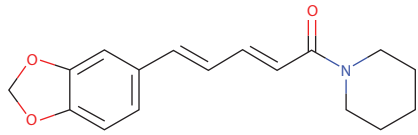
Abstract

Phenotypic drug discovery has never lost its place in the span of drug invention/discovery. This method involves using a known natural product that is proven to have bio-activity, as the base for the invention of new molecules/leads. This chapter reviews the role and the importance of the natural products in the field of medicinal chemistry, basics of drug discovery research with well-known and well-established molecules like morphine, and finally, a discussion on the current research on a molecule named Piperine is made.



Structure of Piperine

Piperine, on structural alteration, shows different bio-activity. In the following, piperine analogs as GABA modulators and as anti-microbials are analyzed. Based on structural-activity relationship analysis of piperine as GABA modulator and anti-microbial agent, the possible pharmacophore of the molecule is predicted as follows,

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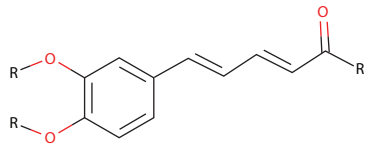


Presumed Pharmacophore

MDP ring connected to the unsaturated chain with a carbonyl functional group. The present chapter will focus on the structural-activity relationship with the examples on bio-activity of the emerging lead molecules from pepper.

Keywords: Piperine, piperine analogs, structure-activity relationship (SAR), GABA, GABA modulation, synergistic effect, pharmacophore, anti-bacterial, anti-fungal, multidrug resistance (MDR), MRSA

List of Abbreviations

Abbreviation	Expansion
CYP	Cytochrome P 450
GABA	Gama Amino Butyric Acid
EPI	Efflux pump inhibitor
MBC	Minimum bactericidal activity
MRSA	Methicillin-resistant Staphylococcus aureus
MDP	Methylene-dioxy phenyl ring
MEC	Minimum efficient concentration
MIC	Minimum inhibitory concentration
SAR	Structure-activity relationship
BBB	Blood-brain barrier

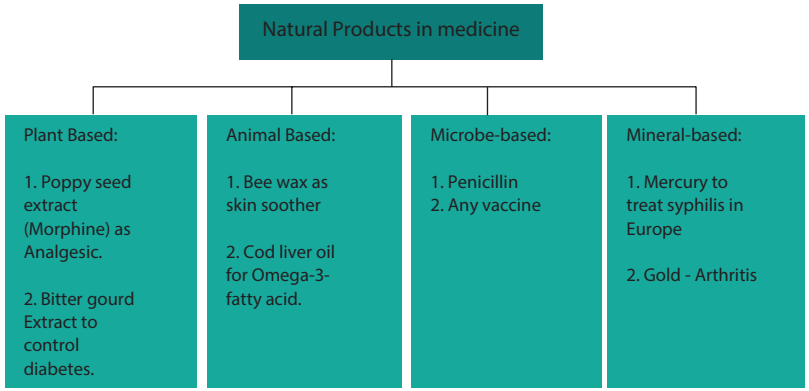
3.1 Introduction: What is a Natural Product?

Nature protects and provides equally for all the creatures she created. Herein, the Darwin's rule kicks in; the ones that understand the nature better are considered to be the fittest, hence the "Fittest Survives". Us, humans, are one of the few fittest living beings on Earth now. While we were in the process of mastering the art of survival, our cerebral capacity developed a lot higher than any other primates or we can say any other living beings. We learnt to derive all our solutions from our surroundings, beginning from our primitive needs like food, shelter, clothing, and medication to

our sophisticated inventions like transportation (metals, petrol, and gas) and dyes.

We learnt to get everything from the nature or to develop from the naturally occurring material. This basically brought us to the top of the food chain and made us better survivors. Though we triumphed to live well on Earth, we still need to keep up our level of survival to meet all the updated checks of nature, up on our population. For instance, along with us, bacteria, virus, and every disease-causing agent evolve, and they learn to fight with our defense system (current medicines) and would eventually win. To overcome this threat, a constant update in the drug library is must. So, medication is one way to keep the risk of our extinction at bay. Acquiring drugs from naturally occurring products would be our first simpler option as it is in our genes.

To make it clearer, let us categorize the natural products that involve in medicinal chemistry into four, as follows:



Now, if we see the story from the genesis. For many decades, from our time as prey, there were diseases—designed to control apostrophe the population of the species; physicians—to tend them; scientists and the drugs they discovered—that made us the fittest.

Throughout the world, every community has/had their own medicinal system which is solely based on their natural resources. When St. John Wart extract was used in the European countries to alleviate headaches, water extract of dried ginger and pepper was used by the Asian countries. Based on the geography, the medicines differed, as did the medicinal system. Some medicinal systems that were followed by the countries rich in natural resources still flourish, e.g., African medicinal system, Chinese medicinal system, and Egyptian and Indian medicinal system.

Among the divisions we saw earlier, plant-based medicines are the major ones; animal-based comes next to that, while in some medicinal systems, metals were used in the medicinal combination to make the drug-combo sustain for a longer period. Microbe-based medicines came in to use only after 1940s, and penicillin was the first known drug from microbe [1].

From the reports, it looks like till 1800s, the medication prescribed by the doctors was mostly water or alcoholic extracts of plant or animal materials. It is the entry of combinatorial chemistry, which brought the synthetic and semi-synthetic analogs inside the picture; after which, the emergence of modern drugs commenced.

The role of the combinatorial chemistry is first marked in the isolation of morphine. Morphine isolation from the poppy seeds was the foremost breakthrough which took chemistry from the level of alchemy to a practical science, and morphine was first isolated by a chemist called Friedrich Wilhelm Adam Serturner in 1805 [2].

The next advancement is the synthesis, and the first drug that was synthesized was acetyl salicylic acid or the famous over-the-counter-drug Aspirin. In ancient days, willow-bark (Figure 3.1) extract was used to alleviate pain in the European countries. Though it reduced pain, there were many side effects like vomiting, ulcer and stomach upset. In 1828, Joseph Buchner extracted the active component from willow-bark extract and named the yellow powder as "salicilin". In 1853, the French chemist, Charles Frédéric Gerhardt, determined the chemical structure of salicylic acid and chemically synthesized acetylsalicylic acid. But, it was in 1894, the young chemist Felix Hoffmann, who joined the pharmaceutical group of Bayer's

Figure 3.1 Willow tree. https://www.wemedia.co.in/article/wm/42678dc3669845898542c2b869bc72ad

and company found acetyl salicylic acid as a potential drug and named it Aspirin. Buchner, Gerhardt, and Hoffmann were the key figures in the development of acetylsalicylic acid as the drug Aspirin. It was then patented by the Bayer's. Aspirin overcame the side effects which the salicilin gave and became a huge hit, and it is the first semi-synthetic drug in the history [3, 4]. After which, many drugs came in to the market which constantly got improved. The upgradation is essential as the side effects would be targeted and removed in the later versions. The best example which would explain the importance of the upgradation of the drug molecules would be penicillin class of drugs—penicillin, ampicillin, amoxicillin, etc. With time, bacteria develop mutant genes to resist the drugs, so when penicillin became ineffective ampicillin was prescribed and the next was amoxicillin, etc.

According to the present-day ways, the drug discovery can be divided in to two basic categories:

(i) Phenotypic drug discovery/reverse pharmacology
(ii) Target-based drug discovery/forward pharmacology

Target-based drug discovery is where the biologists study a disease on molecular level, analyze the proteins governing the pathway of disease, and find the target protein for that disease. Then, a chemist designs a molecule that would bind with the target protein and deactivate that pathway. Phenotypic drug discovery is based on observable traits. For example, an extract that is proven to induce drowsiness may be given treat insomnia or to alleviate pain. The mechanism of action is unknown while there will no compromise in the drug action.

In target-based drug discovery, though the mechanism of action of the drug molecule involved is known, the time required to find the target protein, to synthesize a working drug model, and to analyze the safety of the designed drug would take huge time. While in phenotypic drug discovery, we would know the drug works and that would have been proven to be safe by real time analysis, but the pathway would be not known yet. So, future throughputs to evolve the drugs would be tough if the mechanism of action is not known for long period. Both methods have their own pros and cons.

So, why not combine them both? Say, we

1. Extract the bio-active molecule out of the plant material.
2. Analyze its structure via structure-activity relationship (SAR) theory.
3. By which, the number of proteins to be analyzed as potential targets would be narrowed.

4. While, the active molecule acts as current drug, with the above works, a new structural analog could be synthesized in a very short span of time and could get in to the market before the mother molecule gets outdated.

By this method, we compromise nothing. We would know the time and the science behind the action of the drug which paves the way for the invention of new drug molecules.

Our ancestors have given us a lot of information on how they survived. So, why waste it? Let's make the most out of it! So, for the invention of the new drugs, natural products serve as the inspiration for chemists, with their complex pharmacophores to work on. With these lead molecules new drugs are invented in the modern medication system. With this brief introduction, we belief we embarked why we need to pay attention to the natural products. Now, as the title of the chapter suggests, let's dive in to know about piperine–bio-active molecule from black pepper. Beginning with the importance of black pepper in medicine, the chapter briefs about the

i. Constituents present in the black pepper,
ii. Isolation techniques involved in the isolation of piperine,
iii. Metabolism of piperine in human body,
iv. SAR—explained with morphine as example,
v. Structural analysis of piperine,
vi. Piperine analogs as GABA modulators and anti-microbial agents.

By the end of this chapter, the reader may get an idea about the basics of the drug development research and some of the current research over piperine in the field of medicinal chemistry.

3.2 Black Pepper

The spice, black pepper, is dried (Figure 3.2), unripe berries (Figure 3.3) (peppercorn) of pepper plant. The plant is a climber and a flowering vine (Figure 3.4). The botanical classification of black pepper is given in Table 3.1. Peppercorn has multiple properties, that anchored its place in our daily lives. It is called as "King of Spices" and "Black Gold" for no less reason. In olden days, people used this spice to make their food smell good and they found with pepper added in the food, the expiry date prolonged a little more.

In Siddha, the Indian Medicinal, pepper plays a significant role. It is commonly called "Milagu" in Tamil (Script used to write Siddha system) and "Pippal" in Sanskrit. It belongs to the family Piperaceae and is native to South East Asia. Both long pepper and black pepper are similar in composition just the former is less expensive. Based on the seasons and geographical areas, the concentrations of alkaloids differ.

Figure 3.2 Dried unripe pepper berries.

Figure 3.3 Unripe pepper berries.

Figure 3.4 Pepper vines.

Table 3.1 Botanical classification of black pepper [5].

Kingdom	Plantae
Division	Manoliophyta
Class	Magnoliopsida
Order	Piperales
Family	Piperaceae
Genus	Piper
Species	nigrum

As mentioned before, throughout the world, every community has their own medicinal system which is solely based on the naturally occurring products. Among all, the Indian medicinal system, African and Chinese medicinal systems are still in practice due to their proven benefits.

Pepper is held to be a fantastic bio-availability enhancer, antioxidant, healing, stomachic, laxative, anthelmintic, and carminative; improves the appetite; is useful in bronchitis, abdominal pains, and diseases of the spleen, tumor, and ascites; and relieves biliousness. It is also attributed with numerous diseases of respiratory tract, like cough, bronchitis, and asthma as a counter irritant and analgesic when applied locally for muscular pains and inflammation. Also, as snuff in coma and drowsiness and internally as carminative; for epilepsy; as general tonic and hematinic; as cholagogue in

obstruction of bile duct and gall bladder; as an emmenagogue and abortifacient; as antihelmenthic in dysentery and in leprosy [6, 7].

Black pepper could be found in many medicinal combinations, thus living by its name "The King of Spice".

3.2.1 Constituents of Black Pepper

The black pepper (Piper *nigrum*), contains volatile oils and alkaloids which are responsible for its famous aroma and the physiological activity.

Composition of Indian pepper is reported as follows:

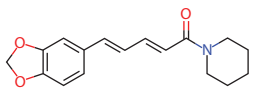
Volatile oil	2%–4% w/w
Alkaloids	5%–9% w/w
Protein	10%–14% w/w
Carbohydrates	60%–70% w/w

Lipids, crude fiber, etc., in smaller quantities. Volatile oil contains a complex mixture of monoterpenes (70%–80%), sesquiterpenes (20%–30%), and small amounts of oxygenated compounds, with no pungent principles present. Concentration and composition vary, depending on sources. Major monoterpenes include thujene, pinene, camphene, sabinene, pinene, myrcene, careen, limonene, and phellandrene. Sequiterpenes include caryophyllene (major componenet), bisabolene, farnesene, humulene, selinene, elemene, cubebene, copaene, myristicin, nerolidol, safrole, pinone, and N-formypiperidine, among others.

3.2.2 Major Alkaloids of Black Pepper

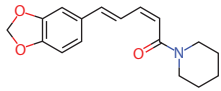
Major alkaloids present in pepper are piperine, piperanine, and chavicine (Figure 3.5) and their composition in pepper is 5%–9% w/w. apart from these major components, other alkaloids such as papavarine, piptigrine, pipnohine, pipyahyine, pellitorine, guineensine, pipercide, refractamide A, dipiperamides D and E (bialkaloids), and nigramides A-S (dimeric amides) are also present in pepper which contribute approximately 1% in total [8]. Piperine, chavicine, isochavicine, and isopiperine are geometrical isomers, and piperanine is dihydro form of piperine.

Previously, chavicine was believed to be responsible for the taste. However, it was reported later that piperine has a strong pungent taste. Also, piperine comes under vanilloid class of compounds same as capsaicin (compound responsible for the pungency in Chilli).



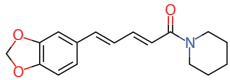
Common Name: Piperine.

IUPAC Name: (2*E*, 4*E*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one



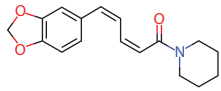
Common Name: Isopiperine.

IUPAC Name: (2*Z*, 4*E*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one



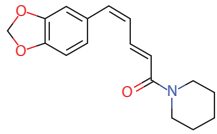
Common Name: Dihydropiperine.

IUPAC Name: (2*Z*, 4*E*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one



Common Name: Chavicine.

IUPAC Name: (2*Z*, 4*Z*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one



Common Name: Isochavicine.

IUPAC Name: (2*E*, 4*Z*)-5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one

Figure 3.5 Major alkaloids present in black pepper.

3.3 Piperine—Active Molecule of Pepper

Piperine is the major constituent of black pepper and it is proven to be the bio-active molecule responsible for the potency of black pepper by various research groups. Piperine is a yellow solid, nearly insoluble in water (at 18°C the solubility is 40 mg/L) [9]. It tastes nothing at first but leaves a burning sensation a few seconds later.

3.3.1 Isolation of Piperine

Peppercorns largely contain volatile (essential) oil and pungent (alkaloids) compounds, which are responsible for their aroma and pungency, respectively. Pepper oil, which is normally extracted by steam distillation of dried pepper corns, does not contain pungent compounds and only represents aromatic and odorous constituents. Because of its aroma, pepper oil is highly valued in the fragrance industry and is used in high-grade toiletry products and the perfume industry as well as the flavor industries.

The peppercorns are ground and dried (steamed or shade dried) to remove the volatile oils [10, 11]. Drying under sunlight might reduce the

yield as the structure of piperine is rich in rotatable olefinic bonds that might undergo photochemical reactions. Typically, the oleoresin offered by major producers for sale is reported to contain 15% to 20% volatile oil and 35% to 55% piperine. Oleoresin is the solvent-extractable portion of pepper which constitutes around 6% to 13% of black pepper and possesses odor, flavor, and pungency. It is obtained by repeated extraction of ground pepper by volatile organic solvents, such as ethanol, acetone, ether, dichloroethane, or ethyl acetate, and subsequent removal of solvent under reduced pressure to trace levels. The organoleptic properties of the so-called oleoresin are determined by its volatile oils and piperine contents whose abundance depends on the variety of pepper and its maturity stage, the used extraction solvent, and the extraction condition [12].

Generally, an ideal extraction method should be comprehensive, rapid, simple, and cheap [13]. Solvent extraction by aliphatic and chlorinated hydrocarbons is among the conventional methods used for piperine extraction [14]. Nevertheless, these solvents are not selective toward piperine and, thus, the extract obtained by this method always contains some major components such as resins and gums. For pharmaceutical applications, the purity of piperine should be 95% to 98%. Therefore, the oleoresin extract with 40% to 50% purity requires further purifications. The most common method for purification of piperine are crystallization from aqueous alcoholic solutions and treatment with aqueous alkali solutions which inevitably reduce the piperine yield. There are many types of solvents used for piperine extraction including dichloromethane, petroleum ether, diethyl ether, and alcoholic solvents like ethanol, hydrotrope solutions, and

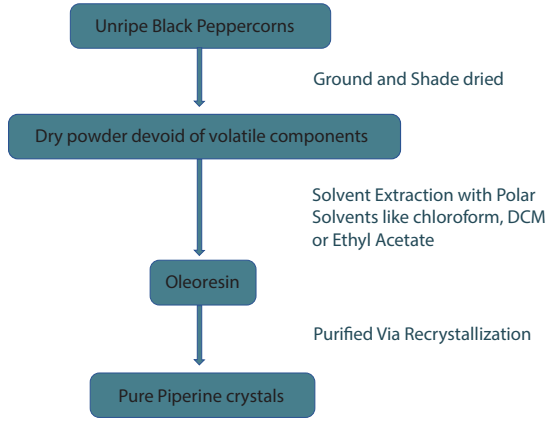


Figure 3.6 Flowchart of extraction of piperine from black pepper.

ionic-based solutions [15]. Figure 3.6 shows the extraction of piperine from black pepper in the form of a flow chart.

In conclusion, the source of black pepper, solvent used both affects the yield. From the studies, we can take that the yield of pure piperine when conventionally extracted comes at an average of around 3 g per kg of peppercorns.

3.3.2 Piperine as Potential Drug

There is huge number of literatures that states the bio-activity of piperine. Hence, it is evident that piperine is responsible for the activity of pepper. In spite of having a huge medicinal benefit, and having isolated in the 1800s, the molecule still has not been fully explored. The piperine scaffold is still under study. But, let's see what has been done so far. Let us go through the

1. Metabolism of piperine,
2. The theory of SAR, and
3. Activity profile of piperine and its analogs for two categories: (i) GABA modulators and (ii) anti-microbial activity.

3.3.2.1 Metabolism of Piperine

The *in vivo* test for piperine metabolism on rat shows that approximately 96% of piperine gets absorbed by the body. The oral administration shows result such that the peak concentration of piperine is attained after 6 hours of administration in the liver, serum, blood, spleen, and tissue. The concentration of piperine decreases gradually from then and on the 5th day from the oral administration it is undetectable in the feces. The reports say that there is no trace of unmodified piperine in the urine of the animal and about 3% of unmodified piperine is detected in the feces, having maximum on the first day. This shows that nearly 96% of the molecule gets absorbed by the intestine as such and then when it reaches the other parts of the body, the molecule gets metabolized by various tissues. There is also a theory due to the increased number of conjugated glucuronides, sulfates, and phenols in the urine for up to 8 days, the researchers suggest that this might be the metabolized products of piperine. The metabolism reported so far is demethylenation of methylenedioxy group: glucouronidation and sulfation in rats. The major metabolite found in the urine sample that is recently detected is 5-(3,4- methylenedioxy phenyl)-2E,4E-pentadienoic acid-N-(3-yl propionic acid)-amide. It is a very interesting result that proves that during the metabolism, a part of the concentration of piperine is not affected much and only the amide ring is being transformed to propionic acid [16–18]. The metabolism

of piperine differs from mice to mice and hence the exact numbers can't be generalized for humans, but we can expect a similar metabolism in our body.

The study states that piperine stays in the physiological system for almost 5 days. It has to be noted that it is potent Cyp inhibitor, any cyp-initiator drug might not be efficient when given along with piperine and the cyp-metabolized drugs might be favored. The bio-availability of the drug is very important because even if the drug is active *in vitro*, it won't be efficient *in vivo* if the bio-availability is low. Piperine, having a very good bio-availability, is a fit pro-drug which could be modified in to a more potent drug.

3.3.2.2 Structure-Activity Relationship

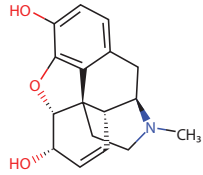
The SAR states the relationship between the chemical structure of a molecule and its biological activity. This idea was first presented by Crum-Brown and Fraser in 1865. The structure-activity analysis enables identifying the group responsible for the particular activity of a molecule in the biological system. So, it can be understood that any molecule having that particular molecular framework responsible for the bio-activity would act in a similar way.

A molecule is active because, when it goes inside the biological system, it binds with a particular protein in a particular way. This is because all proteins are made up of amino acids and only few of the amino acids would be in position for interaction with the molecule while others are engaged. The amino acids responsible for the binding would always bind in a similar fashion with any other molecule with similar electronic crowd, hydrophobic centers, and hydrogen donor/acceptor sites. So, on analyzing the structures of the molecules and their activity over the biological system, the molecular framework responsible for the specific activity can be elucidated. This molecular skeleton which is responsible for the activity of the molecule is called as the pharmacophore.

Chemists sometimes work with these pharmacophores and make new plausible drugs. Let us once again take the first and famous example of Morphine to have a better understanding. In many medicinal systems, Lachryma *Papaveris* (Poppy seeds) had been prescribed for dry cough. The activity of the plant material is due to the presence of a molecule called Morphine, an Opiate, very efficient antitussive and analgesic. Also, it is highly reputed for its narcotic property, which is a huge snag.

3.3.2.2.1 Morphine

Morphine (Figure 3.7) comes under the class Opiates. Morphine was first extracted from opium, a residue that seeps out of damaged poppy seed



- Obtained from Opium, the residue that seeps out of the damaged poppy seed pods.
- A very effective Analgesic
- Highly addictive

Figure 3.7 Morphine.

pods, by a German chemist, Fredrich Serturner, in 1803. The name was coined after the Greek god of dream Morpheus [2].

Opiates are naturally occurring morphine like compounds, and the ones that are synthesized in laboratory with similar pharmacophore are called as Opioids (Figures 3.8–Figure 3.10). Opium has nearly a dozen of compounds like morphine which includes codeine, thebaine, and papaverine. The toxicity and addictiveness of morphine were recognized only after the drug had become an established feature of clinical medicine. The administration of opiates to wounded Civil War soldiers made opiate addiction a significant social problem in the U.S. and prompted the search for nonaddictive synthetic opiates. In the 1890s, for example, the Bayer company introduced heroin, a morphine derivative with two added acetyl groups ($COCH_3$) as a nonaddictive analgesic, as they did with acetyl salicylic acid, the now paracetamol, which was a huge hit. Likewise, in the 1940s, meperidine (Demerol) became the most popular opiate analgesic in American medical practice because it was thought to be nonaddictive. The growing number of Demerol addicts soon convinced the Bureau of Narcotics that it was otherwise [19]. All these structures share a specific molecular framework without which the effect is gone. This framework is called pharmacophore.

3.3.2.2.2 Structures of Some of the Opiates and Opioids

Opioids—naturally compounds:

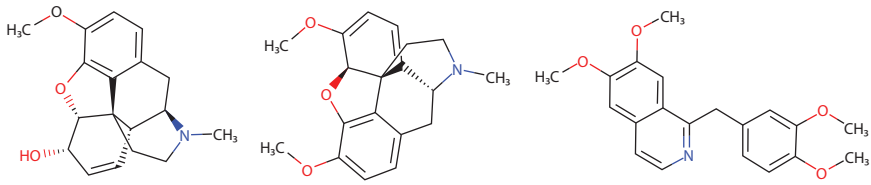


Figure 3.8 Structure of Opiates.

Opioids:

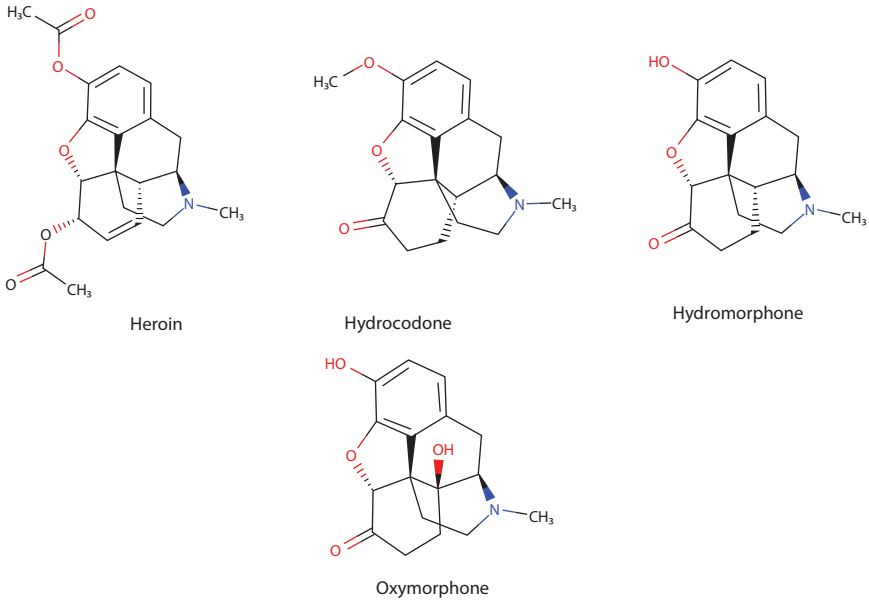


Figure 3.9 Semi-synthetic Opioids.

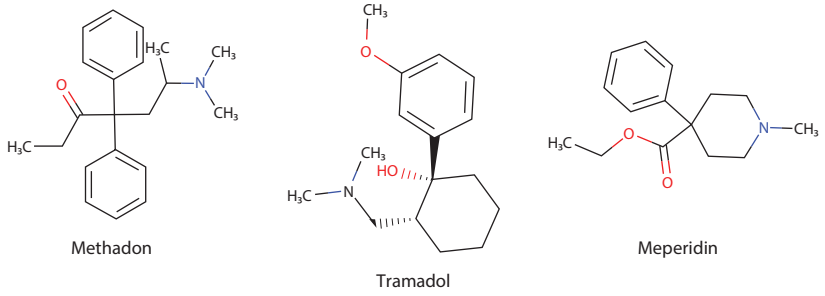


Figure 3.10 Synthetic Opioids.

3.3.2.2.3 Mechanism of Action

On significant research, by testing many opioids and opiates scientists have come to a conclusion that the main receptors of these substances lie in our Central Nervous System, and they named the receptors as MOP - μ (mu) opioid peptide receptor, KOP - κ (kappa) opioid peptide receptor, and DOP - δ (delta) opioid peptide receptor. There are sub-types in these

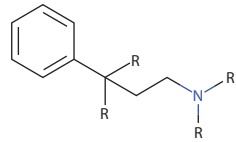


Figure 3.11 Pharmacophore of the Morphine-like compounds.

receptors which we are not going to discuss about as it would fetch us far from the goal, let's go back to SAR and summarize [20].

So, any molecule that contains 1. an Aromatic ring that is, 2. attached to a quaternary carbon which is, 3. attached by a two-carbon spacer to a, 4. Tert-Amine group will be expected to bind at any one the opioid peptide receptor and act as analgesic (Figure 3.11). This is a rule and it is called as "Morphine Rule" [21]. For a molecule to have a particular action, it must have specific pharmacophore. When working with a molecular scaffold, there are high chances of encountering analogs that has completely different behavior in the biological system from the parent molecule itself. Body is full of proteins which make good binding sites for many molecules. Similarly, some molecules which are synthesized might never come in to the picture as it might get rejected in the Phase I metabolism itself. This applies to all the organic molecules. But there aren't many rules like "Morphine's Rule" because not everybody gets famous, and that how life works, which definitely doesn't mean other molecules are any less. So, this technique can be applied in both reverse and forward pharmacology. As it helps,

1. To identify the groups responsible for the activity, it is possible to narrow down the target proteins in the biological system and to find the mechanism of action of the molecules. (reverse pharmacology).
2. To design a completely new molecule, that fit the target in the biological system (synthetic drugs) (forward pharmacology).
3. To alter the existing molecule for better binding or to overcome the existing drawback (semi-synthetic) (reverse pharmacology).

This is a general outline about SAR and we insist to read recent research publications for further understanding. Having that done, now, let's do the analysis in piperine, in the similar way.

3.3.2.2.4 Structure of Piperine

The structure of piperine mainly consists of three hydrogen acceptor sites, three hydrophobic sites, and an aromatic ring. Other than these, there will

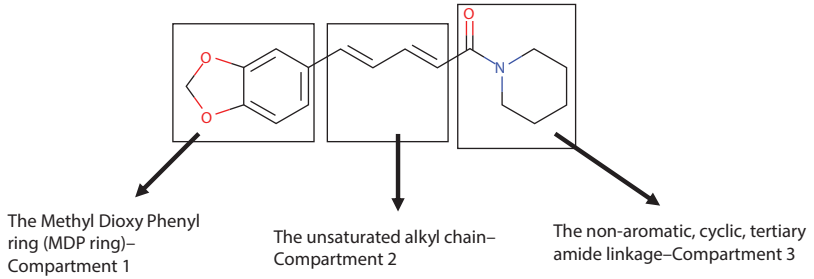


Figure 3.12 Structural dissection Piperine.

be other weak forces of interactions like Vanderwaal's forces. Let's dissect the molecule and name them as Compartment 1, 2, and 3 as given in Figure 3.12. Let's follow the nomenclature till the end.

Piperine complies with Lipinski's rule or Rule of 5. This adds to the pros of using piperine scaffold for further studies. Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria [22]:

- No more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds).
- No more than 10 hydrogen bond acceptors.
- A molecular mass less than 500 Daltons.
- An octanol-water partition coefficient log P not greater than 5.

One of the bio-activities that piperine is very famous for is its bio-availability enhancement property. This activity could be made use to achieve better drug-plasma concentration and to work with a drug that gets eliminated at phase I or II metabolism process. Pepper had been used along with many drugs in Siddha, an ancient medicinal system, but the term "bio-availability enhancer" was coined by Dr. C. K. Atal, the Director of institute RRL Jammu in 1979. Dr. Atal also initiated the bio-enhanced anti-tubercular drug research project using Rifampicin, which is the first modern bio-availability enhanced anti-tuberculosis drug combination. Piperine, though long discovered, is still under exploration, due to its wide range of activity. So, we would see the modern research on piperine and compare the structures of the analogs, to get an idea of the pharmacophore responsible for the particular activity.

3.3.2.3 Piperine and Piperine Analogs

There are lots of research on piperine and its application. As we intend to analyze the pharmacophore of the molecule, let us peek in to some of the

research articles that talk about piperine and its structural analogs. Two trending and less reviewed applications are taken for our section.

3.3.2.3.1 Piperine Analogs as GABA Inhibitor

Gamma-aminobutyric acid, or GABA, is a neurotransmitter that regulates the communication between brain cells. The role of GABA is to inhibit the neuro transmission. There are three classes of GABA receptors: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels, whereas $GABA_B$ receptors are G protein-coupled receptors (or) metabotropic receptors.

GABA plays an important role in behavior, cognition, and the body's response to stress. Research suggests that GABA helps to control fear and anxiety when neurons become overexcited. When neurons get over exited GABA is released by the GABAergic neurons, which binds to the receptors in the brain. In the case of $GABA_A$, the receptor is made up of five subunits forming a central pore which allows the movement of Cl^- ions. When GABA binds to the receptor, the central pore opens and enables the flows of Cl^- ions, causing hyper polarization resulting in less action potential thus cutting out the communication [23, 24].

Each $GABA_A$ receptor contains two $GABA_A$ binding sites and at least three allosteric binding sites for $GABA_A$ modulators. $GABA_A$ modulators bind with the receptor and enhance or diminish the activity of $GABA_A$. So, $GABA_A$ modulators (agonists/enhancers) are given to treat anxiety, depression, epilepsy, etc. For example, alcohol and benzodiazepine are $GABA_A$ agonists, while flumazenil is $GABA_A$ antagonist.

GABA was first identified in 1950 by Eugene Roberts and Sam Frankel. These scientists not only identified Gamma-Amino Butyric Acid but also reported that the amino acid is found in higher concentration only in brain tissues and also in free form. In 1957, Canadian researchers identified the action of GABA. Though it's found 70 years back, it remains still under investigation, as many blanks are yet to be filled.

So, coming to our story, of many uses, pepper was also used in many herbal combinations in Asian medicinal system to alleviate pain, for sedation and in the treatment of epilepsy. There are many reports that states the efficiency of piperine to cross the blood-brain barrier (BBB) in rat and human models [25, 26]. This adds up to the efficiency of piperine as GABA modulator. The first modern article in our time that correlates pepper and GABA is given by Pedersen *et al.* in 2009 [27]. The African traditional medicinal system uses Piper capense L.F. to induce sleep. This point was taken in to account for the studies. The group did bioassay-guided fractionation of the roots of P. *capense* that led to the isolation of piperine and 4,5-dihydropiperine, which has showed moderate affinity toward $GABA_A$

receptor. Having them as pharmacophore, they developed a library of 10 analogs by modifying the alkyl linker, the amide ring, and by halogenating the MDP ring (Figure 3.13). The report states that none of the analogs beat piperine in binding with at the benzodiazepine site in the $GABA_A$ receptor, and the binding affinity of all the compounds were low. They concluded that a strict molecular framework has to be maintained for better binding. Allegedly, for a better binding the group suggested the following:

1. Maintaining the alkyl chain not less than four,
2. Having a double bond near the amide group and avoiding free rotation in the alkyl linker,
3. Having a bulky amine part [27].

Zaugg *et al.* in 2010 [28] did HPLC-activity profiling for 880 plant and fungal extracts and came up with piperine as the main active compound and with other 12 structurally related piperamides which were less active or inactive (Figure 3.13). Through two-microelectrode voltage clamp assay on Xenopus *laevis* oocytes, the activity of the compounds has been analyzed. According to this group, piperine is the effective $GABA_A$ modulator among all the compounds and they found it binds to a benzodiazepine-independent binding site. This result supports the first article, why the affinity toward benzodiazepine site was low. The result includes that the potency of the compound could be increased if the binding of piperine with TVRP1 enzyme and its involvement in phase-I and II metabolism is addressed [28].

Schöffmann *et al.*, in 2014 [29], have built an extensive library of 76 compounds and reported a very interesting SAR theory. They have modified the alkyl chain linker and the amide group leaving the MDP ring untouched. As per Zaugg *et al.*, [28] the same two-microelectrode voltage clamp assay on *Xenopus laevis* oocytes has been used to study the pharmacological activity of the synthesized compounds by Schoffmann *et al.* This study validates the theory of the previous works. Compound no. 23 works better among all the analogs and the strongest modulation of $GABA_A$ is observed with the receptor containing $\alpha_1\beta_2\gamma_{2S}$ subunits and it does not activate TVRP1 enzyme, as activation of TVRP1 might cause unwanted side effects like changes in pain sensation and body temperature. So, 23 induced pronounced anxiolytic action with little sedation. Here, goes the SAR [28, 29].

1. Increase or decrease in the alkyl linker chain length or restricting alkyl liker rotation but maintaining the distance as such in the pharmacophore does not improve the activity of the molecule.

2. Alkyl substituted amines are better being N-Dipropyl amide optimum, neither bulkier nor smaller N- alkyl group worked better. N,N-Dialkyl chain increases the efficacy as well as potency.
3. Introducing heteroatom in the amide ring also decreases the activity. Let's see some of the modifications done by the group that are highly active, moderately active, and not active/deactivating under #2.

We are breaking down the article by Schöffmann *et al.* and Zaugg *et al.*, as the sub-unit used in the activity profiling is more specific and a huge library of molecules are analyzed. The analogues that are to be investigated are categorized and tabulated in the Tables 3.2–3.7.

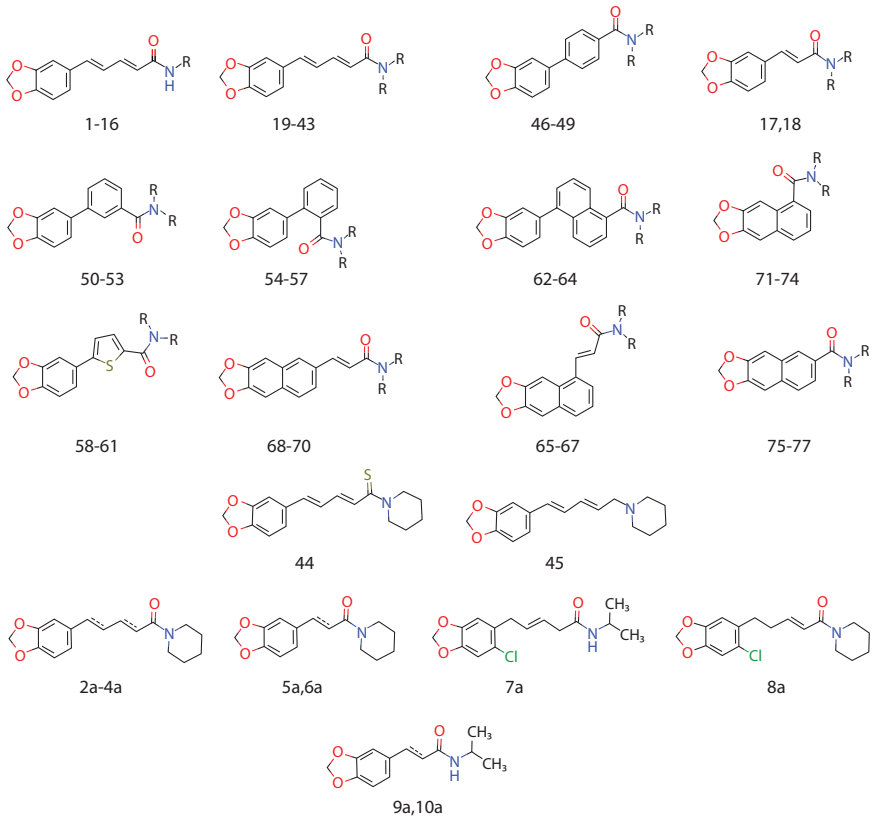
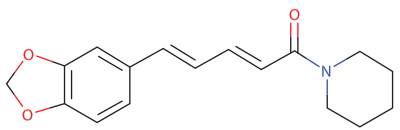
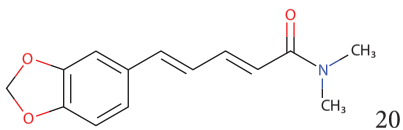
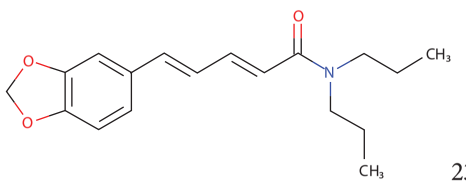


Figure 3.13 General modifications done over piperine scaffold as $GABA_A$ modulators [27–29].

Table 3.2 Structural variation 1: Change in the Compartment 3—Dialkyl amide with various alkyl chain in the place of Piperine Amide and their activity profile [28, 29].

Structure	Activity ($I_{GABA\text{-}max}$ in %)	Analysis
	302 ± 27	Piperine
 20	61 ± 28	Alkyl amide and shorter chain length— decreases the activity
 23	1673 ± 146, Also works as anxiolytic in mice model.	The best of all piperine analogs as $GABA_A$ modulator, with alkyl amide and with optimum chain length— shoots up the activity

(*Continued*)

Table 3.2 Structural variation 1: Change in the Compartment 3—Dialkyl amide with various alkyl chain in the place of Piperine Amide and their activity profile [28, 29]. (*Continued*)

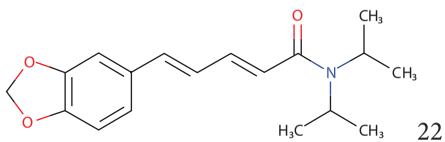
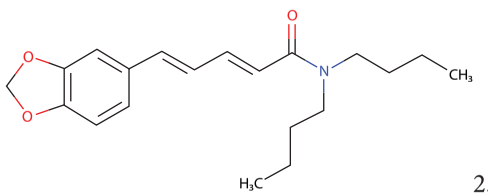
Structure	Activity ($I_{GABA\text{-}max}$ in %)	Analysis
 22	986 ± 244	Chain branching has decreased the modulation activity a bit, but still the second-best analog.
 25	760 ± 47	Increase in the chain length and decrease in the activity

Table 3.3 Structural variation 2: Change in Compartment 3—Substitution of a hetero cyclic amide ring in the place of Piperine Amide part Ring their activity profile decreases the activity [28, 29].

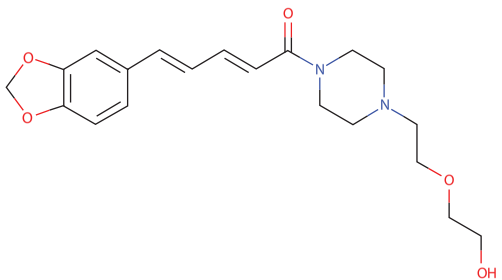
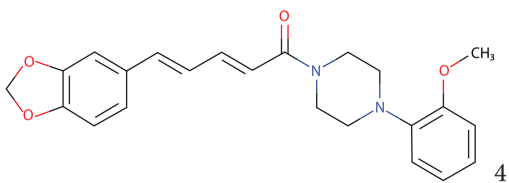
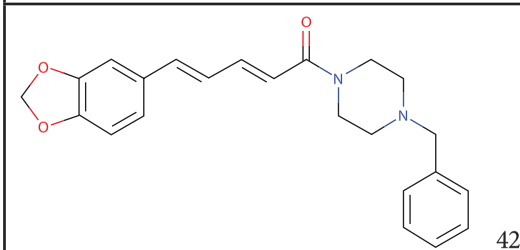
Structure	Activity ($I_{GABA\text{-}max}$ in %)
 40	12 ± 8
 41	5 ± 5
 42	48 ± 12

Table 3.4 Structural variation 3: Change in Compartment 3—Cyclic tertiary amides in the place of Piperine Amide and their activity profile [28, 29].

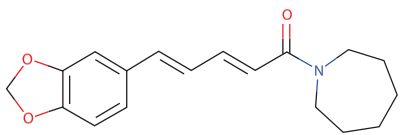
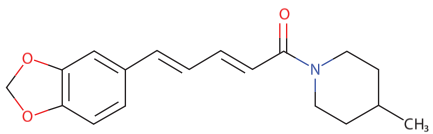
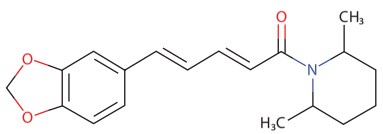
Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)	Analysis
 28	294 ± 66	Ring expansion—nearly equivalent to the activity of piperine
 35	568 ± 54	Methyl group substitution—induces the GABA modulation
 38	218 ± 43	Interestingly, less active than Piperine.

Table 3.5 Structural variation 4: Change in Compartment 3—Secondary Amide functional group in the place of Piperine Amide part increases in polarity leads to decrease in the activity [28, 29].

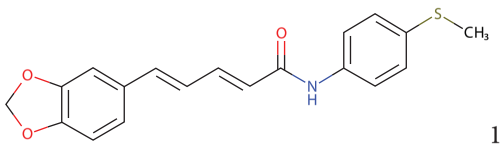
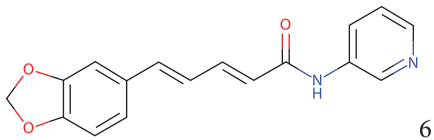
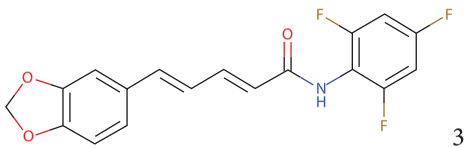
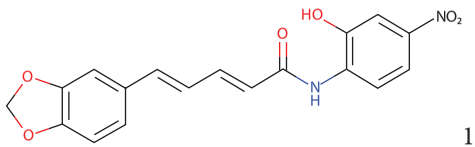
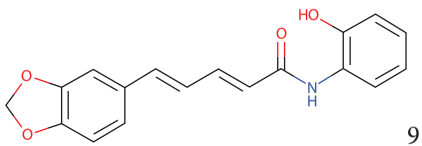
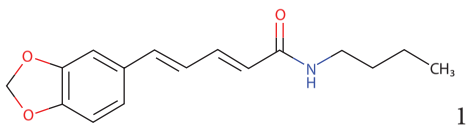
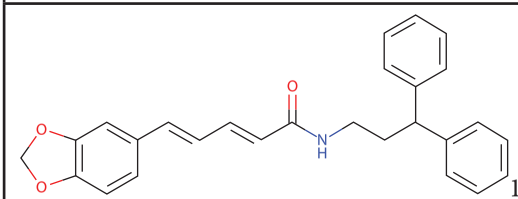
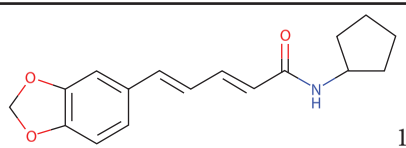
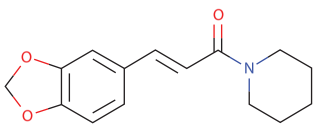
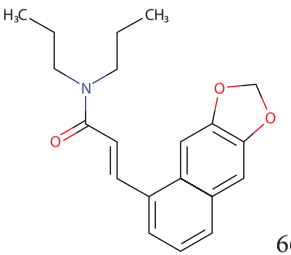
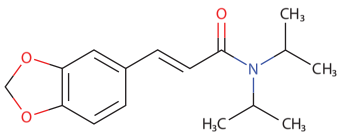
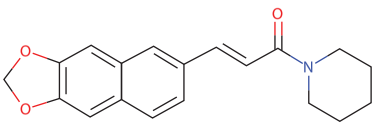
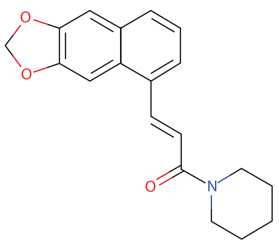
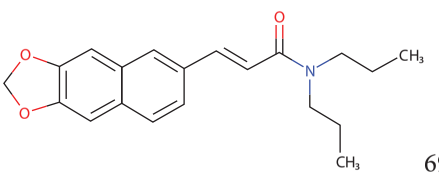
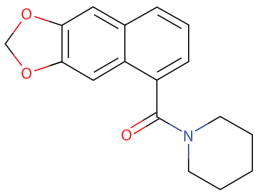
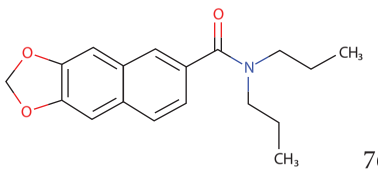
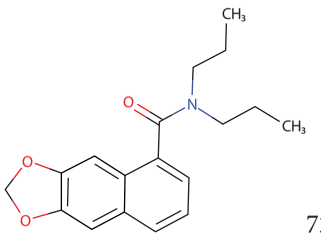
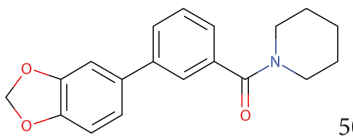
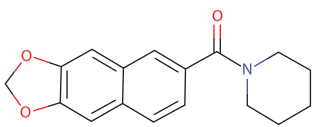
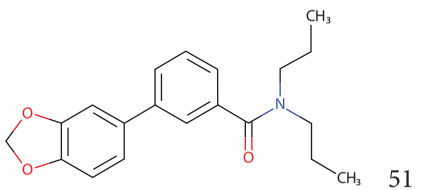
Structure	Activity ($I_{GABA\text{-}max}$ in %)	Structure	Activity ($I_{GABA\text{-}max}$ in %)
 1	0 ± 0	 6	−7 ± 3
 3	5 ± 5	 10	1 ± 7
 9	0 ± 0	 11	51 ± 11
 12	−6 ± 6	 13	33 ± 17

Table 3.6 Structural variation 5: Change in Compartment 2—Modification in the alkyl chain linker of piperine: (a) restriction of rotation of the alkyl chain, (b) introduction of hetero-atoms, and (c) decrease/increase in chain length decreased the activity [28, 29].

Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)	Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)
 17	79 ± 8	 66	67 ± 23
 18	66 ± 30	 68	−1 ± 12
 65	105 ± 18	 69	74 ± 1

(*Continued*)

Table 3.6 Structural variation 5: Change in Compartment 2—Modification in the alkyl chain linker of piperine: (a) restriction of rotation of the alkyl chain, (b) introduction of hetero-atoms, and (c) decrease/increase in chain length decreased the activity [28, 29]. (*Continued*)

Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)	Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)
 71	32 ± 10	 76	122 ± 26
 72	334 ± 23	 50	178 ± 32
 75	58 ± 29	 51	280 ± 52

(*Continued*)

Table 3.6 Structural variation 5: Change in Compartment 2—Modification in the alkyl chain linker of piperine: (a) restriction of rotation of the alkyl chain, (b) introduction of hetero-atoms, and (c) decrease/increase in chain length decreased the activity [28, 29]. (*Continued*)

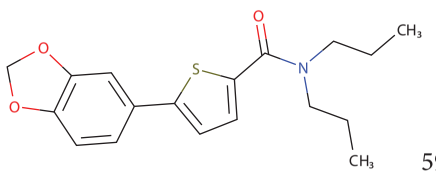
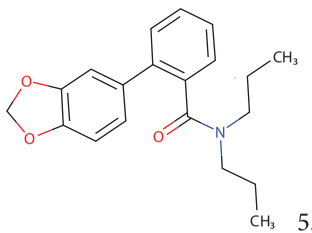
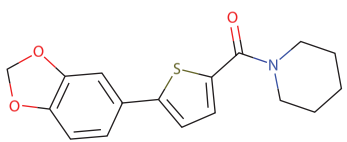
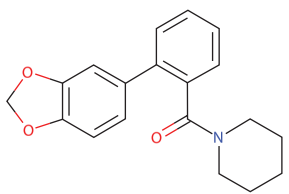
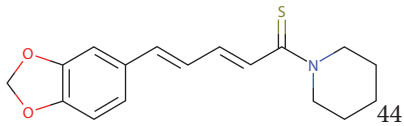
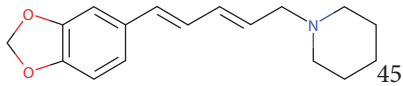
Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)	Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)
 59	−5 ± 12	 55	34±8
 58	134 ± 39	 54	79 ± 24

Table 3.7 Structural variation 6: Change in Compartment 3—Change in functional group of piperine decreases in $GABA_A$ modulation [28, 29].

Structure	Activity ($I_{GABA\text{-}max\ in\ \%}$)
 44	17 ± 17
 45	−16 ± 14

The I_{GABA} enhancement shows a nonlinear (parabolic) function to the number of carbon atoms, with the optimum being dipropyl Dialkyl tertiary amide.

Conclusion:

1. Piperine is a positive $GABA_A$ modulator.
2. It binds with Benzodiazepine independent site in the $GABA_A$ receptor. Strongest modulation of $GABA_A$ is observed with the receptor containing $\alpha_1\beta_2\gamma_2S$ subunits.
3. Compound with N,N-di-propyl amide in the place of piperidine moiety shows best potency so far with anxiolytic property.
4. Regarding the Structural analogs,
   - Change in the MDP ring has not been explored much to come to a conclusion.
   - Topological polar surface of the molecule smaller than 39 works better.
   - Rigidification, reduction in length or increase in length of the alkyl linker decreases the activity.
   - Log P value smaller than 5.2 works better.
   - Conjugation near the amide group is suggested.
   - Di-alkyl amide with di-propyl being the optimum shows better activity.

3.3.2.3.2 Piperine Analog as Anti-Microbial Agent

The next application to be analyzed is piperine against the bad guys like bacteria and fungi.

With piperine amides, anti-bacterial studies are done comparatively more than any other microbes, so, let us begin from there.

There are many anti-bacterial agents in the market. But, these microbes are so tricky to deal with, as they get their next generation upgraded efficiently. Once the bacteria learn to defend a drug, the trait will be passed on to its next generation making them resistant. These are called as resistant strains. Resistance is a part of evolution, which is natural and unavoidable. During mutation, resistance gene is developed. This gene is then transferred to the other non-resistant bacteria or while replication it is carried over to the next generation. Resistance is induced when a bacterium lives under the anti-biotic pressure for longer time or when exposed to insignificant amount of anti-biotic drug. The resistant strains can fight against drugs by producing an efflux pump, by changing the target of the antibiotic or by producing a new enzyme that targets the drug molecule which would inactivate the drug. When a bacterium possesses multiple resistance genes, it becomes multi-drug resistant. Development of resistance by the bacteria cannot be stopped but delayed by using existing anti-microbial drugs only when they are absolutely necessary and by having a constant amendment in the anti-microbial drugs [30–32].

In olden days, this spice was used to control the microbial growth in food that is intended to be stored. In modern science, the first report that speaks about anti-bacterial activity of Piperine that includes the synthetic routes for Piperine is by Linke, Kurz, and Zeiler [33] on 1982. From 1980s, till now, this area is active for the scientific explorers. Piperine has better activity over gram positive than gram negative bacteria. Some researchers experimented the synergistic effect of piperine with existing antibiotics. After several synergism tests, it is concluded that piperine inhibits efflux pump in the bacteria, which potentiates the drug to stay inside the bacterial cell and kill it or prevent its reproduction. There are many efflux pumps but only few bacteria had been analyzed so far. An extensive screening of different bacteria with the Piperine and its analogs is required to understand thoroughly.

3.3.2.4 Synergistic Activity of Piperine

In 2006, Khan *et al.* [34] tested the combination of Piperine and Ciprofloxacin against Staphylococcus aureus, a gram-positive bacterium. Both wild type and the mutant strains were taken under study. In addition, 12.5 μg/ml piperine and 25 μg/ml piperine show two-fold reduction in the MIC of ciprofloxacin (0.12 μg/ml, the MIC of Ciprofloxacin in the absence of Piperine is 0.25 μg/ml) and when the concentration of Piperine is increased to 50 μg/ml, a four-fold reduction in the MIC (0.06 μg/ml) is observed.

This combination also shows activity toward MRSA (resistant strain of S. aureus). The time kill studies shows that the activity of 1 μg/ml ciprofloxacin kills 99.9% bacteria, while the combination of 50 μg/ml piperine and 0.5 μg/ml of ciprofloxacin shows the same level of bactericidal activity. The group also reported that mutation prevention concentration (MPC) to be decreasing when piperine is administered along with ciprofloxacin. The MPC of ciprofloxacin is 4 μg/ml, when combined with 25 μg/ml of piperine MPC of Ciprofloxacin is reduced to 2 μg/ml, and with 50 μg/ml of piperine the MPC of Ciprofloxacin is just 1 μg/ml. The group has also studied the mutation reversal effect using MRSA strains. Using Ethidium Bromide (marker) and reserpine (efflux pump blocker) as controls, the study has been carried out. For mutant strain, there is 512-fold increase in the MIC of Ciprofloxacin from the wild type (MIC for the resistant strain reported is 128 μg/ml), but the combination of 25 μg/ml of piperine reduced the MIC of Ciprofloxacin to 8 μg/ml, which shows the reversal of bacterial resistance toward ciprofloxacin The results show that there is increased accumulation of Ethidium bromide in the resistant strain bacteria in the presence of piperine similar to reserpine, suggesting piperine blocks the efflux pump, as ethidium bromide is a substrate for many MDR pumps including NorA, like reserpine. But, the study concludes with a comparison of a previous study that says p-glycoprotein inhibition causes enhances ciprofloxacin enhancement; thus, their results could state that piperine is yet another p-glycoprotein inhibitor.

The same group in 2008, published a report on the synergistic effect of piperine and its 200 analogs. The structures of the analogs are enclosed, as it is a US pre-grant publication [35]. The group has compared the activity of three analogs that inhibited NorA efflux pump at lower concentrations than piperine and have given a detailed synergistic study as before. This study is focused on NorA. The Staphylococcus aureus bacterial strain with NorA over expression and wild type has been chosen for the study, with same Ethidium bromide marker and Reserpine, Verapamil as standards. All the cells were treated with the marker before analysis. The major refinement in the structure of piperine that led to a significantly higher efflux pump inhibition is as follows:

(i) The introduction of an alkyl group at the C-4 position with ethyl and n-propyl contributes to the maximum.
(ii) Replacement of a piperidinyl moiety by an aromatic amine such as anisidine or toluidine (with ethyl or n-propyl at C-4) shows maximum potentiation. Other basic substituents such as aniline, amino esters, pyrrolidine, azepine, and alkylamines (except isobutyl amine) were less effective.

(iii) Unsaturation is an important contributor to potentiation.
(iv) Amide carbonyl is important for potentiation, as the reduction of this group led to less potentiation.
(v) Retention of 3,4-methylenedioxyphenyl or 4-methoxyphenyl analogs (with C-4 bearing ethyl or n-propyl group) also contributed toward the enhanced potentiation.

Of these points, data for the analysis or the structures are not revealed. While piperine and verapamil showed same eflux pump inhibition (50 μg/ml with Ciprofloxacin shows two-fold increase in the activity), the combination of compound 3 at 6.25 μg/ml (minimum effective concentration/MEC) reduces the MIC of ciprofloxacin to 4 folds in wild-type S. aureus and 8 folds in the resistant strain (refer Table 3.2 for details). Though the homology of mammalian and bacterial cells are not highly identical, the proven mammalian efflux inhibitors like verapamil and reserpine inhibits bacterial efflux pump, which makes these analogs susceptible in the mammalian system also to overcome multi-drug resistant state. Also, as the intensity of the inhibition is measured by the Ethidium Bromide accumulation, by recording the fluorescence emitted by the ethidium bromide-NorA complex (as Ethidium Bromide is a NorA substrate), shows the mechanism of the efflux could be through NorA [36].

Sangwan *et al*. [37] published a set of 38 piperine analogs with the same analysis. This study gives an idea about the pharmacophore of the molecule responsible for the NorA efflux pump inhibition. Among all the analogs, 5 analogs show better efflux pump inhibition with four-fold reduction in the MIC of ciprofloxacin against resistant strain. Twenty-five analogs of 38 has synergistic activity against NorA over expressive bacterial strain and only 7 analogs show synergistic activity against wild-type S. aureus. This could be due to the over expression of the target protein (NorA) in the cell. This may be taken as a proof that efflux inhibition is through NorA. From the analysis of the structures of the analogs, the activity is as follows:

1. Lost when alkyl chain unsaturation is saturated.
2. Increased when the Piperidine ring is replaced with a small chain branched alkyl amine such Iso-propyl amine, isobutyl amine or diisopropyl amine. Straight chain decreases the activity.
3. Increased when anisidinyl group is substituted with an electron withdrawing group such as -CN, with maximum potential at meta position, in the place of piperidine ring.
4. Decreased with the introduction of electron releasing group in the anisidinyl group.

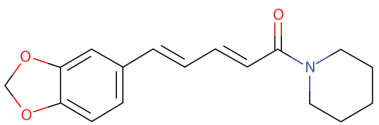
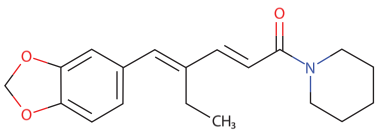
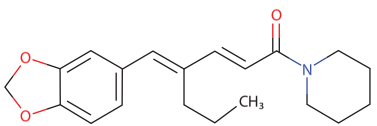
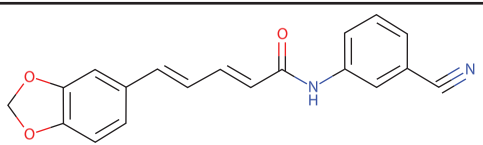
5. Enhanced by four-fold reduction in the MIC of ciprofloxacin with the introduction of ortho, meta, and para phenylacetamide moieties in the place of piperidine ring.
6. Reduced when piperidine ring is replaced with a heterocyclic ring.

Mirza *et al*. [38] report co-administration of piperine with mupirocin. Mupirocin is topical antibiotic drug that works by inhibiting the tRNA and protein synthesis by selectively binding to the bacterial isoleucyl tRNA. S. aureus which is resistant to this strain is tested both *in vitro* and *in vivo*. Similar to ciprofloxacin potentiation, mupirocin potentiation is also observed in MRSA and mupirocin resistant strains. Mupirocin resistance is mediated by the MdeA efflux pump. The MIC of mupirocin against MRSA and wild-type strain is 0.25 μg/ml, with 50 μg/ml of piperine, the MIC of mupirocin against MRSA is reduced to 0.12 and to 0.06 μg/ml in wild type, and the MIC of Mupirocin on MdeA over-expression strain is 256 μg/ml, while in the presence of 50 μg/ml the MIC is reduced to 64 μg/ml (three-fold reduction). Similarly, with 25 and 12.5 μg/ml of piperine the MIC is found to be 64 and 128 μg/ml, respectively, showing the reversal of resistance in the strain. The time kill studies show that MPC of mupirocin is 2 μg/ml. It is reduced to 1 μg/ml when combined with 25 μg/ml of piperine and further reduced to 0.5 μg/ml with 50 μg/ml of piperine. In the *in vivo* studies, four mice among five tested were cured completely at the effective concentration combination of 0.5 (w/v) of mupirocine + 0.5 (w/v) of piperine. Below this concentration, the combo is not effective. Ethidium bromide fluorescene is measured to make sure the potentiation of drug is due to efflux pump inhibition which is positive. Thus, this study proves that piperine also contributes in MdeA efflux pump inhibition.

Mgbeahuruike *et al*. [39] reports that piperine potentiates tetracycline and rifampicin against wild-type S. aureus and Pseudomonas aeruginosa but shows no synergism for the anti-fungal drug itraconazole against the fungi Candida albicans. This adds up as a proof for the theory of efflux pump inhibition. Piperine also shows synergism toward ofloxacin against Mycobacterium tuberculosis [40]. The list of high potentiating analogues with their synergistic activity against the wild type and NorA over expressing *S. aureus* strains are tabulated below in the Table 3.8.

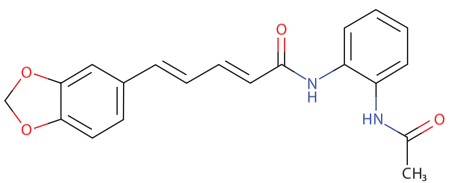
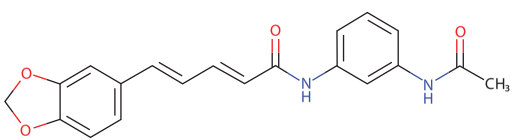
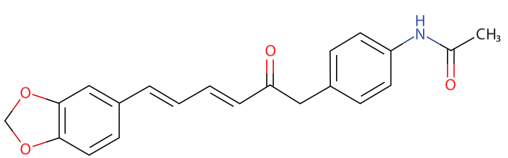
After synergism, many researches focus on the enhancement of the bacterial-kill effect of the piperine. Having a pharmacophore that deflects the efflux pump is a huge positive factor. Yamaguchi and Ozeki in 1985 [41] first performed antibacterial and antitumor activities of piperine. More than 50 research articles speak about the anti-bacterial, anti-tutor

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37].

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
1		2 at MEC 50 µg/ml	2 at MEC 50 µg/ml	Resistant type - NorA over expression.
2		4 at MEC 6.25 µg/ml	8 at MEC 6.25 µg/ml	(Kumar *et al.*, 2008)
3		4 at MEC 12.5 µg/ml	8 at MEC 6.25 µg/ml	
4		0 at MEC 6.25 µg/ml	4 at MEC 6.25 µg/ml	Resistant type - NorA over expression.

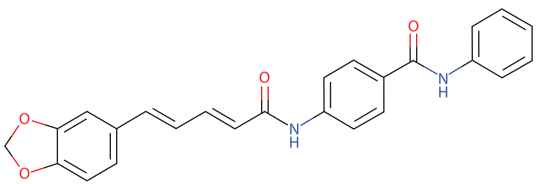
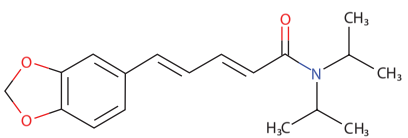
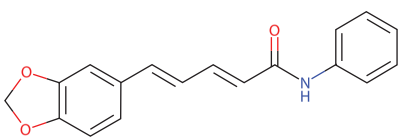
(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
5		0 at MEC 6.25 µg/ml	4 at MEC 6.25 µg/ml	Payare L. Sangwan *et al.*
6		0 at MEC 6.25 µg/ml	2 at MEC 6.25 µg/ml	
7		0 at MEC 6.25 µg/ml	2 at MEC 6.25 µg/ml	

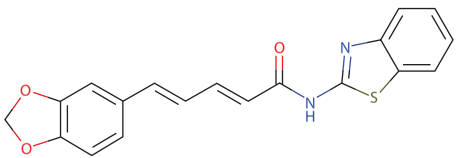
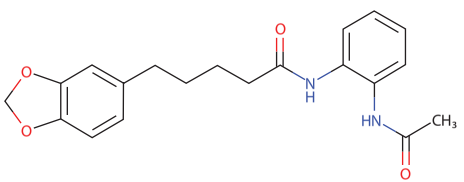
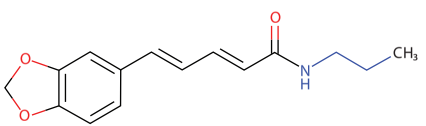
(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
8		0 at MEC 3.12 μg/ml	2 at MEC 3.12 μg/ml	
9		2 at MEC 25 μg/ml	2 at MEC 25 μg/ml	
10		2 at MEC 12.5 μg/ml	2 at MEC 12.5 μg/ml	

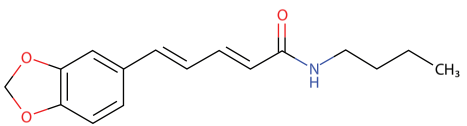
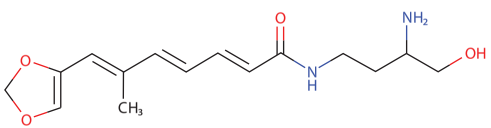
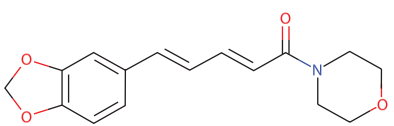
(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
11		2 at MEC 6.25 µg/ml	2 at MEC 6.25 µg/ml	
12		2 at MEC 50 µg/ml	2 at MEC 50 µg/ml	
13		0 at MEC >100 µg/ml	0 at MEC >100 µg/ml	

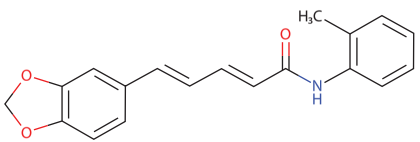
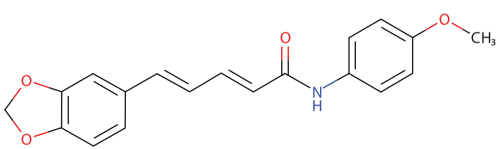
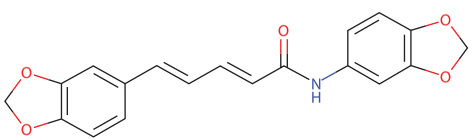
(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
14		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
15		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
16		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	

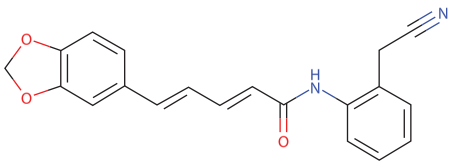
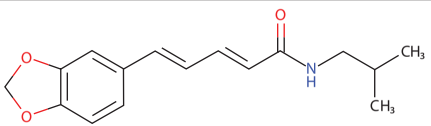
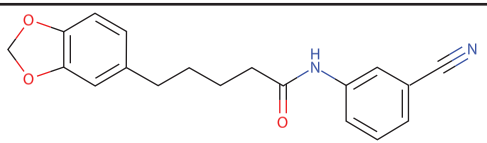
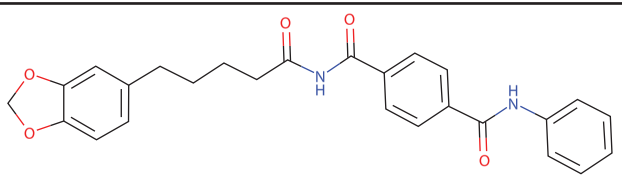
(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
17		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
18		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
19		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	

(*Continued*)

Table 3.8 List of high potentiating piperine analogs against wild type and NorA over expressing Staphylococcus aureus strains [36, 37]. (*Continued*)

	Structure	Wild Type (n- fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Resistant Strain (n-fold reduction in MIC of Ciprofloxacin at MEC of EPI)	Ref
20		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
21		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
22		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	
23		0 at MEC >100 μg/ml	0 at MEC >100 μg/ml	

activity, and the minimum inhibitory concentration, minimum bactericidal concentration of piperine required to achieve it. As the structure responsible for the enhancement of the activity of Piperine is concerned, let us go through the articles that reported piperine analogs.

A set of five analogs are tested against four Gram negative, three Gram positive bacteria, and for their anticancer activity by Umadevi *et al*. in 2013 [42]. This is the first report generated on Piperine analogs against microbes. Let's just focus only on bacteria here, to avoid confusion. They have targeted the piperidine ring moiety and replaced it with 4-Chloro aniline, 4-Bromo Aniline, Histidine, PhenylAlanine, and Trptophan amines. Though all the molecules are more potent than piperine except for Pseudomonas aeruginosa where piperine is the most potent, the common ground for the activity of the molecule is not understood here.

Comparatively, Gram negative bacteria are more susceptible toward the piperine analogs.

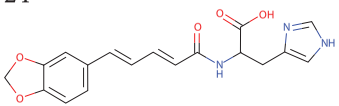
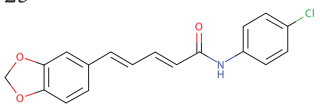
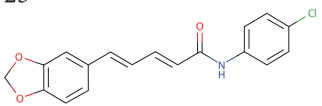
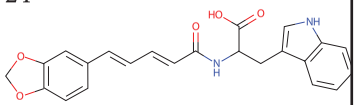
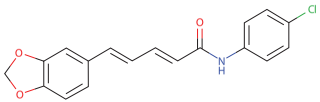
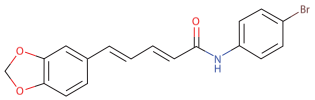
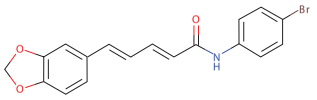
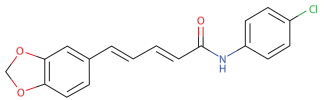
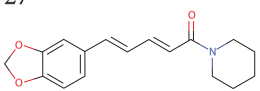
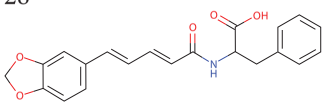
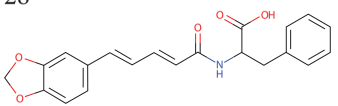
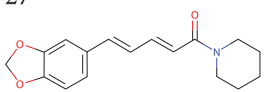
Venugopal *et al*. [43] substituted different amide moiety again and tested the potency of the analogs. α-Aminoacyl phenylalanine pinanediol boronic ester was used in the place of piperidine ring. The variation of this molecule done is having a tetrahydro alkyl chain and tetrabromo alkyl chain in the place of the conjugated alkyl liker. The molecule with the conjugated alkyl linker has worked better among the synthesized, with Klebsiella pneumoniae. Still piperine shows better activity than the reported structures. The group claims the mechanism of action involving t-RNA binding with the analogs as they state in their docking studies.

Then the piperidine ring is replaced with substituted pyridine moieties and by varying the position of amide linkage in the pyridine has been reported. The method of designing is development of hybrid or conjugate molecules. It is binding two biologically active molecule in order to append their goodness. Of eight analogs, the one with amide in the second position and hydroxyl in third position is reported to have better activity against the microbes used for the analysis. Docking models are presented for the bacterial strains. Proteins selected for docking are under different category like DNA binding protein, FTZ polymerization protein, transferase and protein transport [44].

Again, with molecular hybrid concept, six analogs are reported recently in 2019, by Kumar *et al*. 1,2,4 Triazole ring in the place of Piperidine Ring [45].

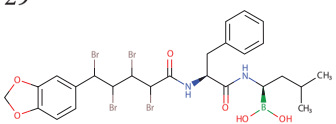
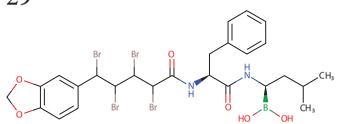
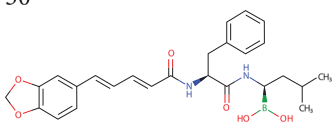
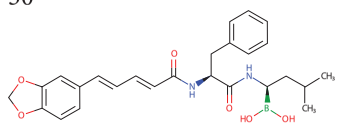
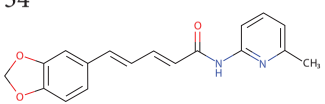
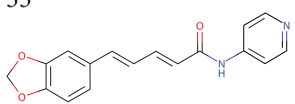
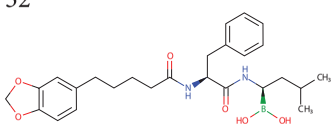
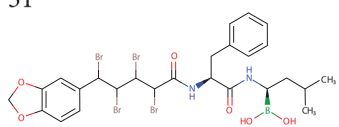
With this, we come to the point where we must see the structures of the potent and inactive molecule. Here in Table 3.9, we categorize molecule that is less potent than piperine as inactive and as molecules equipotent with piperine are not included, we request the readers to jump to the respective journal to get them all.

Table 3.9 Modification of the piperidine ring in piperine [42–45].

Potent structure reported by each group		**Inactive structure reported by each group**	
Gram positive	**Gram negative**	**Gram positive**	**Gram negative**
24  Bacillus subtilis	25  Escherichia coli	25  Bacillus subtilis	24  E. coli
25  Staphylococcus aureus	26  Klebsiella pneumoniae	26  Bacillus subtilis, Pseudomonas aeruginosa	25  Salmonella typhi
27  Pseudomonas aeruginosa	28  Shigella dysentriae	28  S. aureus	27  Shigella dysentriae

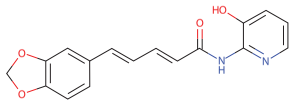
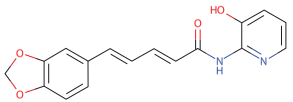
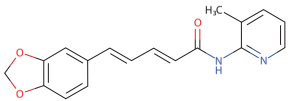
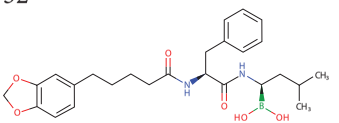
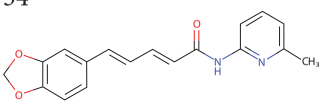
(*Continued*)

Table 3.9 Modification of the piperidine ring in piperine [42–45]. (*Continued*)

Potent structure reported by each group		**Inactive structure reported by each group**	
Gram positive	**Gram negative**	**Gram positive**	**Gram negative**
29  Streptococcus pyogenes	29  Citrobacter koseri, Klebsiella oxytoca, Citrobacter freundii	30  Streptococcus pyogenes, Pseudomonas aeruginosa	30  Shigella dysentriae, Klebsiella pneumoniae, Citrobacter koseri, E. coli, K. oxytoca, Citrobacter freundii
34  Enterococcus faecalis	33  E. coli	32  Streptococcus pyogenes, E. Coli, P. aeruginosa	31  Shigella dysentriae

(*Continued*)

Table 3.9 Modification of the piperidine ring in piperine [42–45]. (*Continued*)

Potent structure reported by each group		**Inactive structure reported by each group**	
Gram positive	**Gram negative**	**Gram positive**	**Gram negative**
35  Enterococcus faecalis, B. cereus, B. subtilis	35  Klebsiella pneumoniae	36  S. aureus	32  Klebsiella pneumoniae, Citrobacter koseri, E. coli, K. oxytoca, Salmonella typhi
			34  Pseudomonas aeruginosa

Conclusion

Regarding Synergism,

- Piperine inhibits efflux pump. Efflux pump is generated by bacteria when foreign materials like drugs enters the cell. By inhibiting the efflux pump, the drug stays inside and kills the bacteria.
- Depending upon the bacterial strain and the drug used, the efflux pump differs. Two drugs ciprofloxacin and mupirocin on S. aureus generates different resistant strains. But both the mutant strains are handled well by the molecule.
- Piperine reverses the resistance of bacteria toward a drug. This is a huge advantage while dealing with multi-drug resistant strains.
- There are not enough structural variations to relate a theory, so for now, we can say,
  - When having a secondary amide, a ring with electron withdrawing group in the meta position
  - Having a propyl chain at C2 position in the alkyl linker
  - Inclusion of halogen
  - Not disturbing the conjugation in the alkyl chain linker
  - And not including a heteroatom works better than piperine.

Regarding Anti-Bacterial Effects

With the existing data we can say,

- Piperine moiety is narrow spectrum anti-biotic.
- Different mechanism of action might be involved with different bacteria.
- Inclusion of halogen works like a charm.
- Saturation of the alkyl linker is not a good move.
- Increasing the number of hydrogen donors and the acceptors increases the potency of the molecule.

Anti-Fungal

Fungal body is not simpler like bacterial cells. They are bit complicated just like human cells. Piperine when tested against Aspergillus flavus, it is

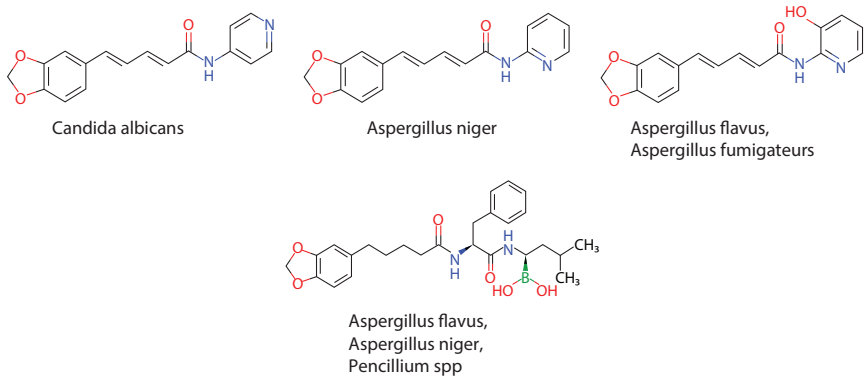


Figure 3.14 Analogs that show potency against Fungal bodies [42–45].

found that piperine inhibits AFB1 production via the perturbation of the oxidative stress balance. Let us look at analogs that worked against fungal body that was reported in the articles we discussed above in Figure 3.14.

Conclusion

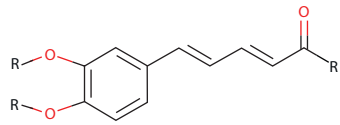
With the current results we can presume, when

- The number of hydrogen interaction units is increased.
- Introduction of a heterocyclic Ring system.
- Retaining the MDP and the alkyl linker in the structure.
- Increases or not decreases the activity of the molecule.

3.4 Overall Summary and Conclusion

Piperine is one of the alkaloids present in black pepper. It is proven to be the active component in the plant material. It has various biological activities and is famous for its bio-enhancement activity, anti-microbial effect, BBB transfer efficiency, etc. One of the modern reasons to quote Piperine to be a pro-drug is because it follows Lipinski's rule.

We analyzed two of its bio-activity in detail—anti-microbial and GABA modulation. From the analysis that we propose, for any application, the piperine moiety should have the MDP ring or at least a substituted/unsubstituted Catechol in its place, an alkene chain liker and the C=O bond. We observe a constant lack of bio-activity in the absence of these groups. So, the pharmacophore of the molecule could be as follows.



Though number of analogs for both the applications has been reported, a perfect working model is still missing. So, the area has so much questions to be addressed. Many of the analogs have not been completely screened. When a research group works on anti-cancer drugs, they only focus on the cancer cell lines, those molecules are left unscreened for the rest of the issues. An analog that is tested for anti-cancer could bind better with CYP and have a better bio-availability enhancement property, but it would have not been tested, which means, still the library of piperine analogs is not analyzed completely. This gap is yet to be filled.

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4

Chemoenzymatic Synthesis of Pharmacologically Active Compounds Containing Chiral 1,2-Amino Alcohol Moiety

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Abstract

Several bioactive molecules with possible pharmaceutical significance and chiral ligands/auxiliaries contain enaniopure vicinal amino alcohols as an essential structural component. While tremendous efforts have already been dedicated all the way through developing economical and more adaptable chemocatalysts as well as reaction media optimization, it still continues to be a challenge in the asymmetric synthesis of these structural moieties due to the complexity of chemicals, poor yield, and serious health risks. Biocatalytic approaches have been progressively more accepted where high regio-/stereoselectivity and environmental friendly/mild conditions are required. Latest progress in biocatalysis involving screening or protein engineering has significantly enhanced the quantity of synthetic prospects from minute optically active multifunctional molecules to very complex analogs. Further, the consideration of biocatalytic methods and the blending of various (bio) catalysts in one-pot practices a spectacular development in synthetic chemistry. In the present chapter, we intend to highlight the significance of 1,2-amino alcohols in medicinal chemistry and as chiral ligands. Moreover, emphasis was put down on chemoenzymatic methods, focusing on the methodology adopted and the kind of biocatalyst(s) employed in the synthesis of some drug molecules containing chiral 1,2-amino alcohols.

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Keywords: Chirality, biocatalysis, 1,2-aminoalcohols, kinetic resolution, enantiomers, green chemistry, chemoenzymatic synthesis

4.1 Introduction

4.1.1 Chirality

Chirality is a general aspect taking place in everyday circumstances: the left and right hand being non-superimposable mirror images of each other are chiral. Chirality is one of the central topics in chemical Science. In fact, ~10% of publications in general chemistry journals in 2016 are associated to the topic in some way [1]. Most biological molecules such as carbohydrates, nucleotides, and amino acids are naturally chiral. The chiroptical effects of these molecules are due to different interaction with plane polarized light [2].

In organic molecules, a carbon atom represents a chiral center (or sometimes a stereogenic center) when it is bound to four nonidentical substituents. For a molecule containing one chiral center, the number of stereoisomers possible is two. Such chiral molecules that are mirror images of one another, but are non-superimposable, are called enantiomers. In molecules containing more than one stereogenic center, nonsuperimposable stereoisomers known as diasteromers can arise that are not mirror images of one another. For a molecule with "n" chiral (steregenic) centers, utmost number of stereoisomers feasible is 2^n.

In bioactive molecules where a chiral center is present, great differences are frequently observed in the pharmacological effects [3] (especially their potency and toxicity) of enantiomers. In some cases, molecules with single enantiomer are normally extra proficient as compared to racemic counterparts [4]; more significantly, even as one enantiomer emerges as an effective medicine, the second one may possibly produces severe side effects [5].

One commonly talked about example is the drug thalidomide **1** (Figure 4.1), which was first marketed in 1957 in West Germany as a sedative under the trade name Contergan. It gained bad name in 1960's, when

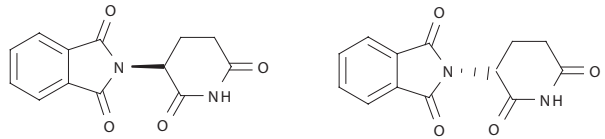


Figure 4.1 Enantiomers of drug thalidomide **1**.

the relationship between severe birth defects like phocomelia in babies and administration of tholidomide to pregnant women was documented [6].

Afterward, it was understood that the desired sedative effect lies with the (*R*)-enantiomer of thalidomide, whereas its (*S*)-enantiomer displays teratogenic effect, which leads to malformation in newborns. The application of single (*R*)-enantiomer in that case would not avert this tragedy due to racemization of enantiomers within the body. The individual enantiomers can racemize to each other due to the acidic hydrogen at the chiral center giving a racemic mixture (50/50: *R*/*S*)—even if the single enantiomer is consumed [7].

Since enzymes are made from chiral subunits and the greater part of transformations in a cell are enzyme catalyzed, the enantiomers of a molecule must be considered as divergent structural units in a biological perspective [8]. In biological systems, enzymes, receptors, and other binding sites distinguish compounds with a specific chirality.

Depending upon the type of substrate/starting material used, the strategies employed for preparing enantiopure molecules can be alienated into three different classes as described in Figure 4.2.

First category involves exploitation of chiral-pool, a source of chiral natural products such as sugars/carbohydrates, α-amino acids, mono terpenes, alkaloids, and some hydroxyacids. Second strategy involves separation of enantiomers through resolution of a racemic mixture. The third

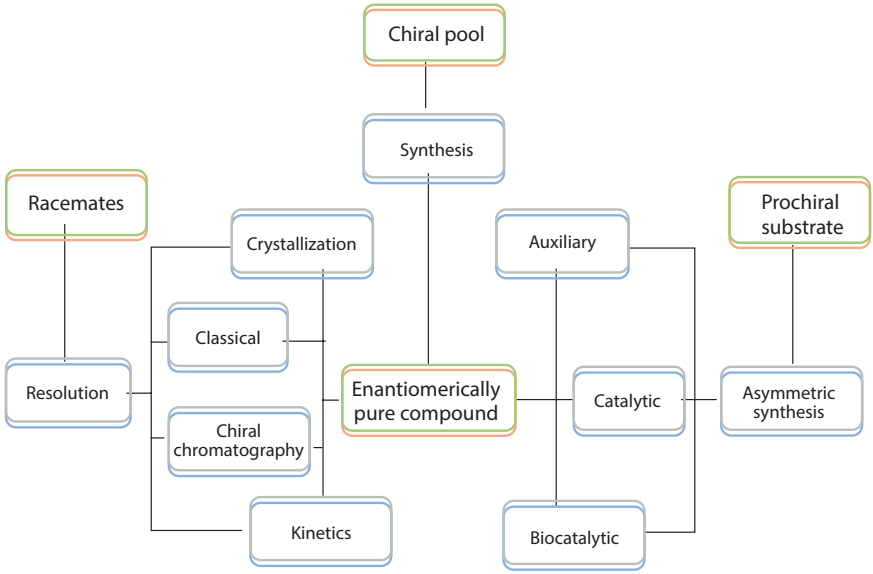


Figure 4.2 Methods used for the synthesis of enantiopure compounds.

category makes use of chiral agents to create a chiral center in a prochiral substrate, i.e., asymmetric synthesis.

4.1.2 Biocatalysis

Biocatalysis has become progressively more frequent in the production of enantiomerically pure compounds. Since many complex bioactive molecules contain one or more stereogenic centers, considerable differences are generally examined for the activities of the enantiomers. This is a common phenomenon usually observed in substances such as drugs, herbicides, vitamins, flavors, and fragrances. The efficient syntheses of these chiral molecules need stereo-/regio-selective catalysts, and enzymes are indeed the most selective catalysts offered. From the last few years, the interest for creating stereogenic centers by applying biocatalytic methods has risen, due to the great demand for optical pure compounds in various fields of industry.

In the pharmaceutical industries, the major cause of waste is the utilization of stoichiometric (mainly inorganic) reagents in synthetic organic chemistry reactions. Biocatalysts have obvious economic and environmental benefits as these are non-toxic and when liberated into aqueous waste

Table 4.1 Classification of enzymes as per IUPAC nomenclature.

S. no.	Class of enzyme	Specific code (EC number)	Reaction catalyzed
1	Hydrolases	EC3	Hydrolysis
2	Oxidoreductases	EC1	Oxidation or reduction
3	Transferases	EC2	Transformation of functional group
4	Lyases	EC4	Breaking of various chemical bonds by means other than hydrolysis and oxidation/additions (C, N, and O) to multiple (especially double) bonds
5	Isomerases	EC5	Isomerization reactions.
6	Ligases	EC6	New chemical bond (C-O, C-N, C-S, etc.) formation.

matter they get quickly degraded [9]. Moreover, biocatalytic reactions are normally achieved under mild conditions and produce a smaller amount of waste than conventional organic syntheses.

The enzymes are biological catalysts that work by lowering the activation energy. The characteristic aspect of biocatalysis is the specificity of enzymes. These are too specific toward their substrates to which they react and therefore the reaction will also be very specific. Enzymes are divided into six different classes depending upon type of reactions they catalyze as per IUPAC nomenclature system. There is a specific code allotted to each enzyme (EC number) that illustrates the reaction type (Table 4.1).

Penicillin antibiotics and other natural products such as lactic acid have been produced since long using fermentations. On the other hand, reports of using isolated enzymes (generally derived from animals/plant sources) became apparent in the second half of the previous century.

4.1.2.1 Biocatalysis is Green and Sustainable

Green Chemistry generally involves renewable resources, diminishes waste material, and circumvents the utilization of unsafe chemicals in the synthesis and exploitation of new chemical products [10]. It is first and foremost pollution avoidance instead of waste remediation. The expression "Green Chemistry" achieved proper recognition after 1998 when 12 principles were published in book Green Chemistry: Theory and Practice by Anastas and Warner [11]. Later in 2005, the American Chemical Society (ACS) and other pharmaceutical companies across the globe established the "ACS GCI Pharmaceutical Roundtable". The broad endeavor was to bring "*key medicines to the patients with a minimal impact on the environment*" [12]. This ought to be accomplished by incorporation of more sustainable; "greener chemistry" substitutes in production of different chemical entities in pharmaceutical industries. The use of enzymes in chemical synthesis presents an enormous potential to achieve the challenges in chemical synthesis as mentioned above [13]. The extensive use of biocatalytic methods in organic synthesis has exceptional advantages such as attaining higher product selectivity and their plentiful economic and environmental benefits. Additional advantages of biocatalytic processes over conventional chemistry encompass amazing stereo-/regio-selectivity, the use of milder/green reaction conditions and decrease in the number of steps involved in a reaction procedure. It has been observed that biocatalysis (Table 4.2) conforms with 10 out of 12 Green Chemistry principles, whereas the left over principles (4 and 10) are not significant as they speak about the product, instead of procedure adopted [14].

Table 4.2 Biocatalysis and green chemistry.

S. no.	Principles of green chemistry	Biocatalytic processes
1	Waste prevention	Considerably diminished waste
2	Atom economy	Additional atom and step economical
3	Less hazardous synthesis	Usually less toxic
4	Design for safer products	Not significant
5	Safer solvents and auxiliaries	Generally carried out in buffer/ water
6	Energy efficiency	Require mild reaction conditions
7	Renewable fee stocks	Biocatalysts are renewable
8	Reduced derivatization	Being regioselective they circumvent multi step processes
9	Catalysis	Biocatalytic reactions
10	Design for degradation	Not significant
11	Real time analysis	Relevant to all biocatalytic methods
12	Inherently safer processes	Environment friendly and mild conditions

The emergent need for more economical processes for the industrial production of enantiopure chiral compounds presented a prospect for the widespread relevance of extremely stereoselective biocatalytic processes [15]. Moreover, biocatalytic strategies are more cost-effective and produce a lesser amount of waste than conventional synthetic chemistry procedures.

4.1.2.2 Industrial Applications of Biocatalysts

The aspiration of industry to decrease production cost and waste while developing new reaction protocols toward chiral precursors often presents challenges and prospects for those in search of developing new methodologies. Applications of enzymes in industry find stimulation in the late 1980s after two significant collective developments which are i) increasing apprehension regarding the abundant quantity of waste produced by

the utilization of classical "stoichiometric" procedures terminated in the "US Pollution Prevention Act of 1990", which paid consideration on the requirement for waste deterrence and ii) government agencies brought up legislation that mandated inclusive testing of the each stereoisomer of chiral drugs [16]. Reports published in the last two to three decades revealed a remarkable increase in the industrial potential and scope of biocatalytic processes, primarily as a straight outcome of significant innovations in molecular biology. Nowadays, merging novel outcomes in DNA sequencing and gene synthesis with directed evolution techniques has facilitated prevalent applications of biocatalytic processes in industry, predominantly in the preparation of chiral building blocks. Literature scan reveals that most of the processes involved in the manufacture of enantiopure APIs (active pharmaceutical ingredients) involve chiral amines or chiral alcohols as key intermediates. Nine of the top 10 drugs contain chiral active ingredient, and for six of them, this ingredient is a single enantiomer small molecule [17].

The preparation of enantiopure drugs is a main challenge in synthetic chemistry, and the industrially significant enzymes together with those useful in detergent, foodstuff, animal feed, and other industries are projected to develop worldwide at a growth rate of 4.7% per annum all the way through 2021. Dewan and coworkers reported that the international market for enzymes rose from approximately $0.5 billion in 1990 to approximately $5 billion in 2016; with an expected spreading out to $6.3 billion in 2021 [18]. Moreover, the rising need for more cost-effective and environment friendly protocols for the preparation of enantiopure chiral drugs [19] presented a prospect for the extensive utilization of highly stereoselective biocatalytic processes [20].

4.1.3 Vicinal Amino Alcohols

The 1,2-amino alcohol unit is a wide spread structural design present in large number of bio-active molecules (natural as well as synthetic) such as β-blockers, antibiotics, and enzyme inhibitors. Bestatin **2** (adjuvant in cancer chemotherapy) [21], serine **3**, threonine **4**, paclitaxel **5**, kinostatins **6**, and sphingosine **7** [22] form an imperative component of naturally occurring amino alcohols (Figure 4.3). Sphingosine is the key component of glycosphingolipids that are crucial in signal transduction processes and also play a significant function in oncogenesis and ontogenesis [23]. Another type of amino alcohols exists where amino residue is enclosed inside the ring, e.g., anisomycin **8** (anticancer agent) [24], cytoxazone **9** (immunomodulator) [25], saquinavir **10** [26], anti HIV agent 11 [27], and Nelfinavir

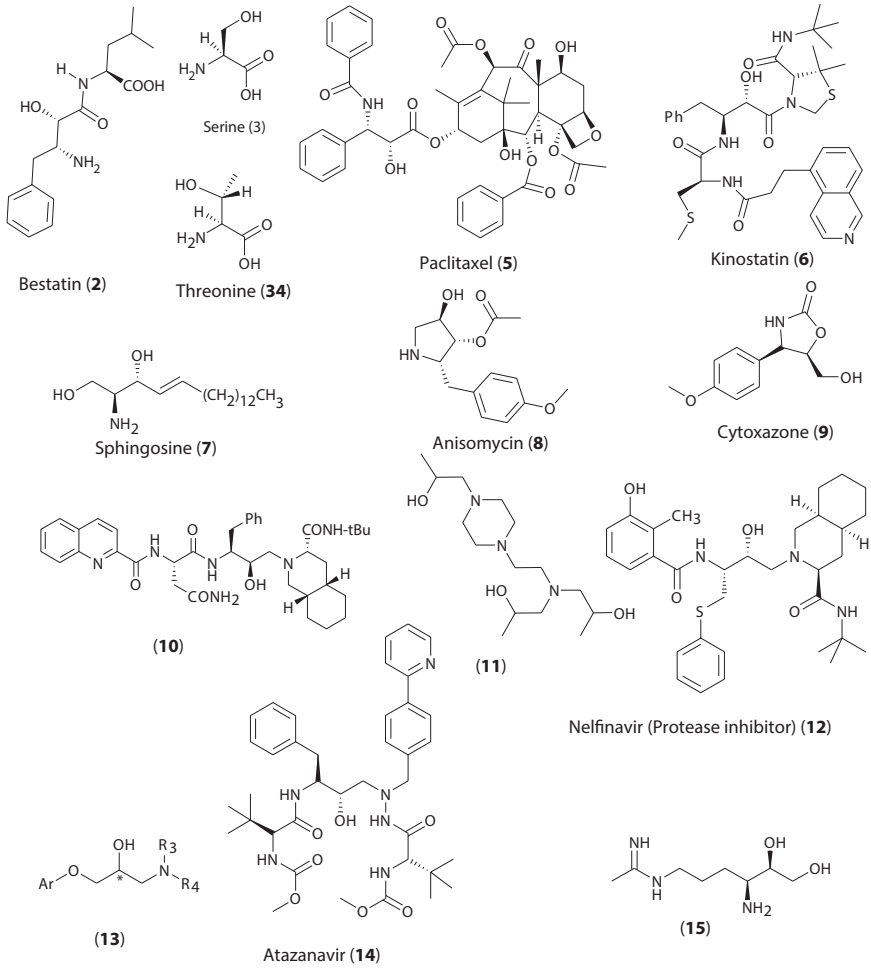


Figure 4.3 Bioactive molecules containing 1,2-amino alcohol moiety.

12 (HIV-Protease inhibitor) [28]. Drug molecules, such as aryloxypropanolamines [29] **13**, Atazanavir **14** (anti-HIV drug) [30], and inhibitor of nitric oxide synthetase **15** [31], also contain vicinal amino alcohol moiety.

A large number of pharmaceuticals contain optically active cyclic vicinal-amino alcohols as the crucial structural components [11]. They are also present in peptide nucleic acids (PNAs) [33] and form an important part of HIV protease inhibitors [34]. Cycloalkane derivatives (Figure 4.4) such as **16** and **17** have novel ion channel properties [35] and anxiolytic

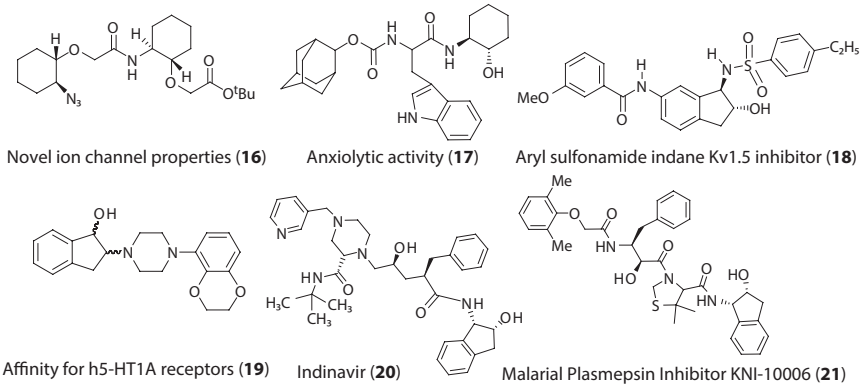


Figure 4.4 Pharmacologically active molecules containing vicinal aminocycloalkanols.

activity [36], respectively. In another report *trans*-2-(arylcycloalkylamine)-1-indanols such as **18** and **19** displayed Kv1.5 inhibitor activity and the utmost affinity for human, recombinant h5-HT1A receptors respectively [37]. Indinavir **20**, which also contains optically pure (1*S*, 2*R*)-1-amino-2-indanol, is a effective inhibitor of the protease of HIV [38]. Another amino indanol derivative, KNI-10006, **21** [39], reported to inhibit effectively Plm II (aspartic proteases) by *K*i significance of 0.5 nM. Presently, almost 82 drugs permitted by FDA contain amino alcohol motif [40].

Another important application of chiral amino alcohols is their role as chiral auxiliaries/ligands in asymmetric reactions such as Henry reactions [41], addition of dialkylzinc to carbonyls [42], carbonyl reductions [43], aldol reactions [44], and pericyclic reactions [45, 46]. Some auxiliaries/ligands for example ephedrine derivative **22** [47], Oxazolidinones **23** [48], oxaborolidines **24** [49], propanolamines **25**, and Bis oxazolines **26** [50], containing amino alcohol moiety are depicted in Figure 4.5.

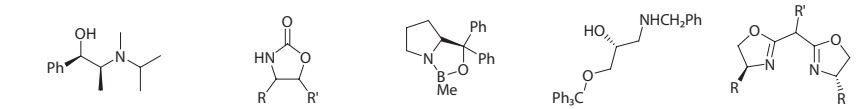


Figure 4.5 1,2-amino alcohols as chiral auxiliaries.

4.2 Synthetic Approaches Toward 1,2-Amino Alcohols

A variety of synthetic routes to *vicinal* amino alcohols are reported owing to existence of this structural moiety in various active pharmaceutical ingredients and in chiral auxiliaries. Vicinal amino alcohols can be prepared synthetically by employing three different strategies such as management of functional groups, adding up of hetero atoms (N, O, S, etc.) to double/triple bond and by coupling reaction (creation of new C-C bond).

In the past few years, numerous reports disclosing the syntheses of optically active 1,2-amino alcohols came into sight [51]. S. C. Bergmeier, in 2000, presented an excellent review highlighting the various synthetic methods toward 1,2-amino alcohols [52]. The synthesis and chemistry of *cis*-1-amino-2-indanol were covered by Gallou and Senanayake in 2006 [53]. Later in 2015, Sehl *et al.* reported in detail the synthetic strategies consisting of asymmetric routes toward 1,2-amino alcohols by utilizing synthetic, biocatalytic and chemoenzymatic methods. Authors described the preparation of all the stereoisomers of phenylpropanolamine by paying a particular attention on one-pot strategies [54].

Synthetic strategies toward the preparation of these compounds usually necessitate protecting groups, costly transition metal catalysts, various steps involved for product recovery and purification [55]. Biocatalytic transformations, in turn, involve green technologies for organic syntheses due to their elevated chemical and energy efficiency. Waste generation can be reduced upto a great extent by performing biocatalytic processes. The enzyme catalyzed reactions primarily occurs by utilizing hydrolases and oxidoreductases, whereas the other four enzyme classes are involved in merely 15% of the investigations.

The significance of biocatalytic methods for *stereo*-/*regio*-selective transformations is now well recognized. In the present work, we seek to illustrate a summary of (chemo)enzymatic routes, focusing on the type of enzyme used and the strategy adopted for the synthesis of drug molecules containing optically active 1,2-amino alcohols.

4.2.1 Chemoenzymatic Synthesis of L-Norephedrine

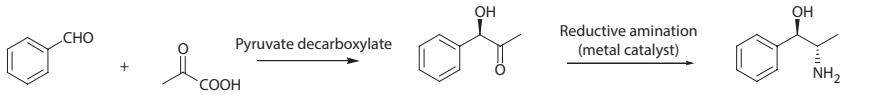
L-Norephedrine, an adrenergic agent used to decrease respiratory infection, is a chiral vicinal amino alcohol isolated from an herb *Ephedra sp*. Nevertheless, it can also be used as a building block to prepare various medicaments, for example, oxyfedrine and ephedrine. L-Norephedrine

can be prepared by methods such as isolation from herb *Ephedra sp*., kinetic resolution of racemic mixtures and by reductive amination during asymmetric synthesis. The major difficulty in asymmetric synthesis of L-norephedrine with optimum optical purity involves the presence of two stereogenic centers. The advancement in biocatalytic strategies for the preparation of L-norephedrine is of immense attention to both industry and academic institutions.

Reports published in the past showed that biocatalytic methods using isolated enzymes or whole cells have certain advantages on stereoselectivity over synthetic methods [56]. In chemoenzymatic methods, biocatalysis can introduce first chiral center, and second one is established via asymmetric synthesis or kinetic resolution of the racemic mixture. For example, Satianegara *et al.* described that transformation of benzaldehyde **27** into L-phenylacetylcarbinol (L-PAC) [57] **28** was achieved using pyruvate decarboxylase from yeast followed by reductive amination of **28** by metal catalysts to afford L-norephedrine **29** (Scheme 4.1) [58].

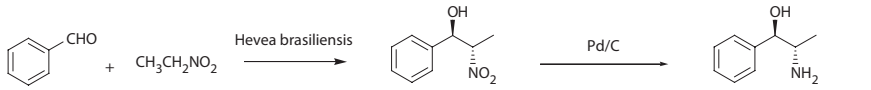
Cyanohydrins are very significant starting materials for the preparation of essential building blocks, ranging from medicaments to those crucial in cosmetics and agrochemicals. Cyanohrdrins can also be transformed into vicinal amino alcohols; therefore, the asymmetric synthesis of these compounds is of great relevance. Biocatalytic processes for the asymmetric synthesis of cyanohydrins involve the use of hydroxynitrile lyases [59]. Enzymes such as *Prunus* sp., *H. brasiliensis*, and *Linum usitassimum* are reported to exhibit hydroxynitrile lyase activity during condensation of hydrocyanic acid (HCN) with carbonyl compounds (e.g., aldehydes or ketones) to yield (*R*)- or (*S*)-α-hydroxy nitriles. Asano and Dadashipour, in 2011, reviewed the biochemistry, screening, and engineering of discovery of hydroxynitrile lyases [60].

Hydroxynitrile lyase from *Hevea brasiliensis* was used to prepare L-norephedrine **29** with an enantiomeric excess of 95% by enantioselective condensation of benzaldehyde **27** with nitroethane (to yield 1*R*,2*S*-2-nitro-1-phenylpropan-1-ol 30) followed by Pd/C catalyzed reduction (Scheme 4.2) [61].



Benzaldehyde **27** L-PAC **28** L-norephedrine **29**

Scheme 4.1 Stereoselective synthesis of L-norephedrine.

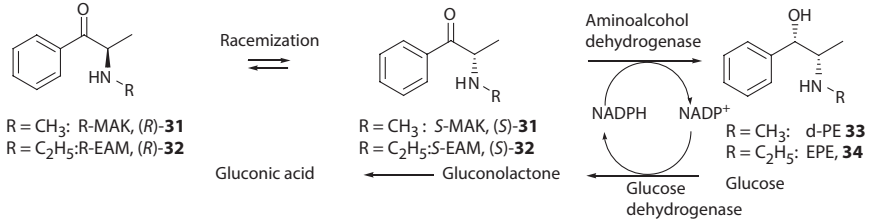


Benzaldehyde **27** 1*R*,2*S*-2-nitro-1-phenylpropan-1-ol **30** L-norephedrine ee 95% **29**

Scheme 4.2 Enantioselective synthesis of L-norephedrine.

A $NADP^+$-dependent L-1-amino-2-propanol dehydrogenase from *Rhodococcus erythropolis* MAK154 was employed by Shimizu *et al.*, in 2006, for preparation of d-pseudoephedrine (d-PE) **33** by enantioselective reduction of (*S*)-MAK {(*S*)-1-phenyl-1-keto-2-methylaminopropane} **31** (Scheme 4.3) [62]. Authors disclosed that, only d-PE is particularly synthesized by the reduction of (*S*)-enantiomer of racemic 1-phenyl-1-keto-2-methylaminopropane(MAK),whereasleftover(*R*)-1-phenyl-1-keto-2-methylaminopropane (MAK) **31** can be impulsively racemized under feeble basic conditions, so it is anticipated that by employing *Rhodococcus erythropolis* MAK154 all the racemic MAK can be exclusively transformed to d-pseudoephedrine (d-PE). In 2008, a recombinant strain *Escherichia coli* coexpressing the amino alcohol dehydrogenase (ADH) gene from *R. erythropolis* MAK154 and the glucose dehydrogenase gene from *Bacillus megaterium* was created and it was observed that 200 mM of racemic MAK.HCl was transformed to 178 mM of d-PE.HCl with 89.1% yield and >99% ee optical purity utilizing cells of recombinant *E. coli* [63]. Since d-PE production was limited to about 40–50 mg/ml, due to inhibition of AADH, hence AADH was randomly mutated for practical application of above reaction [64]. During screening of the mutant library, only two mutated enzymes displayed better tolerability to d-PE **33** as compared to wild type enzyme. Results revealed that EPE (d-2-ethylamino-1-phenyl-1-propanol) **34** can be prepared from (*RS*)-1-phenyl-1-keto-2-ethylaminopropane {(*RS*)-EAM, **32**} by reduction using mutant enzymes.

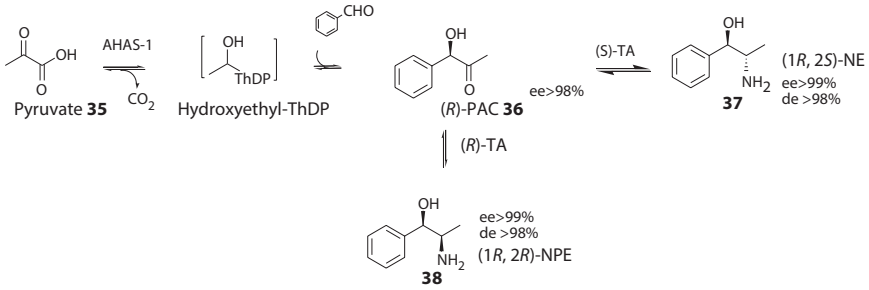
Another biocatalytic approach toward enantiopure (1*R*, 2*R*)-norpseudoephedrine (NPE) **38** (ee > 99%; de > 98%) and (1*R*, 2*S*)-norephedrine



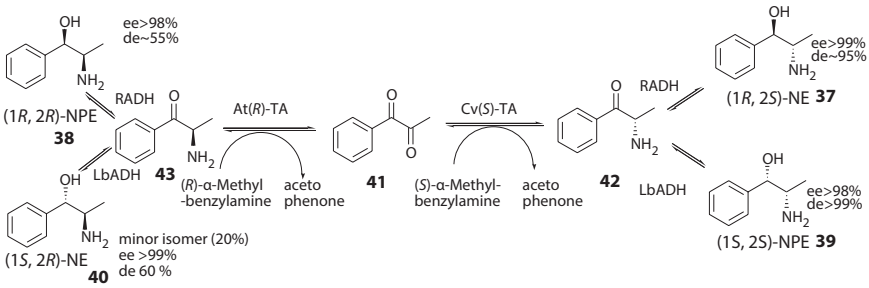
Scheme 4.3 Biocatalytic synthesis of d-PE **33** and EPE **34**.

(NE) **37** (ee > 99%; de > 98%) from pyruvate and benzaldehyde was disclosed by Rother *et al.*, in 2013, using two-step one-pot reaction protocol [65] via intermediate formation of (*R*)-phenylacetyl carbinol (*R*-PAC) **36** (Scheme 4.4) [66]. In the first step, the thiamine diphosphate (ThDP)–dependent acetohydroxyacid synthase-1 (AHAS-1) catalyze the reaction whereas second step is catalyzed by (*S*)- and (*R*)-selective ω-transaminases (Cv-TAs).

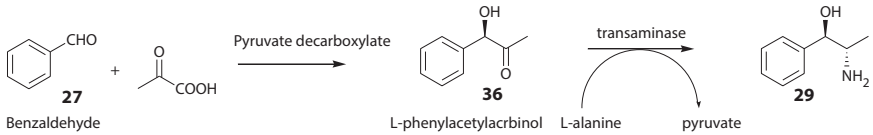
Later on, a *S*-selective thiaminediphosphate (ThDP) carboligase was employed for the preparation of two left over stereoisomers (1*S*, 2*S*)-NPE **39** and (1*S*, 2*R*)-NE **40** via intermediate formation of (*S*)-phenylacetylcarbinol (PAC) but the stereoselectivity was not so good [67]. Therefore to enhance the enantioselectivity a different reaction protocol i.e. "TA-ADH" cascade rather than "carboligase-TA" cascade, containing *S*-selective TA (Cv(*S*)-TA) in permutation with either *R*-selective ADH from *Ralstonia species* or the *S*-selective ADH from *Lactobacillus brevis* could produce (1*R*, 2*S*)-NE **40** and (1*S*, 2*S*)-NPE **39** respectively utilizing 1-phenylpropane-1,2-dione **41** as a substrate (Scheme 4.5) [68].



Scheme 4.4 Reaction sequence depicting one-pot two-step process toward (1*R*, 2*R*)-NPE **38** and (1*R*, 2*S*)-NE **37**.



Scheme 4.5 Reaction sequence for the preparation of isomers of nor(pseudo)ephedrines.



Scheme 4.6 Multienzymatic process for the synthesis of L-norephedrine **29**.

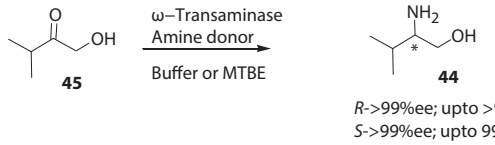
One of the most striking and growing area of biocatalysis involves multienzymatic processes where various kinds of biocatalytic reactions were mingled together to create a sequential procedure. Another stereoselective synthesis of L-norephedrine **29** was achieved by Wo and coworkers in 2014, through a sequential process involving coupling of *S*-selective ω-TA from *Vibrio fluvialis* JS17 (VfTA) and *R*-selective pyruvate decarboxylase from *Saccharomyces cerevisiae* (ScPDC) as described in Scheme 4.6 [69].

4.2.2 Synthesis of Valinol

Valinol **44** represents a typical example of chiral amino alcohols, especially, α-chiral amino alcohols, which was reported to be utilized as organocatalyst in cross aldol reactions [70] and as chiral solvating agent [71], and forms a part of various drugs and drug-like molecules. Some pharmacologically active compounds containing valinol **44** include drugs for the treatment of HIV [72], hepatitis C virus [73], diabetes and obesity [74], as well as analgesic agents [75].

The amino acid valine is generally employed for the production of Valinol. Abundant supply of S-valine leads to the production of *S*-isomer almost exclusively. The most general approach toward **44** still depends on the reduction of valine which is not only costly, in case the nonnatural (*R*)-enantiomer of valine is required, but also requires highly sensitive reagents like $LiAlH_4$.

The preparation of optically pure valinol (ee >99%), can be achieved from the corresponding prochiral hydroxy ketone **45** by utilizing different ω-TAs [76]. Authors disclosed that best results (ee>99% & conversion >99%) were attained for (*S*)-valinol utilizing a TA from *Arthrobacter species* in organic solvents. However, in aqueous buffer, transformations were successfully performed even at 200 mM substrate concentration (20.4 g/L). Depending upon the type of enzyme used, (*R*)- as well as the (*S*)-enantiomer of valinol were effectively synthesized with high enantioselectivity (>99%) (Scheme 4.7).



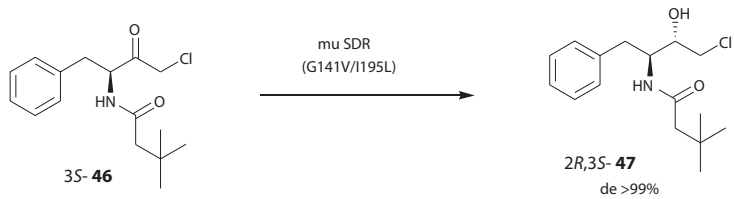
Scheme 4.7 ω-Transaminases catalyzed enantioselective synthesis of (*R*)- or (*S*)-valinol **44**.

4.2.3 Chemoenzymatic Synthesis of Atazanavir

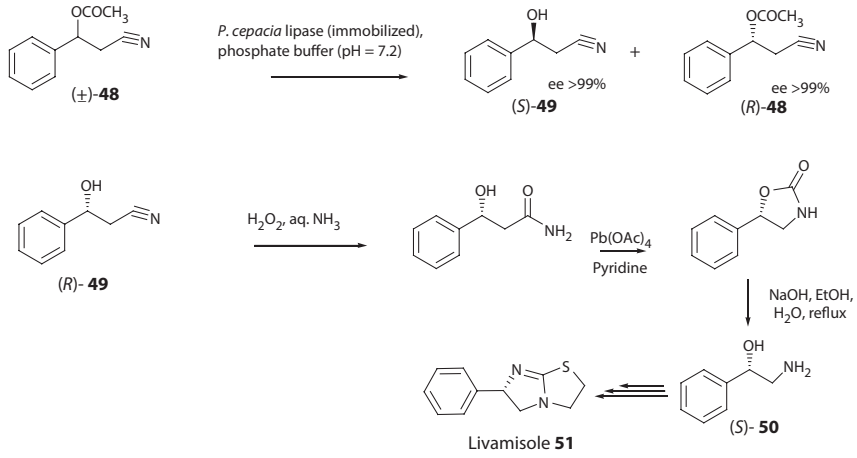
Human immunodeficiency virus (HIV) is a virus that attacks cells which help body to fight against infection; thereby a person becomes more susceptible to secondary infections/diseases. Atazanavir **14** (protease inhibitor) is given along with other antiretroviral drugs for the treatment of infection caused by HIV. This drug is approved by the International Antiviral Society–USA Panel for curing HIV related infections in adults. Wu *et al.*, in 2019, synthesized (2*R*,3*S*)-N-*tert*-butoxycarbonyl-3-amino-1-chloro-2-hydroxy-4-phenylbutane **47**, an intermediate for anti viral drug Atazanavir **14,** via the biocatalytic reduction of (3S)-3-(N-Boc-amino)-1-chloro-4-phenyl-butanone **46** with short-chain dehydrogenase/reductase (SDR). To enhance the activity of dehydrogenase/reductase isolated from *Novosphingobium aromaticivorans* (NaSDR) toward **46**, SDR was engineered via active pocket iterative saturation mutagenesis (ISM) and the mutant (G141V/I195L) thus obtained converted **46** entirely to the corresponding alcohol **47** with a diastereomeric excess of >99% at 30°C and pH 10.0 using 50% toluene (Scheme 4.8) [77].

4.2.4 Chemoenzymatic Synthesis of Levamisole

Levamisole, **51** immunomodulating agent [78], facilitates the restoration/function of some cells (that have been impaired) of the body's defense system. A biocatalytic route toward levamisole **51** has been proposed by Kamal and coworkers via *Pseudomonas cepacia* lipase catalyzed resolution



Scheme 4.8 Biocatalytic synthesis of **47**.



Scheme 4.9 Synthesis of Livamisole **51** involving *Pseudomonas cepacia* lipase catalyzed kinetic resolution.

of 3-acetoxy-3-phenylpropanenitrile **48** followed by its transformation to vicinal amino alcohol **50** as described in Scheme 4.9 [79].

4.2.5 Chemoenzymatic Synthesis of Optically Active (*R*)- and (*S*)-Aryloxypropanolamines

Aryloxy propanolamines (β-adrenergic blocking agents) [80] having general structure **52** as shown in Figure 4.6 attracted much consideration in these days owing to their effectiveness in the treatment of heart diseases, [81], hypertension [82], also other disorders [83].

Their (*S*)-enantiomer, having a strong structural similarity to the adrenergic hormone noradrenaline, is primarily responsible for the curative effect, e.g., (*S*)-propranolol displays 130 times additional activity than (*R*)-propranolol. Keeping into consideration the high biological significance of these compounds, several scientific communities have performed ample research for their preparation in enantiopure form. From the last few years, various biocatalytic processes have been developed for

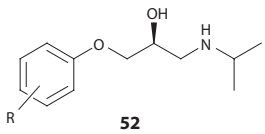


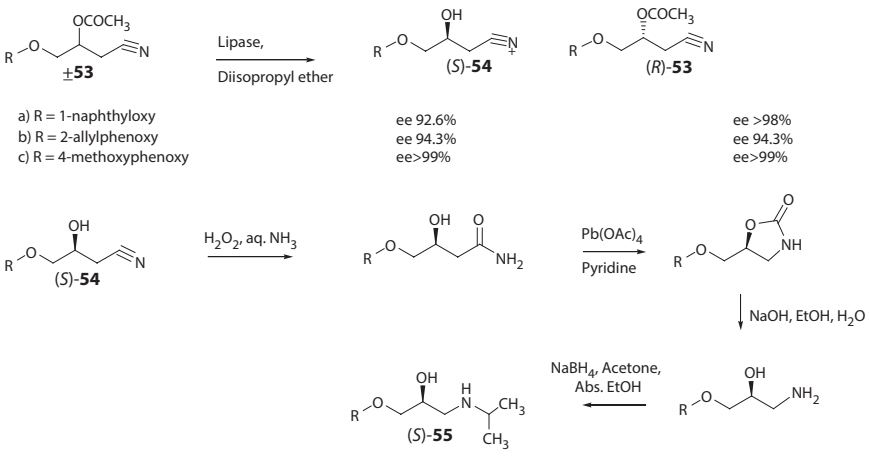
Figure 4.6 Structure of (S)-aryloxypropanolamines.

the enantioselective synthesis of intermediates to β-adrenergic blocking agents [84].

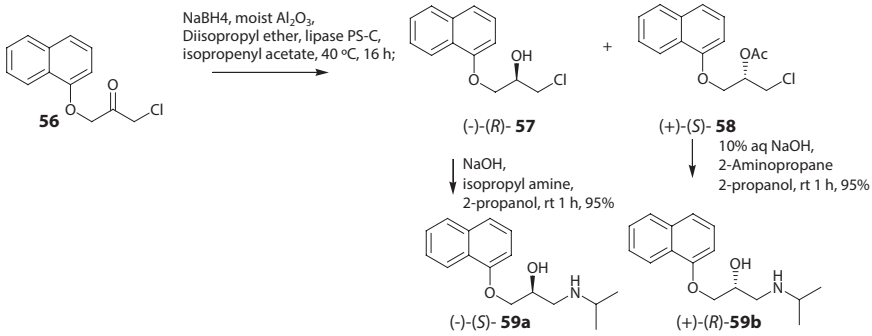
A facile biocatalytic kinetic resolution strategy toward enantiopure β-hydroxy nitriles involving specific lipases was demonstrated by Kamal *et al.* in 2001 [85]. The above strategy was later on effectively exploited for the synthesis of 3-hydroxy-4-aryloxybutanenitrile (**54a-c**), (Scheme 4.10), which can easily be transformed into (*S*)-propranolol, (*S*)-alprenolol, and (*S*)-moprolol by easy reaction procedure in good optical purity and yields [86].

In another report, Kamal *et al.* presented an efficient approach toward the preparation of both *R* and *S* enantiomers of propranolol and sotalol in high enantiopurity by means of one-pot reduction and *in situ* biocatalytic kinetic resolution of the corresponding chlorohydrins [87]. The conversion and optical purity of enzyme catalyzed reactions are monitored on HPLC. Alcohol (−)-**57** (96% ee) and acetate (+)-**58** (>99% ee) were successfully prepared from **56** by one-pot reduction and enzyme catalyzed resolution that can subsequently be transformed to corresponding (−)-**59a** and (+)-**59b** in 95% and 98% ee, respectively by nucleophilic replacement of chlorine with isopropyl amine as described in Scheme 4.11.

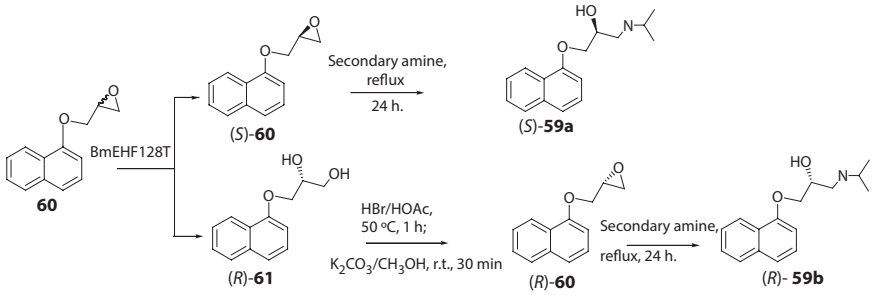
In 2015, (±)-α-naphthylglycidyl ether was successfully resolved using an epoxide hydrolase from *Bacillus megaterium* ($BmEH_{F128T}$). Kinetic resolution in biphasic system produced optically active epoxide (*S*)-**60** (>99% enantiopurity, 45% yield) and 1,2-diol (*R*)-**61** (>99% enantiopurity, 42% yield) which were then transformed into (*R*)-propranolol (**59b**) and (*S*)-propranolol (**59a**) in >99% enantiomeric excess using reaction sequence as described in Scheme 4.12 [88].



Scheme 4.10 Chemoenzymatic approach toward synthesis of aryloxy propanolamines **55**.



Scheme 4.11 Chemoenzymatic approach toward stereoselective synthesis of (*R*)- and (S)-propranolol **59**.



Scheme 4.12 Biocatalytic kinetic resolution approach toward the enantioselective preparation of (*R*)-propranolol (**59b**) and (*S*)-propranolol (**59a**).

β-Nitro alcohols are one of the most studied synthetic intermediates which can undergo multiple transformations, such as transformation to vicinal-amino alcohols. Reports revealed that whole cells of baker's yeast, *Candida parapsilosis and Comamonas testosterone*, were utilized for bioreduction of α-nitro ketones but the stereo selectivity was very poor [89]. The low stereoselectivity may be because of multiple reductases present in the whole cell system. In order to overcome the above drawback of low stereoselectivity the bioreduction was also performed using isolated KREDs [90]. Wang *et al.*, in 2019, disclosed synthesis of various β-nitro alcohols, along with the intermediates of (*S*)-moprolol **63**, (*S*)-toliprolol **62**, and (*S*)-propranolol **59** (Figure 4.7) with 42% to 90% isolated yields. Authors disclosed that YGL039w and SyADH successfully reduced 1-aryloxy-3-nitro-2-propanones **64** to generate enantiomers of resulting β-nitro alcohols **65**, with good to excellent stereoselectivities and yields as depicted in Scheme 4.13 [91].

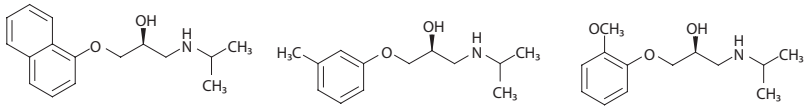
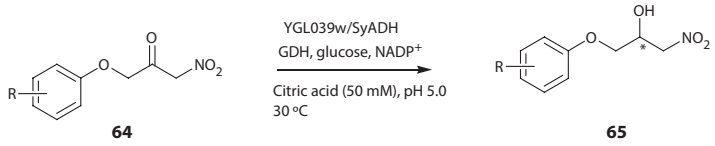
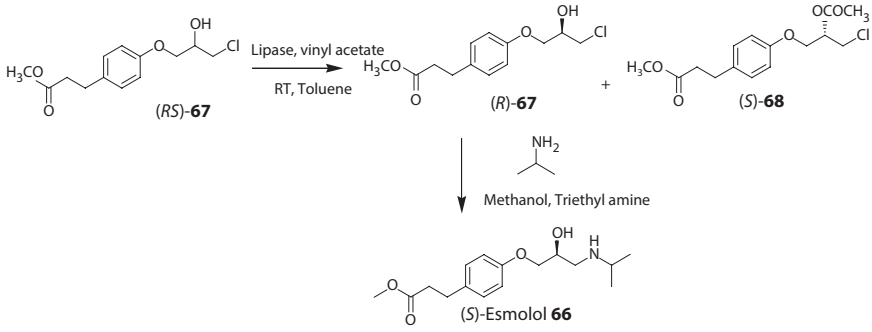


Figure 4.7 Some drug molecules containing aryloxypropanolamine moiety.



Scheme 4.13 Bioreduction of α-nitro ketones.

Esmolol **66** (β1-receptor blocking agent [92]) is a suitable drug used in critical care unit (CCU) for the speedy management of heart beat and/or blood pressure [93]. Currently, esmolol is available in the market as the racemic form; however, studies revealed that (*S*)-enantiomer of esmolol has two times higher potency as a β-blocker than that of (*RS*)-esmolol [94]. Banerjee and coworkers in 2017, investigated a chemoenzymatic approach toward (*R*) and (*S*)-esmolol **66** in good yields (61%–76%) and excellent enantioselectivity (92%–98%) [95]. Authors reported that commercially available hydrolases such as *Pseudomonas cepacia* and *Candida rugosa* displayed specific selectivity for the transesterification of (*RS*)-methyl 3-(4-(3-chloro-2-hydroxypropoxy)phenyl) propanoate **67** to produce enantiopure intermediates (*R*)/(*S*)-methyl 3-(4-(3-chloro-2-hydroxypropoxy)phenyl)propanoate which are vital for the enantioselective preparation of (*R*)/(S)-esmolol **66** (Scheme 4.14).



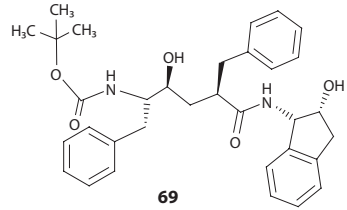
Scheme 4.14 Chemoenzymatic synthesis of (*S*)-esmolol.

4.2.6 Chemoenzymatic Preparation of *Trans*-(1*R*,2*R*)- and *Cis* (1*S*,2*R*)-1-Amino-2-Indanol

Cis-1-amino-2-indanol is a well-recognized precursor in the preparation of active pharmaceutical ingredients (API). With the discovery of L-685,434, an effective HIV-PR inhibitor **69** (Figure 4.8), many asymmetric methods for the preparation of *cis*-1-amino-2-indanol have come into sight. Dorsey and coworkers, in 1994, studied the biomedical characteristics of orally available HIV-PR inhibitor L-735,524, and it ultimately developed into a therapeutic agent Indinavir **20** (marketed as Crixivan) [96]. It was also found an important substituent in malarial Plasmepsin inhibitors [97]. Moreover *cis*-1-amino-2-indanol can also be utilized as chiral auxiliaries/resolving agent in various asymmetric transformations such as carbon-carbon/heteroatom bond formations [98], conjugate additions [99], and pericyclic reactions [100].

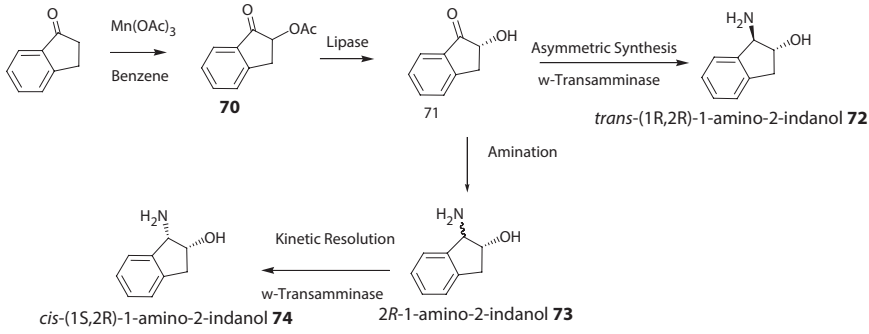
Applications of *cis*-1-amino-2-indanol in active pharmaceutical ingredients and asymmetric synthesis [101] encouraged researchers across the globe to hunt for various synthetic routes, including organic synthesis procedures for their synthesis [102]. In the beginning syntheses of optically active *cis*- and *trans*-aminoindanols was mostly achieved either from chiral starting material or by chemical or enzymatic resolution of racemic precursors. Biocatalytic methods have been adopted as efficient procedures for asymmetric synthesis of *cis*-(1*S*, 2*R*)-1-amino-2-indanol. Merck group used *Culvularia protuberata MF 5400* to convert indene to optically active (1*S*,2*S*)-bromoindanol, which was subsequently transformed to the desired *cis*-amino indanol [103]. Stereoselective *trans*-1-amino-2-indanol (enantimeric excess 99%, yield 50%) and *trans*-2-amino-1-indanol (enantiomeric excess 20%, yield 17%) were prepared by Luna *et al.* by *Pseudomonas cepacia* lipase catalyzed transesterfication of N-benzyloxycarbonyl derivatives [104].

Kim and coworkers, in 2006, disclosed a biocatalytic approach toward *trans*-(1*R*,2*R*)-1-amino-2-indanol, **70** by enzymatic hydrolysis of 2-acetoxyindanone followed by asymmetric transamination of (*R*)-2-hydroxy indanone **71** utilizing (*S*)-1-aminoindan as an amino donor.



69

Figure 4.8 HIV-PR inhibitor L-685, 434 **69**.



Scheme 4.15 ω-TA catalyzed synthesis of *trans*-(1*R*,2*R*)-1-amino-2-indanol and *cis* (1*S*,2*R*)-1-amino-2-indanol.

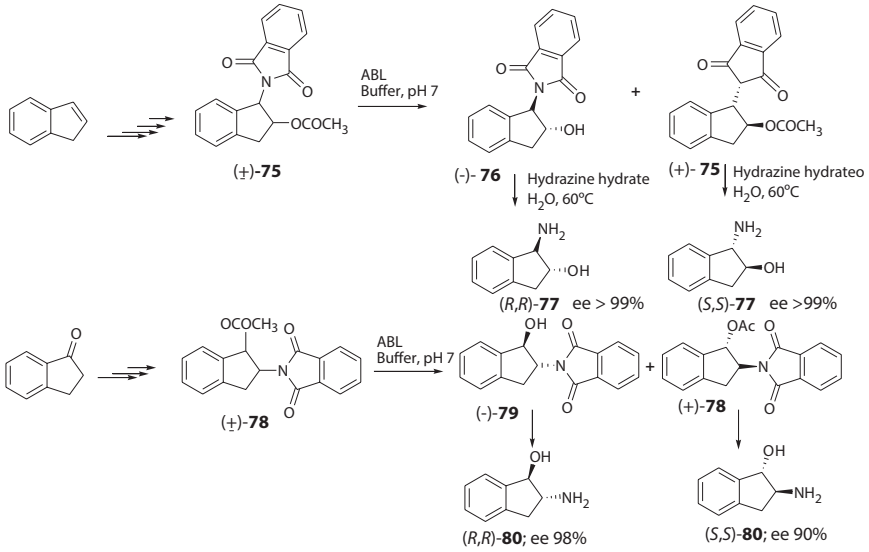
However, *cis*-(1*S*,2*R*)-1-amino-2-indanol **74** was successfully prepared from (2*R*)-1-amino-2-indanol (prepared by reductive amination of (*R*)-2-hydroxy indanone **71** by ω-transamminase (from *Vibrio fluvialis* JS17) catalyzed resolution (Scheme 4.15) [105].

The phthalimide protection strategy involving biocatalytic hydrolysis of *trans*-indanacetates was investigated by Rouf *et al.* for the enantioselective synthesis of *trans*-amino-indanols. Authors disclosed that a lipase from *Arthrobacter sp.* could enantioselectively hydrolyze acetylated *trans*-1-phthalimido-2-indanol **75**, whereas pig liver acetone powder was employed for stereoselective hydrolysis of *trans*-2-Phthalimido-1-acetoxy-oindan **78** (Scheme 4.16). Moreover, to enhance the enantiopurity and rate of hydrolysis, the immobilization of the substrates on a celite support and use of cosolvent (i.e., biphasic medium) was also experienced [106].

4.2.7 Synthesis of Enantiomerically Pure 2-Aminopentane-1,3-Diol and 2-Amino-1,3,4-Butanetriol (ABT)

Chiral 2-amino-1,3-diols play a very important role in the production of active pharmaceutical ingredients [107], and this structural moiety is found in antiviral glycosidase inhibitors [108], antibiotics [109], and sphingolipids [110]. Various chemical methods are reported in the literature to synthesize this category of amino alcohols [111]. Nevertheless, the reaction protocols reported are generally step-intensive and/or use environment unfriendly catalysts and solvents.

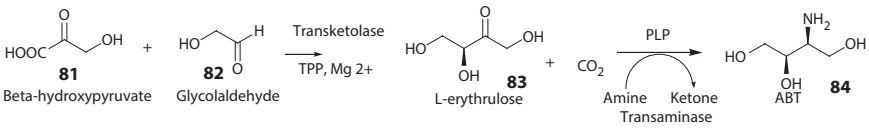
Chiral amino-triols such as (2*S*, 3*R*)-2-amino1,3,4-butanetriol (ABT) finds application in the preparation of statins and eventually for a HIV protease inhibitor, Nelfinavir [112]. It is also utilized in the preparation



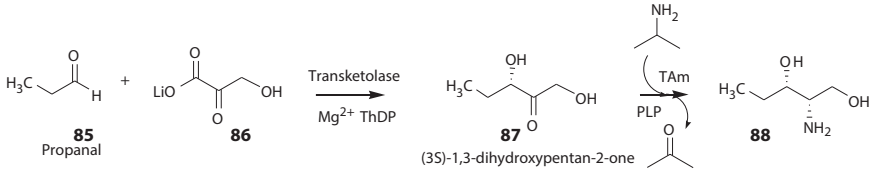
Scheme 4.16 Biocatalytic kinetic resolution of *trans* phthalimido indanols.

of detoxinine [113]. Lye and coworkers in 2007, utilized whole cells of the dual plasmid strain for preparing enantiopure 2-amino-1,3,4-butanetriol (ABT) **84**. L-erythrulose was prepared from glycolaldehyde **82** and β-hydroxypyruvate **81** by using transketolase (TK), which was subsequently transformed by transamination to a single diastereoisomer of ABT using β-alanine: pyruvate aminotransferase (Scheme 4.17) [114].

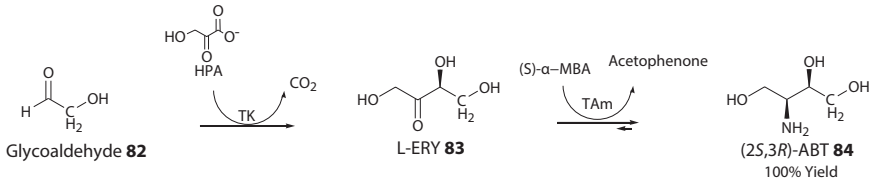
Smith and coworkers disclosed a one-pot reaction protocol toward the preparation of (2*S*,3*S*)-2-aminopentane-1,3-diol **88** using TK in combination with a Tam (Scheme 4.18). Due to ineffectiveness of co-solvent in the reaction catalyzed by CV2025 *ω*-Tam, it was determined to perform the preparation in two steps, i.e., compound (3*S*)-1,3-dihydroxypentan-2-one (DHP) **87** was isolated in the first step catalyzed by TK D469T by solvent extraction followed by CV2025 *ω*-TAm catalyzed second step. Engineering of TK enzyme was also studied so that non-natural substrate propanal **85** can be accepted [115].



Scheme 4.17 Biocatalytic approach toward single diastereomer of 2-amino-1,3,4-buntanetriol (ABT) **84** via de-novo TK-TAm pathway.



Scheme 4.18 TK and TAm catalyzed biocatalytic process for the preparation of 2-amino-pentane-1,3-diol **88**.

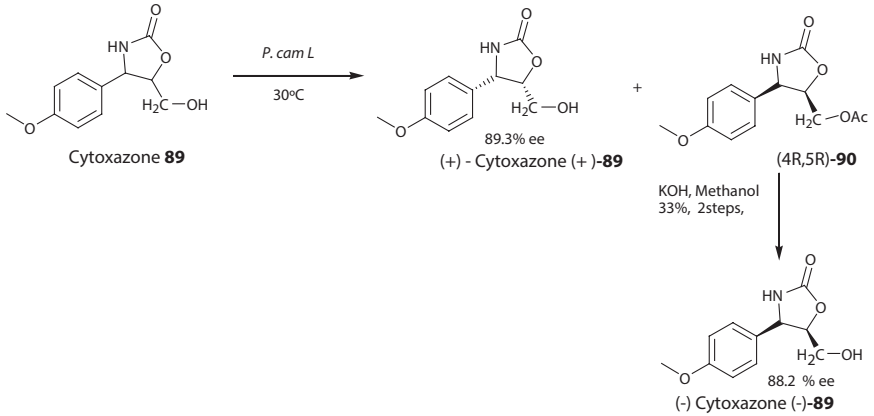


Scheme 4.19 Biocatalytic cascade involving transketolase (TK) and transaminase (TAm) in the preparation of ABT.

Optimization of reaction conditions during the preparation of (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT) **84** and (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) **88** was also examined by different research groups from time to time. Various steps taken for improving the above processes are i) both the enzymes were produced in a single host [116] and ii) using cascading free enzymes in microfluidic devices [117]. Gruber *et al.*, in 2018, demonstrated a two-step biocatalytic preparation of ABT (100% yield) utilizing TK and TA in a continuous flow microreactor system. First step involves the production of L-Erythrulose by TK catalyzed carbon-carbon bond formation between hydroxypyruvate and glycolaldehyde which was followed by second step involving synthesis of chiral amine from keto group by ώ-TA catalyzed conversion (Scheme 4.19) [118].

4.2.8 Synthesis of Optically Active Cytoxazone

Cytoxazone **89** (cytokine modulator) has an oxazolidin-2-one ring which is an essential intermediate in the preparation of β-lactams and renin inhibitors [119]. Various synthetic strategies have been reported for its synthesis. A biocatalytic strategy for the preparation of both the enantiomers of cytoxazone was reported by Sunjic and coworkers in 2001 [120]. Authors disclosed the kinetic resolution of racemic cytoxazone by *Penicillium camambertii* lipase catalyzed transesterification. Acylation of (±)-**89** with vinyl acetate yields the acetate (4*R*,5*R*)-**90** and the (+)-cytoxazone **89** (38%

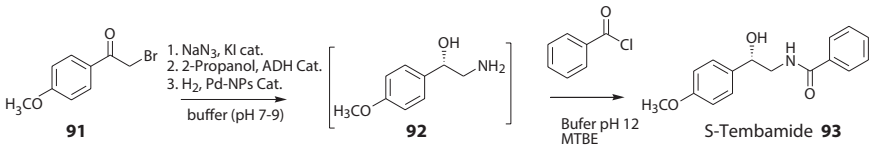


Scheme 4.20 Chemo enzymatic synthesis of the (−) and (+)-cytoxazones **89**.

yield, 89.3% enantiomeric excess). The acetyl derivative of cytoxazone (**90**) was then subjected to alkaline hydrolysis with KOH in MeOH to get (−)-cytoxazone **89** in 33% yield and 88.2% ee (Scheme 4.20).

4.2.9 Chemoenzymatic and Highly Integrated Synthesis of (*S*)-Tembamide

The one-pot reactions are predominantly attractive and have recently brought about outstanding advancements in the use of different catalysts in cascade systems. Getting inspired by the broad significance of cascade processes, a one-pot reaction protocol toward enantiopure vicinal amino alcohols (ee > 99%) from 2-azido ketones or 2-halo ketones was examined by Hollmann and coworkers in 2013. The strategy involves the asymmetric bioreduction of 2-azido ketones employing ADHs. By choosing appropriate ADHs, both the stereoisomers of 2-azidoalcohols can be synthesized in good enantioselectivity which were subjected to Pd nanoparticle-catalyzed hydrogenation reaction to get 1,2-amino alcohols. Thus, this integration of four steps in a single pot demonstrates a highly efficient method for the



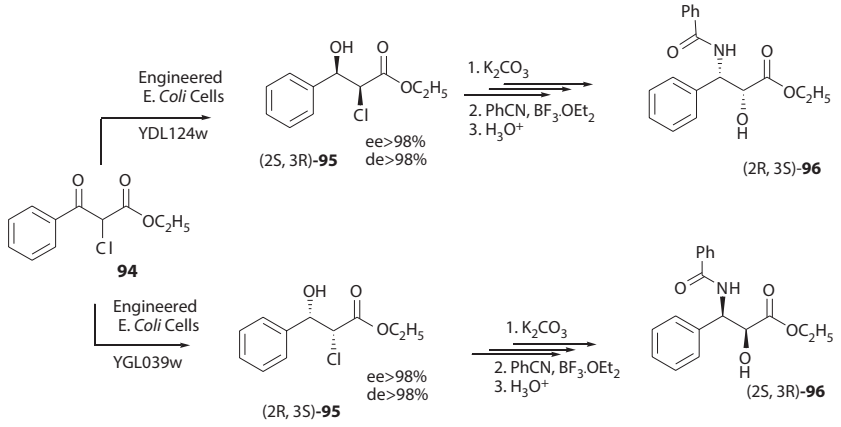
Scheme 4.21 Chemoenzymatic four step one-pot synthesis of (*S*)-tembamide **93**.

preparation of an antiviral compound (*S*)-Tembamide **93** (yield 73%, ee > 99%) (Scheme 4.21) [121].

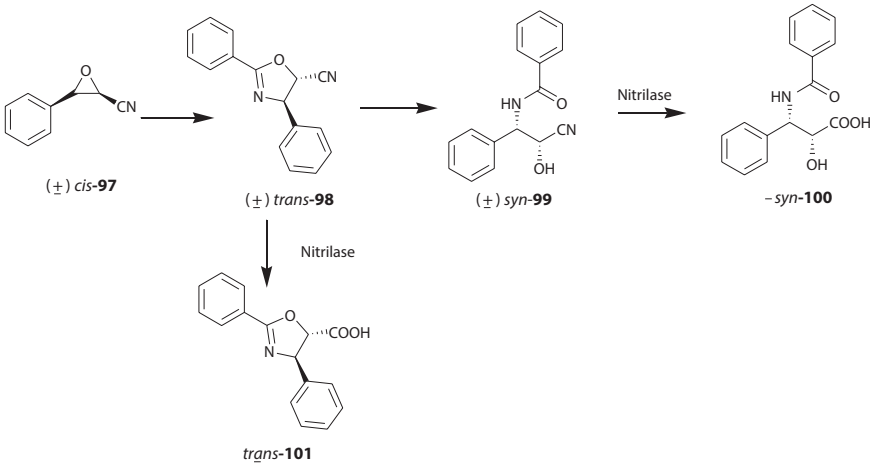
4.2.10 Chemoenzymatic Synthesis of Paclitaxel C_{13} Side Chain

Paclitaxel, sold under the trade name taxol, is a chemotherapy medicament used in anticancer treatment which includes ovarian cancer, gastric cancer, breast cancer, prostate cancer, etc [122]. It is a natural compound (first isolated from *Taxus brevifolia*) synthesized by coupling of N-benzoyl-(2*R*,3*S*)-3-phenylisoserine side chain with the baccatin III. Studies on structure activity relationship suggested that C-13 side chain [(2*R*,3*S*)-N-benzoyl-3-phenylisoserine] is mainly responsible for the pharmacological biological activity of taxol [123]. Various acylases, reductases, lyases, and lipases have been used for the preparation of precursors of C-13 side chain. Kayser *et al.*, in 1999, reported bioreduction of methyl 3-azido-2-oxo-3-phenylpropionate using whole Baker's yeast cells for the synthesis of C_{13} side chain of paclitaxel but the diastereoselectivity in the final product was very poor [124]. Therefore, to enhance the stereoselectivity bioreduction of α-Chloro-*β*-keto ester **94** (Scheme 4.22) was performed with 19 NADPH dependent reductases isolated *Saccharomyces cerevisiae* reductases and it was found that YDL124w produced *syn*-(2*S*,3*R*)-**95** as the only noticeable product while *syn*-(2*R*,3*S*)-**95** and *anti*-(2*S*,3*S*)-**95** in 90:10 was obtained using ADH YGL039w [125].

Klempier and coworkers examined the enzymatic hydrolysis of key intermediate (±)-*syn*-N-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, (racemic *syn*-**99**), and (±)-*anti*-N-(2-cyano-2-hydroxy-1-phenylethyl) benzamide, (racemic *anti*-**99**), by utilizing nitrilases for the preparation



Scheme 4.22 Preparation of (2*R*,3*S*)-N-benzoyl-3-phenylisoserine side chain **96**.



Scheme 4.23 Synthesis of taxol side chain by nitrile transforming enzymes.

of N-benzoyl-3-phenylisoserine side chain [126]. Chemically, (±)-*syn*-**99** and (±)-*anti*-**99** were prepared in a ritter type reaction of racemic *cis*- and racemic *trans*-3-phenyloxirane-2-carbonitrile **97** to dihydrooxazoles (±)-*trans*-**98** and (±)-*cis*-**98** followed by ring opening under acidic conditions. Authors tested four nitrile hydratases, out of which three were successful in hydrolyzing (±)-*syn*-**99** and its epimer (±)-*trans*-**99** (Scheme 4.23). During hydrolysis of (±)-*syn*-**99**, apart from nitrilase PRO-E0263 that gives only preferred carboxylic acid and only slight amounts (< 10%) of the amide by-product, other nitrilases such as NIT-111 and PRO-E0260 provided a significant amount of amide as the by-product. Nitrilase PRO-E0263 also furnished the desired carboxylic acid as the only product. The second side chain precursors (±)-*cis*- and (±)-*trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, **98**, were important molecules for all Co- and Fe-nitrile hydratases tested producing the amides in 30% to 100% yields. The specificity of the substrates for (±)-*cis*-**98** and (±)-*trans*-**98** fluctuate quite significantly as (±)-*cis*-**98** was accepted as substrate by 2 out of 23 nitrilases tested whereas (±)-*trans*-**98** was accepted by eighteen different nitrilases and out of which fifteen gave the desired carboxylic acid **101** as the major product.

4.3 Conclusion

The applications of chiral vicinal-amino alcohols have grown significantly due to the existence of this moiety in imperative structural components

of various bioactive molecules with possible pharmaceutical importance and in chiral ligands/auxiliaries. Asymmetric syntheses of these precursors remain a source of attraction in these days, and various strategies for their enantioselective preparation have already been developed. Hunt for new biocatalysts along with their genetic engineering form the basis of research for various scientific communities. Therefore, the development of new technologies/processes and their commercialization is the need of hour. Recent developments in one-pot processes involving combination of several (bio)catalysts undergo a remarkable progress in organic synthesis. Moreover, cascade processes involving several biocatalysts are mainly attractive because of mild environment friendly reaction conditions, escaping of intermediate extraction/product purification at each step, cost effectiveness, and waste reduction.

This chapter covered the significance of chiral 1,2-amino alcohols in bioactive molecules and as chiral ligands/auxiliaries. Moreover, chemoenzymatic strategies for the preparation of some drug molecules containing these structural components are also discussed.

Although tremendous attempts have been made in the past for the biocatalytic/chemoenzymatic synthesis of optically active 1,2 amino alcohols, the biocatalytic strategies toward enantiopure 1,2-amino alcohols still needs to be explored. The work presented here offers a valuable alternative for the preparation of several classes of compounds as biocatalysis is extremely regio-/stereoselective, reduces number of steps, and avoids protection/deprotection and metal catalysts. It is anticipated that the present endeavor will encourage scientific communities across the globe to observe incredible growth in the development of improved/novel enzymes and to come across more chemoenzymatic strategies toward amino alcohol moiety.

Acknowledgements

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5

1,4-Naphthoquinone: A Privileged Structural Framework in Drug Discovery

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Abstract

Naturally occurring naphthoquinones are known for their various biological and pharmacological activities and has been reported and studied intensely. In this chapter, we have gathered possible literature and representing brief and detailed account of biological activity profile of 1,4-naphthoquinones and their cognate hetero atom (O, N, and S) as well as halo (Cl, Br, and I), cyclic, noncyclic, and five or six membered rings against several pathogenic fungi, bacteria, viruses, plasmodium, leishmanial, and cancer. The literature available suggests that the biological action of 1,4-naphthoquinone is mainly related to redox cycling of naphthoquinone; however, hetero-substituted derivatives of 1,4-naphthoquinone modify the activity comparable to an extent. Quinones are known to have a similar electronic energy level with metals. This feature allows them to form redox isomers by intramolecular electron transfer; hence, quinones are used in many biological processes.

Keywords: 1,4-naphthoquinone, antifungal, antibacterial, antileishmanial, antimalarial, antiviral, anticancer

5.1 Introduction

Naphthoquinone pharmacophore is reported to possess potential antifungal [1], antibacterial [1], anticancer [2], antileishmanial [3], antimalarial

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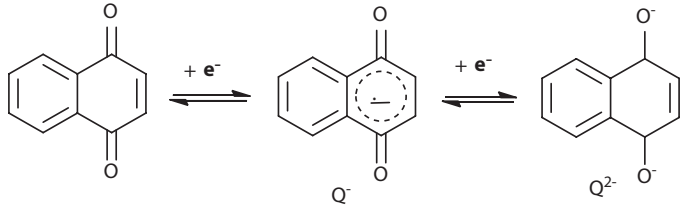

Figure 5.1 Various oxido forms of naphthoquinones.

[4], and antiviral [2] activities. Wide range of naturally occurring quinone extracted from various natural source plants [5], bacteria [6], fungi [7], and algae [8]. However, various synthetic routes, approach, and various synthetic methods have been employed to prepare, and mimic various quinone pharmacophore and has been effectively utilized and explored in drug and medicinal chemistry. Quinone plays vital role in electron transport in biological actions. Quinone-based compound are important model system for electron transfer transformations. In most of cases, pharmacological activity of naphthoquinone has been influenced by redox switching properties, their ability to accept one or two electrons to form corresponding radical anion or dianion. Various oxido forms of parent compound, i.e., 1,4-naphthoquinone is presented in (Figure 5.1). The quinone chemistry relies on either the substituents or variable functional groups being on the quinonic or on alternate rings. That imitates their chemical reactivity, largely in heterocyclic quinones. In the past decades, novel naphthoquinone derivatives containing hetero atoms in their structure were reported with these activities. The substituent present on framework of naphthoquinone modifies its activity [9]. Biological activity of naphthoquinones substituted with hetero atoms such as nitrogen, sulfur, and oxygen as well as the halogens and five or six membered heterocyclic ring are reported deeply. The present aim is to depict detailed account of the biological profile of naphthoquinone based on the literature available to the readers.

5.1.1 Overview

1,4-Naphthoquinone nucleus shows an inclusive range of biological and pharmacological applications. It has been documented for having engrossing and divergent reactivity [10, 11] the naturally occurring quinone and its derivative compounds are tempting synthetic or fabricated targets, due to their biological activity and their engagement in biological processes.

In this chapter, we have provided a detailed biological profile of naphthoquinone and substituted derivatives with respect to antifungal, antibacterial, anticancer, leishmanicidal, antimalarial, and antiviral activities. A large number of biologically active compounds related to quinone structure have been discussed briefly.

5.2 Various Targets of 1,4-Naphthoquinone for Its Actions

5.2.1 Bacterial Topoisomerase II-DNA Gyrase for Antibacterial Action

Type II topoisomerases are universal enzymes which plays a vital role in regulation of replicative DNA synthesis and share constructional and functional homology between various prokaryotic and eukaryotic organisms. This enzyme relieves the pressure as helicase unwound double-stranded DNA. This enzyme allows negative supercoiling of the DNA or relaxes positive supercoils. This is achieved by looping the template to form a crossing, then cutting one of the double helices and going through the other before releasing the break, altering the number of links in each enzymatic step by two. This process takes place in prokaryotes (especially bacteria), whose single circular DNA is cut by DNA gyrase and the two ends are then twisted to form supercoils [12].

5.2.2 Mammalian Topoisomerases I and II for Antitumor Action

Type I topoisomerase cuts a DNA double helix DNA strand, relaxation occurs, and the cut strand is then re-ligated. Type II topoisomerase breaks both strands of a double helix of DNA, passes through it another unbroken helix of DNA, and then re-ligates the cut strands. Both the enzymes are essential for DNA synthesis in mammals [13, 14].

5.2.3 HIV-1 Integrase and Proteinase for or Antiviral Action

For the unification of a double-stranded DNA copy of the viral RNA genome into a host chromosome and for HIV replication, HIV-1 integrase is necessary. HIV-1 protease is liable for the breakage of the big viral precursor polypeptides into functional proteins. Viral assembly and maturation can be blocked by inhibiting HIV-1 protease [15–17].

5.2.4 Dihydroorotate Dehydrogenase for Antimalarial Action

Ubiquinol oxidation is inhibited by the binding of 1,4-naphthoquinone to the cytochrome bc_1 complex's ubiquinol oxidation site, which prevents respiration in these parasites. While the bc_1 complex is vital for energy transduction in all eukaryotic cells, recent studies show critical function of bc_1 complex in *P. falciparum* during the erythrocytic stages of growth is to retain ubiquinone oxidized and therefore available as an electron acceptor for dihydroorotate dehydrogenase (Figure 5.2) [18]. The latter enzyme in the parasite is important for biosynthesis of pyrimidine.

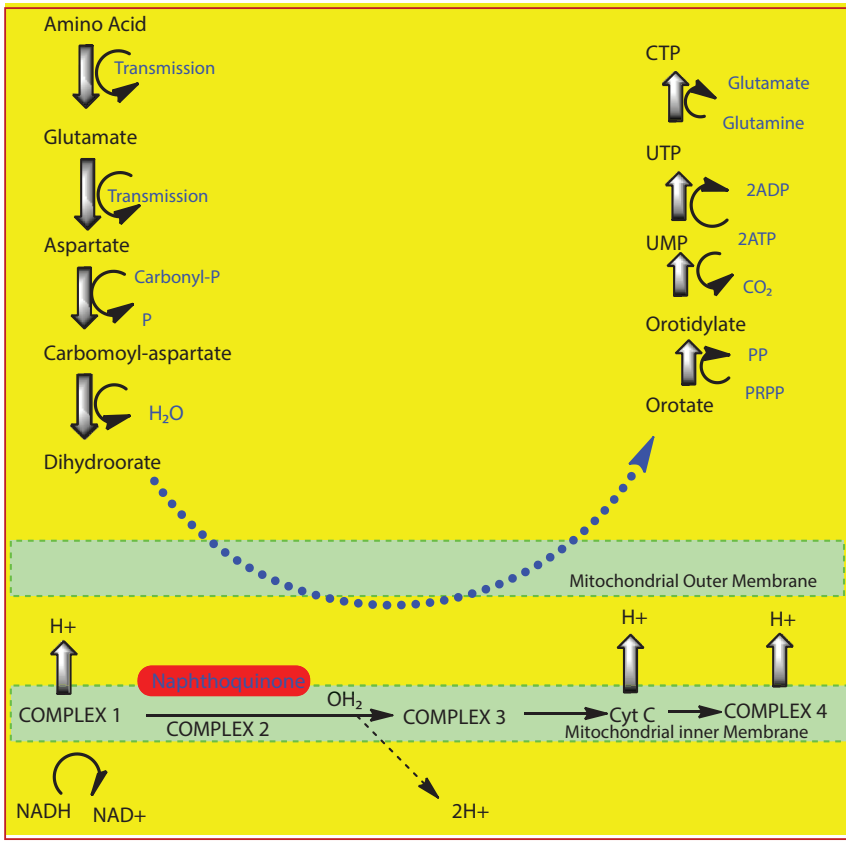

Figure 5.2 Flow chart DHODH dihydroorate dehydrogenase enzyme target in malaria (Ref. 18).

5.2.5 Trypanothione and Trypanothione Reductase (TryR) for Leishmanicidal Action

Dithiol trypanothione is the donor of DNA precursor synthesis derivatives, ascorbate homeostasis, hydroperoxide detoxification, and thiol conjugate sequestration/export [19]. Reduction of trypanothione disulfide is crucial to maintaining a reduced intracellular environment that makes the parasite specific trypanothione reductase an attractive target of the drug molecule [20–23].

5.2.6 Mitochondrial Cytochrome (Coenzyme Q) for Antifungal Action

Naphthoquinones are a semisynthetic antimetabolite of fungal mitochondrial cytochrome, coenzyme Q that is important in electron transport [24]. Coenzyme Q is a lipophilic protein in nearly all cellular membranes in the hydrophobic realm of the phospholipid bilayer [25, 26]. It performs a principle role in normal cell respiration and function, thus disrupting normal cellular functions by shortage of its availability or endogenous development.

5.3 Antifungal Activity

Antifungal activities from the various naturally occurring quinone and their associated hetro atom (O, N, and S), Halo, i.e., (Cl, Br, and I), cyclic, noncyclic, aromatic, etc., compounds have been demonstrated and reported extensively. Numerous review articles have been reported with antifungal profile of naphthoquinones. Ferreira *et al.* has reported a detailed review on naphthoquinones involving natural and synthetic derivatives against potent antifungal activities. Ferreira *et al.* demonstrated that larger no of compounds were evaluated against *Candida alibicans*. Their results demonstrated that more than 30 compounds were showing higher activities to those of currently used antifungal drugs. MIC values (μg ml^{-1}) of various antifungal drugs used have been presented in Table 5.1. And their corresponding molecular structure are presented in Figure 5.3. However, conclusion made by results demonstrates additional studies and new design needs to underline to understand the mode of mechanism behind antifungal activity [26]. Jali *et al.* have reported biological implication of naphthoquinones derivatives with various antifungal activities [27]. Wellington *et al.* studied antimicrobial properties of 1,4-naphthoquinone-2,3-bis-sulfides and 1,4-naphthoquinone sulfides to determine their antimicrobial properties against two bacteria and a fungus [28]. 1,4-naphthoquinone sulfides own

Table 5.1 MIC values (μg ml^{-1}) reported for various drugs against different species of fungi.

Fungi/Drugs	Mico	Nyst	Fluc	Itra	Amph	5-flurocytosine
A. niger	*	*	50	*	2.0	50
A. fumigates	*	*	2.0	*	0.50	*
C. cucumerinum	*	*	*	*	*	*
C. albicans	25	7.80	0.30	4.42	0.45	6.3
C. tropicalis	*	*	6.3	0.17	0.5	6.3
C. neoformans	12.5	3.50	0.75	*	0.78	12.5
C. kefyr	*	*	1.63	0.08	*	*
C. krusei	*	*	2.5	0.02	0.95	6.3
C. dubliniensis	*	*	1.63	0.08	*	*
C. parapsilos	*	*	1.0	0.70	0.4	*
S. schenckii	*	13.72	2.0	*	0.12	*
T. mentagraphytes	<0.78	*	1.0	*	0.422	*

*Activity not reported; Mico, Miconazole; Nyst, Nystatin; Fluc, Fluconazole; Itra, Itraconazole; Amph, Amphotericin B.

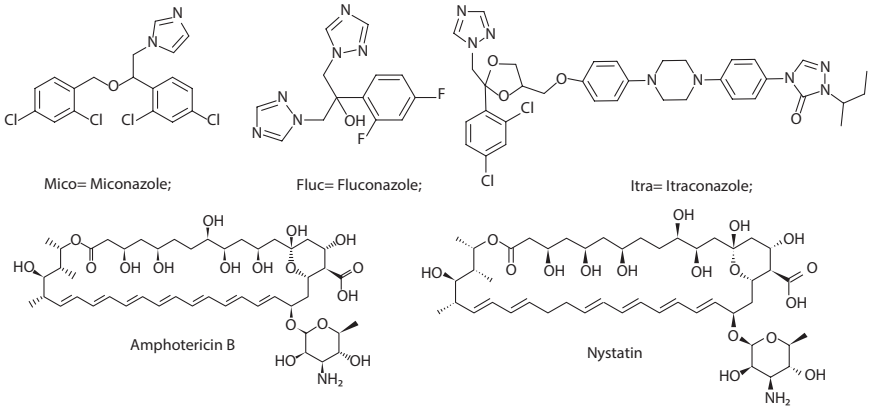

Figure 5.3 Various drugs for antifungal activities.

potent activity with minimum inhibitory concentration (MIC) of 7.8 μg/ml against *Staphylococcus aureus*, MIC of 23.4 μg/ml against *Candida albicans*, which was effective than Amphotericin B (MIC = 31.3 μg/ml), and an MIC of 31.3 μg/ml against *Escherichia coli*. 1,4-Naphthoquinone had an 11.7 μg/ml MIC against *S. aureus* and it also portrays same activity against *E. coli*.

Due to the pharmacological significance covered by three important natural naphthoquinones: β-lapachone (**1**), lapachol (**2**), and lawsone (**3**), curiosity in quinones and the quest for quinones with new biological activities has increased latterly [29]. Plumbagin (**4**) is a natural naphthoquinone that has been examined as an antifungal agent that can be isolated from plants belonging to the families Plumbaginaceae [9], Droseraceae, and Ebenceae. Bis-naphthoquinone (**5**), which has two moieties of naphthoquinones, was isolated from *Ceratostigma plumbaginoides* and showed strong activity against *C. albicans* ATCC 25555 (MIC 0.09 μg/ml and MFC 0.17 μg/ml) using fluconazole and ketoconazole as standard drugs [27].

Hybrid naphthoquinone-anthraquinone **(6)**, newbouldiaquinone A, was isolated from the root bark of *Newbouldia laevis* Seem root (F. Bignoniaceae). Newbouldiaquinone A antimicrobial activity against *C. albicans, C. glabrata,* and *C. krusei* has been evaluated. It revealed strong activities with *C. gabrata*. It is 13 times more active against *C. gabrata* than standard drug nystatin [30]. The deoxyshikonin (**12**), acetylshikonin (**13**), β-hydroxyisovalerylshikonin (**14**), and shikonin (**15**) naphthoquinones were isolated from the *Lithospermum erythrorhizon* (Boraginaceae) roots chloroform extract. These compounds have been tested against *C. albicans* YFC497, YFC803 (azole-resistant), *C. glabrata* YFC 501, *C. krusei* YFC 827, *C. tropicalis* YFC 052, and *C. parapsilosis* YFC 826. Shikonin was found to be four times more potent than fluconazole in fungicide activity (MIC 4 μg/ml). Molecular structure for compound 1 to 14 is represented in Figure 5.4.

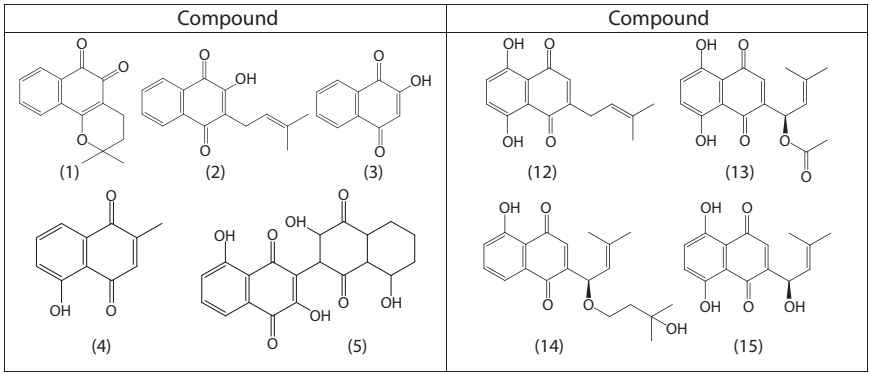

Figure 5.4 Molecular structure for compounds 1 to 14 (Ref. 29, 30).

5.4 Antibacterial Activities

Janeczko *et al*. [31] prepared some new 1,4-naphthoquinone derivatives **(16)** and estimated for antimicrobial activity against eight strains of bacteria (*Proteus*, *Escherichia*, *Klebsiella*, *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Salmonella*, and *Enterococcus*). All 1,4-naphthoquinone derivatives showed marked antimicrobial activity with MIC values ranging from 7.8 to 500 μg/ml. Most of the synthesized compounds exhibited the potent activities against *S. aureus*, with a high point of selectivity.

Riffel *et al*. [32] prepared some novel 1,4-naphthoquinones and evaluated for antibacterial activity against several Gram-positive and Gram-negative bacteria. The most active compound was 5-amino-8-hydroxy-1,4-naphthoquinone **(17)** with inhibition zones of 20 mm at 50 μg/ml against staphylococci, streptococci, and bacilli. Methicillin-resistant *Staphylococcus aureus* and several of this bacterium's clinical isolates were also inhibited. Naphthazarin **(18)**, 5-acetamido-8-hydroxy-1,4-naphthoquinone **(19)**, and 2,3-diamino-1,4-naphthoquinone **(20)** were the subsequent most active compounds. The MIC of the active compounds' MIC has been determined against *S. aureus*, between 30 and 125 μg/ml.

Yildirim *et al*. [33] prepared some novel sulfanyl-1,4-naphthoquinone derivatives and evaluated *in vitro for* antimicrobial activity against 7 bacterial strains (3 Gram-positive and 4 Gram-negative bacteria). The compounds **21** and **22** have been identified as having potent antibacterial efficacy against *S. epidermidis* (MIC of 4.88 and 2.44 μg ml^{-1}, respectively). Both compounds **21** and **22** toxicity was analyzed in detail to compare them with Cefuroxime. The compound 8 was equipotent than that of Cefuroxime. Moreover, three compounds **21**, **22**, and **23** showed outstanding antibacterial activity, and **21** and **22** were two-fold and four-fold more effective than the standard drug Cefuroxime, respectively.

Sharma *et al*. [34] prepared novel 2-amino-1,4-naphthoquinone derivatives (**24**) and evaluated for *in vitro* antimicrobial activity. All these compounds showed high antimicrobial activity against gram positive bacteria *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus aureus*, and gram negative bacteria *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Burkholderia cepacia.*

Iblis *et al*. [35] synthesized monosulfurated 2-chloro naphthalene-1,4-diones (**25**), monosulfurated 2-ethoxy-naphthalene-1,4-diones (**26**), disulfurated naphthalene-1,4-diones (**27**), and symmetrical bis-1,4-naphthoquinones (**28**, **29**) and evaluated for antimicrobial activity. (**25a**), (**26c**), (**27d**), and (**27f**) were found as promising antifungal and antibacterial agents.

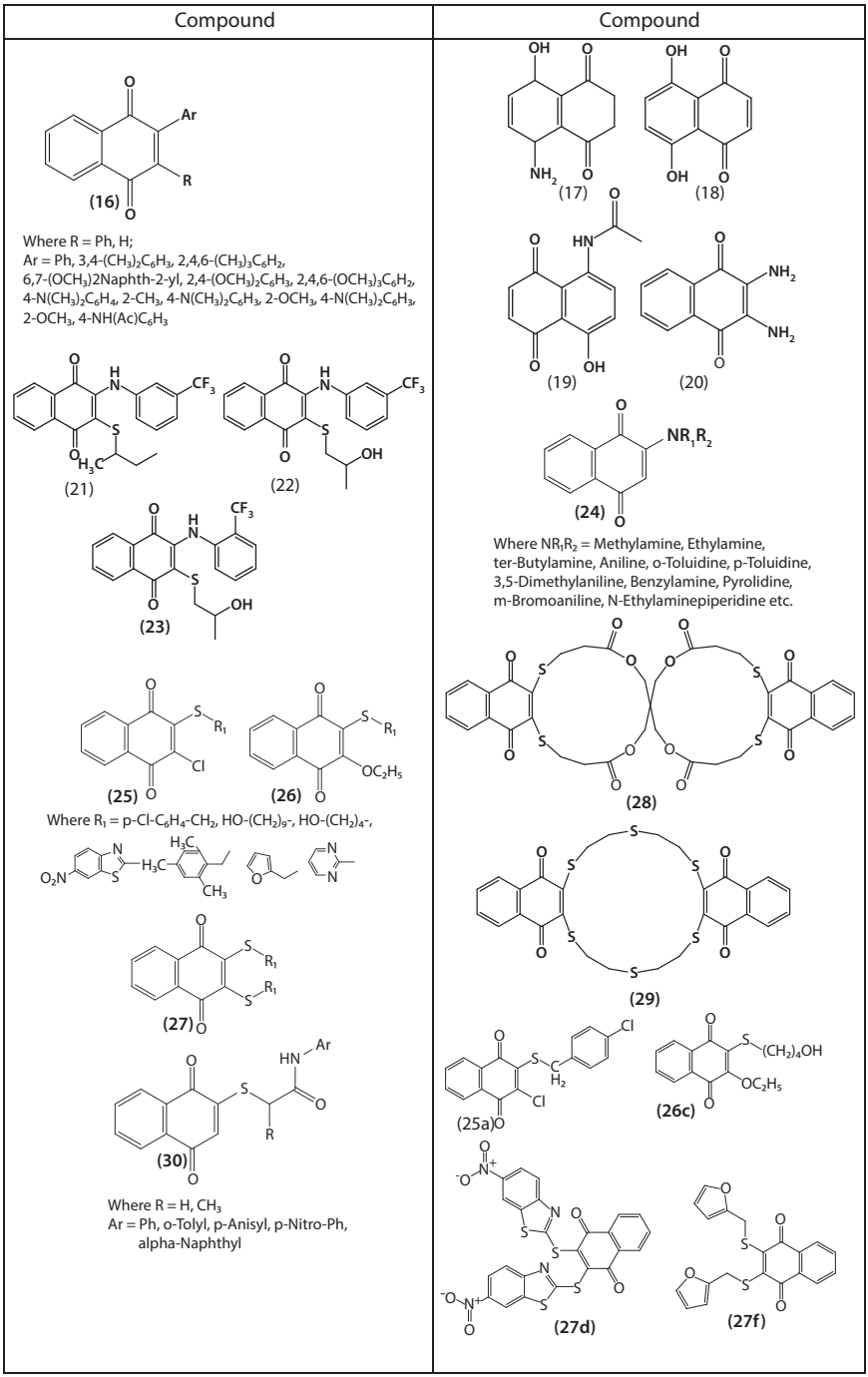

Figure 5.5 Molecular structure for compounds 16 to 30 (Ref. 10, 31–35).

Tandon *et al.* [10] synthesized a series of 1,4-naphthoquinone derivatives (**30**), tested for antibacterial activities, and discussed the structure-activity relationships (SARs) of these compounds. The data indicate that these compounds showed marked *in vitro* antibacterial activity. Important molecular structure for compounds (**16-30**) is presented in (Figure 5.5).

5.5 Anticancer Activity

Kumar *et al.* [11] firstly synthesized 3 naphthoquinones, i.e., lawsone (**31**), lapachol (**32**), and β-lapachone (**33**). In addition, derivatives of 2-alkyl and 2-benzyllawsone was prepared and assessed for anticancer activity. Four derivatives showed significant activity against cancer and the strongest analog, i.e., compound **34** showed potential activity (IC_{50} = 5.2 μM) against FaDu (Hypopharyngeal carcinoma) cell line. Compound **34** induced apoptosis by caspase activation and cell cycle arrest in FaDU cells in the S phase. It also showed marked topoisomerase-II inhibitory activity. Compound **34** in Swiss albino mice up to 1,000 mg/kg oral dose were found to be safe.

Da Cruz *et al.* [12] synthesized 1,2,3-triazole-, arylamino-, and thio-substituted naphthoquinones and examined against multiple human cancer cell lines (ovarian, blood, breast, colon, central nervous system, prostate cancers, and melanoma). Some compounds displayed IC_{50} below 2 μM. Non-tumor cells such as human peripheral blood mononucluear cells (PBMCs) and two murine fibroblast lines (L929 and V79 cells) have also been tested for the cytotoxic ability of the studied naphthoquinones. α-Lapachone- and nor-α-lapachone–based 1,2,3-triazoles (**35** and **36**) and arylamino-substituted naphthoquinones (**37**) showed potent anticancer activity against various cell lines of cancer.

Delarmelina *et al.* [13] synthesized a set of 2,3-(substituted)-1,4-naphthoquinones and assessed for their cytotoxic capacity on human triple-negative breast (MDA-MB-231), lungs (H460), and ovarian (A2780) cancer cell lines. Compounds **39** and **40** showed IC_{50} values of 1.60×10^{-5} mol L^{-1} and 2.16×10^{-5} mol L^{-1} for MDA-MB-231, compounds **38** and **39** showed IC_{50} values of 3.048×10^{-5} mol L^{-1} and 4.24×10^{-6} mol L^{-1} for H460; and **39** and **41** showed IC_{50} values of 3.89×10^{-6} mol L^{-1} and 2.68×10^{-6} mol L^{-1} for A2780. They performed a docking study showing the pharmacophoric conformation of these compounds against the therapeutic targets PI3K and topoisomerase II.

Wang *et al.* [14] synthesized a set of 1,4-naphthoquinone derivatives bearing a 2-*O*-alkyl-, 3-*C*-alkyl-, or 2/3-*N*-morpholinoalkyl group and assessed for their anticancer activity against 5 human cancer cell lines

SW480 (colorectal adenocarcinoma), HT29 (colorectal adenocarcinoma), HL60 (leukemia), HepG2 (hepatocellular carcinoma), and MCF-7 (breast adenocarcinoma) by the MTT assay. The most cytotoxic to these cell lines was found to be 2-hydroxy-3-farnesyl-1,4-naphthoquinone (**42**). Annexin V-FITC/propidium iodide staining detected cell death showed that compound **42** proficiently caused dose-dependent apoptosis of HT-29. Compound **42** efficiently prevents the proliferation of colon cancer cells and can be a strong anticancer agent.

Kanaan *et al*. [15] evaluated the *in vitro* effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) **(43)** on estrogen-positive MCF-7 and estrogen-negative MDA-MB-436 and Hs-578T human breast cancer cell lines. Further, the *in vitro* activity of this compound on apoptosis and cell cycle regulation were evaluated. DCDMNQ's effect on MCF-7, Hs-578T, and MDA-MB-436 cells showed substantial antitumor activities with IC_{50}s, of 0.6 ± 0.02, 3.1 ± 0.4, and 1.4 ± 0.25 μM, respectively. Cell cycle analysis indicated that in MCF-7 and MDA-MB-436 cell lines DCDMNQ prevented progression through the cell cycle in a time-dependent fashion. In the S-phase of the cell cycle, DCDMNQ arrested cells with the highest percentage of cells in the S-phase by day 5. This cell-cycle arrest was supported by DCDMNQ-induced inhibition of topoisomerase I. In addition, DCDMNQ mediated apoptosis time-dependent in both estrogen-positive and negative cell lines. Nonetheless, in the MDA-MB-436 cell line, the highest ratios of apoptotic cells were found.

Prachayasittikul *et al*. [16] synthesized and evaluated a series of 2-substituted amino-3-chloro-1,4-naphthoquinone derivatives against four cancer cell lines including HepG2, A549, HuCCA-1, and MOLT-3, for their anticancer effect. *m*-acetylphenylamino-1,4-naphthoquinone (**44**) with IC_{50} values of 4.758, 2.364, and 12.279 μM was found to be the most active cytotoxic activity against the cell lines HepG2, HuCCA-1, and A549. On the other hand, *p*-acetylphenylamino-1,4-naphthoquinone (**45**) displayed the most strong cytotoxic activity with an IC_{50} of 2.118 μM against the MOLT-3 cell line.

Pingaew *et al*. [17] prepared a new series of 1,4-naphthoquinone-sulfonamide derivatives and assessed for their anticancer and antimalarial activities. All 1,4-naphthoquinone-sulfonamides displayed a wide spectrum of anticancer activities against four cancer cell lines, i.e., A549, MOLT-3, HuCCA-1, and HepG2. Most compounds exhibited good anticancer activity than that of the reference compound etoposide against HepG2 cell lines. Compound **46** was the most effective cytotoxic compound without affecting normal cell. Compounds **47** and **48** were shown to be the most potent anticancer compounds.

Mallavadhani *et al*. [18] prepared 6 new 5,6-fused hybrids such as dihydrobenzofuran-quinone (**49a** and **49b**), benzofuran-quinone (**50a** and **50b**), and chromene-quinone (**51a** and **51b**) of juglone-based 1,4-naphthoquinones and evaluated for anticancer activity *in vitro* against 7 human cancer cell lines, i.e., breast (MCF-7, MDA-MB-453, and MDA-MB-231), cervix (ME-180 and HeLa), prostate (PC-3), and colon (HT-29) by using MTT assay. Outcomes displayed that most of the synthesized compounds showed substantial anticancer activity. Particularly, compounds **51a** and **51b** demonstrated potent activities against prostate and breast cancer cell lines respectively as the reference drug etoposide. Flow cytometric analysis exposed apoptosis induced by compounds **51a** and **51b** arrested the cell cycle in PC-3 and MDA-MB-453 cells respectively at the G2/M phase.

Kishore *et al*. [19] synthesized a set of 7-methyljuglone derivatives from 5-hydroxy-7-methyl-1,4-naphthoquinone (7-methyljuglone) and assessed for anticancer activity on four human cancer cell lines including MCF-7, HeLa, SNO, and DU145 by MTT assay. Most of them showed significant toxicity with lower IC_{50} values on cancer cell lines. The most effective derivative (**52**) displayed the toxicity with IC_{50} value of 5.3 and 6.8 μM on HeLa and DU145 cell lines accompanied by compounds (**53**) with IC_{50} values of 10.1 and 9.3 μM, respectively. The SAR shows that the fluoro substituents at position C-8 while hydroxyl substituents at positions C-2 and C-5 played a major role in cytotoxicity.

Ourique *et al*. [20] evaluated the antitumor effects of juglone **(54)** and Q7 **(55)** in combination with ascorbic acid (Vit. C). The study was conducted using Ehrlich ascites tumor-bearing mice. Samples of ascitic fluid were collected to evaluate the activity of antioxidant enzymes, carbonyl proteins, and GSH. GLUT1, proteins driving cell cycle (cyclin A, p16, and p53), hypoxia inducible factor HIF-1α, and apoptosis (Bcl-xL, Bax, and poly-ADP-polymerase PARP) were assessed by western blot. Tumor cells were categorized by cell cycle stage using flow cytometry and type of cell death using acridine orange/ethidium bromide. A glucose uptake analysis was conducted using liquid scintillation using Ehrlich tumor cells cultured with ^{14}C-deoxyglucose. Treatments resulted in increased protein carbonylation and antioxidant enzyme activity and decreased levels of GLUT1, HIF-1α, GSH, and glucose uptake in tumor cells. In G1, they also induced an increased number of tumor cells, p16 and p53 activation and decreased-cyclin A, but only when combined with Vit. C. In combination with Vit. C, compounds **54** and **55** caused inhibition of tumor progress *in vivo* by activating apoptosis and cell cycle arrest associated with glycolytic metabolism uncoupling, HIF-1 suppression and oxidative stress.

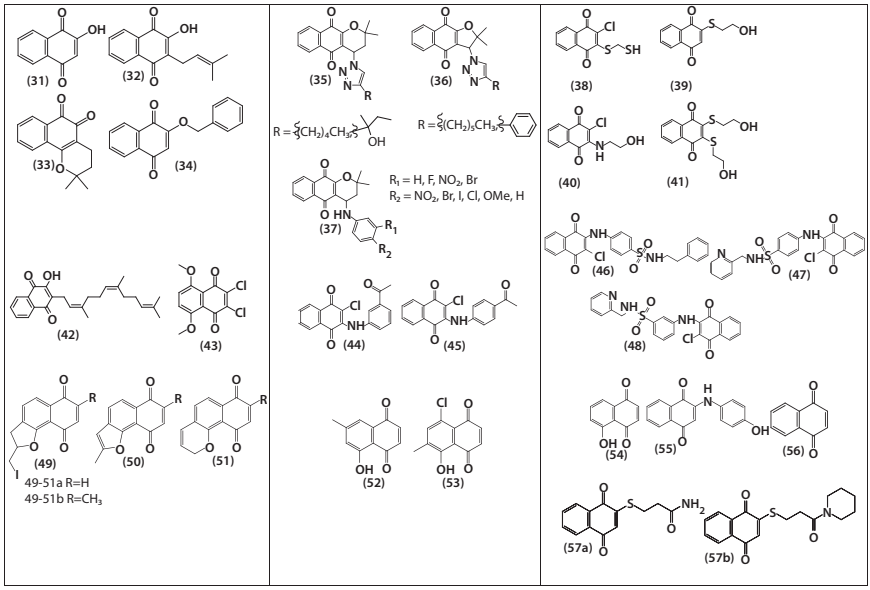

Figure 5.6 Molecular structure for compounds 31 to 57 (Ref. 1, 11–21).

Kayashima *et al.* [21] evaluated the antiangiogenic and anticancer effects of naphthoquinones and its analogs. Out of 13 compounds, one compound **(56)** strongly repressed both angiogenesis and human colon cancer cell (HCT116) growth. The effects of 1,4-naphthoquinone on the development of human umbilical vein endothelial cell (HUVEC) tube, proliferation and chemotaxis were studied to explain the antiangiogenic mechanism. Compound **56** stopped HUVEC functions.

Tandon *et al.* [1] synthesized some 2-substituted-1,4-naphthoquinones, S-(1,4-naphthoquinon-2-yl)-mercaptoalkanoic acid amides **(57)**, related benzoquinone and naphthoquinone derivatives, and 2,3-disubstituted 1,4-naphthoquinones and assessed for their anticancer, antibacterial, antifungal, and antiviral activities. Results showed that compound **57a** and **57b** showed good anticancer activities against Lymphoid Leukaemia P 388. Molecular structure for compounds (31-57) is presented in (Figure 5.6).

5.6 Antileishmanial Activity

De Araujo *et al.* [22] evaluated 2-N,N'-dialkylamino-1,4-naphthoquinones, 2-N-morpholino-, 2-N-thiomorpholino, 2-N-piperidino, and 2-N-(N^4-methyl)-piperazino naphthoquinones **(58a-n)** derived from nor-lapachol

and lawsone for *in vitro* leishmanicidal activity. The compounds having larger alkyl groups and N-methyl-piperazino moiety **(58d, 58h, 58i,** and **58k)** displayed toxic effects similar to the reference drug pentamidine. Though, the rest of the compounds from this series exhibited no harmful effect on the host cell. These cytotoxic compounds **(58d, 58h** and **58i)** had distinct leishmanicidal activity against *L. amazonensis* promastigotes, and treatments with six other compounds **(58d, 58e, 58f, 58h, 58k,** and **58n)** had significant leishmanicidal effect against *L. chagasi* promastigotes. In the assay against *L. chagasi* amastigotes, eight compounds **(58a, 58b, 58c, 58d, 58h, 58i, 58k,** and **58m)** showed significant activity. In addition, the compounds **(58a, 58b, 58c,** and **58m)** displayed effect against amastigotes of *L. chagasi* and not being toxic to the host cell.

Teixeira *et al*. [23] tested the *in vitro* and *in vivo* leishmanicidal activity of lapachol **(59)** and compared its efficacy with a standard drug, sodium stibogluconate. It was evaluated *in vitro* against intracellular amastigotes of *Leishmania* (Viannia) *braziliensis* (LVb), and *in vivo* in an animal model (hamster) to try to reproduce the leishmanicidal activity. *In vitro*, compound **59** showed an antiamastigote effect, whereas *in vivo* it did not stop the growth of LVb-induced lesions at an oral dose of 300 mg/kg/day for 42 days. Sodium stibogluconate confirmed a substantial antiamastigote effect *in vitro* for LVb and apparent clinical cure *in vivo* (60 mg/kg/day).

De Araujo *et al*. [24] synthesized a variety of eight substituted bis-2-hydroxy-1,4-naphthoquinones through lawsone condensation with various aromatic and aliphatic aldehydes under mild acidic conditions and evaluated for leishmanicidal activity *in vitro* against *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes; 6/8 compounds showed good activity without major toxic effects. The compound with the highest activity was used for an *in vivo* experiment with *Leishmania amazonensis*. Results showed that **60a, 60e,** and **60h** are leishmanicidal drug candidates.

Kayser *et al*. [25] synthesized a series of monomeric and dimeric naphthoquinones and evaluated for antileishmanial activity *in vitro* using both a direct cytotoxicity assay against extracellular promastigotes of *Leishmaniadonovani*, *L. infantum*, *L. major*, and *L. enriettii* and a test against intracellular amastigote *L. donovani* residing within murine macrophages. Numerous naphthoquinones demonstrated to be active and their EC_{50} values ranged from 0.9 to 17.0 mg/ml. Compounds **61** and **62**, dimeric naphthoquinones, exhibited the highest toxicity for intracellularly persisting *L. donovani* parasites with EC_{50} values of 15.0 and 14.2 μg/ml, respectively. The monomeric naphthoquinones **63**, **64**, **65**, and **66** also showed marked effects against promastigotes. Compounds with

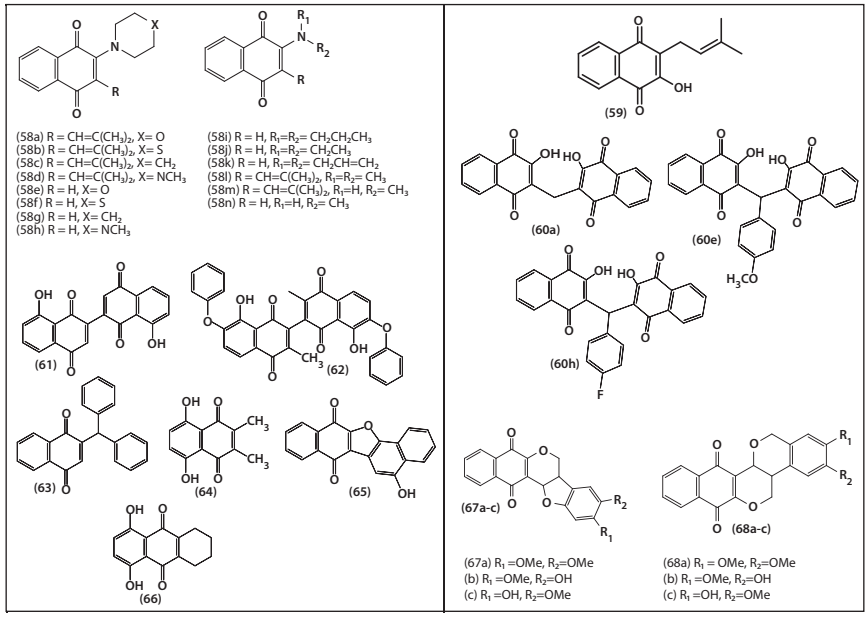

Figure 5.7 Molecular structure for compounds 58 to 68 (Ref. 22–26).

antileishmanial activity showed moderate (EC_{50} > 25 mg/ml) to pronounced (EC_{50} < 10 mg/ml) toxic effects when tested against a panel of human cancer cell lines (KB, SKMel, A 549, MDA) and murine bone marrow culture-derived macrophages (BMMF) as mammalian host cell controls. Pterocarpanquinones **(67a-c)** and a homologous series of derivatives **(68a-c)** were synthesized and evaluated on breast cancer cells (MCF-7) and on the parasites *Leishmania amazonensis* and *Plasmodium falciparum*, in culture. Compounds **67a-c** were more potent than **68a-c** on *Leishmania amazonensis* [26]. Molecular structure for compounds (**58-68**) is presented in (Figure 5.7).

5.7 Antimalarial Activity

Pingaew *et al.* [17] prepared a new series of 1,4-naphthoquinone-sulfonamide and evaluated for their antimalarial activities. The compound **69** with 6,7-dimethoxy groups exhibited the most potent antimalarial activity with IC_{50} value of 2.8 μM.

Kashyap *et al.* [36] prepared a novel series of quinoline-lawsone hybrid compounds **(70a-f)** and assessed *in vitro* for their antimalarial activity. The molecular properties of hybrid compounds were also studied *in silico* for

drug-likeness assessment based on Lipinski's rule of five. The study showed that all the compounds showed activity against both chloroquine sensitive (RKL-2) and chloroquine resistant (RKL-9) strains of *Plasmodium falciparum* substantially lower than the reference drug, Chloroquine. All the compounds demonstrated same degree of activity with IC_{50} values 0.391–1.033 μg/ml at the dose measured against sensitive strain. In addition, four compounds were also tested against resistant strain showed activity at the same dose with IC_{50} values ranged from 0.684 to 1.778 μg/ml. The IC_{50} values for chloroquine against sensitive and resistant strains of *P. falciparum* were found to be 0.0391 and 0.305 μg/ml, respectively. It is evident from the findings that the compound with a small alkyl bridge moiety diaminoethyl has better activity data against both sensitive and resistant strains (IC_{50} = 0.391 and 0.684 μg/ml, respectively) than the majority of the synthesized compounds.

Sharma *et al*. [37] prepared some new 2-hydroxy-1,4-naphthoquinone-4-hydroxyaniline hybrid mannich bases and assessed for their *in vitro* antimalarial activity. The design strategy of novel hybrid mannich bases involves fusion between pharmacophoric structures of lawsone and mannich substituted 4-hydroxyaniline. The study reveals that all the tested compounds showed some degree of *in vitro* antimalarial activity against chloroquine sensitive (RKL-2) strains of *Plasmodium falciparum* which was significantly less as compared to the reference drug, Chloroquine. Though, **71a** and **71f** showed superior activity than rest of the compounds. Compound **71f** was found to possess higher effectiveness than compound **71a** against chloroquine resistant (RKL-9) strains of *Plasmodium falciparum* which was considerably less as compared to the standard. Molecular structure for compounds (**69-71**) is presented in (Figure 5.8).

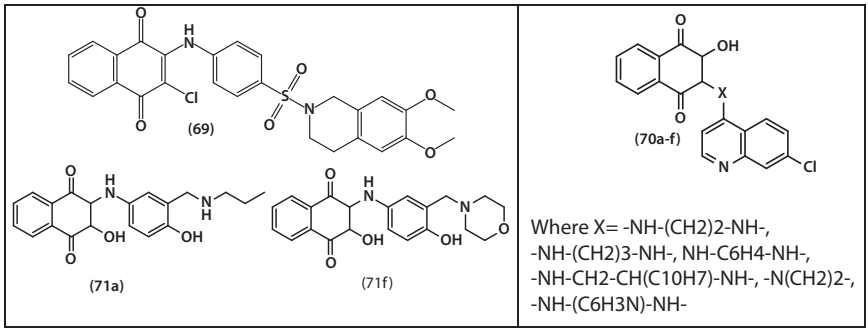

Figure 5.8 Molecular structure for compounds 69 to 71 (Ref. 17, 36, 37).

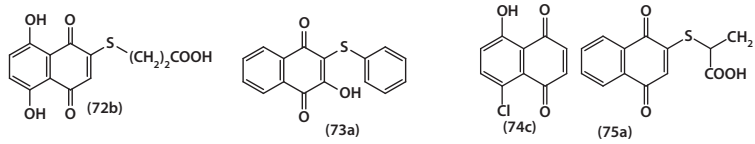

Figure 5.9 Molecular structure for compounds 72 to 75 (Ref. 10, 38).

5.8 Antiviral Activity

Tandon *et al.* [38] synthesized some 2-substituted-1,4-naphthoquinones **(72)**, S-(1,4-naphthoquinon-2-yl)-mercaptoalkanoic acid amides, related benzoquinone and naphthoquinone derivatives and 2,3-disubstituted 1,4-naphthoquinones (**73**) and assessed for their anticancer, antibacterial, antifungal, and antiviral activities. Results showed that compounds **72b** and **73a** exhibited *in vitro* antiviral activity against Influenza-A Virus and Herpes Simplex Virus.

Tandon *et al.* [10] prepared a set of 1,4-naphthoquinones, assessed for antibacterial and antiviral activities and SAR of these compounds were also discussed. Compounds **74c** and **75a** showed inhibitory effect against RNA dependent RNA polymerase induced poliovirus type 2 infected *HeLa cells.* Molecular structure for compounds (**72-75**) is presented in (Figure 5.9).

5.9 Conclusion

In summary, a large number of biologically active compounds have been discussed in relation to quinone structure. Naphthoquinones were found to be favorable group of compounds, as shown by the broad range of biological activities including antifungal, antibacterial anticancer, antileishmanial, antimalarial, and antiviral activities. 1,4-naphthoquinones are widely distributed in nature and have been used in traditional medicine since ancient times. Naphthoquinones chemistry depends on whether the substituent is on the quinonic or neighboring rings. This is expressed, particularly in heterocyclic quinones, in their chemical reactivity. Study shows that naphthoquinones substituted with heteroatom such as oxygen, nitrogen, and sulfur along with halogen and 5 or 6 membered rings, their derivatives results in the increase in activity as discussed. The mechanism of antimicrobial action of naphthoquinones reflected to redox cycling.

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6

Design and Synthesis of Spirobiisoxazoline Derivatives

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Abstract
Spirobiisoxazoline dibenzoquinone derivatives were synthesized starting from 2,5-dimethoxybenzaldehyde in a six-step synthetic sequence. The key step is [3+2] double 1,3 dipolar cycloadditions of oxime chloride with allenoate. This reaction was performed under mild reaction conditions using Na_2CO_3 at ambient temperature. This is the first innovative synthesis of the spirobiisoxazoline dibenzoquinone system where quinone ring is alkylated to isoxazoline moiety.

***Keywords*:** Spirobiisoxazoline, dibenzoquinone, oxime chloride, allenoate, [3+2] double 1,3 dipolar cycloadditions

6.1 Introduction

Cancer causes 12% of all deaths worldwide, and annually, more than 12 million patients are newly diagnosed with cancer and seven million deaths are recorded. The most common cancers are lung, liver, colon/rectum, and esophagus in men and breast, lung, stomach, colon/rectum, and liver cancers in women.

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Chemotherapy, one of the leading cancer therapies over other existing approaches such as radiotherapy, surgery, and targeted therapy, has been widely utilized for the last few decades. The process of carcinogenicity presents a major challenge to scientific research and industry and provides limited tools for its control activities. However, the indiscriminate cytotoxicity of most of the chemotherapeutic agents between cancer and normal cells often causes various frequent side effects like weakness, stroke, hair loss, bone and back pain, blood clots, and fatigue, and also some types of tumors are still difficult to be treated. These risk factors permit the search for potential new non-surgical practices that can either arrest the growth or kill cancer cells.

Natural products and their synthetic analogs are the most frequently used drugs for cancer treatment. Natural isoxazolines and spirocyclic isoxazoline derivatives have emerged as promising drug candidates due to their significant biological activities resulting in an overall antiproliferative effect on a variety of cancer cell lines. The natural product 11-deoxyfistularin-3 contains two spirocyclic isoxazoline moieties and is cytotoxic toward estrogen-dependent MCF-7 breast cancer cells (LD50 = 17 mg/L). Other closely related natural products such as Psammaplysin A, Aerothionin, Homoerotic-nin, Fistularin-3, and 11-Ketofistularin-3 (Figure 6.1) also have spirocyclic isoxazolines as a functional unit.

Quinones have attained great interest from a medical and toxicological perspective due to their unique reactivity and high prevalence in the environment. Natural and synthetic quinonoid compounds are well known for antitumor activity. Many drugs such as Daunorubicin, Doxorubicin

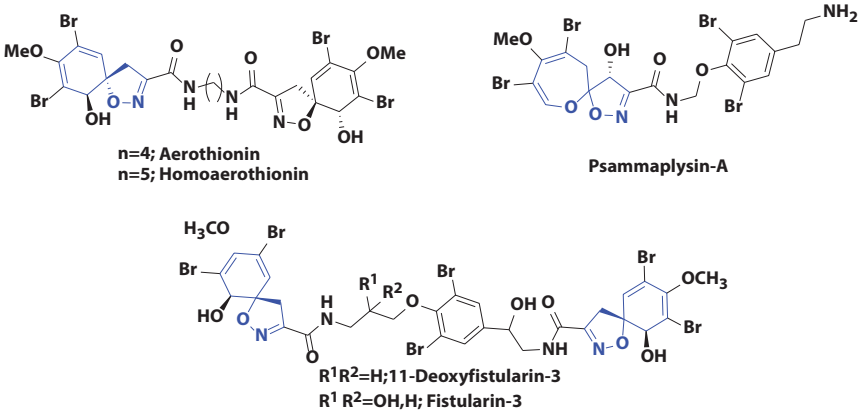


Figure 6.1 Spirocyclic isoxazolines.

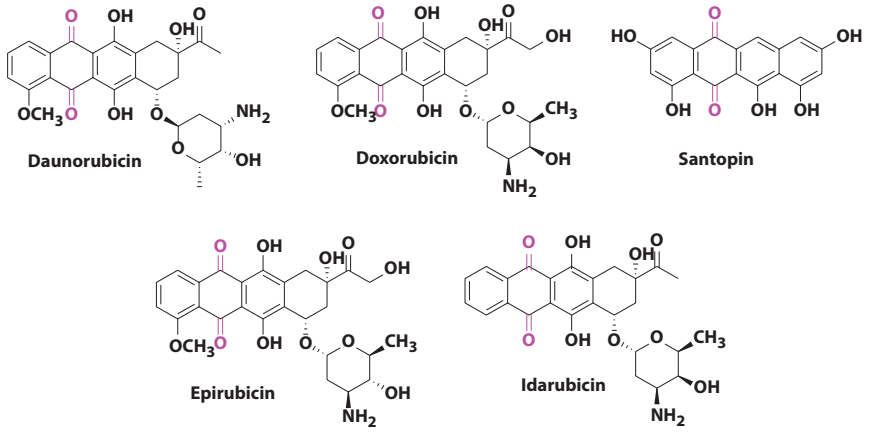


Figure 6.2 Quinone drugs.

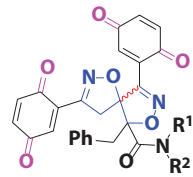


Figure 6.3 Spirobiisoxazoline dibenzoquinone derivatives.

(Figure 6.2), Epirubicin, Mitomycin, Mitoxantrone, and Saintopin are known for the treatment of solid cancers.

Encouraged by the diverse biological activities of spiroisoxazoline and quinone, the synthesis of a new group of compounds spirobiiosxazolines dibenzoquinone derivatives have been designed (Figure 6.3) *via* [3+2] double 1,3 dipolar cycloaddition of oxime chloride with allenoate.

6.2 Literature Review on Spiroisoxazolines

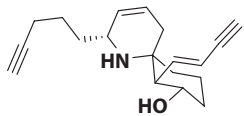
6.2.1 Chemistry

Compounds contain spiro systems endure fascinate significant attention due to their various biological activities. Spiro-heterocyclic compounds dwell in a unique place in the kingdom of natural and synthetic organic chemistry. Spirocyclic compounds have attracted the interest of the chemists for more than a century. In 1900, Bayer made the first spiran which

was called a bicyclic hydrocarbon connected by a single carbon. The ring planes are nearly perpendicular to each other [1] due to the tetrahedral nature of the spiro-linked carbon. Spirocyclic compounds have significant conformational features and structural insinuations for biological systems [2]. The presence of the sterical constraint spiro structure in various natural products also stems from the interest in the investigation of spiro compounds [3].

Carbocyclic and heterocyclic spiro compounds are the structural features of some natural products such as sesquiterpenes and alkaloids. Numerous spiro compounds have been isolated from plants and living organisms and they also serve as building blocks in drug preparation. Many nitrogen-containing spiro heterocycles have been found to play fundamental roles in biological processes and have exhibited important pharmacological activities.

The presence of chiral spiro carbon is one of the important criteria for the biological activities of spirocyclic compounds. The retention of neurotoxic properties of perhydrohistrionicotoxin **2** an analog of a natural product **1** is clear evidence of the role of spiro carbon in steering the biological activity [4].



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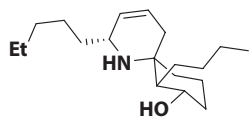


2

Five-membered ring systems containing one oxygen and one nitrogen atom adjacently are isoxazoles, isoxazolines, and isoxazolidines and are collectively referred to as isoxazole systems. Since 1898, the partially saturated systems isoxazolines are known in the literature. In the year 1960, more attention has been paid to these systems in the following order: 2-isoxazolines > 4-isoxazolines > 3-isoxazolines. In recent years, there has been increased attention drawn to the synthesis of isoxazolines as a new source of antibacterial agents. Isoxazoline derivatives have been reported to hold antifungal antibacterial, antitumor, antiinflammatory, antiviral, and anticancer activities. Spiroisoxazolines are the fused ring systems of isoxazolines and different heterocyclic or carbocyclic nuclei. Both isoxazoline and spiroisoxazoline scaffolds are featured in a series of natural and synthetic products with wide-reaching biological activities, as well as useful synthetic intermediates for organic synthesis [5].

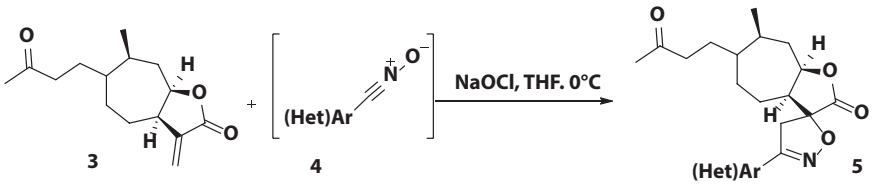
Spiroisoxazolines display interesting plant growth regulatory [6], herbicidal [7], and antitumor [8] activities. Antitubercular and antimicrobial activity [9] of some spiroisoxazoline derivatives have been reported recently. The results clearly show that spiroisoxazoline-based compounds can become drugs of immense use in the above-mentioned areas.

6.2.2 Previous Approaches

Accordingly, the spiroisoxazoline skeleton has been developed over the years *via* several cycloadditions/cyclizations. The strategy based on the cycloaddition of nitrile oxide is very viable and reliable by independent research groups.

1,3-Dipolar cycloadditions are an important type of cycloaddition reactions, in which regioselectivity, diastereoselectivity, and enantioselectivity must be considered. The regioselectivity is controlled by steric and electronic effects [10]. 1,3-Dipolar Cycloaddition was discovered by Curtius and Buchner in the 1880s [11] which involve the combination of 1,3-dipoles and dipolarophiles to generate five-membered rings.

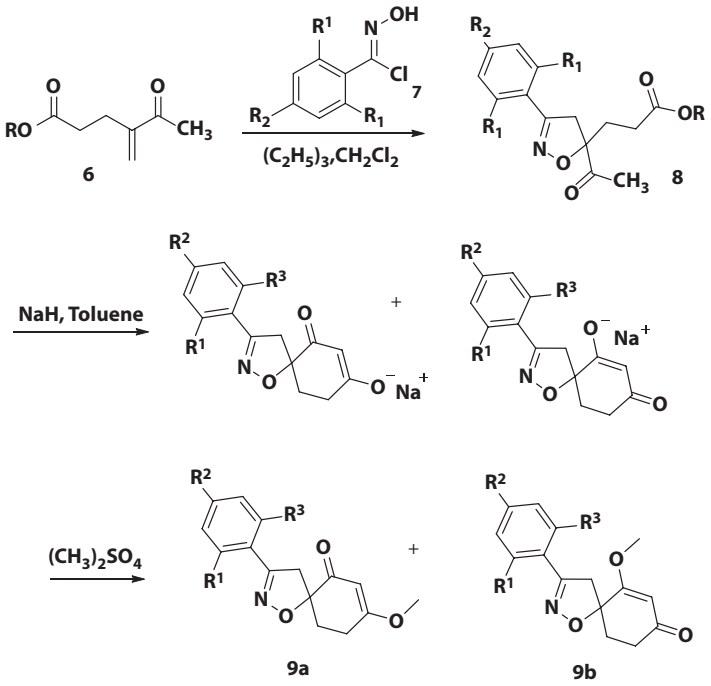
Mohamed Zaki *et al.* [12] synthesized spiroisoxazolines derivatives of tomentosin **5** from various nitrile oxide **4** with tomentosin **3** with exocyclic ring double bond through a 1,3-dipolar cycloaddition.



Nitrile oxides were prepared from various oximes from bleach and bleach was used initially to produce chlorooxime and induce dehydrohalogenation to give nitrile oxide. Experimentally, a bleach solution was dropwise added to a mixture of aldoxime and tomentose in THF at 0°C. Only one diastereoisomer was formed and it is isolated and characterized by NMR and mass analysis.

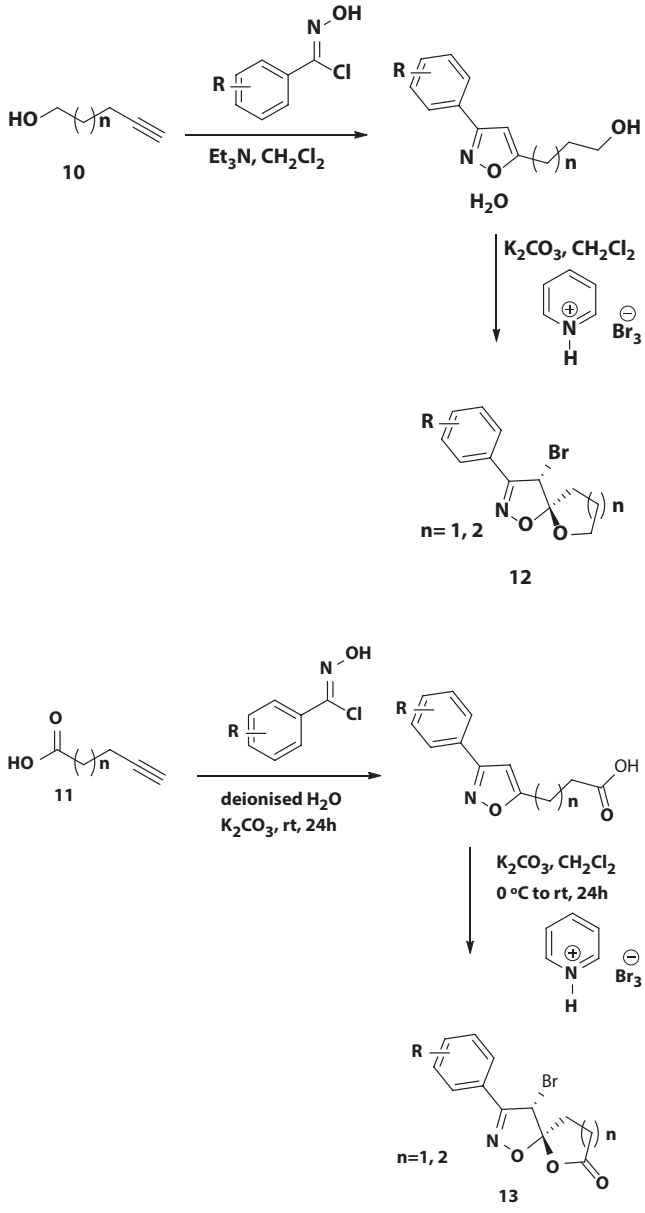
Erick D. Ellis *et al.* [13] reported the synthesis of spiroisoxazolines **9a** and **9b** in two steps starting from a disubstituted geminal alkene. The desired 5,5-disubstituted isoxazoline, **8**, was synthesized regioselectively through nitrile oxide-mediated 1,3-dipolar cycloaddition with a disubstituted geminal alkene, **6**, and chlorooxime, **7**. Regioisomeric spiroisoxazolines,

9a and **9b**, were constructed through an intramolecular cyclization and methylation synthetic sequence.



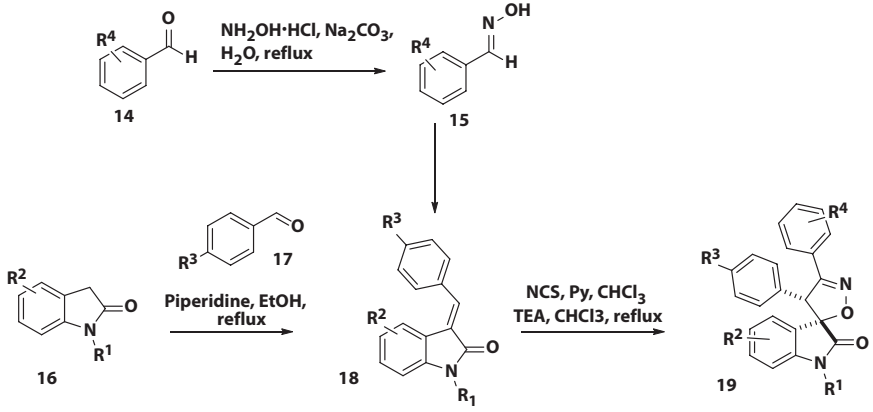
When isoxazolines were reacted with sodium hydride in toluene to get spiroxazolines by intramolecular cyclization and the corresponding enolates were methylated with dimethyl sulfate to afford the desired regioisomeric spiroisoxazolines **9a** and **9b**.

Prasanta Das *et al*. [14] synthesized a series of structurally diverse 4-Bromo spiro-isoxazolines **12** (furan-based) possessing a variety of aliphatic and aromatic substituents at the 3 positions *via* 1,3-dipolar cycloaddition followed by intramolecular cyclization of a pendant hydroxyl **10** or carboxylic acid group **11**. They have explored the synthesis of spiro-isoxazolines with an inherent lactone ring system **13**.

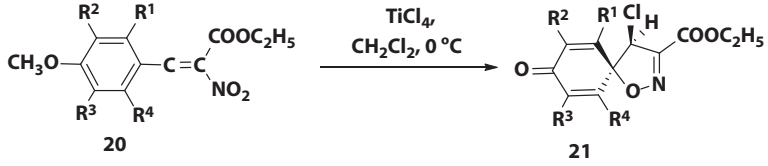


Carlos *et al*. [15] reported the synthesis and biological evaluation of 18 spiroisoxazoline oxindoles derivatives as p53–MDM2 interaction inhibitors. Screening of these compounds in a HepG2 cell line revealed that compounds with bromo or chloro at position 6 of the oxindole aromatic ring were more active than nutlin-3. Spiroisoxazoline oxindoles **19** were

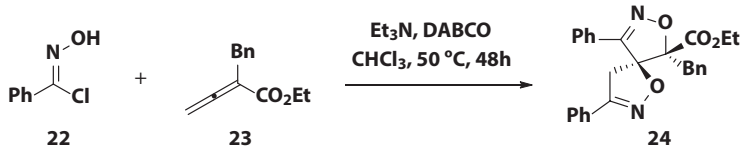
synthesized *via* 1,3-dipolar cycloaddition between 3-methylene indolin-2-ones **18** and nitrile oxides. Aldoximes **15** were synthesized by the reaction of NH2OH with the corresponding aldehyde **14**. Then, aldoximes were reacted with NCS to form chlorooxime which is directly added to olefine to form the final spirooxindole compound **19**. In these cases, the 1,3-dipoles nitrile oxide was generated *in situ* through dehydrohalogenation of the chlorooxime in the presence of a base like triethylamine. Intermediates were synthesized by aldolic condensation of substituted indolin-2-ones **16** with different aromatic aldehydes **17** and piperidine as a base.



Hirotani *et al*. [16] accomplished novel synthesis of spiroisoxazoline **21** from ethyl 3-ary-2-nitroacrylates **20** through titanium tetrachloride-induced *ipso* attack by oxygen of nitro group at 0°C in CH_2Cl_2 in good yields.



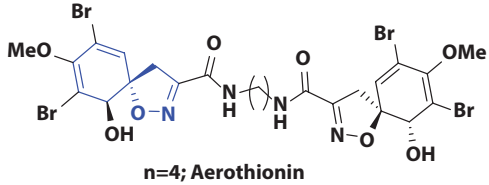
Zecchi *et al*. [17] demonstrated the synthesis of spirobiisoxazoline as a minor product *via* the cycloaddition between nitrile oxide and substituted allene. Xinye Shang *et al*. [18] synthesized spirobiisoxazoline **24** *via* the double 1,3-dipolar cycloaddition of nitrile oxide **22** with allenoate **23** in one pot. The reaction between oxime halides and 2-substituted beta-2, 3-dienoates was carried out in the presence of DABCO and Et_3N. The reaction sequence proceeded smoothly to furnish a wide range of spirobiisoxazolines in 55%–90% yields *via* a double [3+2]-cycloaddition.



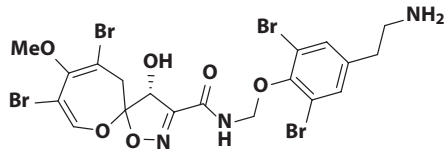
6.2.3 Biological Importance

Many synthetic and naturally occurring spiroisoxazolines exhibit biological activity against a variety of diseases, microorganisms, and enzymes. The spiroisoxazolines 11-deoxyfistularin [19] and pure alidin [20] be cytotoxic against cancer. Furthermore, other spiroisoxazolines are aerothionin [21], aplysinamisines I-III, and displays antifungal, antibiotic, or antimycobacterial activity.

Many spiroisoxazoline compounds within the psammaplysin and ceratinamide families have the potential to serve as a structural template that could lead to synthetic equivalents that target a variety of diseases.



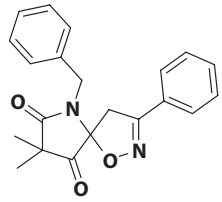
n=4; Aerothionin
n=5; Homoaerothionin



psammaplysin-A

A series of spiroisoxazoline natural products were isolated from marine sponges [22]. These spiroisoxazolines contain a spiro skeleton of an isoxazoline ring and a cyclohexadiene moiety. This skeleton results in the extensive structural diversity seen in this class of natural products [23] which exhibits various biological activities, such as cytotoxicity and anti-inflammatory properties [24]. Synthetic analogs of spiroisoxazolines have also been reported for the inhibition of Mycobacterium tuberculosis detoxification enzyme mycothiol-S-conjugate amidase [25].

The introduction of spiro-heterocyclic frameworks has stimulated specific interest among medicinal chemists. For example, the Ding and Kumar groups [26] recently reported spiro-isoxazoline and spiro-isoxazolidine derivatives of parthenin, a-santonin, artemisinin, and promising anti-cancer activities were obtained. Nigar Najim *et al.* [27] reported the screening of the potential anticancer and neuroprotective activities of novel compounds **25** and **26** against normal and cancer cell lines, including normal lung tissue (Hs888Lu), differentiated (SH-SY5Y), neuroblastoma (SH-SY5Y), lymphoma (U937), leukemia (HL-60), and lung cancer (A549). The results showed that **25** and **26** are not toxic to the neuron cells, and **26** were able to protect against cell loss induced by H_2O_2. Thus, this compound may have a role in the prevention of neurodegenerative disease. Since the compounds **25** and **26** are toxic only in U937 cells with IC50 values 119 and 92 μM, respectively, and then, this is very interesting as it may suggest a tumor-specific mechanism of action.



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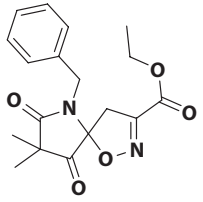
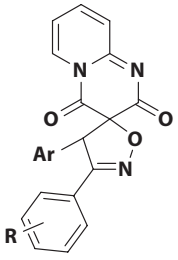


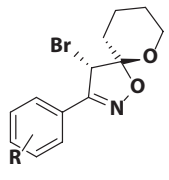
26

Abha Bishnoi *et al.* [28] synthesized compound **27**. Among the synthesized compounds, one having benzo[*d*] [1,3]dioxole as substituents exhibited excellent activity against both bacterial and fungal strains. The zone of inhibition of these substituents was quite considerable against the Gram+ve *S. aureus* (18 and 24 mm), *B. subtilis* (21 and 20 mm), and the fungus *C. albicans.*



27

Das *et al*. [14] reported substituted furan and pyran based spiro-isoxazolines **28** and **29** and efficacy as antiproliferative agents against breast cancer cell lines MCF-7(ER+), MDA-MB-231(ER–, PR-, HER2-), and the other two being the prostate cancer cell lines PC3, and DU-145 (both AR- and PSA-). Spiro-isoxazoline derivatives bearing a *p*-chloro or an *o*-dichloro aromatic substituent at the 3-position of the isoxazoline showed considerable antitumor activities in all four cell lines with an IC_{50} value ranging from 43 to 56 μM.



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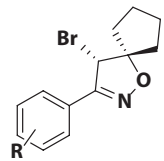
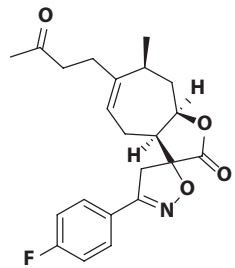


29

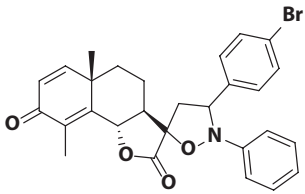
Zaki *et al*. [12] synthesized interesting potential spiro-isoxazolidine and isoxazoline derivatives of tomentose **30** *via* 1,3-dipolar cycloaddition of respective nitrones and nitrile oxides to the natural compound.



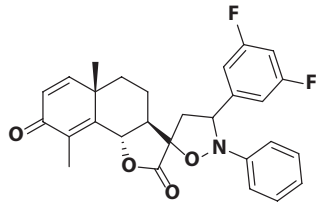
30

Khazir *et al*. [29] synthesized novel spiro-isoxazoline and spiro-isoxazolidine derivatives of α-santonin and tested for their anticancer activity against six human cancer cell lines. All the synthesized compounds were assayed for *in vitro* cytotoxicity against a panel of six human cancer cell lines including HCT-1 (colon), PC-3 (prostate), THP-1 (leukemia), MCF-7 (breast), Hep-2 (liver), and A549 (lung). Mitomycin, Adriamycin, and 5-FU were taken as reference compounds. From the IC50 values, it is clear that the majority of the compounds showed significant cytotoxicity against prostate, leukemia, breast, and cervix derived cancer cell lines. However, it may be noted that among all the tested spiro derivatives of a-santonin spiro-isoxazoline derivative and spiro-isoxazolidine derivatives **31** and **32**

showed comparatively more potent IC_{50} value against HCT-1, PC-3, and MCF-7 cancer cell lines.



31



32

6.3 Literature Review on Quinones

6.3.1 Chemistry

Quinones are a class of natural and synthetic compounds that have been used for several beneficial effects and endowed with rich and interesting chemistry. Quinones are abundant in nature and occur mainly in flowering plants and some fungi. Quinones are not aromatic but electrophilic Michael acceptor and stabilized by conjugation. Depending on the quinone and the site of reduction, the reduction can either rearomatize the compound or break the conjugation and conjugate addition always breaks the conjugation. Michael's addition of quinones with cellular thiols and amines made them more toxic to the cells. The knowledge of the essential chemical reactivity of quinones is relevant to understand their physiological and toxicological properties. Quinones have two properties that are essential for understanding their biological effects. First, quinones can undergo reversible oxido-reduction reactions and second many of them can undergo nucleophilic attack due to their electrophilic character.

Many drugs such as Daunorubicin, Doxorubicin, Epirubicin, Mitoxantrones, Mitomycin, and Saintopin are used for the treatment of solid tumors. The cytotoxicity of quinones is due to the inhibition of DNA topoisomerase-II [30]. However, the contributions of chemical reactivity and pathways of metabolism are difficult to understand due to the complex structure of the quinones. The quinoids undergo enzymatic reduction *via* one or two electrons to give the corresponding semiquinone radical or hydroquinone. The semiquinone radical anion gives its extra electron to molecular oxygen to give the parent quinone and superoxide radical anion under aerobic conditions. The reaction sequence was initiated by the bioreduction of the quinone followed by oxidation with dioxygen of the

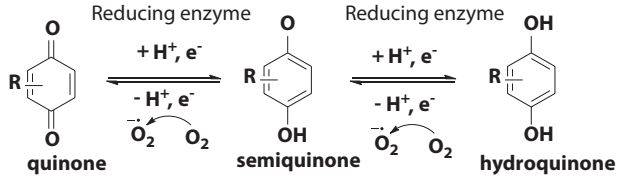


Figure 6.4 One- and two-electron reduction of benzoquinone. NQO1: NAD(P)H: quinone acceptor oxidoreductase.

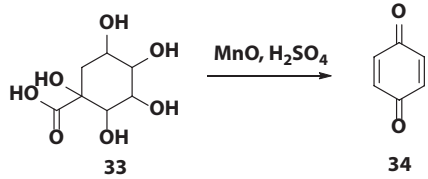
radical anion intermediate. This process is known as redox-cycling and it continues until the system becomes anaerobic. The hydroquinone formed in the redox cycle was excreted by the detoxification pathway. Both the semiquinone and the superoxide radical anion can produce the hydroxyl radical, which causes DNA strand break.

Quinones feature three readily accessible oxidation states, namely, fully oxidized quinone, one-electron reduced semiquinone, and two-electron-reduced hydroquinone (Figure 6.4), and they are capable of mediating both closed and open-shell redox processes.

6.3.2 Synthetic Approach

Based on the literature review, there are varieties of methods reported by various researchers for the synthesis of quinone and different quinone derivatives.

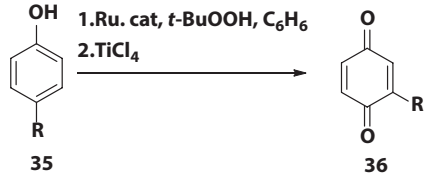
Pardasani *et al.* [31] reported the synthesis of benzoquinone **34** by the oxidation of quinic acid **33** with manganese dioxide and sulfuric acid. This reaction sequence involves dehydration, decarboxylation, and oxidation reactions.



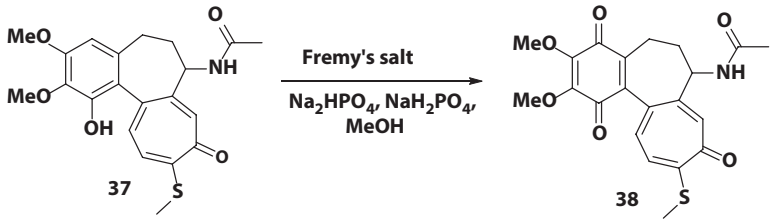
33 34

Murahashi *et al.* [32] reported the Ruthenium catalyzed the oxidation of para-substituted phenols **35**. Phenols were treated with *tert*-butyl hydroperoxide in benzene or EtOAc as a solvent and then treatment with $TiCl_4$ gives high yields (70%–80%) of 2-substituted benzo-1,4-quinones

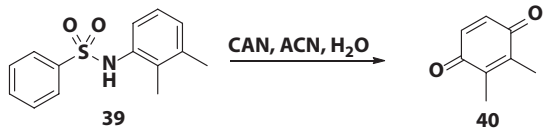
36 with the 4 substituent of the phenol migrating to the 2-position of the benzo-1,4-quinone.



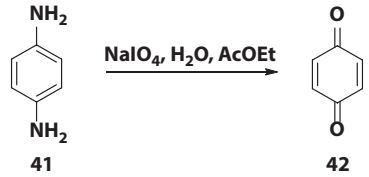
Guan *et al*. [33] reported the oxidation of the highly oxygenated aromatic ring of 1-demethylthiocolchicine **37** to the 1,4-quinone **38** by Frémy's salt.



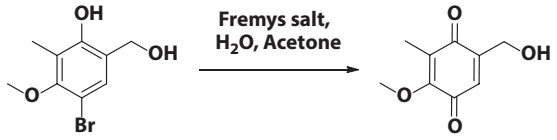
Hewson *et al*. [34] reported the oxidation sulphonamides **39** to benzoquinone **40** with CAN in acetonitrile and H_2O as an efficient oxidant system.



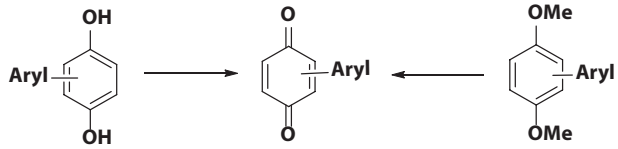
Telvekar *et al*. [35] synthesized quinone **41** from aryl diamines **42** using sodium periodate, ethyl acetate, and water at room temperature in good yield.



Lipshutz *et al*. [36] reported the oxidation of *p*-bromophenol **43** to benzoquinone **44** in acetone and water at rt in good yields.



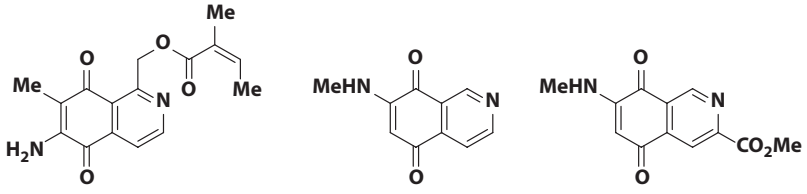
For oxidation of hydroquinones **45** to quinones **47**, oxidants utilized are polymer-supported Ru (II)/dm-Pheox complex in THF (yield 99%) [37], Cerium (IV)/SiO_2 reagent in DCM (yield 97%) [38]. 1,4-Dimethoxybenzenes **46** are also readily oxidized to benzo-1,4-quinones **37**; CAN in acetonitrile-water is the most commonly reported oxidant [39].



6.3.3 Biological Importance

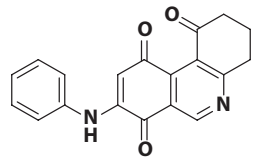
Quinones have attained great interest due to their unique reactivity and high occurrence in the environment. Natural and synthetic quinonoid compounds are well-known substances that possess a variety of biological properties such as antibacterial, antifungal, antiprotozoal, and antitumor activity.

Among the broad variety of *N*-heterocyclic quinones with anticancer activity, there are several examples of naturally occurring aminoquinones containing the isoquinolinequinone scaffold such as Cribrostatin **48**, Caulibugulone A **49**, and Mansouramycin C **50** [40].



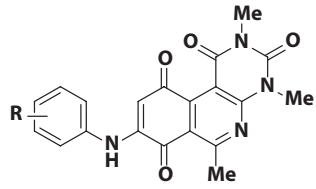
Valderrama *et al.* [41] synthesized phenylamino-3,4-tetrahydro phenanthridine-1,7,10 (2H)-trione derivatives **51** and screened using MTT colorimetric method *in vitro*, against normal cell line (MRC-5) and three human

tumor cells: AGS SK-MES-1 lung, gastric adenocarcinoma, and J82 bladder carcinoma. Most of the compounds exhibited promising cytotoxicity.



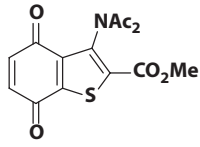
51

Vásquez *et al.* [42] synthesized 8-aminopyrimido[4,5-c] isoquinolinequinone derivatives **52** and were regioselectively synthesized. Cytotoxic activity was evaluated against one normal cell line (MRC-5 lung fibroblasts) and human cancer cell lines AGS, human gastric adenocarcinoma; SK-MES-1, human lung cancer cells, J82, human bladder carcinoma, and HL-60, human leukemia. Many compounds exhibited antitumor activity against human lung cancer cells and AG Shuman gastric adenocarcinoma.



52

Jaime *et al.* [43] reported the synthesis and *in vitro* leishmanicidal and anti-HTLV-1 activities of benzo- and naphtho[2,3-*b*]thiophene-4,7-quinones **53-55** containing the *ortho* aminoester functionality on the thiophene ring. These compounds showed moderate cytotoxic activity.



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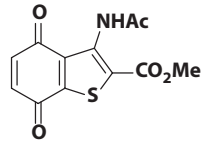

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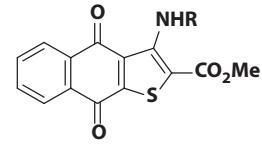
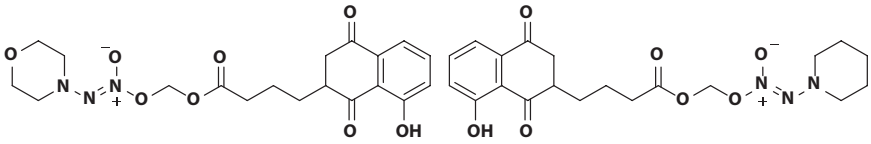


55

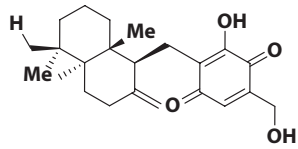
Bao *et al.* [44] synthesized 12 novel plumbagin hybrids and evaluated their inhibitory effects on human cancer cell lines (MDA-MB-231, HCT-116, A549, and HepG2 cells) and two normal human cells (HK-2 and WRL-68 cells). Compounds **56** and **57** exhibited superior potencies

compared to their parent compound (IC_{50} values of 3.48–6.68 mM) against the tested cancer cell lines and weak inhibitory effects on normal cells.



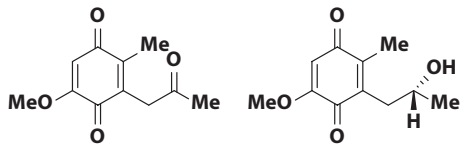
56 57

Wijeratne *et al*. [45] isolated sesquiterpene quinones (tauranin) **58** from *Phyllosticta spinarum* and *Platycladus orientalis*. Tauranin is reported to have apoptotic activity and antiproliferative toward several cancer cell lines.



58

Wang *et al*. [46] isolated new anserinone A **59** and B **60** from the liquid cultures of the coprophilous fungus *Podospora anserina*. They exhibited cytotoxic, antifungal, and antibacterial activities.



59 60

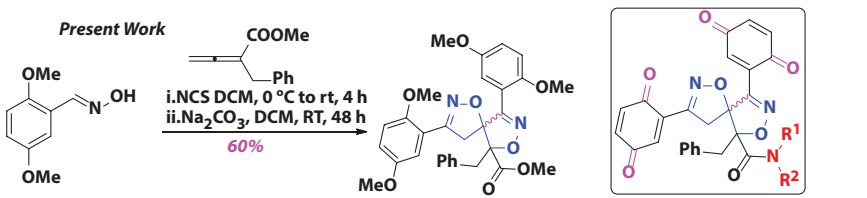
6.4 Review on 1,3 Dipolar Cycloadditions of Oxime Chloride With Allenoates

Several cycloadditions/cyclizations to approach the isoxazoline and spiroisoxazoline skeleton have been established over the years. One of these, the strategy based on the cycloaddition of nitrile oxide, has been revealed to be very feasible and trustworthy by many research groups [47]. Whereas, in the above reports, only limited reaction partners (alkenes) were reacted with nitrile oxides to construct the isoxazoline frameworks. Very limited reports are addressed on the formation of two rings *via* double-intramolecular 1,3-dipolar cycloaddition of diene for a spirocyclic compound [48]. Therefore, evolving further approaches is still highly desirable.

Many cycloaddition reactions of allenoates involved only carbon-carbon double bonds have been reported [49]. Zecchiet *et al.* (1976) reported the minor formation of spirobiisoxazoline from the cycloaddition between nitrile oxide and substituted allene. Guo *et al.* [50] have reported an efficient double 1,3-dipolar cycloaddition of allenoates with nitrile imines for the construction of spirobidihydro pyrazoles. Recently Xinye Shang *et al.* [51] addressed the double 1,3-dipolar cycloaddition involved DABCO combined with Et_3N, 2-substituted buta-2,3-dienoates reacted with oxime chlorides to afford spirobiisoxazolines.

6.5 Present Work; Spirobiisoxazoline

In recent years, the strategy employing to design new bioactive hybrid molecules through the conjugation of different pharmacophores to get compounds with more efficiency in biological activity became significant. Keeping because of diverse biological activities associated with spirocyclic compounds, it was thought to construct a novel system which may combine bioactive quinone and spirobiisoxazoline rings in a single molecular framework to see the additive effects toward their biological activities. Based on this strategy, we designed and synthesized new ***Spirobiisoxzoline Dibezoquinone Derivatives*** to become a significant drug candidate. For the synthesis of spirobiisoxazoline, we developed a simple and mild new methodology with inorganic base mediated [3+2] double 1,3 dipolar cycloaddition reaction of oxime chloride with allenoate.



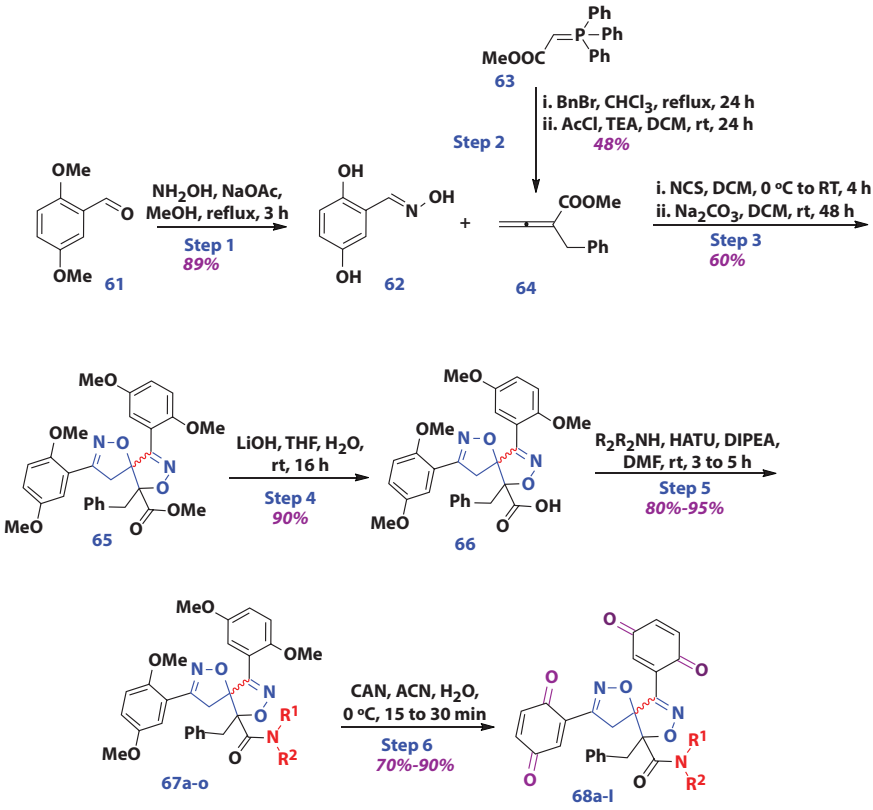
Spirobiisoxazoline Dibenzoquinone Derivarives

6.5.1 Results and Discussion

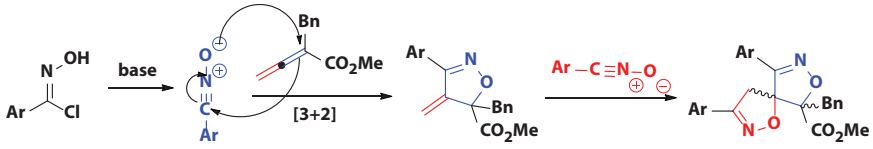
6.5.1.1 Synthetic Studies

For the synthesis of spirobiisoxazoline dibenzoquinone derivatives **196a-l**, 2,5-dimethoxybenzaldehyde **61** was reacted with NH2OH in MeOH at reflux afforded oxime **62** (**89%**). Oxime **62** was treated with NCS in DCM

afforded oxime chloride which on further treatment with allenoate **64** resulted in dimethoxy spirobiisoxazoline **65**. The synthetic strategy as depicted in Scheme 6.1. Therefore, a reactive intermediate nitrile oxide could be easily generated *in situ* from oxime chloride in the presence of Na_2CO_3 in DCM, and the nitrile oxide was expected to react with allenoate to furnish the desired dimethoxy spirobiisoxazoline **65** as a diastereomeric mixture (9:1, 58 %) by double 1,3-dipolar cycloaddition (Scheme 6.2).



Scheme 6.1 Synthesis of spirobiisoxazoline dibenzoquinone derivatives (**68a-l**).



Scheme 6.2 Reaction mechanism of double 1,3 dipolar cycloaddition.

In this investigation to optimize the reaction condition, the reaction of oxime chloride precursor with allenoate **64** was chosen as the model reaction with the use of Na_2CO_3 as the base, the reaction was performed in DCM at rt for 48 hours to give the corresponding spirobiiosxazoline in 60% yield (Table 6.1**, entry 5**). Further investigation on a combination of different solvents such as ACN, DCM, $CHCl_3$, and THF with different bases like Na_2CO_3, K_2CO_3, Cs_2CO_3, and NaOH indicated that the reaction media played an important role in the process (entries 1–16). An improvement of the yield was achieved when the reaction was carried out in DCM with Na_2CO_3 (60%) at rt for 48 hours. After that, the reaction with K_2CO_3 in $CHCl_3$ gave a good yield (52%), and the yields with remaining systems were not desirable. The reaction with NaOH as the base is poor yielding.

The base-catalyzed hydrolysis of compound **65** gave the key intermediate carboxylic acid derivative **66**, which on coupled with different aliphatic and

Table 6.1 Optimization of reaction conditions for spirobiisoxazoline.

S. no.	Solvent	Conditions	Yield
1	ACN	Na_2CO_3, RT, 64h	32%
2	ACN	Cs_2CO_3, RT, 48h	22%
3	ACN	K_2CO_3, RT, 48h	32%
4	ACN	NaOH, RT, 48h	10%
5	DCM	Na_2CO_3, RT, 48h	60%
6	DCM	Cs_2CO_3, RT, 48h	25%
7	DCM	K_2CO_3, RT, 48h	48%
8	DCM	NaOH, RT, 48 h	15%
9	$CHCl_3$	Na_2CO_3, RT, 48h	40%
10	$CHCl_3$	Cs_2CO_3, RT, 48 h	28%
11	$CHCl_3$	K_2CO_3, RT, 48h	52%
12	$CHCl_3$	NaOH, RT, 48h	12%
13	THF	Na_2CO_3, RT, 48h	25%
14	THF	Cs_2CO_3, RT, 48h	10%
15	THF	K_2CO_3, RT, 48h	10%
16	THF	NaOH, RT, 48h	traces

aromatic amines using HATU as coupling reagent afforded amide derivatives **67a-l** Table 6.1 (**85%–95%**). Oxidation of amide derivatives (**67a-o**) with ceric ammonium nitrate in ACN/water at 0°C resulted in spirobiisoxazoline dibenzoquinone derivatives **68a-l**; as yellow solids with very good yield (**70%–90%**) (Table 6.2). However, quinones **68m**, **68n**,

Table 6.2 Dimethoxy spirobiisoxazoline derivatives (**67a-o**).

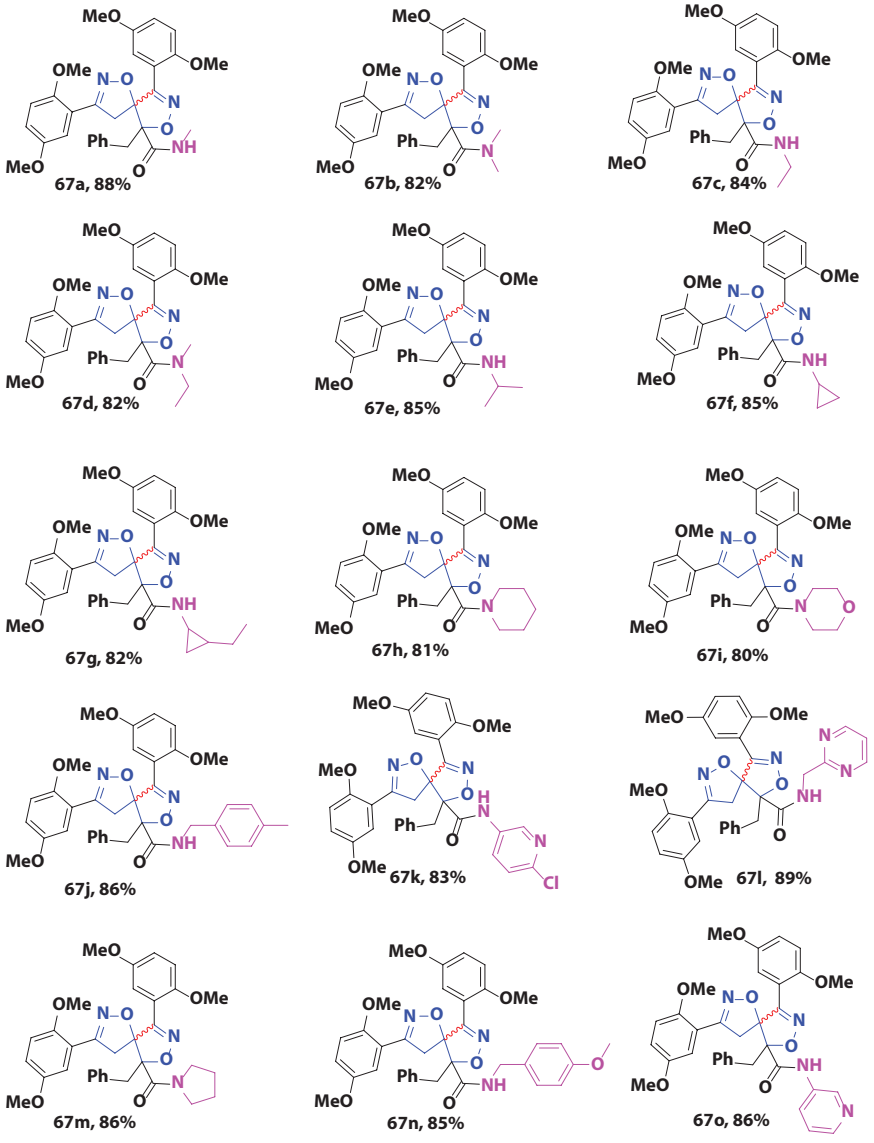
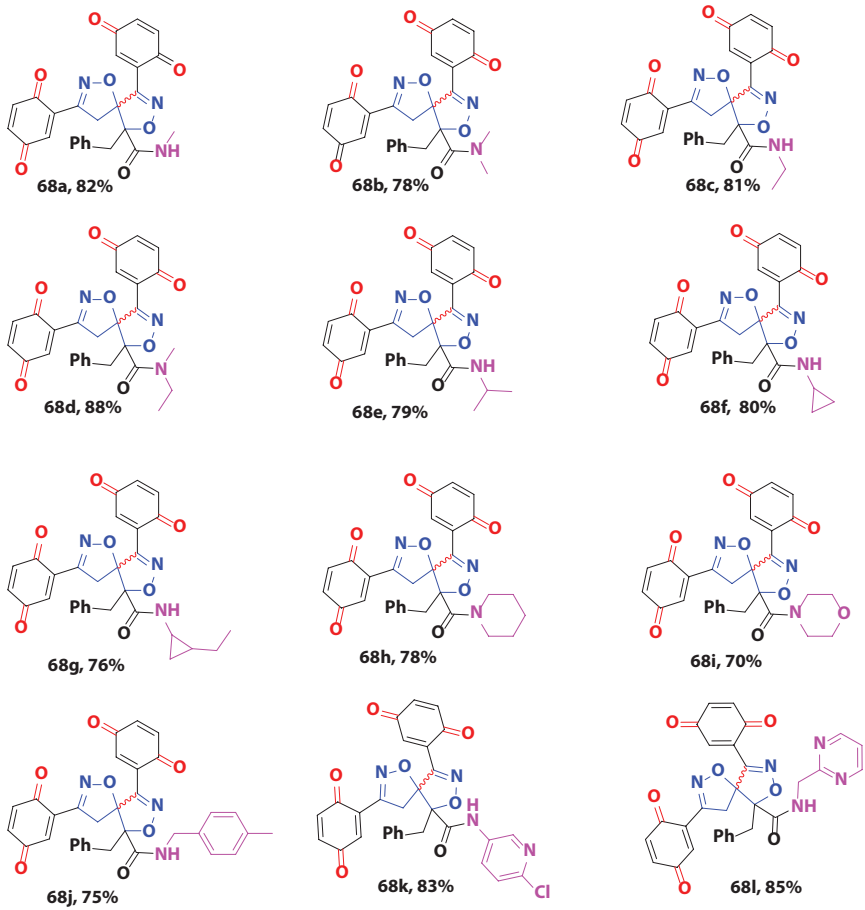


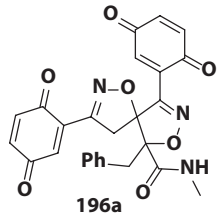
Table 6.3 Spirobiisoxazoline dibenzoquinone derivatives (**68a-l**).



and 68o obtained with some of the amides **m** (pyrrolidine) **n** (4-methoxybenzyl), and **o** (3-pyridyl) were unstable even at 0°C, and thus, we were unsuccessful in isolating these compounds (Table 6.3).

6.5.1.2 Spectral Analysis

The compounds were elucidated by FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectral (HRMS) analyses. To assign the FT-IR bands and NMR signals, compound **68a** has been chosen as a representative.



196a

FT-IR and Mass Spectral Analysis

The FT-IR spectral data indicate the presence of characteristic functional groups in the title compounds. For all novel compounds **68a-l** synthesized, a strong absorption band appeared around 1,710 and 1,558 cm^{-1} is due to the presence of quinone and amide carbonyl groups. All the observed IR bands are in support of the evidence for the expected functional groups present in synthesized compounds **68a-l**. The observed m/z values of all compounds **68a-l** are in good agreement with the proposed molecular formula.

^{1}H NMR Spectral Analysis

The ^{1}H NMR spectra of compounds **67a-o and 68a-l** have been recorded in $CDCl_3$ and analyzed. The signals were assigned based on their position, multiplicity, and integral values. Compound **67a** is chosen as a representative compound to explain the spectral features of the **67a-o** compounds. The ^{1}H NMR spectrum of compound **67a** was recorded at 400 MHz. A sharp four singlets, with three proton integrals, appeared at 3.77–3.74 ppm and are claimed for the 12 dimethoxy protons of the phenyl ring. The 11 protons as multiplet between 7.31 and 6.83 ppm are corresponding to phenyl rings. The protons at 3.99–3.95 (d, J = 18.8 Hz, 1H), 3.67–3.63 (d, J = 18.4 Hz, 1H), 3.43–3.31 (d, J = 13.2 Hz, 1H), and 3.06–3.02 (d, J = 13.2 Hz, 1H) are of $-CH_2$ protons of benzyl and spirobiisoxazolines. Compound **68a** is chosen as a representative compound to explain the spectral features of the **68a-l** compounds. The ^{1}H NMR spectrum of compound **68a** was recorded at 400 MHz and it was confirmed by the disappearing of four singlets, with three proton integrals appeared at 3.83 and 3.89 ppm. The multiplets between 7.32 and 6.25 ppm are corresponding to a total of 11 protons are assignable to the phenyl rings of spirobiisoxazoline dibenzoquinone derivatives.

In the ^{13}C NMR spectrum of compound **68a**, the signal observed in the far downfield region at 186.2 and 184.6 ppm is assigned to the four

quaternary carbonyl carbons in the quinone ring. The carbon signal at 165.9 is due to the resonance of the carbonyl carbon of the amide group. The ^{13}C shifts found at 153.6 and 153.0 ppm are attributed to quaternary carbons of the spiroisoxazole nitrogen attached quaternary carbons. Phenyl and quinone sp^2 carbons are observed at 136.9 and 127.4 ppm. The resonances of $-CH_2$ of isooxazoline and chiral carbons are observed at 100.5 and 94.7 ppm, respectively. –Benzyl $-CH_2$ is at 39.5 ppm and methyl carbon at 26.4 ppm is observed.

6.5.2 Experimental Section

Synthesis of 2,5-dimethoxybenzaldehyde oxime 62: To a solution of 2, 5 dimethoxy benzaldehyde **61** (16g, 96.38 mmol, 1 eq) in MeOH were added $NH_2OH.HCl$ (115.65 mmol, 1.2 eq), and catalytic NaOAc (0.1 eq), heated to reflux for 5 hours. The progress of the reaction was monitored by TLC analysis. The solvent was evaporated and the crude compound was quenched into ice-cold water and solid precipitated out was filtered to afford oxime **190** as pale yellow solid (15.5 g, 89 %).

Methyl 6-benzyl-3,9-bis(2,5-dimethoxyphenyl)-1,7-dioxa-2,8-diazaspiro [4.4]nona-2,8-diene-6-carboxylate 65: To a solution of oxime **62** (6 g, 33. 14 mmol, 1 eq) in DCM (60 ml) was added NCS (33. 14 mmol, 1 eq) at 0°C and stirred at rt for 4 hours. Subsequently, this solution of chloro oxime was slowly added to allenoate **64** (16.57 mmol, 0.5 eq) and Na_2CO_3 (66.29 mmol, 2 eq) dissolved in DCM (60 ml) and stirred at rt for 48 hours. Once starting material consumed (monitored by TLC, LCMS), the mixture was concentrated and the residue was purified through flash column chromatography (EtOAc/hexane) to afford dimethoxy spirobiisoxazole **65** as an off-white solid (7.4 g. 42 %).

6-Benzyl-3,9-bis(2,5-dimethoxyphenyl)-1,7-dioxa-2,8-diazaspiro[4.4]nona-2,8-diene-6-carboxylic acid 66: To a solution of dimethoxy spirobiisoxazole **65** (6.7 g, 12.24 mmol, 1 eq), in THF (60 ml) and water (30 ml) was treated with LiOH (24.49 mmol, 2 eq) at rt. The reaction mixture was stirred at rt for 16 hours. Later, THF was evaporated under vacuum, diluted with water (10 ml) and acidified with 20% hydrochloric acid, the solid precipitated out was filtered and then dried to give dimethoxy spirobiisoxazole acid **66** as a white solid (6 g, 92%).

General Procedure for Amide Coupling (67a): To a solution of dimethoxy spirobiisoxazole acid **66** (300 mg, 0.566 mmol, 1 eq), in dry DMF (3 ml) were added HATU (0.849 mmol, 1.5 eq), DIPEA (1.68 mmol, 3 eq) and corresponding amine (1.1 eq) and stirred for 4–5 hours at rt. Then, the

reaction mixture was diluted with cold water and the solid precipitated out was filtered and dried to afford amide derivative **67** as a pale brown solid.

General Procedure for Oxidation with CAN (68a): To a solution of amide **67** (200 mg, 0.366 mmol, 1 eq), in ACN:H_2O in 1:1 (6 ml) was added ceric ammonium nitrate (1.83 mmol, 5 eq) at 0°C and stirred at rt for 30 min. The reaction mixture was diluted with water and the solid precipitated out was filtered and dried to give quinone derivative **68** as a colored solid (based on the amide **67** used).

6.6 Conclusion

We synthesized a series of novel spirobiisoxazoline dibenzoquinone derivatives *via* [3+2] double 1,3 dipolar cycloaddition reaction in very good yields. We developed a facile, efficient, one-pot method for the synthesis of these derivatives from oxime chloride with allenoate. Further, a literature survey also reveals no report on the synthesis of spiroxazoline with the dibenzoquinone novel nucleus yet so far. Hence, spiroisoxazoline scaffolds are featured in a range of natural and. synthetic products with wide-reaching biological activities and useful synthetic intermediates for organic synthesis; spirobiisoxazoline dibenzoquinone–based compounds can become drugs of immense use.

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7

Potential of Metal Complexes for the Treatment of Cancer: Current Update and Future Prospective

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Abstract

Cancer is the second most common cause of death in humans worldwide after cardiovascular diseases, for which chemotherapy is one of the most effective treatment strategies. The discovery of platinum-based drugs as a chemotherapeutic regimen has greatly improved the cure rate for cancer. However, the growing resistance of cancer toward existing platinum drugs led to exploration of alternative non-platinum metal complexes with minimal side effects. In this context, many target specific metal-based complexes have been synthesized by redesigning or tailoring the entirely new complex with enhanced cytotoxicity profile while simultaneously reducing toxicity. The cytotoxic potency of a metal complex is strictly dependent on choice of ligand framework; therefore, metals incorporated in a ligand scaffold hold promise for the improved pharmacokinetic profile and better efficacy of drugs. This chapter aim to focus on designed metal-based anticancer complexes and their molecular target to give a strategic outlook to highlight the need for next-generation drug with minimized toxicities in clinical cancer care.

Keywords: Cancer, coordination complexes, anticancer agents, cisplatin, platinum anticancer drugs, DNA, cancer cell lines, organometallic complexes

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7.1 Introduction

Cancer is a chronic disease in which cells undergo uncontrolled growth, invasion, and sometimes proliferation in distant organs (metastasis), leading to death [1]. World Health Organization (WHO) estimated 18.1 million diagnosed cases of cancer in 2018 globally, which is anticipated to increase to 29.4 million by 2040 [2]. Significantly, the global burden of cancer has increased, accounting for one in six deaths worldwide. Basically, surgery, chemotherapy, radiations, and immunotherapy are the traditional and most widely used treatment modalities for cancer. However, the most viable treatment is chemotherapy that involves use of anticancer drug either alone or in combination to block the unwanted proliferation of cancer cells.

Metal-based drugs are well-established chemotherapeutic agents existed for decades in the first-line therapy to fight against wide variety of different cancer phenotypes [3–5]. Metals endowed with unique characteristics such as redox activity, variable coordination modes, and reactivity toward several organic substrates. In fact, metals incorporated in a bioactive ligand scaffold turned out to be as potential anticancer drugs with improved pharmacokinetic profile and better efficacy. Besides metal-ligand synergistic effect, ligands play undeniable role in biological activity, ranging from outer-sphere recognition of the target site to anticancer activity of their own. Hence, synthesis and evaluation of novel anticancer metal-based drugs is still one of the most active fields in medicinal inorganic chemistry [6–8]. Metal-based therapeutics received recognition from ancient Egyptians, Chinese, and Greeks who have utilized metal drugs of gold and arsenic to cure many diseases [9, 10]. While, the modern era of medicine was ushered with the discovery of arsenic-based organometallic complex, arsphenamine (Salvarsan), a potent antibiotic used to treat syphilis, by German Nobel laureate Paul Ehrlich [11]. Undoubtedly, the field of medicinal inorganic chemistry has gained prominence by the serendipitous discovery of an archetypical inorganic anticancer drug-cisplatin, (cis-diamminedicholroplatium(II) [cis-$(NH_3)_2PtCl_2$]), CDDP by B. Resenberg in 1965 [12]. CDDP was the first platinum (Pt) drug with anticancer activity approved by the U.S. Food and Drug Administration (FDA; 1978) which represented a milestone in the field of anticancer drug development. Presently, it is used alone or in combination with other systemic chemotherapeutic drugs against wide range of cancers including bladder, testicular, cervical, metastasized ovarian, and nonsmall lung cancer. It is also used to treat malignant mesothelioma, squamous cell carcinoma of the head and neck, neuroblastoma, tumors of the brain, and esophageal cancer [13]. The mechanism for the cytotoxicity of CDDP is thought to be based

on the formation of platinum-DNA adduct, which consequently activates apoptosis (Figure 7.1) [14]. The square-planar geometry of CDDP facilitates ligand substitution reaction (aquation) accompanied by replacement of chloride ligand by a water molecule. CDDP is administered directly into the bloodstream, where the Cl ion concentration is high (~100 mM) resulting in suppression of aquation reaction [15]. However, in the cytoplasm where the Cl ion concentration is below 20 mM, the hydrolysis of CDDP occurs efficiently, substituting one or both chloride ions by water molecules and resulting in the formation of aquated CDDP, cis-$[Pt(NH_3)_2Cl\,H_2O)]^+$. This positively charged aquated CDDP attracts the polyanionic nuclear DNA, leading to formation of Pt-DNA adducts especially via 1,2-intrastrand cross-link at N7 of purine bases, guanine, and adenine [16, 17]. The formation of these Pt-DNA adducts distort structure of helical DNA in a substantial manner, ultimately leads to cancer cell apoptosis.

Although CDDP is most widely used anticancer drugs, but due to Pt center, it not only binds with DNA but also with donor atom containing proteins, particularly to sulfur and seleno amino acids. It is therefore claimed that <10% of CDDP binds covalently to DNA, whereas 75%–85% binds to various proteins resulting in "off-target" severe side effects including nephrotoxicity [18, 19], neurotoxicity [20], ototoxicity [21], myelosuppression, and vomiting. Thus, it is not surprising that to overcome these limitations of CDDP and to broaden the range of treatable cancers, several Pt analogs have been evaluated clinically as anticancer agents in chemotherapeutic regimens (Scheme 7.1).

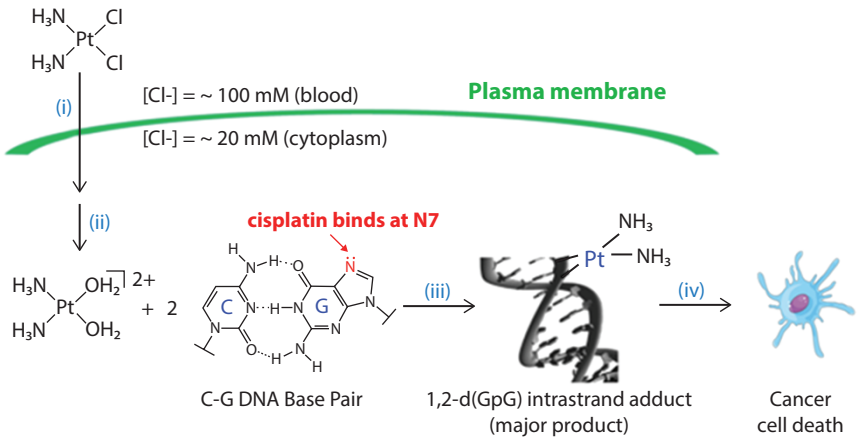
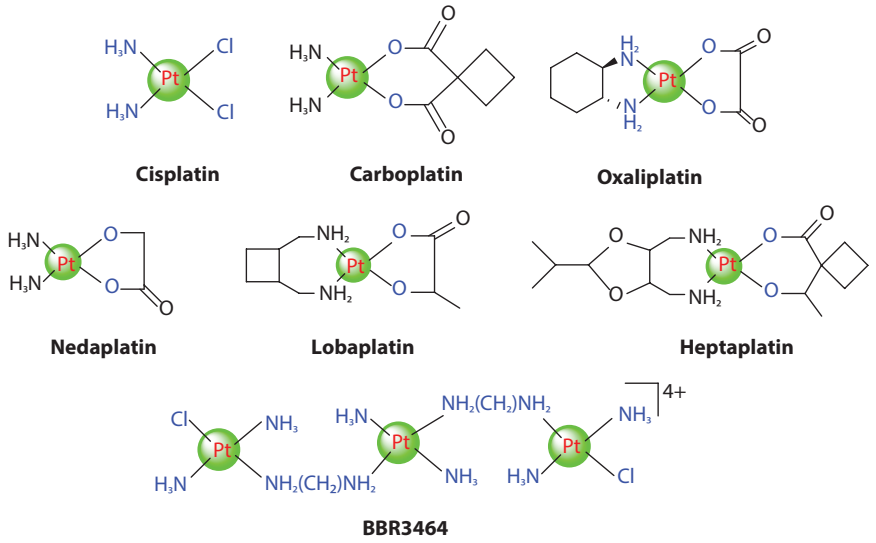


Figure 7.1 Stepwise mechanism of action of cisplatin (i) cellular uptake, (ii) aquation via ligand substitution reaction, (iii) formation of DNA adduct, and (iv) apoptosis.



Scheme 7.1 Platinum(II)-based anticancer drugs.

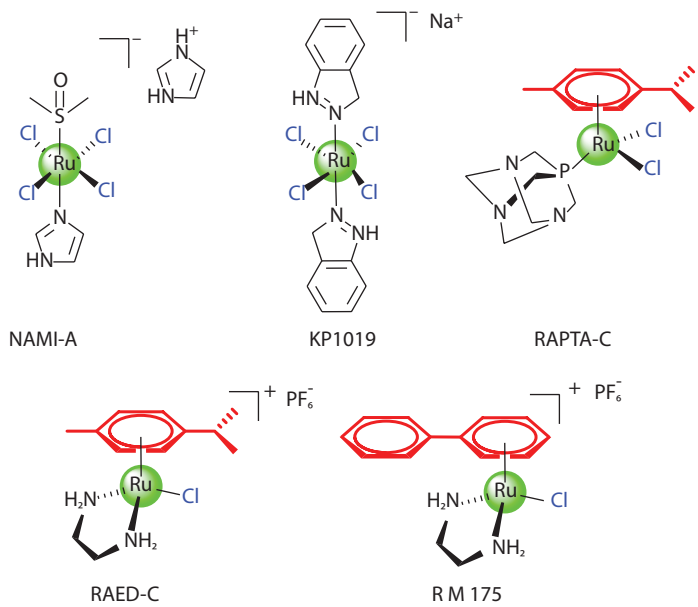
In order to increase the effectiveness and to reduce the systemic toxicities of platinum drugs modulation in the ligands framework was carried out by various eminent research groups which led to the development of second- and third-generation platinum derivatives. Carboplatin, second-generation platinum drug, is less toxic than CDDP due to its dianionic biscarboxylato leaving group, which results in slower rate of aquation. Carboplatin is used primarily for the treatment of ovarian cancer as well as cancers of head and neck, endometrium, cervix, testes, breast, lung, and bladder [22]. Further, advanced or metastatic colorectal cancers are treated with oxaliplatin, the third-generation Pt derivative. Furthermore, multinuclear platinum complexes are new class of anticancer agents different from their mononuclear counterparts in terms of distinct DNA binding pattern. Among such complexes, BBR3464 is a trinuclear platinum complex, in which Pt centers are linked by an alkanediamine chain, and is in preclinical evaluation particularly for cisplatin resistant cancer cell lines [23]. However, only three platinum containing drugs, namely, cisplatin, carboplatin, and oxaliplatin have gained worldwide approval for treating cancers in human, while three others, nedaplatin, lobaplatin, and heptaplatin received regional approval for clinical use specifically only in Japan, South Korea, and China, respectively. Several mechanistic studies showed that carboplatin and cisplatin exhibit similar kind of adduct formation with DNA via covalent bonds with the N7 of purine bases, thereby causing interference in normal transcription and DNA replication mechanisms

eventually leading to cell death. Conversely, oxaliplatin works through a distinctive mechanism of action in which instead of cell death through DNA damage, it induces ribosome biogenesis stress [24]. However, the mechanism of action of nedaplatin is associated with the inhibition of selenoenzyme thioredoxin reductase (TrxR). Recently, several findings have shown that the mechanism of action of these platinum metallodrugs is associated with their ability to modulate the immune system [25, 26].

Another promising approach includes nonconventional Pt(IV) complexes, which act as prodrugs due to their diverse pharmacological properties such as liophilicity, stability, and reduction potential which make them kinetically more inert and less reactive toward biomolecules. Pt(IV) complexes have ability to get activated to Pt(II) inside the cancer cells where they interact with DNA. Iproplatin, tetraplatin, ormaplatin, and satraplatin are few Pt(IV) drugs which are in clinical trials. Regardless, the clinical and commercial success of current Pt drugs, downsides associated with them has stimulated an extensive search for unconventional chemotherapeutic strategies [27]. Transition metals other than platinum such as ruthenium, gold, palladium, iridium, rhodium, iron, osmium, and tin have been evaluated for their cytotoxicity on a panel of cancer cell lines which have shown significant results coupled with minimal side effects in comparison to platinum-based drugs [28].

In fact, well tailored Ru(II)-complexes have came out as one of the best alternatives by following rationales such as favorable kinetic aspects, potential to overcome Pt-associated multi-drug resistance, inherently less toxicity toward healthy cells and higher cytotoxicity for cancer cells. Importantly, ruthenium complexes might cause cytotoxicity due to interaction with multiple targets including DNA, proteins [29, 30] and enzymes [31, 32], and encourage various activities, *viz*., accumulation in cellular organelles and enhancement of oxidative stress [33, 34], photoactivation [35], and disruption of the cellular redox balance [36, 37], resulting in comparable or superior cytoxicity profile against a wide spectrum of cancer phenotypes. Notably, Ru(III) complexes act as "prodrug" that are activated *in vivo* by reduction to Ru(II) active species in the hypoxic environment of cancer cells [38]. In this class of complexes, success of KP1019 [trans-$RuCl_4$(1H-indazole)$_2$] and its Na^+ analog KP1339, [trans-$RuCl_4$(1H-indazole)$_2$] at preclinical and clinical trials has led interest with improved cytotoxicity. Interestingly, KP1339 (IT-139) has shown long-lasting responses in patients with solid tumors, whereas clinical evaluation of NAMI-A, [trans-$RuCl_4$(1H-imidazole) (DMSO-S)] failed due to severe side effects. Moreover, several promising organometallic Ru(II)-arene complexes have shown significant antimetastatic, antiangiogenic,

and anticancer activities on variation in their structure at ancillary ligands [39, 40]. The representative metallodrugs among this class are RAPTA-C ([RuII(η^6-*p*-cymene)(PTA)Cl]$^+$), PTA = 1,3,5-triaza-7-phosphatricyclo [3.3.1.1]decane), READ-C ([RuII(η^6-*p*-cymene)(en)Cl]$^+$) and RM175 ([RuII(η^6-*p*-biphenyl)(en)Cl]), en = ethylenediamine) (Scheme 7.2) [41, 42]. Structurally, organometallic Ru(II)-arene complexes offer hydrophobic arene ligand which influences the cellular uptake along with kinetic reactivity of Ru(II) complexes, while the coordinated ligand offer intrinsic control over the reactivity and selectivity toward biomolecules. Moreover, ruthenium arene compounds have ability to mimic platinum drugs in targeting DNA due to their similar ligand exchange kinetics [43, 44]. However, diverse range of modifications have been employed at both the arene moiety and the ancillary ligand systems in an effort to precisely tune anticancer activity of Ru(II)-arene complexes [45–47]. Pettinari *et al.* studied Ru(II)-arene-acylpyrazolonato complex and revealed remarkable cytotoxicity on wide range of cancer cell lines, *viz.*, HeLa, MCF-7, HepG2, and HCT-116 with IC_{50} = 13–30 μM [48]. Further, Hartinger *et al.* reported synthesis and antiproliferative activity of RuII(η^6-arene) compounds carrying bioactive flavonol ligands on different cancer cell phenotypes (CH1, SW480, A549, 5637, LCLC-103H, and DAN-G) and results exhibited good IC_{50} values in the range of 0.86–19 μM [49]. Moreover, literature reports has also



Scheme 7.2 Promising Ru(II)/(III) anticancer agents.

shown that ruthenium polypyridyl complexes such as Δ-$[Ru(bpy)_2(UIP)]^{2+}$ [where UIP = 2-(5-uracil)-1H-imidazo-[4,5-*f*][1,10]phenanthroline] or $[Ru(bpy)_2(dpq)]^{2+}$ [50] exhibit anticancer activity due to their interaction with DNA by intercalation and can also induce mitochondria-mediated and caspase dependent apoptosis.

Copper is one of the essential transition elements in the human physiology which is associated with its redox properties accessible within the cellular potential range. Several studies have demonstrated that copper accumulates in cancer cells due to selective permeability of the cell membranes and thus cancer cells could get targeted by copper complexes [51]. There has been a tremendous drive for the design and synthesis of copper complexes as anticancer agents which can be witnessed by work described in literature reports [52–54]. Several copper complexes such as (i) copper-thiosemicarbazone complexes [55, 56], (ii) copper-Schiff base complexes [57], (iii) copper complexes with quinolones, flavones, and benzimidazoles [58, 59], (iv) copper-phenanthroline and bipyridine complexes [60], and (v) binary/ternary copper complexes with amino acids [61] exhibited good selectivity against tumors and various have shown efficiency to overcome cisplatin resistance. Cu(II) complexes possess a broader spectrum of activity and lower toxicity than platinum drugs along with the capability to overcome inherited and/or acquired resistance to cisplatin. It is well documented that copper derivatives interact with DNA double helix noncovalently rather than forming coordinate covalent adducts with DNA as in case of platinum drugs, which consequently trigger cell death. The noncovalent DNA interactions include intercalative, electrostatic, and groove binding of metal complexes along the major or minor DNA groove [62]. However, cellular constituents other than DNA such as topoisomerases [63] or proteasome mutiprotein complex [64] could also be possible biochemical targets for these copper complexes. Moreover, it is assumed that redox cycling ability of copper between Cu(I) and Cu(II) give rise to production of reactive oxygen species (ROS), which are fatal to cancer cells [65]. Remarkably, Ruiz-Azuara *et al.* [66] have synthesized a class of cationic Cu(II) complexes containing diimine donor ligands, [Cu(N-N)(A-A)] $[NO_3]$}, where N-N represents neutral diimine donors, either phen or bipy, A-A stands for uninegative N-O or O-O donors, either aminoacid or acetylacetonate. These mixed chelate Cu(II) complexes are called as Casiopeinas (Cas) and have proved their significant anticancer activity in preclinical trials. Moreover, two of them Cas II-gly, [Cu(1,4-dimethyl-1,10-phen)(gly) NO_3] and CasIII-ia, [Cu-(acetylacetonato)(4,4'-dimethyl-2,2'-bipy](NO_3), have entered in Phase I clinical trial (Figure 7.2). In fact, anticancer results from CasIII-ia elucidated apoptotic mediated cancer cell death.

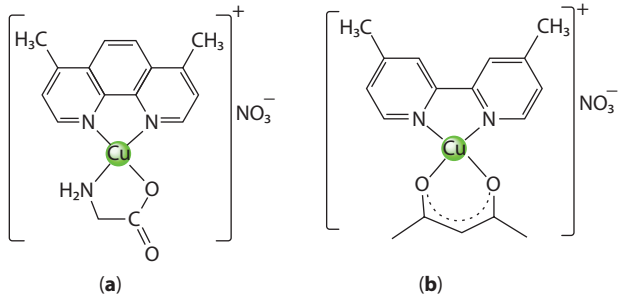


Figure 7.2 Casiopeinas complex (a) Cas II-gly and (b) Cas III-ia.

After platinum, gallium is the most used metal ion among metal-based anticancer agents [67, 68]. A diverse range of gallium complexes have been characterized and reported to display cytotoxicity against different cancer cell lines. It has been shown that oral administration of Ga is less toxic, which allows its bioavailability in cancer cells [69, 70]. The first-generation $Ga(NO_3)_3$ has reached phase II clinical trial with promising results in the treatment of bladder carcinoma, urothelium carcinoma, and lymphomas. Other complexes such as gallium chloride, gallium maltolate, and tris(8-quinolinolato)gallium (III) (KP46) are also under clinical trials [71–73]. Since, Ga(III) shares chemical similarity with Fe(III), so it is thought that mecahnism of action of Ga(III) is associated with its competition to Fe(III) for transferrin binding in biological system [74]. Moreover, Ga(III) complexes cause inhibition of enzyme ribonucleotide reductase (RR), which is responsible for conversion of ribonucleotide to deoxyribonucleotide [75].

Gold complexes are promising class of effective metal-based anticancer agents owing to their strong cancer cell growth inhibitory effect. Gold derivatives exhibit noncisplatin like mechanism of action. Several classes of Au(I)/(III)-based drug candidates have been designed and evaluated for anticancer activity, *viz*., phosphane Au(I) compounds, Au(III) dithiocarbamate, Au(III) porphyrins, Au(III) complexes with bipyridyl-type ligands, and organogold(III) complexes, Au(III) N-heterocyclic carbenes, cyclometallated Au(III) complexes, and Au(I) alkenyls [76–79]. Although, specific mechanism of action of these cytotoxic gold derivatives is still unclear; however, studies have indicated that the main target of gold complexes are selenocysteine (Sec)-dependent enzymes, such as glutathione peroxidase (GPx) and TrxR [80, 81]. TrxR inhibition by gold complexes can disturb mitochondrail function and induce elevated ROS-levels, leading to cell death by apoptosis or necrosis. Tetracetyl-b-D-thioglucosegold(I) triethylphosphine, auranofin, (Figure 7.3a) is an antiarthritic agent which has paved the way for researchers to discover new gold complexes for the treatment of

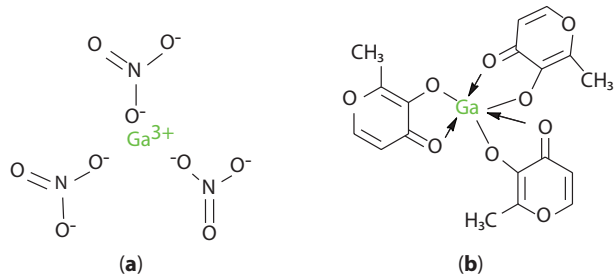


Figure 7.3 Structure of (a) gallium nitrate and (b) gallium maltolate.

cancer. In fact, this Au(I) derivative is under clinical trials against chronic lymphocytic leukemia, lung cancer, and glioblastoma. Although mode of action of auranofin is not clear, however, it is considered that cytotoxicityof auranofin could be due to inhibition of TrxR and glutathione reductase (GR). Phosphanegold(I) thiolate derivatives comprise a family of promising anticancer agents in which P-Au-S linkage is present, which is responsible for enhanced cytoxicity as compared to thiolates. Other promising series of gold(I) phosphine complexes with 1,2-bis(diphenylphosphine)ethane have shown pronounced cytotoxicity results especially in few cisplatin resistant cancer cell lines. More recently, Rubbiani *et al.* [82] described a series of three structurally related Au(I) complexes with benzimidazole derived NHC ligands that inhibited TrxR and thus suggested significant antiproliferative activity of Au(I) derivatives. Another gold complex under clinical investigation is aurothiomalate which is currently tested against advanced nonsmall cell lung cancer. Its mechanism of action is related to inhibition of protein kinase Ciota (PKCiota) signaling.

Notably, Au(III) complexes have attracted curosity as potential anticancer agents since they are isoelectroonic and isostructural (square-planar) with Pt(II). The first promising results were obtained by Au(III) complexes of 2-[(dimethylamino)methyl]phenyl] (damp) ligands, which exhibited anticancer activity on different cancer cell lines comparable or better than standard drug cisplatin, along with cytotoxicity against cisplatin resistant cell lines. Fregona *et al.* [83] prepared Au(III) dithiocarbamota complexes that have shown cancer cell death after generating ROS, in particular H_2O_2. It was suggested that activity of these dithiocarbamato complexes could be due to a selenoenzyme TrxR. Furthermore, porphyrinato Au(III) derivatives developed by Che and Sun [84], which include the complex $[Au(TPP)]^+$ (Figure 7.3b) have also exhibited profound cytotoxicity in nasopharyngeal and heptocellular carcinoma, colon, neuroblastoma, melanoma, and promyelocytic leukemia cancer.

In the past few years, various studies have revealed the potential of rhenium complexes as promising antiproliferative agents [85–87]. Compared to novel Pt(II) drugs, Re complexes possess favorable properties which make them amenable for use, *viz*., (i) they can bind covalently to DNA nucleobases, (ii) the ligand substitution kinetics are similar as of platinum-based drugs, (iii) they can prepare "cold" Re complexes which are used as luminescent probes [88] for cell-imaging inside the cells, and (iv) they can also prepare "hot" analogs that can be applied for radio-imaging as well as for cancer therapy. Earlier Re complexes were prepared as luminescent probes; however, their anticancer activity was discovered only after screening the potential compatability with biological systems. Potentially, vast range of Re complexes are synthesized from chemically robust $Re(CO)_3$ core (Figure 7.4), and most of them have displayed cytotoxicity on various human cancer cell lines comparable or more than that of standard anticancer drug cisplatin [89–91]. Many excellent reviews on anticancer and other therapeutic potency of Re complexes have been published [92, 93] mainly focusing on their cellular uptake, localization, and cytotoxic activities. Although considerable number of Re(I) complexes have been screened, but their mechanism of cytotoxicity has not yet been clarified.

Morgant and d'Angelo *et al.* [94] synthesized novel Re(I) tricarbonyl complexes $Re(CO)_3Cl(PhSe(CH_2)_2SePh)$, $Re(CO)_3Cl(PhSe(CH_2)_3SePh)$, $Re(CO)_3Cl(HO_2C\text{-}CH_2Se(CH_2)_2SeCH_2\text{-}CO_2H)$, and $Re(CO)_3Cl(HO_2C\text{-}CH_2Se(CH_2Se(CH_2)_3\text{-}SeCH_2\text{-}CO_2H)$, with diseleno ligands Re-$(CO)_3$(Se-Se) Cl (Figure 7.5) and structurally characterized them by means of physiochemical measurements, including X-ray diffraction analyses. The antiproliferative activity of these complexes were tested on cancer cell lines; HT 29

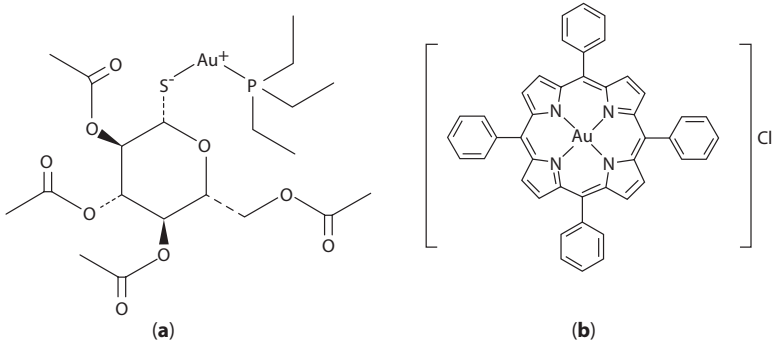


Figure 7.4 Gold complexes (a) aurofin and (b) $[Au(TPP)]^+$.

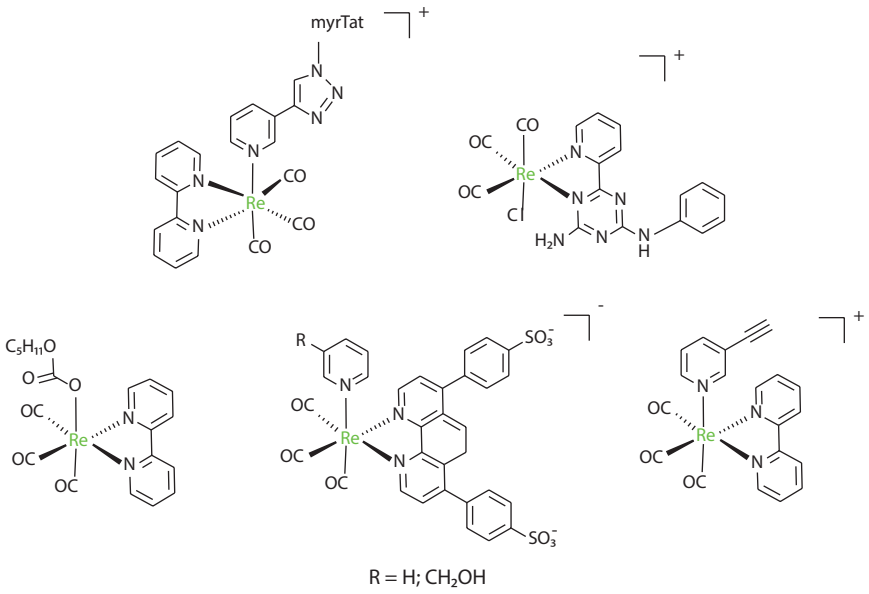


Figure 7.5 Cytotoxic Re complexes derived from $Re(CO)_3$ core.

(colorectal cancer), MCF-7 (hormone dependent brest cncer), A549S (luung adenocarcinoma), and HeLa (solid uterine carcinoma). The results exhibited significant activity (4 μM) against MCF-7, while no toxicity toward HT-29, A549 and HeLa cell lines.

The pioneering work of Kopf and Kopf-Maier [95] and Kepplar *et al.* [96] on titanocene dichloride $Ti(\eta^5\text{-}C_5H_5)_2Cl_2$ and budotitane (Figure 7.6), respectively led these complexes into human clinical trials as anticancer agents. These titanium-based complexes has attracted continuous attention of researchers since they have shown to exhibit cytotoxic activity toward cisplatin-resistant cancer cell lines with reduced side effects [97–99]. Titanocene dichloride was the first non-platinum metal complex to enter

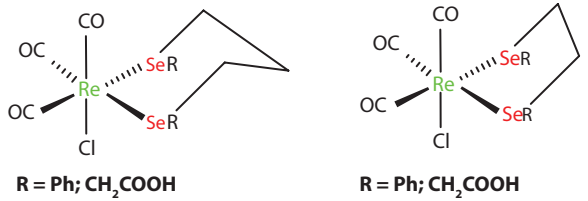


Figure 7.6 Re(I) tricarbonyl complexes with Se-Se bidentate linkage.

clinical trials in 1993, however phase II clinical trials in patients were not sufficiently promising and thus has been discontinued from further clinical trials. The hydrolytic instability problems and lack of standard formulation are contributing factors for discontinuation of titanocene dichloride from clinical trials [100, 101]. Similarly in case of budotitane, rapid hydrolysis in aqueous environment combined with its hindered pharmaceutical formulation [102], impeded its clinical trials beyond phase I. The close structural similarity of both Cp_2TiCl_2 and budotitane to cisplatin invoked DNA as the probable target of these anticancer drugs, however several studies proposed that inhibition of enzymes including protein kinase C and DNA topoisomerases II could be the mode of action of their anticancer activity. Motivated by the interesting cytoxicity results of titanocene dichloride and budotitane complexes, several Ti(IV) complexes have been synthesized and explored for their anticancer activity, which exhibited potent *in vitro* and *in vivo* cytotoxicity than standard chemotherapeutics, against different cell lines [103, 104]. Notably, many reviews on titanium complexes have been published dealing with their role in the treatment of cancer [105, 106].

Iron is an essential element in biological system that plays crucial involvement as cofactor in metabolic pathways. The ability of iron to switch between oxidation states Fe(II) and Fe(III) under physiological conditions enables it to exhibit anticancer activity [107]. Firstly, anticancer properties of iron complexes were revealed in ferrocenium picrate and ferrocenium trichloroacetate salt (Figure 7.7), which could be attributed to the formation of ROS leading to oxidative DNA damage. One of the notabe examples of anticancer compound involving iron complexation is bleomycin. Bleomycin is a glycopeptide comprising of N-terminal metal-binding domain, with clinical efficacy against several cancer phenotypes especially testicular carcinoma, squamous cell carcinomas, and malignant limphomas [108, 109]. The proposed anticancer mechanism of action of bleomycin includes formation of reactive species, causing DNA cleavage. Another

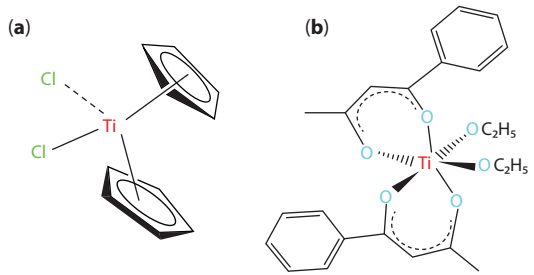
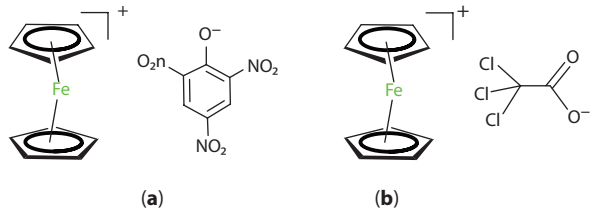


Figure 7.7 Anticancer drugs (a) Titanocene dichloride and (b) Budotitane.



(**a**) (**b**)

Figure 7.8 Structure of (a) ferrocenium picrate and (b) ferrocenium trichloroacetate.

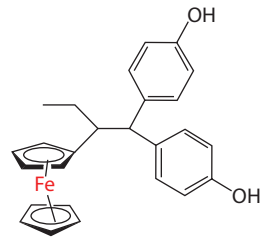


Figure 7.9 Structure of ferrocifen.

noteworthy examples are organometallic ferrocifen analog of tamoxifen (anti-estrogen) (Figure 7.8) and its derivatives, which are found to possess distinguished mode of antiproliferative activity [110].

The activity of ferrocifens against estrogen receptor, ER(+) cancer cell lines can be rationalized by invoking a mechanism similar to tamoxifen. Moreover, ferrocifen is also active against highly invasive ER(−) MDA-MB-231 breast cancer cells (Figure 7.9), which do not respond to tamoxifen. Moreover, a polypyridine iron(II) complex, Fe(II)-N_4Py [N_4Py = N,N-bis(2-pyridylmethyl)-N-bis(2pyridyl)methylamine] is a synthetic bleomycin mimetic [111], that has ability to cleave DNA efficiently under aerobic conditions and thus induce cell death via nuclear DNA damage.

7.2 Conclusion and Future Prospective

Metal complexes owing to variations in coordination numbers and geometries, accessible redox and oxidation states, tunable ligand substitution reactions offer a promise for the treatment of various diseased conditions. It was by the discovery of cisplatin which not only cured cancers but also opened up new prospects toward the development of metallodrugs with potential biomedical applications. During the past decades, many

platinum complexes have been evaluated as robust antitumor agents; however, only carboplatin and oxaliplatin have entered worldwide in clinical use. Moreover, despite the successes made with the current platinum drugs against the treatment of cancer, there are still some major drawbacks, *viz*., systemic toxicity and acquired or intrinsic resistance associated with these drugs. Therefore, in order to overcome these complications, rational design of metal complexes with potential antitumor properties and reduced toxic issues is still a challenge. In recent years, several classes of non-platinum(II) complexes (Ru, Ga Fe, Cu, Ti, and Re) which could overcome cisplatin-resistance have also been designed. Further, the success of ruthenium complexes against cancer treatments has inspired to develop non platinum complexes as anticancer agents. Non-platinum complexes are expected to show different modes of action with diminished side effects than cisplatin-based classical chemotherapy. Thus, elucidation of the detailed mechanism of anticancer agents will lead to future developments in metal-based drugs. Novel anticancer metallodrugs are expected to increase the lifespan of cancer patients and further improve the quality of life.

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8

Design, Synthesis, and Biological Evaluation of Aziridynyl Quinone Derivatives

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Abstract

Aziridinyl quinone isoxazole hybrids (**67a**-**67j**) were synthesized *via* Claisen reaction, cyclization, alkoxy carbonylation, hydrolysis, oxidation, and aziridine insertion. All the compounds were evaluated for anti-biofilm, antimicrobial, and cytotoxic activities. Among the tested compounds, compound **67h** showed good antibacterial and anti-biofilm activities with MIC value of 3.9, 3.9, 3.9, and 7.8 μg/ml, respectively, and IC_{50} values of 1.9, 2.5, 2.8, and 5.1 μM, respectively, against *S. aureus* MLS-16 MTCC 2940, *B. subtilis* MTCC 121, *S. aureus* MTCC 96 and *K. planticola* MTCC 530, and also exhibited potent antifungal activity against *C. albicans* MTCC 854, *C. albicans* MTCC 227 and *C. krusei* MTCC 3020, equipotent to standard miconazole (MIC value and 7.8 μg/ml). All the synthesized compounds exhibited promising cytotoxicity against PC3 and A549 cell lines (IC_{50} values between 1 and 4 μM). Compounds **67b** and **67j** exhibited an IC_{50} value of 0.5 μM which was similar to that of Mitomycin C against the PC3 cell line.

Keywords: Cytotoxicity, quinone diaziridinyl hybrids, anti-biofilm, antifungal, antibacterial activity

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8.1 Introduction

Medicinal chemistry is the branch of science that provides drugs either through discovery or design. Drugs are chemical components that help to restore the health to the diseased individuals and play an indispensable role in modern medicine. Classical drugs were mostly discovered either by modification of natural substances or by comprehensive chemical synthesis. In the present millennium, new borderline investigations such as molecular pharmacology, molecular biology, biomedicine, bimetallic chemistry, bioinformatics, cellular biology, genetics, and others have begun to hold the interest of medicinal chemistry scientists. The drug is as old as a disease because many new diseases have been arising along with globalization. In spite of several drugs available for medical use, the development of resistance to many old and new drugs, a sincere continuous effort by man to control and cure diseases, has created a substantial medical need for new classes of drugs. This has led to a search for new drugs or suitable derivatives of existing drugs. The tendency of resistance created the urgent requirement for safe, new, and more effective drugs with a lack of many side effects. A potential approach to overcome the resistance problem and the rising of new diseases is to design innovative agents with different means of action. Therefore, recent efforts have been focused on exploring novel drugs. In recent years, a new strategy has gained importance which involves the development of hybrid molecules through the conjugation of different pharmacophores in the same structure. This strategy is expected to lead the molecules with more efficiency in biological activity. However, there is a growing demand for the synthesis of new active molecules to identify potential leads that can control the specific disease without any side effects with a careful justification of the hybridization partners is essential.

Alkaloids are one of the attractive natural products leading to drug development. Indeed, many alkaloids and their derivatives are widely used as clinical medicines. Among them, the natural products containing quinone nucleus (**Doxorubicin, Epirubicin, and Idarubicin**) and aziridinyl quinone (Figure 8.1) (**Mitomycin**) represent the medicinally and pharmaceutically important class of compounds. However, even though they have been used for antimicrobial and antitumor activities, problems such as toxicity and resistance have served to stimulate an intense demand and all out-research efforts for the discovery of a new class of novel anti-tumor and antimicrobial agents. Since the last few years, considerable progress has been observed in the screening of many quinones and aziridinyl quinone moieties.

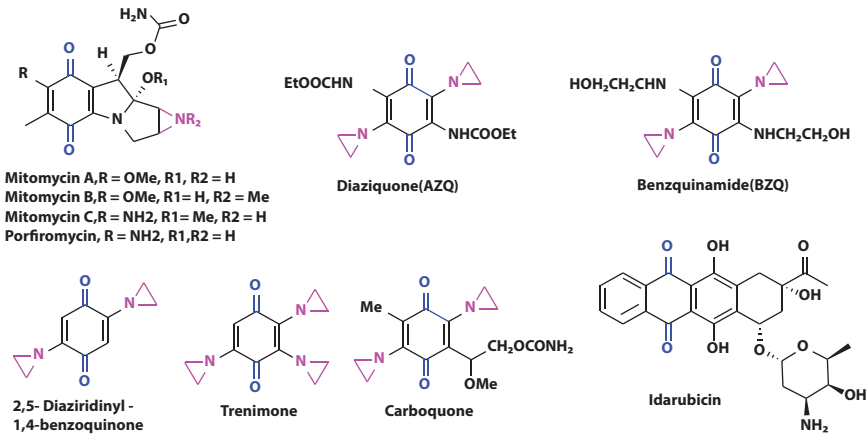


Figure 8.1 Diaziridinyl quinones.

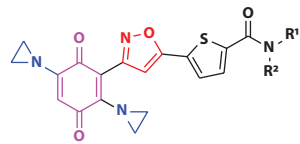


Figure 8.2 Aziridinyl quinone isoxazole hybrids.

Considering the significant pharmacological and biological activities of aziridine, quinone, and isoxazole derivatives, we have selected these functional units to discover and develop a new class of hybrid molecules. In our on-going research program, we have designed the synthesis of novel aziridinyl quinone isoxazole derivatives (Figure 8.2) as a new class of hybrid molecules to identify biologically potent compounds.

8.2 Aziridines

8.2.1 Literature Review

Three-membered heterocycles are an important functional unit among the wide variety of heterocyclic compounds due to its wide variety of biological activity. They are more reactive due to ring strain so that aziridine and its derivatives play a significant role as an intermediate in the synthesis of organic [1], pharmaceutical [2], and natural product intermediates due to

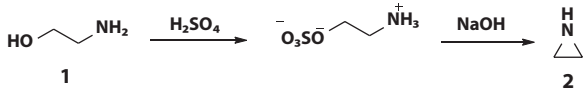
its high reactivity. Aziridine constitutes the main class of versatile molecules among three-membered heterocycles with two methylene groups and one amine group. They are nitrogenous analogs of epoxides and valued reactive species in many amino acid derivatives and azomethine ylides synthesis.

Aziridine

8.2.2 Synthetic Approach

Synthesis of aziridine derivatives is one of the major interests in the field of medicinal chemistry. Aziridines can be synthesized in numerous ways including SN_2 displacement, 1-4 addition, and the addition of carbenes, electrophiles, nitrenes, and reduction.

The Wenker synthesis is the conversion of beta-amino alcohol **1** to an aziridine **2** under acidic condition conc. H_2SO_4 [3]. The reaction involves two steps: the first step involves the reaction of ethanolamine with H_2SO_4 at the temperature of 250°C to give the sulfate mono-ester. The salt formed in the first step reacts with NaOH to form aziridine **2** in the second step. It was also observed that the reaction at a lower temperature of 140–180°C reduced charring, thereby the yield of sulfate mono-ester was improved.



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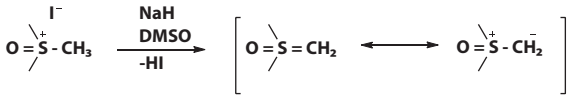
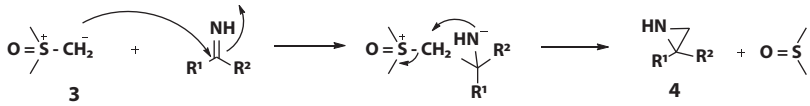
The Corey-Chaykovsky reaction [4] was employed in modern organic synthesis to prepare substituted aziridines by reacting sulfur ylides with electrophile imine. Dimethylsulfoxonium methylidene is the commonly used sulfur ylides. Deprotonation of sulfonium halides with strong bases like NaH in DMSO results in sulfur ylides *in situ*.



Resonance stabilized sulfur ylide

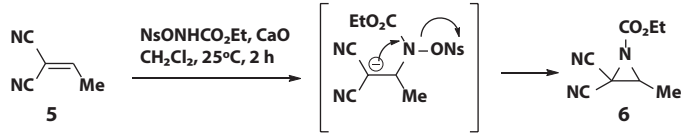
Electron withdrawing groups like phenyl, carbonyl, and ester stabilize sulfur ylides and the electron-donating groups destabilize the ylides.

Dimethylsulfoxonium methylide is more stable than dimethyl sulfonium methylide due to the presence of the S=O group. Negatively charged ylide carbon acts as a nucleophile and form bond with electrophilic carbon attached to "N" atom in the first step. Then, ylide carbon becomes electrophilic and is attacked by the negatively charged "N" atom (acting as an intramolecular nucleophile) to accomplish ring closure to furnish aziridine ring **4**, and sulfoxide acts as leaving the group.



Ylide carbon acting as nucleophile Ring closure step

Fioravanti *et al.* [5] reported the synthesis of aziridine **6** from electron-deficient olefins **5**. The dicyanoalkene (derived from the Knoevenagel condensation of malononitrile with acetaldehyde) undergoes a facile aza-Michael addition of ethyl nosyloxycarbamate in the presence of calcium oxide to give a β-aminocarbanion intermediate, which quickly cyclizes to the corresponding dicyanoaziridine in excellent yield.

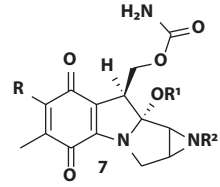


8.2.3 Biological Importance

The potency of aziridines is mainly based on toxicity rather than specific activity and the ring strain making them attractive for pharmacological activity. Aziridine acts as an electrophile and is subject to attack on nitrogenous bases in DNA base pairs results in ring-opening by pharmacological activity which leads to potential mutagenicity [1]. Since the last decades, aziridine-containing compounds have attained great interest as both immunomodulatory and anticancer agents [6]. Ring strained property of aziridines is attractive for the study of its reactivity and pharmacodynamic action.

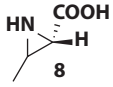
Many known aziridines containing natural products, such as Mitomycin [7], Porfiromycin [8], and Carzinophilin A [9], are known as antitumor agents. The activity of Mitomycin C depends on the ring-opening of aziridine and interaction with nucleobases like guanine in the DNA alkylation.

These result in covalent interstrand DNA-DNA crosslink formation, inhibition of replication, and finally to cell death.

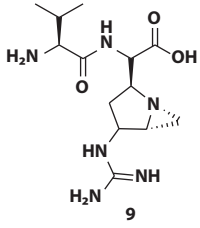


Mitomycin A,R = OMe, R^1, R^2 = H
Mitomycin B,R= OMe, R^1= H, R^2 = Me
Mitomycin C,R= NH_2, R^1= Me, R^2 = H
Porfiromycin, R= NH_2, R^1, R^2 = H

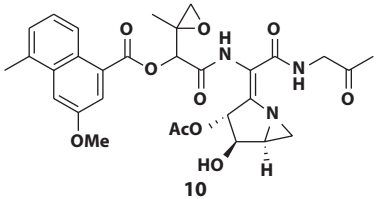
Azirinomycin **8** is active against *S. aureus* followed by *Ss faecalis*, *Proteus vulgaris*, and *B. subtilis*. The methyl ester of azirinomycin exhibited antibiotic activity against both gram-positive and gram-negative bacteria [10].

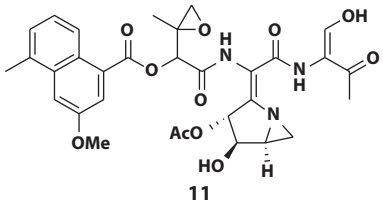


The alkaloid antibiotic Ficellomycin **9** produced by *Streptomyces ficellus* inhibited the growth of gram +ve bacteria *in vitro* and *in vivo* during the treatment of *S. aureus* infections in mice [11].

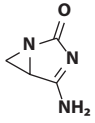


Anticancer drugs, Azinomycin A **10** and **11** are active against both Gram-positive and Gram-negative bacteria. Aziridine derivative, azicemicin A and B, demonstrated antibacterial activity against *Mycobacterium smegmatis*, *Escherichia coli* NIHJ, *Corynebacterium bovis*, and *Micrococcus luteus* [12].





Amide derivatives of aziridines are of significant interest with Imexon **12** as well-known molecules. It is an anticancer agent and active against human myeloma cells. It interacts with cellular thiols and decreases the amount of glutathione and cysteine production in target cells which results in elevated levels of reactive oxygen species (ROS). As a result, mitochondria swell and cytochrome c is released, caspase 3 and 9 are activated, and cells enter an apoptotic pathway [13].



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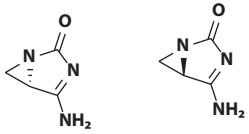
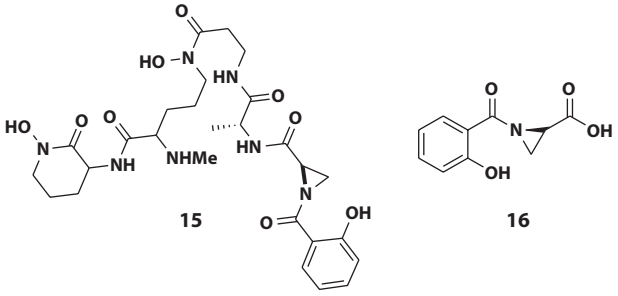
More recently, (5R)- and (5S)-imexons **13, 14** known for the treatment of cancer [14].



13 **14**

Some aziridine ring containing natural peptides such as madurastatin A1 and B1, **15, 16** are Ser and salicylic acid consisting of compounds. They have shown antibacterial activity against *Micrococcus luteus* [15].



15 **16**

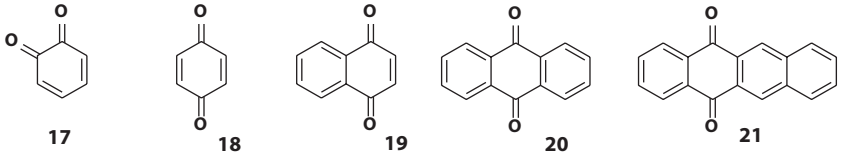
8.3 Quinones

8.3.1 Literature Review

Quinones are a class of natural and synthetic compounds that have been used for several beneficial effects and endowed with rich and interesting

chemistry. Quinones are abundant in nature and occur mainly in flowering plants and some fungi [16]. Quinones are not aromatic but electrophilic Michael acceptor and stabilized by conjugation. Depending on the quinone and the site of reduction, the reduction can either rearomatize the compound or break the conjugation and conjugate addition always breaks the conjugation. Michael's addition of quinones with cellular thiols and amines made them more toxic to the cells.

Quinones are characterized by a common basic structural pattern: an *ortho*- or a *para*-substituted dione conjugated either to an aromatic nucleus 1, 2, and 1,4 benzoquinones) **17**, **18**, or to a condensed polycyclic aromatic system, such as naphthoquinones **19**, anthraquinones **20**, and anthracyclinones **21**.



The knowledge of the reactivity of quinones is relevant to understand their physiological and toxicological properties. The two essential properties of quinones are to understand the biological effects are; undergo oxido-reduction reactions and second many of them can undergo nucleophilic attack due to their electrophilic character.

Many quinone drugs such as Daunorubicin, Doxorubicin (Figure 8.3), Epirubicin, Mitoxantrones, Mitomycin, and Saintopin are used for the treatment of solid tumors. The cytotoxicity of quinones is due to the inhibition of DNA topoisomerase-II [17]. However, the contributions of

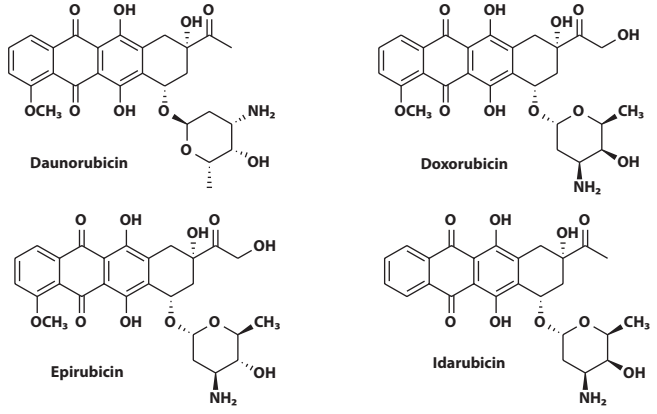


Figure 8.3 Quinone anticancer drugs.

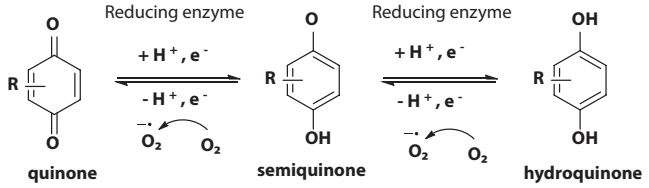


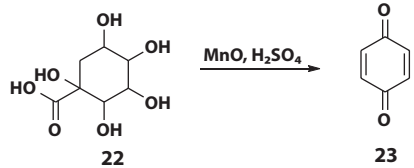
Figure 8.4 One- and two-electron reduction of benzoquinone. NQO1: NAD(P)H: quinone acceptor oxidoreductase.

chemical reactivity and pathways of metabolism are difficult to understand due to the complex structure of the quinones. The quinoids undergo enzymatic reduction *via* one or two electrons to give the corresponding semiquinone radical or hydroquinone. The semiquinone radical anion gives its extra electron to molecular oxygen to give the parent quinone and superoxide radical anion under aerobic conditions. The reaction sequence was initiated by the bioreduction of the quinone followed by oxidation with dioxygen of the radical anion intermediate. This process is known as redox-cycling and it continues until the system becomes anaerobic. The hydroquinone formed in the redox cycle was excreted by the detoxification pathway. Both the semiquinone and the superoxide radical anion can produce the hydroxyl radical, which causes DNA strand break [18].

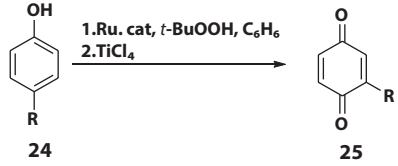
Quinones feature three readily accessible oxidation states, namely, fully oxidized quinone, one-electron reduced semiquinone, and two-electron-reduced hydroquinone (Figure 8.4), and they are capable of mediating both closed and open-shell redox processes.

8.3.2 Synthetic Approach

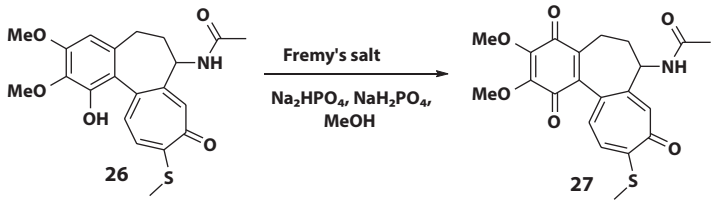
Based on the literature review, there are varieties of methods reported by various researchers for the synthesis of quinone and different quinone derivatives. Pardasani *et al.* [19] reported the synthesis of benzoquinone **23** by the oxidation of quinic acid **22** with manganese dioxide and sulfuric acid. This reaction sequence involves dehydration, decarboxylation, and oxidation reactions



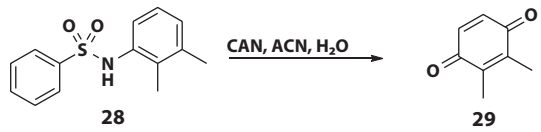
Murahashi *et al.* [20] reported the Ruthenium catalyzed the oxidation of para-substituted phenols **24**. Phenols were treated with *tert*-butyl hydroperoxide in benzene or EtOAc as a solvent and then treatment with $TiCl_4$ gives high yields (70%–80%) of 2-substituted benzo-1,4-quinones **25** with the 4-substituent of the phenol migrating to the 2-position of the benzo-1,4-quinone.



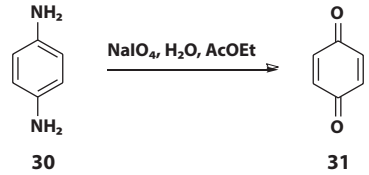
Guan *et al.* [21] reported the oxidation of a highly oxygenated aromatic ring of 1-demethylthiocolchicine **26** to the 1,4-quinone **27** by Frémy's salt.



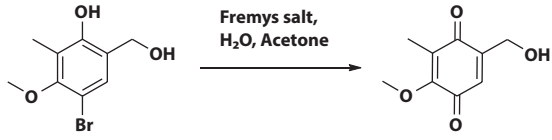
Hewson *et al.* [22] reported the oxidation sulphonamides **28** to benzoquinone **29** with ceric ammonium nitrate in acetonitrile and water as an efficient oxidant system.



Telvekar *et al.* [23] synthesized quinone **31** from aryl diamines **30** using sodium periodate, ethyl acetate, and water at room temperature in good yield.

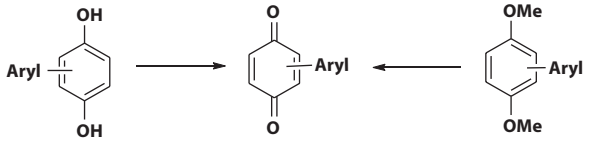


Lipshutz *et al.* [24] reported the oxidation of *p*-bromophenol **32** to benzoquinone **33** in acetone and water at rt in good yields.



32 33

For oxidation of hydroquinones **34** to quinones **36**, oxidants utilized are polymer-supported Ru (II)/dm-Pheox complex in THF (yield 99%) [25], Cerium (IV)/SiO_2 reagent in DCM (yield 97%) [26] (Fischer *et al.*, 2013), $H_2SO_4/H_2O_2/I_2$/MeOH [27], Ag_2O, K_2CO_3/Benzene (yields 50%–90%) [28], $HCl/H_2O_2/FeCl_3/H_2O$/AcOH (91% yield) [29], R:$PhI(O_2CCF_3)_2$, S: DMF [30], 1,4-Dimethoxybenzenes **35** are also readily oxidized to benzo-1,4-quinones **36**; CAN in acetonitrile-water is the most commonly reported oxidant.



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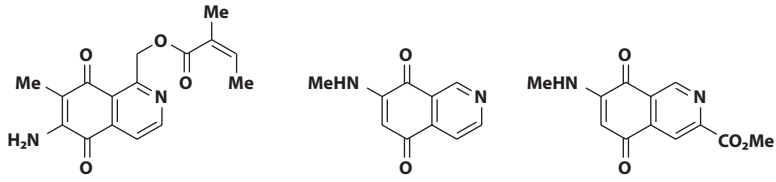
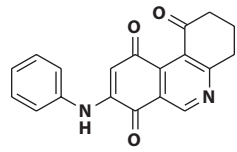
8.3.3 Biological Importance

Quinones have attained great interest from a medical and toxicological perspective due to their unique reactivity and high prevalence in the environment. Natural and synthetic quinonoid compounds are well-known substances that possess a variety of biological properties such as antibacterial, antifungal, antiprotozoal, and antitumor activity. Among the broad variety of *N*-heterocyclic quinones with anticancer activity, there are several examples of naturally occurring aminoquinones containing the isoquinolinequinone scaffold such as Cribrostatin **37**, Caulibugulone A **38**, and Mansouramycin C **39** [31].



37 38 39

Valderrama *et al.* [32] synthesized phenylamino-3,4-tetrahydro phenanthridine-1,7,10 (2H)-trione derivatives **40** and screened using MTT colorimetric method *in vitro*, against normal cell line (MRC-5) and three human tumor cells: AGS SK-MES-1 lung, gastric adenocarcinoma, and J82 bladder carcinoma. Most of the compounds exhibited promising cytotoxicity.



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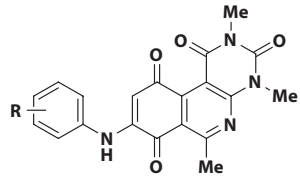
Vásquez *et al.* [33] synthesized 8-aminopyrimido[4,5-c] isoquinolinequinone derivatives **41** and were regioselectively synthesized. Cytotoxic activity was evaluated against one normal cell line (MRC-5 lung fibroblasts) and human cancer cell lines AGS, human gastric adenocarcinoma: SK-MES-1, human lung cancer cells, J82, human bladder carcinoma, and HL-60, human leukemia. Many compounds exhibited antitumor activity against human lung cancer cells and AG Shuman gastric adenocarcinoma.



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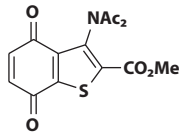
Jaime *et al.* [34] reported the synthesis and *in vitro* leishmanicidal and anti-HTLV-1 activities of benzo- and naphtho[2,3-*b*]thiophene-4,7-quinones **42-44** containing the *ortho* aminoester functionality on the thiophene ring. These compounds showed moderate cytotoxic activity.



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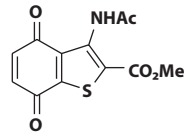

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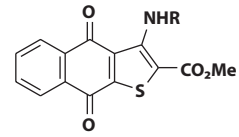
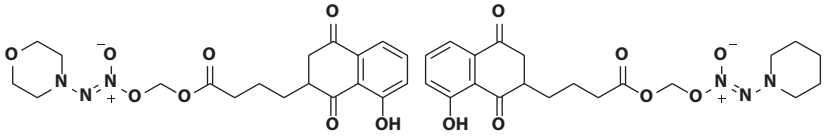


44

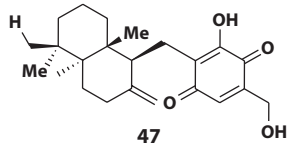
Bao *et al.* [35] synthesized 12 novel plumbagin hybrids and evaluated their inhibitory effects on human cancer cell lines (MDA-MB-231,

HCT-116, A549, and HepG2 cells) and two normal human cells (HK-2 and WRL-68 cells). Compounds **45**, **46** exhibited superior potencies compared to their parent compound (IC_{50} values of 3.48–6.68 mM) against the tested cancer cell lines and weak inhibitory effects on normal cells.



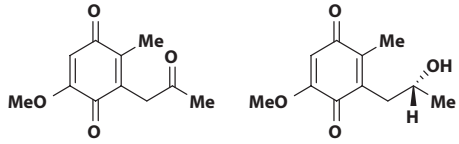
45 **46**

Wijeratne *et al*. [36] isolated sesquiterpene quinones (tauranin) **47** from *Phyllosticta spinarum* and *Platycladus orientalis*. Tauranin is reported to have apoptotic activity and antiproliferative toward several cancer cell lines.



47

Wang *et al.* [37] isolated new anserinone A **48** and B **49** from the liquid cultures of the coprophilous fungus *Podospora anserina*. They exhibited cytotoxic, antifungal, and antibacterial activities.



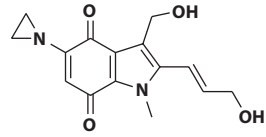
48 **49**

8.4 Aziridinyl Quinone Derivatives

DNA cleaving reagents have attracted continuous and extensive interest due to their wide applications in the fields of molecular biological technology and drug development. Mitomycin species were significant as antimicrobials and DNA cleaving reagent due to the presence of aziridinyl quinone as a biological functional unit.

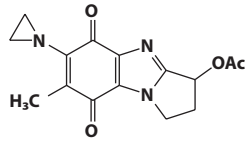
Mitomycins (Figure 8.5) was first discovered in *Streptomyces* sp. by Hata and coworkers, in 1956 and Mitomycin C was isolated in 1958 by Wakaki and his group from *Streptomyces caespitosus*. Mitomycin C and porfiromycin have shown substantial anticancer activity. Mitomycin C has proved to be most effective for the frontline treatment of a small number of solid tumors, such as superficial bladder cancer [38] pancreatic [39]. Studies on the structure-activity relationship (SAR) of mitomycins were especially useful for understanding the role of aziridine moiety. Studies carried on about 70 mitomycin derivatives concluded that the aziridine, quinone, and C-10 carbamate groups were required for mitomycin antibacterial activity [40]. Several studies have shown that all these three key constituents play a functional role in the cytotoxic action of Mitomycin C. The mechanisms of activation of mitomycines are complex and are still under debate.

Apaziquone **50** is an indolequinone bioreductive prodrug and analog of mitomycin C with potential radiosensitization with antineoplastic activities. It is activated by DT-diaphorase expressed by bladder tumor cells to form cytotoxic alkylating agents [41].

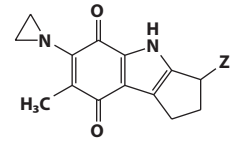


50

Fused benzoquinones, pyrrolobenzimidazoles **51**, and indolobenzoquinones with aziridine moiety **52** bearing antitumor activity which has been broadly studied by Skibo's research group [42].



51



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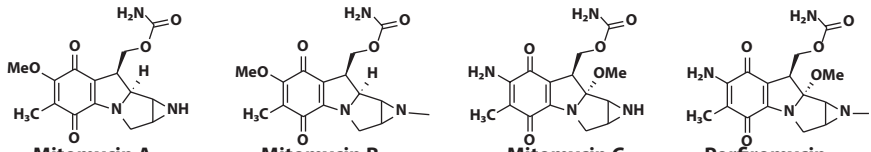


Mitomycin A **Mitomycin B** **Mitomycin C** **Porfiromycin**

Figure 8.5 Mitomycin derivatives as anti-tumor agents.

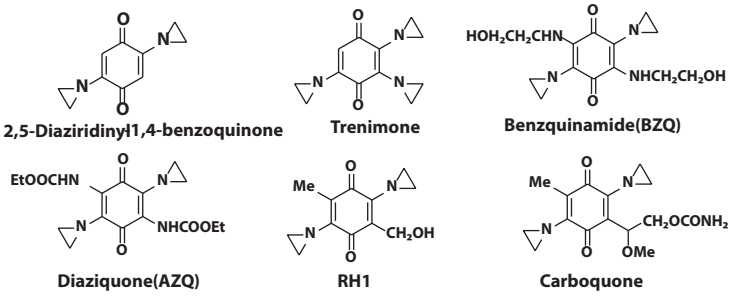


Figure 8.6 Di- and ltriaziridinyl benzoquinones.

The diaziridinyl benzoquinones are the simplest molecules in the aziridinyl quinones category and are gone for more important studies of quinone-DNA interactions. 2, 5-Diaziridinyl-1,4-benzoquinone (Figure 8.6) and its analogs were synthesized by Petersen and co-workers in the early fifties. Trenimon was the most broadly studied compound (Figure 8.6) and for the treatment of leukemia, breast cancer, and cervix carcinoma in Germany [43].

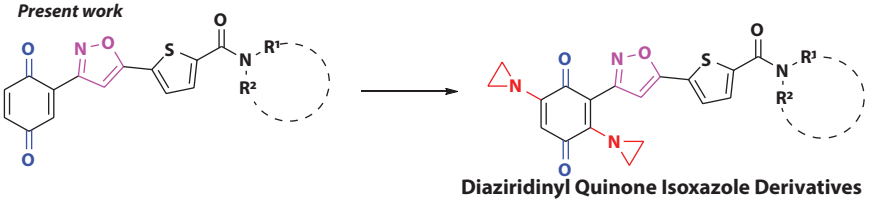
Nakao and co-workers synthesized more than forty diazidinyl benzoquinones to study the alkylating ability of the carbamate side chain of Mitomycin C. Diaziridinyl quinones found to be active against mice bearing L1210 tumors and this resulted invention of Carboquone (Figure 8.6) as a potential active unit. This quinone still using in combination therapy for prostate cancer in Japan and ovarian cancer in Finland [44].

In the late 1970s, Driscoll and co-workers designed diaziridinyl quinones as potential anti-tumor agents for the central nervous system to cross the blood-brain barrier. The most active compound identified was Diaziquone (AZQ) and effective against both intraperitoneal and inter-cerebral implanted tumors [45]. A more water-soluble analog, Benzquinamide (BZQ), was identified from these studies [46]. The AZQ underwent phase II clinical trials against a regular primary brain tumor and advanced large bowel cancer [47]. Recently, RH1 emerged as a promising and potential antitumor agent, demonstrating its activity at submicromolar concentrations, and encouraging results were noticed in preclinical and phase-I clinical trials [48].

8.4.1 Present Work

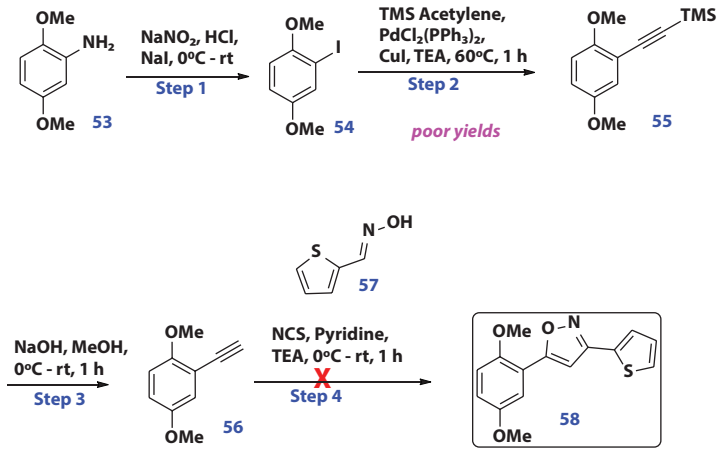
Aziridines, aziridinyl quinones, and isoxazole containing various molecular fragments have exhibited an enormous array of therapeutic benefits.

Considering its significant chemical pharmacological and biological activities and clinical diversity, we have selected these biologically active functional units to discover and develop a new class of hybrid molecules with more potency. As a part of the on-going research program, we designed the synthesis of novel ***Diaziridinyl Quinone Isoxazole Derivatives*** as novel hybrid molecules to identify more potent biologically active compounds. These molecules have salient features such as the potential to enhance efficacy, improve safety cost-effective, and reduce the tendency to elicit resistance relative to the parent drugs.



8.4.2 Synthetic Studies

For the preparation of the target compounds, we have chosen a strategy as shown in Scheme 8.1 which is based on our previous 1,3-dipolar cycloaddition reaction protocol [49]. To begin with, 2,5-dimethoxyaniline **53** was treated with $NaNO_2$/HCl/KI at 0°C to room temperature, to obtain



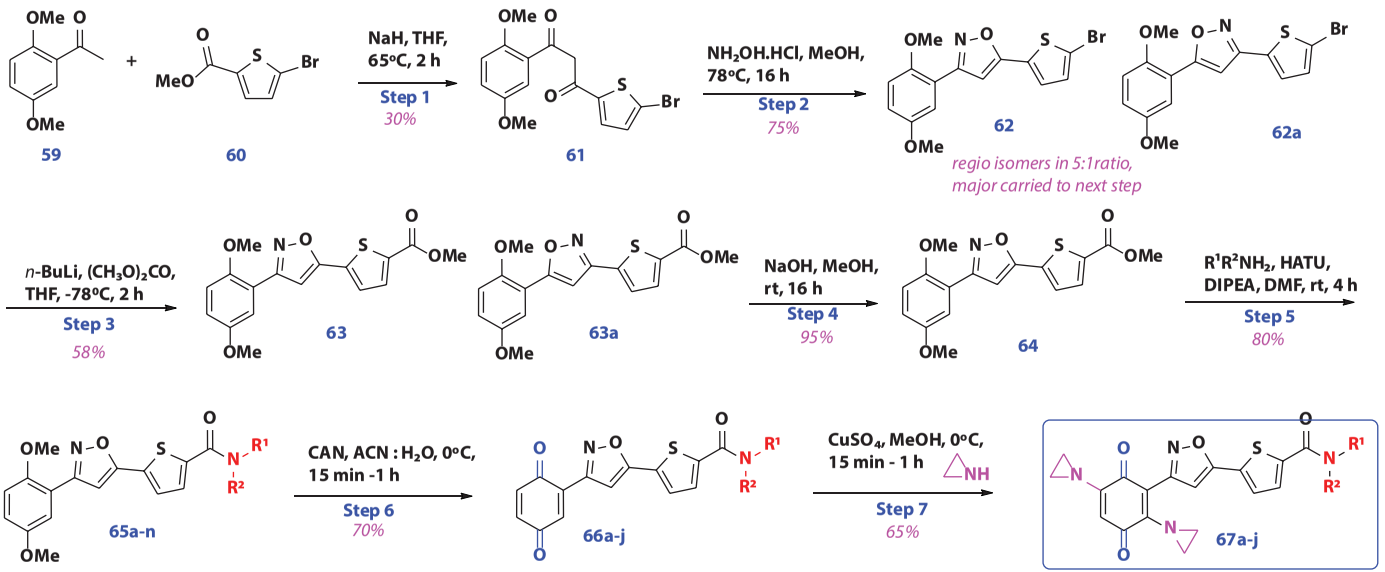
Scheme 8.1 Attempted 1,3-dipolar cycloaddition protocol.

the 2,5-dimethoxy iodobenzene **54**. Compound **54** was subjected to Sonogashira coupling with TMS acetylene using $Pd(PPh_3)_2Cl_2$, CuI, and Et_3N at room temperature followed by TMS deprotection with NaOH in MeOH at room temperature, in one pot reaction which afforded compound **56**. But the yield was poor (**20%**) due to the formation of major dimerized by-products. Since the iodo derivative is poor yielding, we also tried to prepare compound **54** by using commercially available 2,5-dimethoxy bromobenzene by similar Sonogashira protocol but the starting material was intact at 60°C and des-bromo compound 1,4-dimethoxybenzene formation was observed at 80°C–90°C.

On the other hand, we treated 2-thiophene carboxaldehyde with hydroxylamine hydrochloride and catalytic NaOAc in MeOH as a solvent at reflux conditions to afford compound **57**. Compound **57** was later subjected to the key 1,3-dipolar cycloaddition reaction with 2,5-dimethoxyphenyl acetylene **56** in the presence of $NaOCl/Et_3N$ in DCM as solvent at 0°CC, but the isoxazole derivative **58** was not formed using the Huisgen's one-pot protocol and we observed multiple spots in TLC. NCS/Et_3N in $CHCl_3$ at 0°C conditions was also used to prepare isoxazole derivative **58**; however, it was observed that the chlorooxime reverted to oxime derivative **57** and the decomposition of phenylacetylene derivative was observed even at −10°C. Furthermore, attempted the reversible addition of chlorooxime to the solution of phenylacetylene and triethylamine in dry DCM at −10°C, and all of these attempts to prepare isoxazole derivative **58** were unfruitful. Finally, the reaction worked out with NCS/Et_3N using the catalytic amount of pyridine (**10%**) but poor yielding.

The synthetic pathway that was depicted in Scheme 8.2 was chosen. 2,5-diethoxy acetophenone **59** and methyl-2-bromothiophene-5-carboxylate **60** were subjected to Claisen reaction using sodium hydride in THF at reflux gave β-diketone compound **61** as keto and enol mixture (**30%**).

The crude keto and enol mixture **61** was used as such without any further purification in the next step. Compound **61** was treated with $NH_2OH.HCl$ and a catalytic amount of conc. HCl in MeOH at reflux, which afforded isoxazole key intermediate as a mixture of two regioisomers in 9:1 ratio **62** and **62a** (LCMS). The regio mixture **62** and **62a** was treated with *n*-BuLi at −78°C, quenched with dimethyl carbonate in THF afforded methyl ester derivative of **63** and **63a** as a mixture of regioisomers. The two regioisomers **63** and **63a** were separated by flash column chromatography obtained compound **63** as major isomer (**48%**) and compound **63a** as minor isomer (**10%**). These two regioisomers **63** and **63a** were characterized and confirmed by ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC experiments.



Scheme 8.2 Synthesis of diaziridinyl quinone isoxazole derivatives **(67a-j)**.

8.4.2.1 Confirmation of Regioisomers 63 and 63a

The HMBC, HSQC, and ^{13}C spectroscopic data were used to characterize the structures of two regioisomers **63** and **63a**. Heteronuclear Multiple Bond Coherence (HMBC) spectrum enabled to obtain proton-carbon long-distance couplings over 2 to 3 bonds to assign the ^{13}C chemical shifts of **C-4** (163.26 ppm) and **C-7** (160.59 ppm) of regioisomer **63** and the ^{13}C chemical shifts of C-4 (166.72 ppm) and **C-7** (157.67 ppm) for other regioisomer **63a**. HMBC spectroscopy of compounds **63** and **63a** where the delay in pulse sequence was optimized for 8 Hz showed both two and three bond coupling cross-peaks, for compounds **63** and **63a**, and we could easily discern the 2 and 3 bond coupling of the isoxazole proton (**H6, 7.06 ppm**) and the phenyl proton (**H1, 7.51 ppm**) to the **C4** carbon and 2 and 3 bond coupling of the isoxazole proton (**H6, 7.06 ppm**) and the thiophene proton (**H10, 7.51 ppm**) to the **C7** carbon, respectively, shown as magenta color peaks in Figures 8.7 and 8.8. Here, we assume the

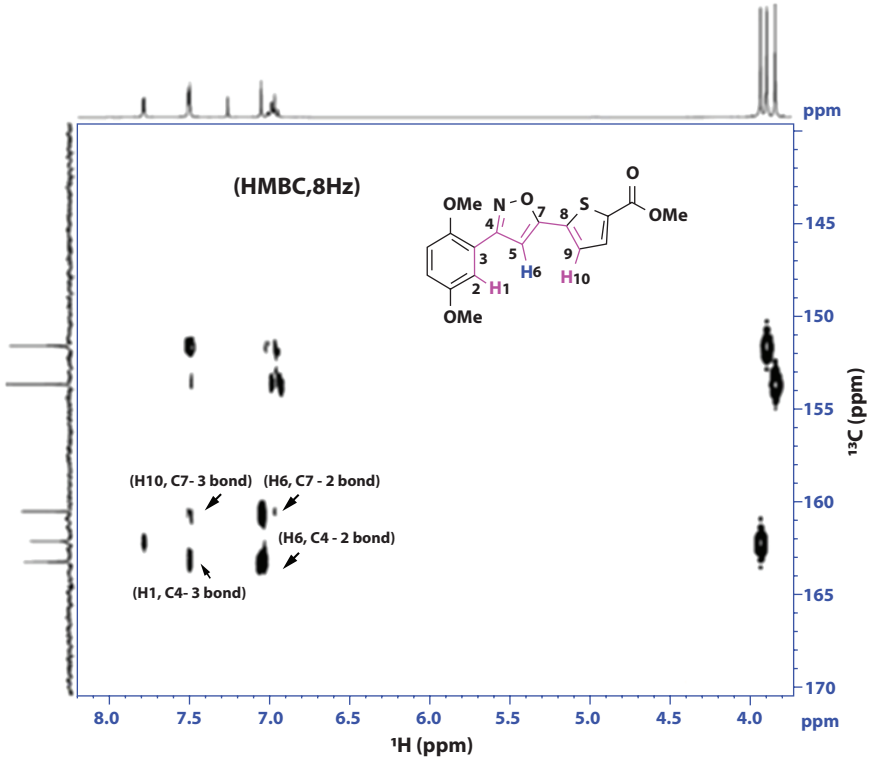


Figure 8.7 HMBC Connectivity Spectrum of Compound **63** at 8 Hz (key correlation peaks are colored magenta).

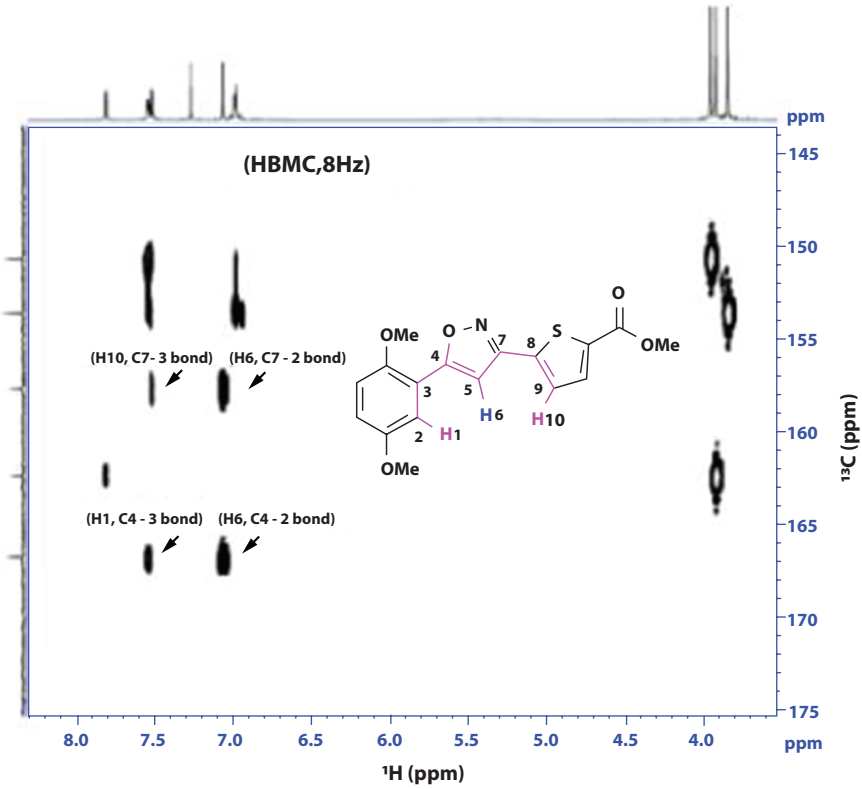


Figure 8.8 HMBC Connectivity Spectrum of Compound **63a** at 8 Hz (key correlation peaks are colored magenta).

structures theoretically based on the difference in chemical shift values of C4 and C7 in both isomers where the difference is low (2.03 ppm) which is considered as compound **63**, and where the difference is more (9.03 ppm) is considered as compound **63a**. Further, our assumption was confirmed more authentically through single-crystal XRD (Figure 8.9) recorded for the compound **185h** (CCDC accession no. 1423565).

The major isomer ester derivative **63** was carried to subsequent steps. The base mediated hydrolysis with NaOH of compound **63** gave the key intermediate carboxylic acid derivative **64**, which was subjected to acid-amine coupling with a series of aliphatic, aromatic, acyclic, and cyclic,

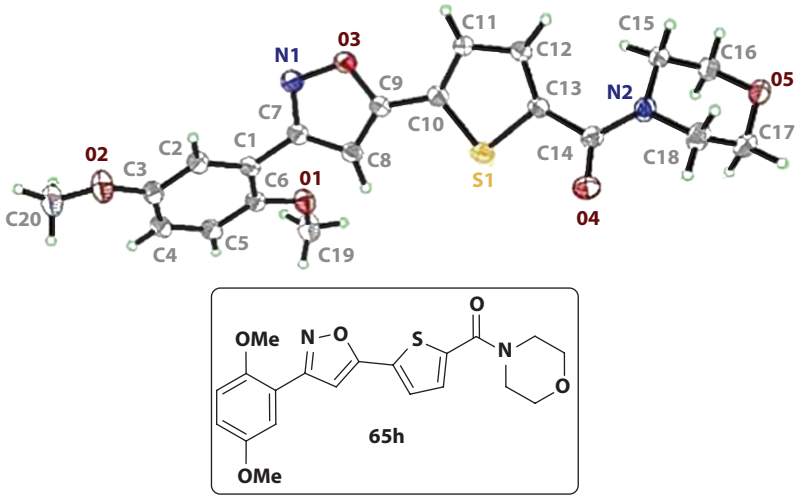


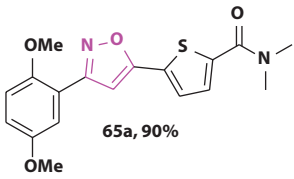
Figure 8.9 A view of **65h** showing the atom-labeling scheme. Displacement ellipsoids are drawn at the 30% probability level and H atoms are represented by circles of arbitrary radii.

primary/secondary-amines using HATU as a coupling reagent, afforded amide derivatives **65a-n (50%–95%)**, Table 8.1.

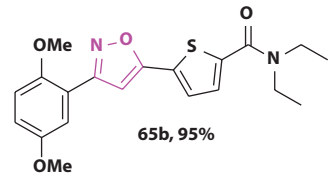
The oxidation of amide derivatives **65a-n** with ceric ammonium nitrate in ACN/water at 0°C afforded quinone derivatives **66a-j;** Table 8.2 as yellow to orange solids in very good yields (**55%–88%**)**.** However, oxidation of some amides **k** (4-methoxybenzyl), **l** (N-methylpiperazine), **m** (cyclopropyl), and **n** (2-fluorophenyl) was unsuccessful and the corresponding quinone derivatives **66k**, **66l**, **66m**, and **66n** were found to be unstable even at 0°C.

The key reaction is the insertion of aziridine into the quinone ring system. The quinone derivatives **66a-j** were treated with freshly prepared aziridine [50] in $CuSO_4$/MeOH resulting in diaziridinyl benzoquinone isoxazole derivatives (**67a-j**) Table 8.3 as an orange to red solids with moderate to excellent yields (**50%–90%**). We also noticed that the aromatic quinones gave good yields as compared to the aliphatic quinones. Here, a mixture of monosubstituted and disubstituted aziridinyl derivatives was expected; however, exclusively diaziridinyl analogs were obtained.

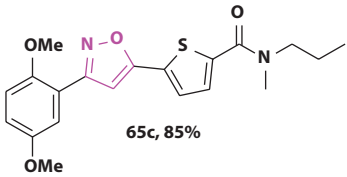
Table 8.1 Dimethoxy isoxazole derivatives **65a-n**.



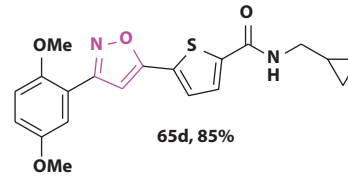
65a, 90%



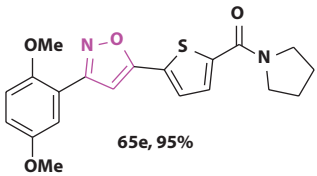
65b, 95%



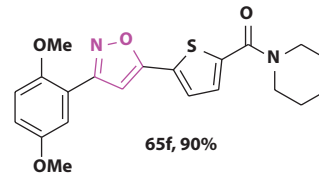
65c, 85%



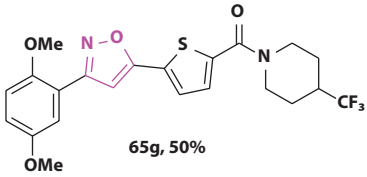
65d, 85%



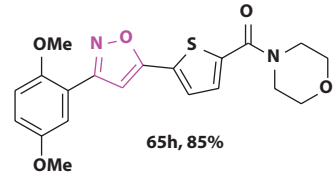
65e, 95%



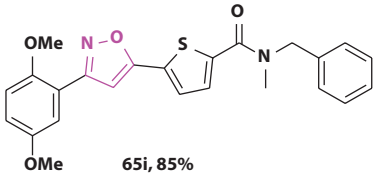
65f, 90%



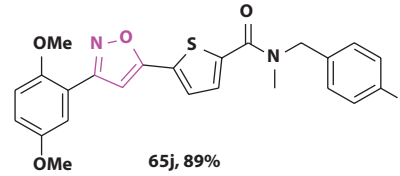
65g, 50%



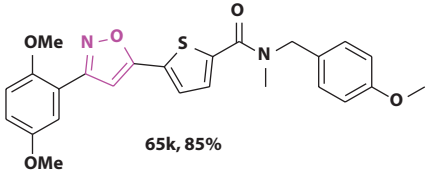
65h, 85%



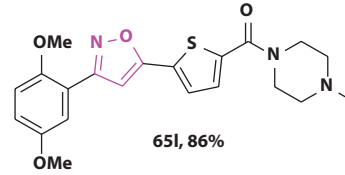
65i, 85%



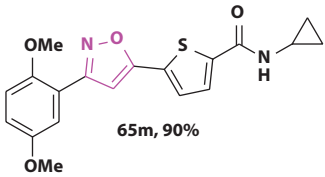
65j, 89%



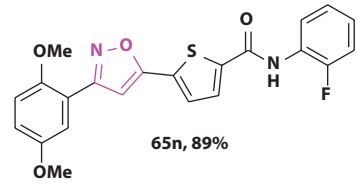
65k, 85%



65l, 86%

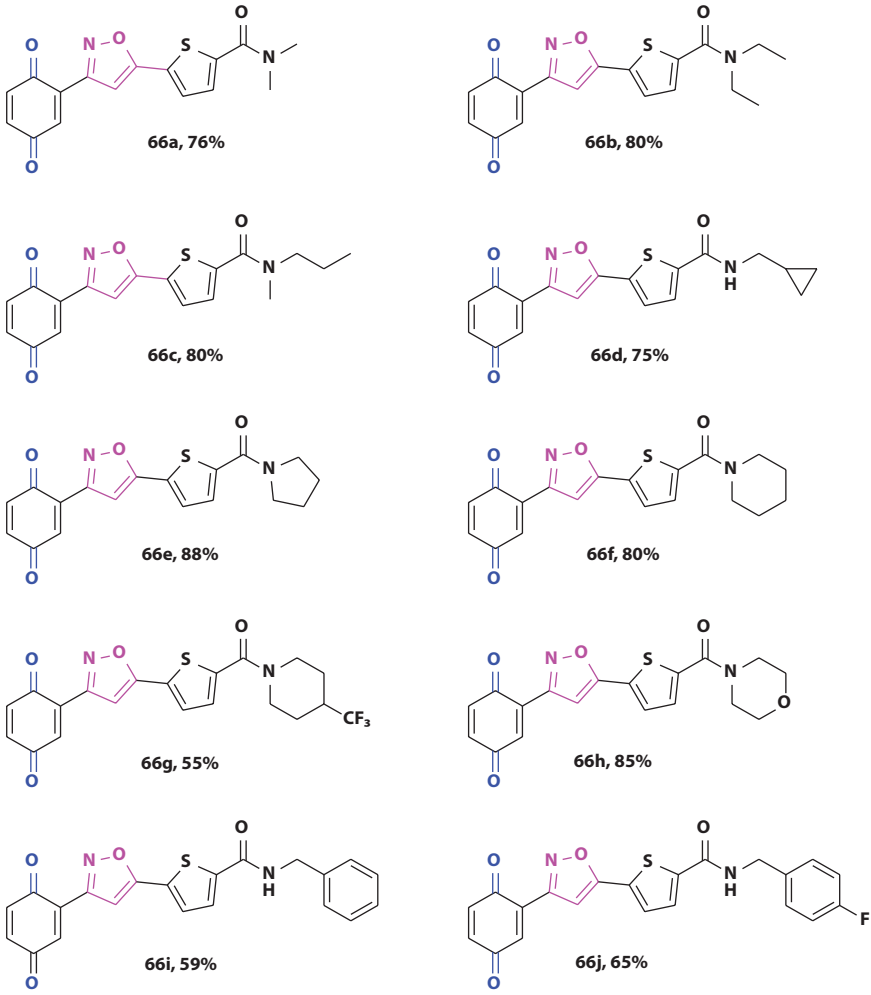


65m, 90%



65n, 89%

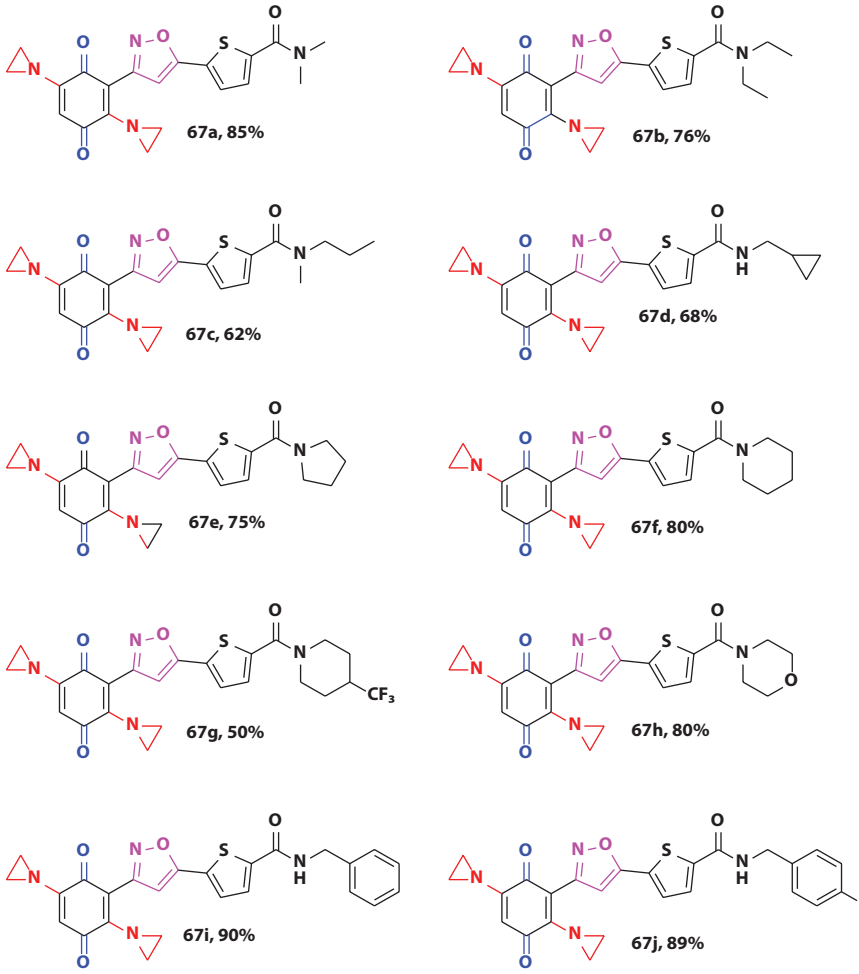
Table 8.2 Quinone isoxazole derivatives **66a-j**.



8.4.2.2 Confirmation of Regioselectivity for Diaziridinyl Compounds

The HMBC spectroscopic data was used to confirm the introduction of aziridine (Figure 8.10) at positions 3 and 6. The NOESY (Figure 8.11) spectrum clearly explains the substitution regioselectivity in compound **67h**. HMBC spectroscopy of compound **67h** showed both two and three bond coupling cross-peaks. The absence of a correlation of proton (H7, 6.037 ppm) to the C-2 (112.402 ppm) confirmed the structure of **67h** with

Table 8.3 Diaziridinyl quinone isoxazole derivatives **(67a-j)**.



diaziridine at positions 3 and 6. NOESY data also supported the structure by showing a special correlation of H7 (6.037 ppm) proton with one of the aziridine moiety C8 and C8a.

8.4.3 Biological Evaluation

All the synthesized diaziridinyl quinone isoxazole hybrid derivatives were evaluated for various biological activities such as antimicrobial, minimum

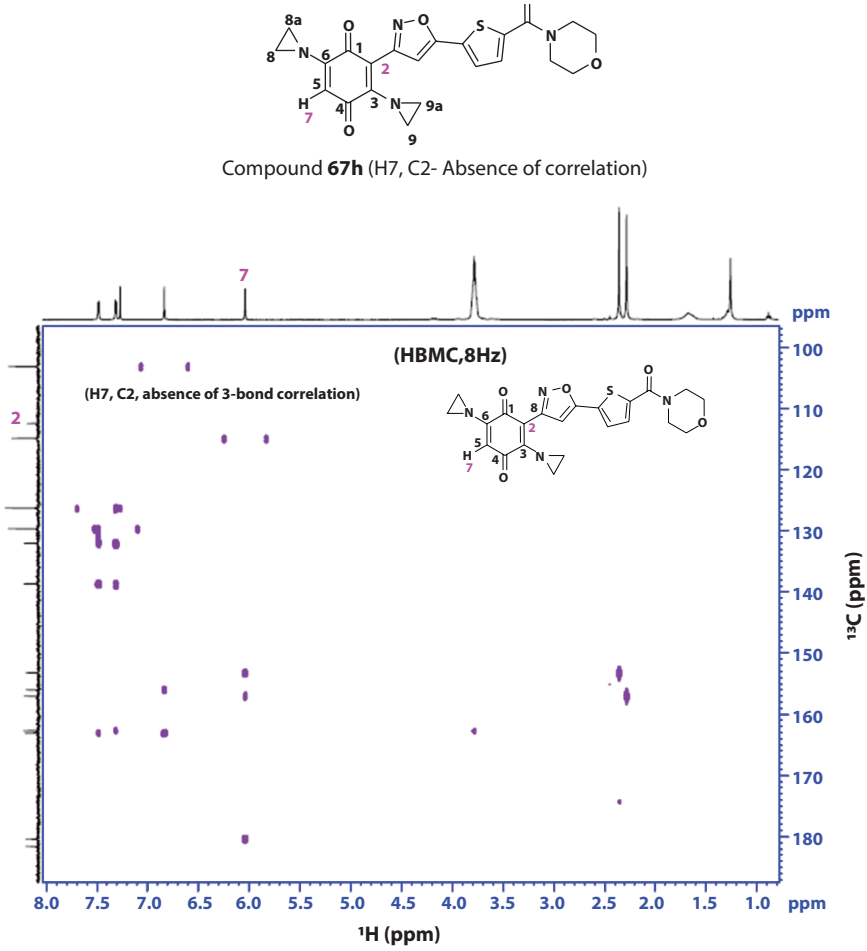


Figure 8.10 HMBC connectivity spectrum of diaziridinyl compound **67h**.

bactericidal concentration (MBC), anti-biofilm, antifungal, minimum fungicidal concentration (MFC), and cytotoxicity on two cell lines (A549 and PC3).

8.4.3.1 Antibacterial Activity

Compounds **67a-j** were screened for *in vitro* antibacterial activity against various Gram-positive and Gram-negative bacterial strains such as *Micrococcus luteus* MTCC 2470, *S. aureus* MTCC 96, *S. aureus* MLS-16 MTCC 2940, *B. subtilis* MTCC 121, *E. coli* MTCC 739, *P. aeruginosa*

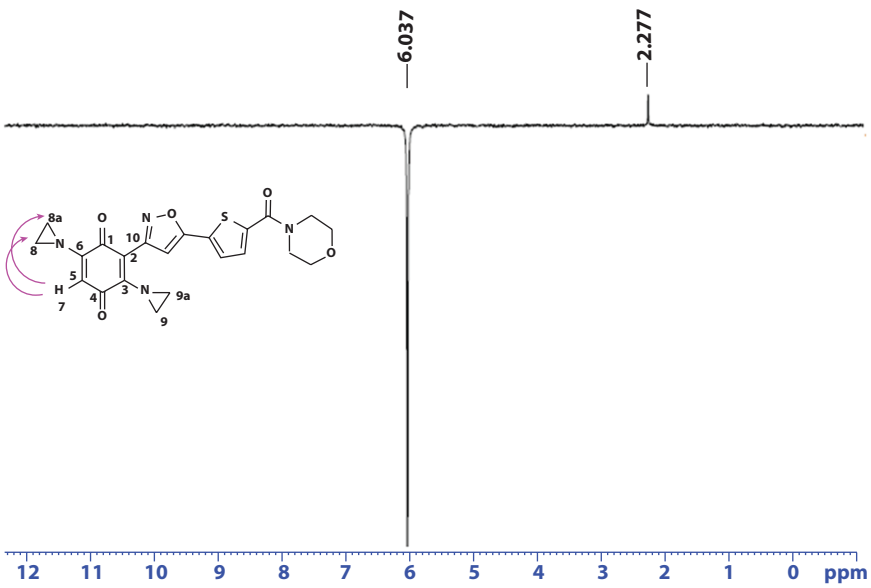


Figure 8.11 NOESY spectrum of diaziridinyl compound **67h**.

MTCC 2453, and *Klebsiella planticola* MTCC 530. Compounds **67a**, **67b**, **67c**, **67e**, **67f**, **67g**, and **67h** exhibited promising activity (MIC values ranging between 3.9 and 62.5 μg/ml) against all tested bacterial strains except *E. coli* MTCC 739 and *P. aeruginosa* MTCC 2453. The compounds **67a**, **67c**, **67f**, **67g**, and **67h** exhibited good activity (MIC value of 3.9 μg/ml) specifically toward *Bacillus subtilis* MTCC 121 and compound **67h** proved promising and potent exhibiting antibacterial activity against *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940 and *Bacillus subtilis* MTCC 121 with MIC value of 3.9 μg/ml and toward *Klebsiella planticola* MTCC 530 with MIC value of 7.8 μg/ml. Based on the SAR, it was observed that compounds **67a**, **67b**, and **67c** exhibited variable activities as compared to the compound **67h** which has a morpholine substituent attached to the basic diaziridinyl quinone isoxazole scaffold, having two electronegative atoms in the ring probably contributing to the antibacterial activity. The antibacterial activity results in this regard are tabulated in Table 8.4.

8.4.3.2 Minimum Bactericidal Concentration

Further, all the synthesized compounds were screened for the MBC against all the bacterial strains except *Escherichia coli* MTCC 739 and *Pseudomonas*

Table 8.4 Antimicrobial activity of target compounds **(67a-j)**.

Test compounds	Minimum inhibitory concentration (µg/ml)							
	Staphylococcus aureus **MTCC 9**	***Bacillus subtilis*** **MTCC 121**	***S. aureus*** **MLS16 MTCC 2940**	***Micrococcus luteus*** **MTCC 2470**	***Klebsiella planticola*** **MTCC 530**	***Escherichia coli*** **MTCC 739**	***Pseudomonas aeruginosa*** **MTCC 2453**	***Candida albicans*** **MTCC 3017**
67a	15.6	3.9	15.6	62.5	7.8	>125	>125	15.6
67b	7.8	7.8	7.8	15.6	15.6	>125	>125	15.6
67c	7.8	3.9	15.6	15.6	7.8	>125	>125	7.8
67d	>125	15.6	>125	>125	>125	>125	>125	15.6
67e	15.6	7.8	15.6	31.2	15.6	>125	>125	31.2
67f	15.6	3.9	15.6	62.5	7.8	>125	>125	15.6
67g	>125	3.9	>125	31.2	>125	>125	>125	31.2
67h	3.9	3.9	3.9	15.6	7.8	>125	>125	7.8
67i	31.2	31.2	62.5	15.6	>125	>125	>125	>125
67j	62.5	15.6	31.2	31.2	>125	>125	>125	>125
Mitomycin C (Standard)	1.9	0.45	1.9	0.9	1.9	3.9	1.9	>500
Miconazole (Standard)	–	–	–	–	–	–	–	7.8
Ciprofloxacin (Standard)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	–

Table 8.5 Minimum bactericidal concentration of target compounds **67a-j**.

Minimum bactericidal concentration (μg/ml)					
Test compounds	***Bacillus subtilis* MTCC 121**	***Staphylococcus aureus* MTCC 96**	***Staphylococcus aureus* MLS16 MTCC 2940**	***Micrococcus luteus* MTCC 2470**	***Klebsiella planticola* MTCC 530**
67a	7.8	31.2	31.2	125	31.2
67b	31.2	15.6	31.2	62.5	62.5
67c	7.8	15.6	31.2	31.2	15.6
67d	31.2	>125	>125	>125	>125
67e	15.6	31.2	31.2	62.5	31.2
67f	15.6	31.2	62.5	125	15.6
67g	7.8	>125	>125	62.5	>125
67h	3.9	7.8	3.9	31.2	15.6
67i	125	62.5	125	62.5	>125
67j	62.5	>125	>125	>125	>125
Mitomycin C (Standard)	0.9	3.9	1.9	1.9	3.9
Ciprofloxacin (Standard)	1.9	0.9	1.9	0.9	1.9

aeruginosa MTCC 2453 in comparison to ciprofloxacin as standard. The activity data to this regard is shown in Table 8.5. Based on the MBC results, it was observed that most of the compounds showed good to moderate bactericidal activity against all the tested bacterial strains with MBC values ranging between 3.9 and 62.5 μg/ml. Among the tested compounds, the compound **67h** was exhibited quite promising with MBC values of 3.9, 7.8, and 3.9 μg/ml against *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, and *Staphylococcus aureus* MLS-16 MTCC 2940, respectively. Further, the compounds **67a**, **67c**, and **67g** also showed good results against *Bacillus subtilis* MTCC 121 with MBC values of 7.8 μg/ml. The compounds like **67b** and **67c** exhibited specific bactericidal activity against *Staphylococcus aureus* MTCC 96 with MBC values of 15.6 μg/ml, while compounds **67c**, **67f**, and **67h** exhibited bactericidal activity against *Klebsiella planticola* MTCC 530 with MBC values of 15.6 μg/ml. Other compounds exhibited moderate bactericidal activity against all the tested bacterial strains (MBC values ranging between 31.2 and 125 μg/ml).

8.4.3.3 Biofilm Inhibition Assay

Biofilms are surface-attached structured communities of bacteria surrounded in a self-produced extracellular polymeric matrix, which is composed of proteins, polysaccharides, DNA, and other components that provide mechanical strength and stability to the biofilm so that it can withstand the shear forces [51]. Several factors including the substratum, structure, availability of nutrients, the microbial species involved, the EPS contribute to biofilm formation and final architecture [52]. Bacterial biofilms cause chronic infections in humans through hospital and community environments. Due to high antibiotic resistance exhibited by them and the ability to resist the body's defense system as well as phagocytosis, they are important from a clinical observation [53]. The biofilm-related infections due to the indwelling implants such as prostheses, heart valves, stents, vascular grafts, and urinary catheters have expanded the importance from a biomedical point [54]. Based on these facts, the novel compounds that can specifically target and inhibit the biofilm formation would be motivating as compared to the rational use of antibiotics. In the present study, steps were undertaken to investigate whether the synthesized diaziridinyl quinone isoxazole hybrids exhibited a specific anti-biofilm activity or whether this observation was simply related to the general toxic effect on the Gram-positive and -negative bacterial strains.

To this regard, the selected compounds **67a**, **67c**, **67d**, **67f**, **67g**, and **67h** exhibiting good antimicrobial activity were further screened

Table 8.6 Biofilm inhibition assay of target compounds **67a**, **67c**, **67d**, **67f**, **67g**, and **67h**.

Test compounds	IC_{50} values (in μM)				
	Bacillus subtilis **MTCC 121**	***Staphylococcus aureus*** **MTCC 96**	***Staphylococcus aureus*** **MLS16 MTCC 2940**	***Micrococcus luteus*** **MTCC 2470**	***Klebsiella planticola*** **MTCC 530**
67a	2.2 ± 0.12	9.8 ± 0.15	10.1 ± 0.25	38.9 ± 0.52	6.1 ± 0.26
67c	2.2 ± 0.15	4.6 ± 0.24	10.6 ± 0.35	8.9 ± 0.22	3.5 ± 0.15
67d	9.8 ± 0.35	–	–	–	–
67f	2.8 ± 0.14	11.2 ± 0.24	9.8 ± 0.18	35.6 ± 0.48	5.4 ± 0.21
67g	2.1 ± 0.17	–	–	16.2 ± 0.28	–
67h	2.8 ± 0.22	2.5 ± 0.11	1.9 ± 0.11	9.8 ± 0.33	5.1 ± 0.31
Mitomycin C (Standard)	0.3 ± 0.07	1.1 ± 0.09	0.9 ± 0.11	0.9 ± 0.09	1.1 ± 0.12
Ciprofloxacin (Standard)	0.5 ± 0.11	0.7 ± 0.08	0.6 ± 0.12	0.8 ± 0.08	0.6 ± 0.09

for anti-biofilm activity [55] against *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS16 MTCC 2940, *Micrococcus luteus* MTCC 2470, and *Klebsiella planticola* MTCC 530 which are important nosocomial pathogens having a biofilm-forming ability. The results summarized in Table 8.6, clearly reveal that not much information on the SAR can be highlighted at this stage; however, it was observed that compounds **67a**, **67c**, **67f**, and **67h** exhibited promising activity (IC_{50} values ranging between 1.9 and 38.9 μM) toward all the tested bacterial species. Compound **67d** and **67g** showed specific activity toward *Bacillus subtilis* MTCC 121, and *Micrococcus luteus* MTCC 2470. Based on the SAR, it was observed that the basic diaziridinyl quinone isoxazole the scaffold of these compounds **67d** and **67g** possess different substituents such as cyclopropyl and trifluoromethyl piperidine amides which antagonize the biofilm formation and probably may be contributing to the anti-biofilm activity. The activity data to this regard is shown in Table 8.6.

8.4.3.4 Antifungal Activity

Different *Candida* species are important opportunistic fungal pathogens among patients with transplantation procedures, immunosuppression, cancer chemotherapy, chronic indwelling devices, broad-spectrum antibiotics, and/or among HIV-infected individuals [56]. Among the many pathogenic *Candida* species, most notably *Candida albicans* is a dimorphic fungus behaving as a commensal or an opportunistic pathogen causing both superficial and systemic infections [57]. Different *Candida* strains can form biofilms and candidiasis is usually associated with indwelling medical devices [56]. Based on the results depicted in Table 8.7 of the present study, it was found that only compounds **67c** and **67h** showed promising antifungal activity against *Candida albicans* MTCC 3017 (MIC value of 7.8 μg/ml) comparable to the standard miconazole drug. However, Mitomycin C was not active against this fungal strain even at the maximum tested concentration (>500 μg/ml). Considering this fact, we further screened these two compounds against a series of other *Candida* strains of clinical importance available in our laboratory. It was interesting to note that these two compounds exhibited antifungal activity against all the tested *Candida* strains. However, compound **67h** showed equipotent antifungal activity (MIC value of 7.8 μg/ml) against *C. albicans* MTCC 227, *C. albicans* MTCC 854, and *C. krusei* MTCC 3020, while compound **67c** exhibited promising activity only against *C. aaseri* MTCC 1962 (MIC value of 7.8 μg/ml) as compared to miconazole drug. Based on the SAR, it was observed that compound **67h** has a morpholine substituent attached to the basic

Table 8.7 Antifungal activity of target compounds **67a** and **67h**.

Test compounds	Minimum inhibitory concentration (μg/ml)												
	C.a MTCC 183	*C.a* MTCC 227	*C.a* MTCC 854	*C.a* MTCC 1637	*C.a* MTCC 3018	*C.a* MTCC 3958	*C.a* MTCC 4748	*C.a* MTCC 7315	*C.a parapsilosis* MTCC 1744	*C.a aaseri* MTCC 1962	*C.a glabrata* MTCC 3019	*C.a krusei* MTCC 3020	*Issatchenkia hanoiensis* MTCC 4755
67c	31.2	62.5	62.5	62.5	31.2	62.5	31.2	15.6	15.6	7.8	15.6	15.6	15.6
67h	15.6	7.8	7.8	31.2	62.5	15.6	31.2	62.5	15.6	31.2	31.2	7.8	15.6
Miconazole (Standard)	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

*C.a = *Candida albicans*

diaziridinyl quinone isoxazole scaffold, having two electronegative atoms in the ring, while compound **67c** has methyl and propyl substituents which probably may be contributing to the antifungal activity. The antifungal activity results in this regard are tabulated in Table 8.7.

8.4.3.5 Minimum Fungicidal Concentration

Based on the antifungal activity results, the selected compounds **67c** and **67h** were further evaluated for MFC against different *Candida* strains in comparison to the standard miconazole drug. Both the tested compounds showed MFC values ranging between 15.6 and 125 μg/ml. However, the standard miconazole drug exhibited MFC values ranging between 7.8 and 15.6 μg/ml. Among them, the compound **67h** proved promising against *Candida albicans* MTCC 3017 (MFC value of 15.6 μg/ml). The MFC activity data to this regard is represented in Table 8.8.

8.4.3.6 Cytotoxic Activity

The cytotoxic effect of the synthesized isoxazole amide hybrids **65a-m**, quinone isoxazole hybrids **66a-k**, and diaziridinyl quinone isoxazole hybrids **67a-j** were evaluated using the XTT-based colorimetric assay on A549 and PC3 cell lines. Based on the cytotoxicity results presented in Table 8.9, it was observed that among the isoxazole amide hybrids **65a-m** screened, compound **65b** showed IC_{50} values of 11 and >30 μM, while compound **65m** showed promising cytotoxicity (IC_{50} values of >30 and 7.8 μM, on A549 and PC3 cell lines, respectively. While the other compounds showed moderate cytotoxicity with IC_{50} values of >30 μM. Further, when the isoxazole amide hybrids were subjected to oxidation, they were converted to quinone isoxazole hybrids. When the quinone isoxazole hybrids **66a-k** were subjected to cytotoxic evaluation, based on the SAR, it was observed that the potency of some of the compounds **66a**, **66b**, **66c**, **66e**, **66i**, and **66k** was enhanced by the presence of highly reactive quinone moiety. The compound **66a** showed the IC_{50} value of 7.2 μM and the compound **66b** showed promising cytotoxicity (IC_{50} value of 10 and 8.9 μM), while compound **66i** showed the IC_{50} value of >30 and 11 μM, on A549 and PC3 cell lines, respectively.

Based on the cytotoxicity results presented in Table 8.9 for diaziridinyl quinone isoxazole hybrids **67a-j**, it was observed that the compounds showing IC_{50} values ranging between 1 and 4 μM proved promising. Based on the SAR, it was noticed that the diaziridinyl moiety attached to the quinone isoxazole scaffold enhances the potency of the different derivatives **67a-j**.

Table 8.8 Minimum fungicidal concentration of target compounds **67c** and **67h**.

Test compounds	Minimum inhibitory concentration (µg/ml)												
	C.a MTCC **183**	*C.a* MTCC **227**	*C.a* MTCC **854**	*C.a* MTCC **1637**	*C.a* MTCC **3018**	*C.a* MTCC **3958**	*C.a* MTCC **4748**	*C.a* MTCC **7315**	*C. parapsilosis* MTCC **1744**	*C.aaseri* MTCC **1962**	*C.glabrata* MTCC **3019**	*C.krusei* MTCC **3020**	*Issatchenkia hanoiensis* MTCC **4755**
67c	31.2	62.5	62.5	62.5	31.2	62.5	31.2	15.6	15.6	7.8	15.6	15.6	15.6
67h	15.6	7.8	7.8	31.2	62.5	15.6	31.2	62.5	15.6	31.2	31.2	7.8	15.6
Miconazole (Standard)	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

Table 8.9 Cytotoxic effects of the compounds **(65a-m)**, **(66a-j)**, and **(67a-j)**.

Test compounds	IC_{50} (μM)	
	A549 (lung carcinoma cell line)	**PC3 (prostrate carcinoma cell line)**
65a	>30	>30
65b	11	>30
65c	>30	>30
65d	>30	>30
65e	>30	>30
65f	>30	>30
65g	>30	>30
65h	>30	>30
65i	>30	>30
65j	>30	>30
65k	>30	>30
65m	>30	7.8
66a	>30	7.2
66b	10	8.9
67c	>30	<30
67d	>30	>30
67e	>30	<30
67f	>30	>30
67g	>30	>30
67i	>30	11
67j	>30	>30
67k	>30	<30
67a	1.5	>30

(*Continued*)

Table 8.9 Cytotoxic effects of the compounds **(65a-m)**, **(66a-j)**, and **(67a-j)**. (*Continued*)

Test compounds	IC_{50} (μM)	
	A549 (lung carcinoma cell line)	**PC3 (prostrate carcinoma cell line)**
67b	0.9	**0.5**
67c	2.7	**0.6**
67d	2.1	3.5
67e	1.7	1.0
67f	1.7	21.3
67g	3.7	2.0
67h	1.5	1.4
67i	7.3	1.2
67j	15.3	**0.5**
Puromycin	0.6	0.7
Mitomycin C	0.5	0.5

Compound **67b** exhibited promising cytotoxicity toward PC3 cells (IC_{50} value of 0.5 μM), while toward the A549 cell line the IC_{50} value was 0.9 μM. Based on the SAR, we expected that the potency of compound **67b** was further increased by the presence of diethyl amide along with the presence of diaziridine moiety. The compound **67b** (IC_{50} value of 0.5 μM) also exhibited equipotent cytotoxicity as compared to reference drug Mitomycin C (IC_{50} value of 0.5 μM) and more potent than the other reference drug Puromycin (IC_{50} value of 0.7 μM) toward both PC3 cell line. The compound **67a** did not show cytotoxicity toward PC3 cells even at 30 μM concentration. It was also observed that the basic diaziridinyl quinone-isoxazole scaffolds **67a** and **67b** compounds possess dimethyl amide and diethylamide substituents, respectively, and probably the length of the aliphatic chain (enhanced lipophilicity) may be contributing to the considerable difference in the cytotoxicity of these two compounds. Similarly, compound **67c** exhibited cytotoxicity toward PC3 cells (IC_{50} value of 0.6 μM), and the potency of **67c** was observed to be very close to the reference drug Mitomycin C (IC_{50} values of 0.5 μM). Interestingly, compound **67f** showed selectivity toward

A549 when compared with PC3 cells, 1.7 and 21.3 μM, respectively (~12 fold). Based on the SAR, it was observed that the basic diaziridinyl quinone isoxazole scaffold **67f** possess six-membered cyclic amide substituents, probably the size of the cyclic ring contributed to the cytotoxicity selectively toward A549 when compared with PC3 cells. Similarly, compound **67j** showed selectivity toward the PC3 cell line when compared to the A549 cell line, 0.5 and 15.3 μM (~30 fold selective), respectively. We also noticed that the presence of fluoro benzyl amide substituent on the basic scaffold contributed to the cytotoxicity selectively toward the PC3 cell line and was equipotent to Mitomycin C (IC_{50} value of 0.5 μM). The reference compounds Puromycin showed IC_{50} values of 0.6 and 0.7 μM, respectively, while Mitomycin C showed IC_{50} values of 0.5 μM toward both A549 and PC3 cell lines.

8.4.4 Experimental Section

8.4.4.1 Chemistry

A series of novel diaziridinyl quinone isoxazole hybrids **67a-j** were synthesized starting from 2, 5-dimethoxy acetophenone 1 *via* Claisen reaction, cyclization, alkoxy carbonylation, hydrolysis, oxidation, and aziridine insertion.

1-(5-Bromothiophen-2-yl)-3-(2,5-dimethoxyphenyl)propane-1,3-dione (61). To a suspension of NaH (83.3 mmol, 1.5 eq) in dry THF (50 ml) was added a solution of 2,5- diethoxy acetophenone **59** (10 g, 55.5 mmol, 1 eq) and 2-bromo,5-thiophene carboxylic acid ethyl ester **60** (66.6 mmol, 1.2 eq) dissolved in 50.0 ml of dry THF in dropwise for 1 h under an inert atmosphere. Then, the mixture was refluxed for 2 h. The progress of the reaction was monitored by TLC analysis. Then, the reaction mixture was allowed to cool to room temperature, 30 ml of cold water was added, and later 30 ml of 3 mol/L hydrochloric acid was added. The organic layer was separated, washed with water (100 ml) and brine (100 ml), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford crude *β*-diketone **61** as a yellow solid (6 g; 16.3 mmol; **30%**), which was used directly for the next step without any further purification.

5-(5-Bromothiophen-2-yl)-3-(2,5-dimethoxyphenyl)isoxazole (62). To a solution of crude *β*-diketone **61** (6 g, 21.68 mmol) in MeOH (80 ml) was added NH_2OH-HCl (43.3 mmol, 2 eq) and conc. HCl (catalytic) and heated to reflux for 16 h. The progress of the reaction was monitored by TLC analysis. The reaction mixture was concentrated, and the crude compound obtained was dissolved in EtOAc and washed with water (100 ml).

Dried over anhyd. $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by flash column chromatography using silica gel (60–120 mesh), eluted with 5% EtOAc and hexane to give an isomeric mixture of isoxazole **62 and 62b** in 9:1 ratio (4.5 g; 12.32 mmol, **75%**).

Methyl 5-[3-(2,5-dimethoxyphenyl) isoxazol-5-yl]thiophene-2-carboxylate (63). To a solution of compound **62** and **62a** (4.5 g, 12.32 mmol, 1eq), in dry THF (100 ml) was added *n*-BuLi (1.6 ml in THF, 24.65 mmol, 2 eq), slowly at −78°C under an argon atmosphere and stirred for 30 min. Then, dimethyl carbonate (24.65 mmol, 2 eq) was added slowly and stirred at −78°C for 2 h. The progress of the reaction was monitored by TLC analysis. The reaction mixture was cooled down to room temperature and added aq. a solution of ammonium chloride (10%, 200 ml). The reaction mixture was diluted with EtOAc (200 ml), the organic layer was washed with water (200 ml) and brine (100 ml), then dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (petroleum ether/EtOAc (94: 6 → 88: 12) to give isoxazole methyl ester derivatives as yellow solids (**63:** 2 g, 5.79 mmol, **48%**); **63a**: 400 mg, 1.16 mmol, **10%**).

5-[3-(2, 5-Dimethoxyphenyl) isoxazol-5-yl]thiophene-2-carboxylic acid (64). To a solution of isoxazole methyl ester **63** (5 g, 14.59 mmol, 1 eq), in MeOH (30 ml) was added 2N NaOH solution (43.77 mmol, 3 eq) at ambient temperature. The reaction mixture was stirred at room temperature for 16 h. The progress of the reaction was monitored by TLC analysis. Later, MeOH was removed under vacuum, water (10 ml) was added and acidified with 20% hydrochloric acid, the solid precipitated out was filtered and then dried to give isoxazole acid **64** as a white solid (4.5 g, 13.5 mmol, **95%**).

General procedure for amide coupling (**65a**). To a solution of isoxazole acid derivative **185** (0.624 mmol, 1 eq), in dry DMF (5 ml) were added HATU (0.95 mmol, 1.5 eq), DIPEA (1.87 mmol, 3 eq) and a corresponding amine at room temperature and left it stirring for 4 h at room temperature. The progress of the reaction was monitored by TLC analysis. The reaction mixture was diluted with cold water and the solid precipitated out was filtered and dried to afford amide derivative **65a** as a solid.

General procedure for oxidation with CAN (**66a**). To a solution of amide **65a** (0.534 mmol, 1 eq), in ACN: water in 1:1 (6 ml) was added ceric ammonium nitrate (1.6 mmol, 2.5 eq) at 0°C and stirred at the same temperature for 1 h. The progress of the reaction was monitored by TLC analysis. The reaction mixture was diluted with water and the solid precipitated out was filtered and dried to give quinone derivative **66a** as a colored solid (based on the amide **65** used).

General procedure for the synthesis of airidine derivatives (**67a**). To a solution of quinone derivative **66a** (0.24 mmol), in dry MeOH (5 ml) were

added $CuSO_4$ (0.046 mmol, 0.2 eq) and Aziridine (1.16 mmol, 5 eq) at 0°C and stirred at the same temperature for 30 min. The progress of the reaction was monitored by TLC analysis. The reaction mixture was diluted with water and the solid precipitated out was filtered and dried to obtain diaziridine derivative **67a** as a colored solid (based on the quinone **66** used).

8.4.4.2 Biological Studies

8.4.4.2.1 Antibacterial Assay

The antimicrobial activity of the diaziridinyl quinone isoxazole hybrids was determined using well diffusion method [58] against different pathogenic reference strains procured from the Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the Muller-Hinton agar Petri plates, with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland). Wells of 6.0 mm in diameter were prepared in the medium plates using a cork borer and the synthesized diaziridinyl quinone isoxazole hybrids at a dose range of 125–0.97 μg ml^{-1} was added in each well under sterile conditions in a laminar airflow chamber. A standard antibiotic solution of Ciprofloxacin, Mitomycin C, and Miconazole at a dose range of 125–0.97 μg ml^{-1} and the well-containing methanol served as positive and negative controls, respectively. The plates were incubated for 24 h at 37°C for bacterial and 30°C for *Candida albicans* and the well containing the least concentration showing the inhibition zone was considered as MIC. All experiments were carried out in duplicates and mean values are represented.

8.4.4.2.2 Minimum Bactericidal Concentration Assay

MBC assay was performed in sterile 2.0 ml microfuge tubes against a panel of pathogenic bacterial strains, including *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, and *Klebsiella planticola* MTCC 530, cultured overnight in Mueller Hinton broth. Serial dilutions of test compounds at different concentrations ranging from 0 to 125 μg ml^{-1} were prepared in Mueller Hinton broth. To the test compounds, 100 μl of overnight cultured bacterial suspensions were added to reach a final concentration of 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard) and incubated at 37°C for 24 h. After 24-h incubation, MBC was determined by sampling 10 μl

of suspension from the tubes onto Mueller Hinton agar plates and were incubated for 24 h at 37°C to observe the growth of test organisms. MBC is the lowest concentration of test compound required to kill a particular bacterial strain. All the experiments were carried in duplicates and mean values are represented.

8.4.4.2.3 Biofilm Inhibition Assay

The diaziridinyl quinone isoxazole hybrids were screened in sterile 96 well polystyrene microtiter plates using the modified biofilm inhibition assay [55] against a panel of pathogenic bacterial strains including *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Pseudomonas aeruginosa* MTCC 2453 and *Klebsiella planticola* MTCC 530, which were cultured overnight in tryptone soy broth (supplemented with 0.5% glucose). The test compounds of predetermined concentrations ranging from 0 to 250 μg ml^{-1} were mixed with the bacterial suspensions having an initial inoculum concentration of 5×10^5 cfu ml^{-1}. Aliquots of 100 μl were distributed in each well and then incubated at 37°C for 24 h under static conditions. The medium was then discarded and washed with phosphate-buffered saline to remove the non-adherent bacteria. Each well of the microtiter plate was stained with 100 μl of 0.1% crystal violet solution followed by 30 min incubation at room temperature. Later, the crystal violet solution from the plates was discarded, thoroughly washed with distilled water for 3 to 4 times, and air-dried at room temperature. The crystal violet stained biofilm was solubilized in 95% ethanol (100 μl) and the absorbance was recorded at 540 nm using TRIAD multimode reader (Dynex Technologies, Inc, Chantilly, VA, USA). Blank wells were employed as a background check. The inhibition data were interpreted from the dose-response curves, where the IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were carried out in triplicates and the values are indicated as mean ± S.D.

8.4.4.2.4 Antifungal Activity

The antifungal activity of the synthesized diaziridinyl quinone isoxazole hybrids was determined using well diffusion method against different *Candida* strains such as *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. aaseri* MTCC 1962, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and

Issatchenkia hanoiensis MTCC 4755 procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the Muller-Hinton agar Petri plates with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard). Wells of 6.0 mm in diameter were prepared in the media plates using a cork borer and the synthesized compounds dissolved in 10% DMSO at a dose range of 125–0.97 μg/ml was added in each well under sterile conditions in a laminar airflow chamber. Standard antibiotic solutions of Miconazole at a dose range of 125–0.97 μg $well^{-1}$, served as a positive control, while the well containing DMSO served as a negative control. The plates were incubated for 24 h at 30°C for different *Candida* strains. The well containing the least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in duplicates and mean values are represented.

8.4.4.2.5 Minimum Fungicidal Concentration Assay

MFC assay was performed in sterile 2.0 ml microfuge tubes. Different *Candida* strains such as *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. aaseri* MTCC 1962, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020, and *Issatchenkia hanoiensis* MTCC 4755 were cultured overnight in Sabouraud dextrose broth. Serial dilutions of test compounds in different concentrations ranging from 0 to 150 μg ml^{-1} were prepared in Sabouraud dextrose broth. To the test compounds, 100 μl of overnight cultured bacterial suspensions were added to reach a final concentration of 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard) and incubated at 30°C for 24 h. After 24-h incubation, the MFC was determined by sampling 10 μl of suspension from the tubes onto fresh plates of Sabouraud dextrose agar to observe the growth of the fungi. The plates were incubated for 24 h at 30°C. All the experiments were carried in duplicates and mean values are represented, where MFC is the lowest concentration of compound required to kill a particular *Candida* strain.

8.4.4.2.6 Cytotoxicity Evaluation

The cytotoxic activities of the synthesized isoxazole amide hybrids (**65a-m**), quinone isoxazole hybrids (**66a-j**), and diaziridinyl quinone isoxazole hybrids (**67a-j**) were tested using an XTT based colorimetric cellular

cytotoxicity assay [59] against A549 derived from human alveolar adenocarcinoma epithelial cells (ATCC No. CCL-185) and PC3 derived from human prostate cancer cells (ATCC No. CRL-1435) cell lines procured from American Type Culture Collection (ATCC). The second-generation tetrazolium dye, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) is bioreduced to form an orange colored formazan derivative which is quantified by measuring the color intensity at 450 nm. The cell lines were cultured in DMEM medium, supplemented with 10% heat-inactivated FBS, in a humidified 5% CO_2 atmosphere. Cells when reached to 80-90% confluency, were detached from the flasks by trypsinization, neutralized, counted, and then seeded with appropriate cell numbers (A549—5,000 cells/well; PC3—3,000 cells/well) in a 96 well clear bottom tissue culture plates. After 24 h, the cells were treated with diaziridinyl quinone isoxazole hybrid derivatives and incubated at 37°C for 72 h in a 5% CO_2 incubator. After incubation, the XTT reagent was added and incubated for 2 h. The absorbance was measured at 450 nm using a SpectraMax^{384} plate reader. The IC_{50} values (50% inhibitory concentration) were calculated from the plotted absorbance data for the dose-response curves. The cytotoxic potency (IC_{50} values in μM) of the compounds was determined by analyzing the data using GraphPad Prism statistical tool.

8.5 Conclusion

A series of novel diaziridinyl quinone isoxazole hybrids **67a**-**j** were synthesized starting from 2,5-dimethoxy acetophenone *via* Claisen reaction, cyclization, alkoxy carbonylation, hydrolysis, oxidation, and aziridine insertion. The formation of diaziridinyl quinone hybrid molecules *via* a simple synthetic sequence is a valuable approach in the development of novel lead compounds. All the synthesized compounds were evaluated for their antibacterial, MBC, antifungal, MFC, anti-biofilm, and cytotoxic activities. All the compounds showed good to very good activity. Among the screened compounds, **67h** exhibited promising antimicrobial activity against all the tested bacterial pathogens except *Escherichia coli* and *Pseudomonas aeruginosa*. Compound **67h** also showed antifungal activity against a range of *Candida* strains. Further, compounds **67c** and **67h** exhibited promising anti-biofilm activity against all the tested pathogens. All the synthesized compounds exhibiting cytotoxicity against A549 and PC3 cell lines with IC_{50} values ranging between 1 and 4 μM proved promising. Compounds **67b** and **67j** exhibited equipotent activity as compared to Mitomycin C against the PC3 cell line.

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9

Exploring the Promising Anticancer and Antimicrobial Potential of Bioactive Triazoles and Their Related Compounds

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Abstract

The increasing incidences of multidrug resistance toward conventional antibiotics and anticancer agents have increased the demand for the discovery and development of new antimicrobial and anticancer agents. Triazoles and their derivatives have gained prominent importance because of their wide range of biological activities. Noticeably, many triazole-based compounds have been utilized for the treatment of various types of diseases, which has shown their great potential as promising medicinal agents. Furthermore, triazole ring-based compounds have the ability to bind readily with a variety of enzymes and receptors in biological systems through different non-covalent interactions and thus exhibit numerous biological activities. Research on triazole-based derivatives as potential drug candidates for various diseases of great concern has been an extremely active area, and many significant advances have been made, which include their use as antiviral, antibacterial, antifungal, anticonvulsant, anti-inflammatory, antituberculosis, analgesic, antidiabetic, anti-obesitic, antihistaminic, antihypertensive, anti-neuropathic, and anticancer agents. This book chapter will delineate the most recent information regarding triazoles and their derivatives with great pharmacological profiles in the fields of antimicrobial and anticancer research. It is anticipated that this book chapter will also provide a stimulus for new ideas in pursuit for the rational design of more active and less toxic triazole-based drug candidates.

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***Keywords*:** Triazoles, heterocyclic aromatic compounds, antimicrobial, anticancer

9.1 Introduction

Microbial infections and cancers are of a great concern to the humanity because they are collectively responsible for causing huge mortality and greater economic losses in countries all over the world. Reports reveal that microbial infections and cancer result in large proportion of deaths worldwide and multi-drug resistance is further aggravating the problem [1]. Presently available antimicrobial and anticancer agents are having several limitations that necessitate the development of new drug candidates to escape the development of resistance and other drug related side effects. Exhaustive research efforts are ongoing all over the world to discover benign and more promising antimicrobial and anticancer agents. Among the range of therapeutic methods to wipe out microbial infections and cancers, the assessment of using heterocyclic compounds have shown tremendous potential due to their varied applications in the fields of drug design and discovery [2, 3]. Several five-membered heterocyclic compounds, such as oxazoles, imidazoles, thiazoles, oxadiazoles, and thiadiazoles, are common and typically possess proficient biological activities. In recent decades, a huge number of publications have revealed that the triazole ring is an important structure with broad-spectrum biological activity.

Triazoles, also known as pyrrodiazoles, are a class of organic heterocyclic compounds comprising of five membered rings bearing three nitrogen (N) atoms and two carbon (C) atoms as part of the five-membered aromatic ring [4]. Triazole is a white, pale yellow crystalline solid with the molecular formula $C_2H_3N_3$ and exist as either one of the two isomeric forms which differ in the relative positions of the three nitrogen atoms as shown in Figure 9.1. Each isomer has two tautomer's which differ by the nitrogen to which hydrogen is bonded (Figure 9.2).

Figure 9.1 Structures of the two isomers (1,2,3-triazole and 1,2,4-triazole) of triazole.

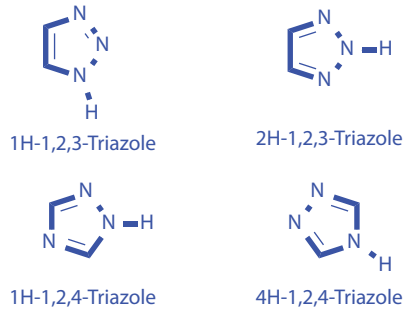


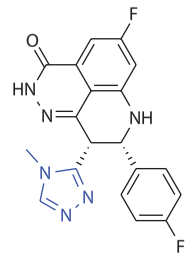
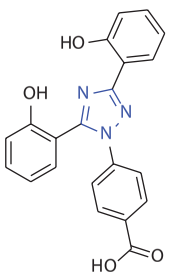
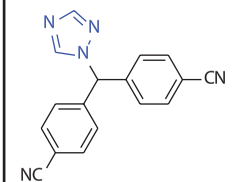
Figure 9.2 Tautomeric structures of 1,2,3-triazole and 1,2,4-triazole.

Among the two isomers (1,2,3-triazole and 1,2,4-triazole) of triazole, heterocycles derived from 1,2,4-triazole have received considerable attention due to their intriguing biological profiles. 1,2,4-triazole derivatives have demonstrated anti-inflammatory, antimicrobial, anticonvulsant, antitubercular, anticancer, and analgesic applications [5–8]. Furthermore, an important feature of triazoles is the aromatic and the electron rich nature of these scaffolds which allows them to interact with different enzymes and other receptors in biological systems. Additionally, triazoles are isostere of imidazole, pyrazole, thiazole, oxazole, amide moiety, etc., and can be employed as linkers to combine various bioactive fragments to generate promising drug candidates [6]. Furthermore, linkers are important in designing targeted drug delivery agents. The stability provided by the triazole ring affords an exceptional opportunity in engaging it as a linker, which is why several homo- and heterodimers of active pharmacophores as triazole linked conjugates have been reported [7].

The incorporation of 1,2,4-triazole core into various therapeutically efficient drug candidates has influence the polarity, lipophilicity, and hydrogen bonding capacity of molecules [9]. Some of the modern day drugs contain fused heterocycles and a triazole moiety are estazolam (sedative, hypnotic, and tranquilizer), alprazolam, trazodone (antidepressant and anxiolytic), triazolam, trapidil (hypotensive), terconazole (antifungal), hexaconazole (antifungal), etizolam (amnesic, anticonvulsant, anxiolytic, sedative, hypnotic, and skeletal muscle relaxant), rilmazafon (hypnotic and anxiolytic), and rizatriptan (antimigrane agent) [4, 10]. Some important triazole-containing compounds as clinical drugs are summarized in Table 9.1.

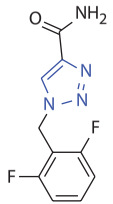
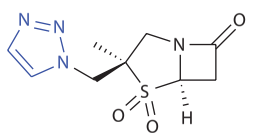
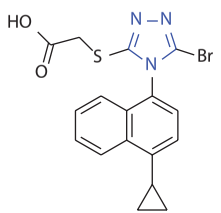
Triazoles have a mechanism of action that is similar to the imidazoles. They potentially suppress ergosterol biosynthesis (a major membrane sterol in fungi) by blocking the cytochrome P450-dependent enzyme C-14 alpha-demethylase, being essential for transforming lanosterol to

Table 9.1 Structures of some important triazole-containing compounds as clinical drugs.

Entry	Molecular structure	Drug name	Brand name	Target	Medicinal indication
1		Talazoparib	Talzenna	Poly [ADP-ribose] polymerase 1	Treatment of germline BRCA mutated HER2 negative, locally advanced or metastatic breast cancer. Approved by FDA on October 16, 2018.
2		Deferasirox	Exjade	Iron	Used for the treatment of chronic iron overload due to blood transfusions (transfusional hemosiderosis).19 First oral medication approved in the USA for this purpose.
3		Letrozole	Femara	Cytochrome P450 19A1	Adjuvant treatment of hormonally responsive breast cancer. Ovulation induction in women with polycystic ovarian syndrome (PCOS).

(*Continued*)

Table 9.1 Structures of some important triazole-containing compounds as clinical drugs. (*Continued*)

Entry	Molecular structure	Drug name	Brand name	Target	Medicinal indication
4		Rufinamide	Banzel, Inovelon	Glutamate receptor activity	Medication to treat seizure disorders like Lennox–Gastuat syndrome.
5		Tazobactam	NA	Beta-lactamase SHV-1 activity	Used in combination with piperacillin to broaden the spectrum of piperacillin antibacterial action.
6		Lesinurad	Duzallo, Zurampic	Urate transmembrane transporter activity	Oral uric acid transporter 1 (URAT1) inhibitor indicated for the treatment of hyperuricemia associated with gout.

ergosterol. Several drug interactions and toxic properties are linked to the interaction with human enzymes which rely on cytochrome P450. Some 1,2,4-triazole derived compounds are also found to be openers of large conductance Ca^{2+}-activated Potassium (Maxi-K) channels [11]. Anticancer profiles of triazole derivatives can most possibly be allocated to their huge attraction toward anticancer targets, such as tumor necrosis factor TNFα, JNK-stimulating phosphatase-1 (JSP-1), anti-apoptotic bio-complex Bcl-XL-BH3, integrin avb3 receptor, etc. [5]. Furthermore, some triazole derivatives act as potential inhibitors and target the epidermal growth factor receptor (EGFR) which is a receptor tyrosine kinase of the ErbB family that controls several complex biological processes, comprising adhesion, cell cycle regulation, cell motility as well as apoptosis, angiogenesis, and metastasis [12].

Several efforts have been taken on to alter structures of effective triazole-based drugs to enhance their antibacterial, antifungal, and anticancer potency and selectivity. In addition, it is deemed interesting to club other chemotherapeutically active moieties into the main structure with the hope of imparting some synergism to the target compounds [13]. Many triazole-based compounds are examined for their biological applications and are considered as an attractive area in the research and development of new drug candidates. In this book chapter, we tried to cover the most recent information regarding triazoles and their derivatives as promising new antimicrobial and anticancer scaffolds.

9.2 Anticancer Triazole Derivatives

Cancer is a dilapidating disease which has crumbled our society without any borders of rich and poor. The World Health Organization (WHO) has listed cancer as a chronic degenerative noncommunicable disease and a top cause of death globally. Attempts to find new therapies and strategies to tackle cancer are of great importance [14, 15]. Lung, breast, colon, and melanoma cancers are most common in the developing and underdeveloped countries. Chemotherapy is a widely used to cure several types of cancers [16]. Furthermore, combination chemotherapy has proved to be effective with different mechanisms of action being assumed to treat cancer.

1,2,3-triazole acts as attractive groups to link different biologically useful scaffolds into one molecule to create advanced multifunctional compounds [17]. The greater dipole moment of 1,4-substituted 1,2,3-triazoles helps them to be used as hydrogen bond acceptor, thereby favoring their binding to biologically active sites and improves solubility. On the basis

of biological inferences, it is predicted that the combination of theophylline and nucleoside pharmacophore units with 1,2,3-triazole linkage, an efficient platform to produce new theophylline, 1,2,3-triazoles linked nucleoside hybrids framework with amazing anticancer and antimicrobial activity could be achieved. Theophylline acetylene and other derivatives (Figure 9.3) were prepared and assessed for anticancer potential using four different cell lines viz. colon (HT-29), lung (A549), breast (MCF-7), and melanoma (A375). The IC_{50} of the all the compounds and the reference drugs are shown in Table 9.2. Interestingly, compounds **2**, **3**, **4**, and **5** showed the highest activity in lung cancer cells with IC_{50} values of 1.56, 2.56, 2.89, and 3.57 μM, respectively [18].

1,2,3-triazole-pyrimidine hybrids (Figure 9.4) assessed for their anticancer properties toward a panel of four cancer cell lines (EC-109, MGC-803, MCF-7, and B16-F10) have shown promising results. Most compounds exhibited reasonable activity toward all the tested cancer cell lines. Compound **6** showed most promising anticancer potential that too in micromolar range (IC_{50} of 1.42 to 6.52 μM). Further mechanistic studies exposed that compound **6** prominently inhibited the proliferation of

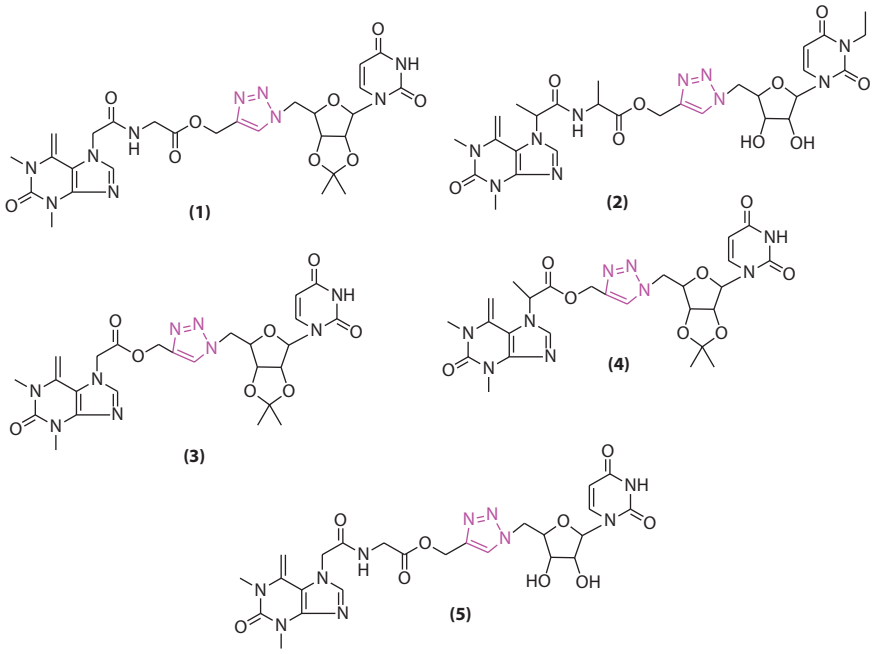
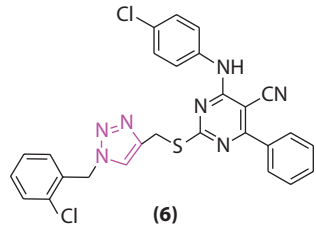


Figure 9.3 Structures of important theophylline containing 1,2,3-triazole nucleoside derivatives (**1-5**).

Table 9.2 Anticancer activity (IC_{50}) of the prepared compounds (**1-5**).

Compound	A549 (lung)	HT-29 (colon)	MCF-7 (breast)	A375 (melanoma)
1	**5.67**	59.3	**12.60**	59.8
2	**1.56**	**2.71**	**3.80**	52.3
3	**2.89**	62.8	**2.13**	68.4
4	**2.56**	**2.19**	**1.89**	**4.89**
5	**3.57**	**2.90**	**2.10**	**5.81**

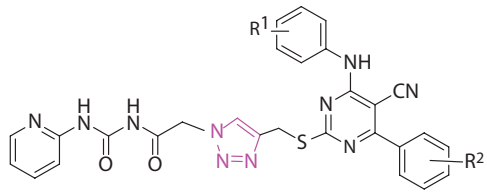


(6)

Figure 9.4 Structure of novel 1,2,3-triazole-pyridimine hybrid **6** revealed about seven-fold more potent than 5-Fu against EC-109.

EC-109 cancer cells by initiating apoptosis and arresting the cell cycle at G2/M phase [19].

1,2,3-triazole–pyrimidine–urea derivatives (Figure 9.5) were examined for anticancer profiles against four selected cancer cell lines (EC-109, MGC-803, MCF-7, and B16-F10). Most compounds displayed moderate to powerful activity toward the investigated cancer cell lines. In particular, **7**,

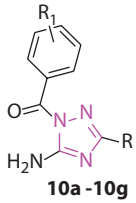


Compound	R^1	R^2
7	p-CH_3	m,p,m-TriOCH_3
8	p-$CH(CH_3)_2$	p-CH_3
9	p-$CH(CH_3)_2$	p-Br

Figure 9.5 Structures of 1,2,3-triazole-pyrimidine-urea hybrids (**7-9**).

8, and **9** displayed outstanding growth inhibition in B16-F10 with IC_{50} values of 32, 35, and 42 μM, respectively. Flow cytometry studies confirmed concentration-dependent cellular apoptosis by compound **7** [20].

Some novel hybrids of 1,2,4-triazole/isothiocyanates (Figure 9.6) obtained by the tethering of two bioactive units, 1,2,4-triazole and isothiocyanates in one compacted structure to provide synergism have been reported. The synthesized compounds **(10a-10g)** were analyzed via different sophisticated spectroscopic techniques and were evaluated for antitumor properties via *in vitro* cytotoxicity assays on human cancer cell lines. Furthermore, the inhibitory activity of most active compounds (**10a**-**10g**) in MTT assay was studied against BRAF, EGFR, and tubulin: three well-known anticancer targets. The results showed a strong tubulin inhibition triggered by compounds **10c** and **10d**. Furthermore, **10c** displayed the best EGFR inhibition with an IC_{50} value of 3.6 μM (Table 9.3) [21].



Compound	R	R_1
10a	C_6H_5	4-OCH_3
10b	3′4′5-tri(OCH_3)C_6H_2	4-OCH_3
10c	Pyridine-3-yl	4-OCH_3
10d	Pyridine-3-yl	4-OCH_3
10e	3′4′5-tri(OCH_3)C_6H_2	3′4′5-tri(OCH_3)
10f	4-Cl-C_6H_4-	3′4′5-tri(OCH_3)
10g	3-Cl-C_6H_4-	3′4′5-tri(OCH_3)

Figure 9.6 Structures of 1,2,4- triazole/isothiocyanates (**10a**-**10g**).

Table 9.3 Antiproliferative activities (IC_{50} μM) of compounds (**10a-10g**).

IC_{50} ± SEM (μM)				
Compound	**Panc-1**	**PaCa-2**	**HT-29**	**H-460**
10a	2.5 ± 1.1	1.5 ± 0.7	1.9 ± 1.6	1.6 ± 0.7
10b	0.2 ± 0.1	0.8 ± 0.5	1.6 ± 0.4	1.3 ± 0.2
10c	1.5 ± 0.9	1.0 ± 0.1	1.4 ± 0.7	1.7 ± 0.5
10d	1.9 ± 1.5	1.3 ± 0.9	1.7 ± 0.9	1.6 ± 0.7
10e	4.2 ± 0.8	6.9 ± 2.4	4.7 ± 1.8	5.8 ± 3.5
10f	5.5 ± 1.5	5.2 ± 2.7	4.9 ± 0.6	4.1 ± 1.5
10g	3.2 ± 1.9	2.7 ± 0.5	4.3 ± 0.5	2.6 ± 0.3

1,2,3-triazole-dithiocarbamates (Figure 9.7) have been found to display promising anticancer activities in four selected human tumor cell lines (MCF-7, MGC-803, EC-109, and PC-3). Most compounds in this series displayed moderate to strong anticancer activity in MCF-7 and MGC-803 cell lines. Compounds **11a** and **11b** exhibited outstanding anticancer activity with IC_{50} values ranging from 0.73 to 11.61 μM and 0.49 to 12.45 μM, respectively. In particular, compound **11a** was found to be more potent than 5-fluorouracil toward all selected cancer cell lines. Flow cytometry showed that treatment of MGC-803 with **11b** resulted in cell cycle arrest at G2/M phase along with an upsurge in apoptotic cell death after 12 h [22].

A series of N-substituted-3-mercapto-1,2,4-triazoles (**12a, b**), triazolo[1,3,4]thiadiazines (**13a, b**) and triazolo[1,3,4]thiadiazoles (**14a-d** and **15a-d**) were formed from isonicotinic acid hydrazide (Figure 9.8). These compounds were screened for their *in vitro* anticancer potential toward six selected cancer cell lines and normal fibroblasts, which revealed these compounds may act as capable units for further modification to obtain new promising class of anticancer agents [23]. Seven of the studied

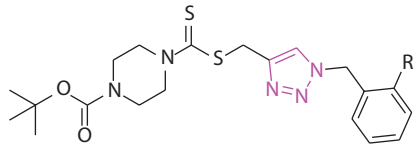


Figure 9.7 Structures of 1,2,3-triazole-dithiocarbamate hybrids (**11a** and **11b**).

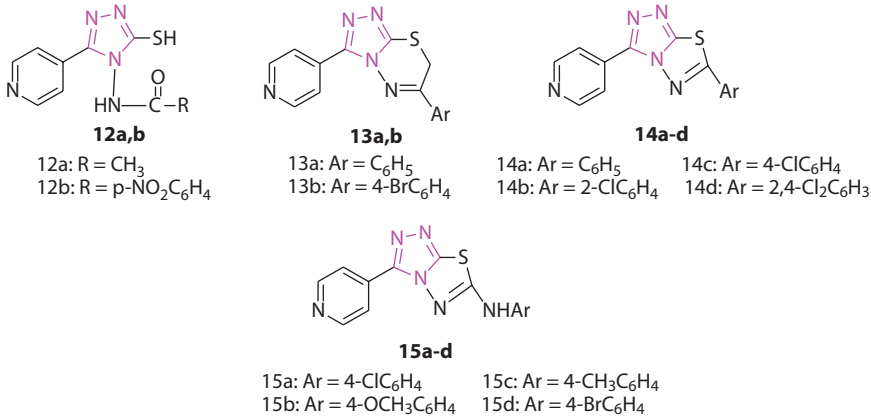


Figure 9.8 Structures of compounds: **12a**, **b**; **13a, b**; **14a-d**; and **15a-d**.

compounds (**12a**, **b**, **13a**, **14c**, and **15b-d**) displayed remarkable cytotoxicity toward most of the selected cell lines. Compound **14c** demonstrated comparable cytotoxic activity to standard CHS 828 in gastric cancer cell line with an IC_{50} value of 25 μM. Normal fibroblast cells (WI38) were affected to lesser degree, presenting IC_{50} values > 10,000 μM.

Glycosyl triazole linked 18β-glycyrrhetinic acid (GA) hybrids were prepared by 1,3-dipolar cycloaddition reaction between propargyl ester of 18β-GA and per-O-acetylated glycosyl azide derivatives (Figure 9.9), employing the concept of "Click chemistry". The obtained triazole hybrids were de-O-acetylated to offer **16a–h** and **17a–c** with free hydroxyl units in the carbohydrate moieties, further assessed for their anticancer profile in normal kidney epithelial (NKE) cells and human cervical cancer (HeLa) cells. GA, **16d**, **16g**, and **17c** displayed proficient anticancer activities [24].

A copper nanoparticle catalyzed click reaction was used to synthesize chalcone linked-1,2,3-triazole derivatives. Cytotoxicity effects of the prepared compounds were estimated by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay toward a panel of human cancer cell lines (MCF-7, MIA-Pa-Ca-2, A549, and HepG2) to expose their anticancer activity. Compound **18** (Figure 9.10) emerged to be the most potent compound in all the selected cancer cell lines with IC_{50} values in the range of 4–11 mM and displayed better or similar activity to the reference drug in all the selected cell lines. Cell cycle investigation exposed that compound **18** induced apoptosis and G2/S arrest in MIA-Pa-Ca-2 cells and also it activates mitochondrial loss in pancreatic cancer MIA-Pa-Ca-2 cells. Furthermore, **18** triggers caspase-3

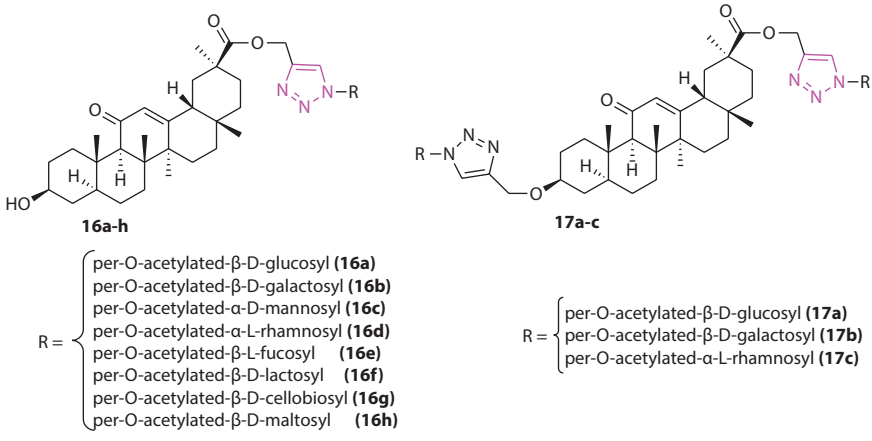


Figure 9.9 Structures of compounds (**16a-h**; and **17a-c**).

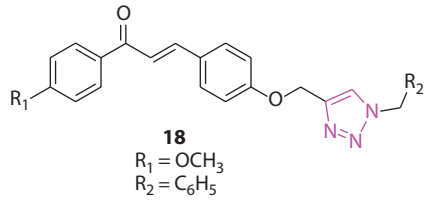


Figure 9.10 Structure of chalcone linked-1,2,3-triazole **(18)**.

and PARP-1 cleavage, which increases in a dose dependent manner. Compound **18** appeared as a significant compound, thus could be ideal molecule in the development and identification of 1,2,3-triazole derived anticancer agents [25].

1,2,3-triazole substituted N-phenyl nitrone derived compounds, obtained through a Schiff's base reaction, reduction, and then followed by oxidation of 1-substituted-1,2,3-triazole-4-carbaldehydes have been found as important scaffolds. Likewise, N-alkyl nitrone substituted 1,2,3-triazole scaffolds were prepared in a single step involving the reaction of 1-substituted-1,2,3-triazole-4-carbaldehydes and N-alkylhydroxylamine hydrochlorides. The obtained compounds were evaluated for anticancer potential toward various cancer cell lines. Among the screened compounds, **19a**, **19b**, **20a**, **20b**, and **20c** (Figure 9.11) displayed strong activity toward all the selected cell lines (COLO 205, PC-3, A549, and MDA-MB 231) having IC_{50} values of <15 μM (Table 9.4) [26].

Substituted 1,2,3 triazole derivatives obtained from 2,6-di-substituted purine and several substituted aromatic azides were studied for *in vitro* cytotoxicity toward several human cancer cell lines like IMR-32 (neuroblastoma), HCT-1 (colon), and A-549 (lung) and THP-1 (leukemia). Bioassay findings exposed that majority of the prepared compounds showed good strength toward selected cancer cell lines, but few of the derivatives like **21a**, **21b**, and **21c** (Figure 9.12) emerged as more powerful compounds,

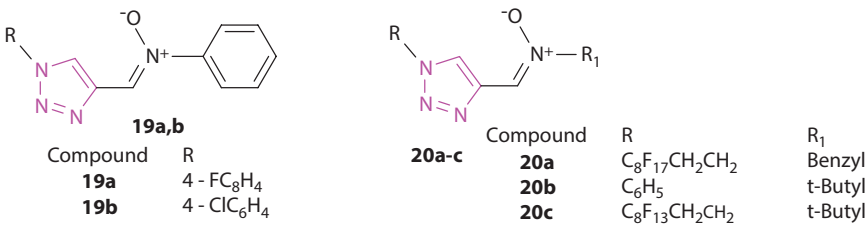


Figure 9.11 Structures of 1,2,3-triazole substituted-N-phenyl nitrone derivatives (**19a, b**) and -N-alkyl nitrone derivatives (**20a-c**).

Table 9.4 Anticancer activity of compounds **19a, b** and **20a-c**. IC_{50} values in μM.

Compound	A549	COLO 205	MDA-MB 231	PC-3
19a	12.5 ± 0.22	10.2 ± 0.27	9.8 ± 0.09	11.4 ± 0.32
19b	10.5 ± 0.24	9.7 ± 0.42	8.9 ± 0.43	11.1 ± 0.36
20a	8.8 ± 0.19	9.4 ± 0.18	8.4 ± 0.33	8.1 ± 0.32
20b	17.4 ± 0.17	15.2 ± 0.15	14.8 ± 0.28	15.4 ± 0.34
20c	11.5 ± 0.41	9.5 ± 0.34	9.8 ± 0.32	11.2 ± 0.22
Doxorubicin	0.8± ± 0.22	0.7 ± 0.42	0.8 ± 0.44	0.6 ± 0.28

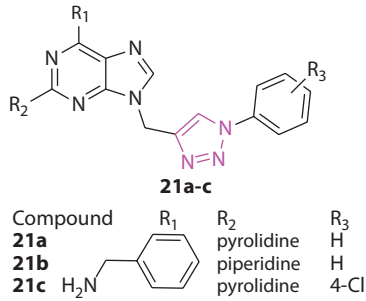


Figure 9.12 Structures of 2,6,9-Tri-substituted analogues of purine (**21a-c**).

with compound **21a** displaying IC_{50} values of 0.08 and 0.4 μM toward THP-1 and A-549 cell lines, respectively [27].

Hydrazide-hydrazones containing 1,2,4-triazole ring has been obtained from (S)-naproxen (**22a-f**) (Figure 9.13) and then assessed for their anticancer potential toward three cancer cell lines (DU-145, LNCaP, and PC3) employing the commonly used MTT colorimetric assay. Compound **22a**

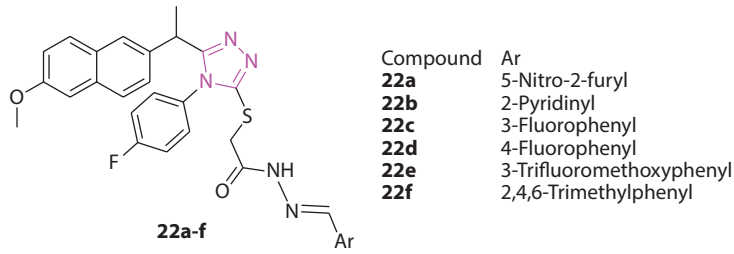


Figure 9.13 Structures of hydrazide-hydrazones with a 1,2,4-triazole ring (**22a-f**).

displayed most potent activity toward PC3, DU-145, and LNCaP cancer cell lines with IC_{50} values of 34.5, 26.0, and 48.8 μM, respectively. Compounds **22b**, **22e**, and **22f** displayed anticancer properties toward PC3 and DU-145 cancer cell lines with IC_{50} values of 43.0, 36.5, and 29.3 μM and 49.8, 49.1, and 31.6 μM, respectively. Compounds **22c** and **22d** displayed anticancer properties toward PC3 cells with IC_{50} values of 43.4 and 34.5 μM, respectively. *Ex vivo* studies revealed compound **22a** accumulated in the prostate was larger than free dye, showing that the compound **22a** could be an efficient candidate for treating prostate cancer [28].

A library of spirochromenocarbazole joined 1,2,3-triazoles were prepared (**25a-g**) *via* click chemistry-based one-pot, five component reaction between malononitrile, N-propargyl isatins, aralkyl, 4-hydroxycarbazole, and sodium azide (NaN_3) by means of heterogeneous catalyst (cellulose supported CuI nanoparticles, Cell-CuI NPs) (Figure 9.14). Antiproliferative potential of the prepared complexes was examined toward a number of selected cancer cell lines like MDA-MB-231, MCF-7, PANC-1, HeLa, A-549, and THP-1. Most compounds showed promising anti-proliferative effects toward breast (MDA-MB-231 and MCF-7) and cervical (HeLa) cancer cells having IC_{50} values lower than 10 μM. Compounds **25a** and **25b** exhibited excellent antiproliferative properties with an IC_{50} value of

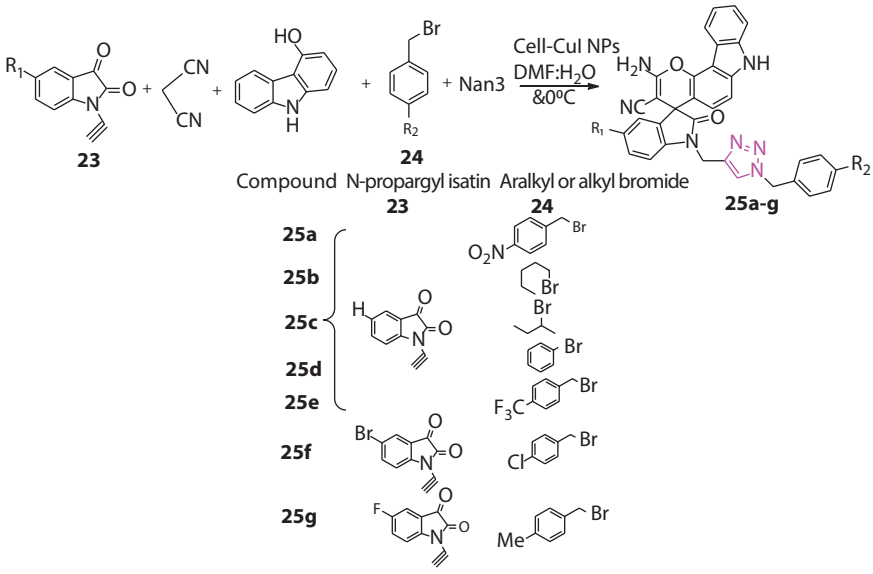
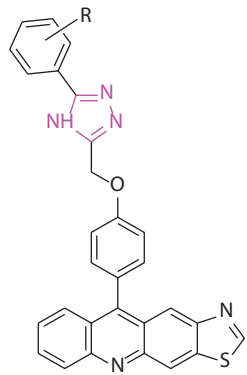


Figure 9.14 Structures of spiro [indoline-3,4′-pyrano[3,2-*c*] carbazole tethered 1,2,3-triazoles (**25a-g**).

2.13 and 4.80 μM, respectively, against MCF-7 cells. In MDA-MB-231cell line, compounds (**25c**, **25b**, and **25f**) displayed good antiproliferative properties with compound **25c** being the most potent one having an IC_{50} value 3.78 μM. On the other hand, compounds **25d**, **25e**, **25f**, and **25g** displayed brilliant antiproliferative activity with IC_{50} values 4.05, 3.54, 3.83, and 3.35 μM, respectively, against cervical cancer (HeLa) cells. All the screened compounds were nontoxic toward human umbilical vein endothelial cells. Furthermore, studies by fluorescence microscopy and AO and EtBr staining of the active compounds ($IC_{50} < 5$ μM) revealed apoptosis as the mechanism of cell death [29].

Another library of fused acridine holding 1,2,4-triazole hybrids (**26a-j**) (Figure 9.15) was assessed for their anticancer profiles against a panel of human cancer cell lines (A549, MCF7, A375, and HT-29). The synthesized compounds exhibited IC_{50} values in the range of 0.11 ± 0.02 to 13.8 ± 0.99 μM in comparison to the standard drug (0.11 ± 0.02 to 0.93 ± 0.056 μM range). Among the selected compounds, **26d**, **26f**, **26g**, **26h**, **26i**, and **26j** showed more potent activity [30].

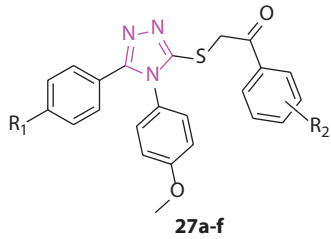
Several 3-alkylsulfanyl-1,2,4-triazole scaffolds (Figure 9.16) were prepared and estimated for anticancer activity in A549, MCF7, and SKOV3 cell lines employing the most commonly used MTT assay wherein compound **27e** demonstrated excellent anti-proliferative activities toward the cell lines whereas **27b** and **27e** displayed effective anti-proliferative properties in SKOV3 cell line with IC_{50} values of 0.81 and 0.53 μM, respectively. Moreover, compound **27e** induced prominent cell cycle arrest at the G2/M phase in SKOV3 cell lines in a concentration dependent manner. Furthermore, molecular docking studies of derivatives confirmed their suitable candidature to be explored for further anticancer activity [31].



26a-j

Compound	R
26a	H
26b	4-methyl
26c	4-methoxy
26d	3′4′5-trimethoxy
26e	4-bromo
26f	4-chloro
26g	4-fluoro
26h	4-trifluoromethyl
26i	4-nitro
26j	4-cyano

Figure 9.15 Structures of fused acridine containing 1,2,4-triazole derivatives (**26a-j**).



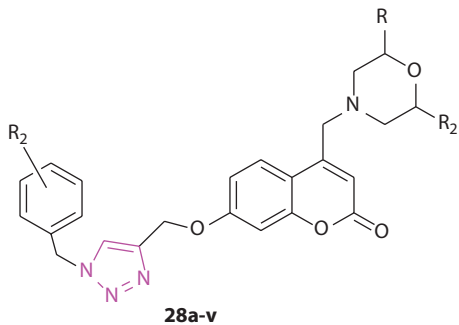
27a-f

Compound	R_1	R_2
27a	H	H
27b	H	3,4,5-$(OME)_3$
27c	H	4-Cl
27d	3,4,5-$(OME)_3$	H
27e	3,4,5-$(OME)_3$	3,4,5-$(OME)_3$
27f	3,4,5-$(OME)_3$	4-Cl

Figure 9.16 Structures of 3-alkylsulfanyl-1,2,4-triazole derivatives (**27a-f**).

A library of novel morpholines linked coumarin-triazole derivatives (**28a-v**) were synthesized (Figure 9.17) and examined for their antiproliferative properties against human cancer cell lines, (MDA-MB-231, MG-63, A549, HCT-15, and HepG2), using MTT assay. Of all the compounds, **28n** presented substantial growth inhibition toward MG-63 cells presenting IC_{50} value of 0.80 ± 0.22 μM. Furthermore, initiation of apoptosis by **28n** on MG-63 cells established by morphological changes, arrest of sub G1 phase, and decline in mitochondrial membrane potential and a rise in ROS levels. The *in vitro* Gal-1 expression in cell culture supernatant of MG-63 cells treated with compound **28n**, exhibited dose dependent reduction [32].

Another library of compounds containing substituted coumarin 1,2,4-triazole–based N-heterocyclic carbene (NHC) derivatives and their Ag(I) complexes (**29a-f**), and Au(I)-NHC (**30a-f**) complexes were prepared



28a-v

Compound	R	R_1	R_2
28a	H	H	H
28b	H	H	4-NO_2
28c	H	H	2,4-dichloro
28d	H	H	4-CN
28e	H	H	4-Br
28f	H	H	3-OCH_3
28g	H	H	4-OCH_3
28h	H	H	napthyl
28i	H	H	4-F
28j	H	H	3,5-dimethyl
28k	H	H	3-Cl
28l	CH_3	CH_3	H
28m	CH_3	CH_3	4-NO_2
28n	CH_3	CH_3	2,4-dichloro
28o	CH_3	CH_3	4-CN
28p	CH_3	CH_3	4-Br
28q	CH_3	CH_3	3-OCH_3
28r	CH_3	CH_3	4-OCH_3
28s	CH_3	CH_3	napthyl
28t	CH_3	CH_3	4-F
28u	CH_3	CH_3	3,5-dimethyl
28v	CH_3	CH_3	3-Cl

Figure 9.17 Structures of morpholines linked coumarin-triazole hybrids (**28a-v**).

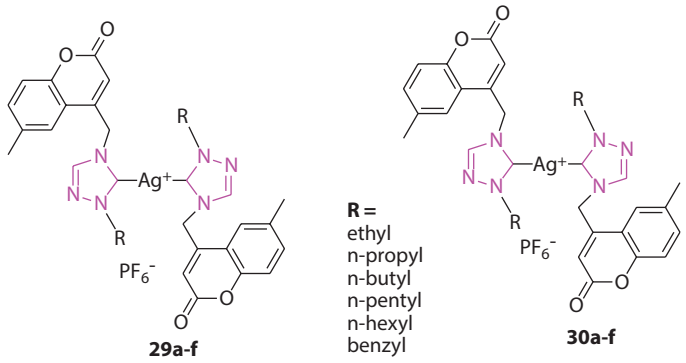


Figure 9.18 Structures of substituted coumarin 1,2,4-triazole–based N-heterocyclic carbene (NHC) derivatives and their Ag(I) complexes (**29a-f**), and Au(I)–NHC (**30a-f**) complexes.

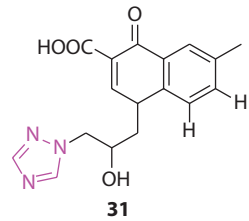
and thoroughly analyzed (Figure 9.18). *In vitro* anticancer profiles of the silver(I) complexes (**29a-f**) and gold(I) complexes (**30a-f**) toward two cancer cell lines, the colon cancer (HT-29) and the breast cancer (MCF 7) cell lines were observed by Sulforhodamine B assay. Ag(I) complexes showed advanced anticancer properties than the Au(I) complexes against both tested cell lines, excluding complex **29f** toward MCF 7 cells. Remarkably, complex **29e** displayed maximum anticancer activity in comparison to other obtained complexes with the GI_{50} value of 0.3540 ± 0.032 and 8.5983 ± 0.98 μM toward the HT-29 and MCF 7 cell lines, respectively [33].

9.3 Antimicrobial Triazole Derivatives

Triazole derivatives are holding a unique position in heterocyclic chemistry due to their efficient biological profiles. The small and the simple triazole nucleus which is present in compounds is involved in research work aiming at evaluating the new products possessing anti-microbial activity. Due to the huge interest in 1,2,4-triazoles, several 1,2,4-triazole–based compounds are now available in the pharmaceutical market like fluconazole (Doern) and triazolam. Undoubtedly, the interest in the field of microbial chemotherapy is growing due to quite a lot of reasons like rise and re-emergence of new pathogens and the exigent problem of evolving resistance to existing drugs. Thus, there is an unending need for new antimicrobial drugs that are more selective with lower side effects. Seemingly, 1,2,4-triazoles are now receiving more attention in the field of microbial chemotherapy due to their wide-ranging biological profiles.

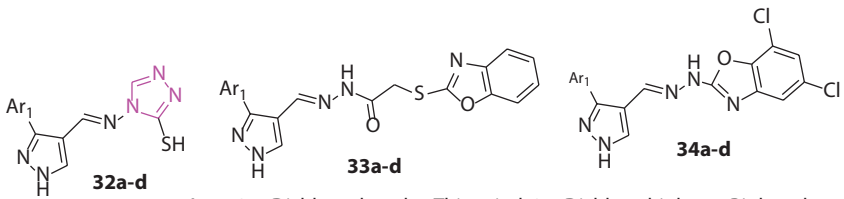
Some quinolone triazoles have shown promising antimicrobial properties toward seven bacterial and four fungal strains. Most of the newly synthesized derivatives showed excellent antimicrobial properties toward the tested strains including multi-drug resistant *S. aureus* (MRSA) compared to the reference drugs Fluconazole, Norfloxacin, and Chloromycin. The initial examinations of compound **31** by fluorescence and UV-vis spectroscopic methods (Figure 9.19) with calf thymus DNA exposed an efficient intercalation of the compound **31** with DNA, resulting in compound **31**—DNA complex that blocks DNA replication, thus explaining its interesting antimicrobial activity [34].

New Schiff bases and hydrazones from substituted pyrazole (**32a-d**, **33a-d**, and **34a-d)** (Figure 9.20) were prepared and examined for analgesic activity, displaying moderate to good activity in comparison to Pentazocin. Compound **32c** containing a 2,5-dichlorothiophene substituent on pyrazole ring exhibited remarkable analgesic activity. Antimicrobial assessments of the new compounds exposed **32c** and **33d** containing 2,5-dichlorothiophene and 2,4-dichlorophenyl substituent on pyrazole ring displayed promising antimicrobial profiles. However, compounds **32a-d** containing triazole moiety displayed good analgesic and antimicrobial properties in comparison to **33a-d** and **34a-d**, containing benzoxazole substituent. As regards the relationships between the structure of the heterocyclic moiety



31

Figure 9.19 Structure of quinolone triazole hybrid (**31**).



32a-d **33a-d** **34a-d**

Ar_1 = 2-4-Dichlorophenyl, 4-Thioanisyl, 2,5-Dichlorothiphene, Biphenyl

Figure 9.20 Structures of 1,2,4-triazole and benzoxazole derivatives bearing substituted pyrazole moiety (**32a-d**, **33a-d**, and **34a-d**).

and the observed antimicrobial/analgesic properties, it displayed diverse biological properties. Likely for this situation the idea of the heterocyclic ring and the presence of various substituents caused a specific difference in activity. It can be concluded from the results obtained from antimicrobial and analgesic screening, that a combination of two different heterocyclic systems, namely, pyrazole and 1,2,4-triazoles has boosted the pharmacological properties and hence further modifications are to be carried to obtain more efficient antibacterial and analgesic agents [35].

A series of 8-trifluoromethylquinoline clubbed with 1,2,3-triazole derivatives (**35a-c**, **36a-d**, and **37a-c**) (Figure 9.21) were obtained by multi-step reactions using click chemistry approach. The final compounds were evaluated for their *in vitro* antimicrobial activity by zone of inhibition (ZOI) method. Of the compounds, **35c** and **36b** displayed modest inhibition activity toward all the selected bacterial strains. The higher antibacterial profiles of **35c** and **36b** were mainly because of the substitution of the phenyl ring of 1,2,3-triazole and ethyl 8-(trifluoromethyl) quinoline-3-carboxylate by chlorine. Furthermore, the increase in the length of alkyl chain at 4th position of 8-trifluoromethylquinoline in the 1,2,3- triazole ring and the electron donating nature decreases antimicrobial activity. However, the presence of halogen (chloro and dichloro) substitution and electron withdrawing group (NO_2) enhance the antimicrobial properties of the obtained derivatives [36].

1,2,3-triazole coupled diaryl sulfone derivatives (Figure 9.22) have been prepared under the ultrasound irradiation by copper catalyzed

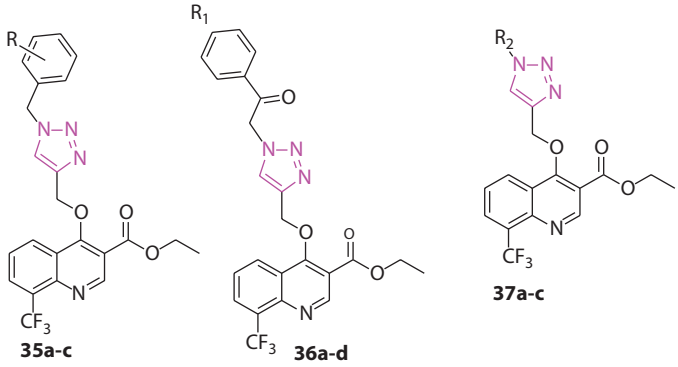


Figure 9.21 Structures of 1,2,3-triazol-8-trifluoromethyl-quinoline-3-carboxylic acid ethyl ester derivatives (**35a-c**, **36a-d**, and **37a-c**).

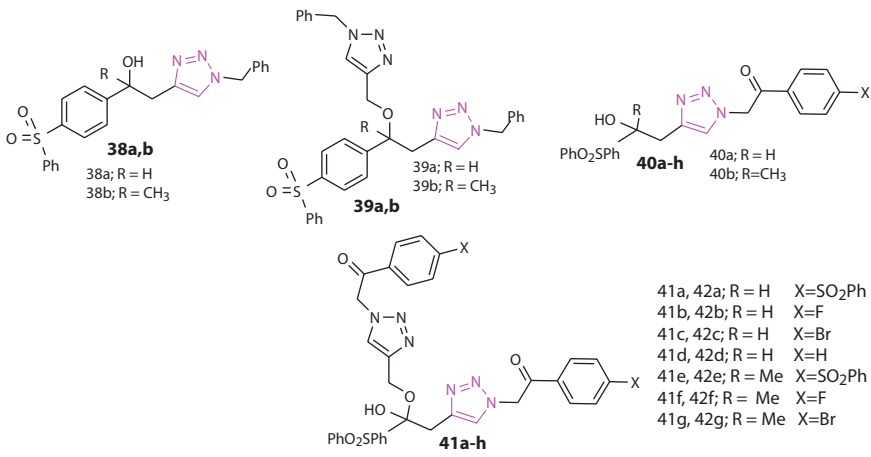
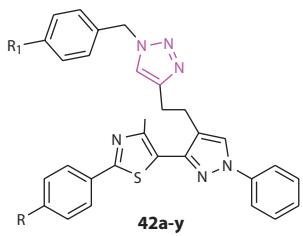


Figure 9.22 Structures of 1,2,3-triazoles coupled diaryl sulfone derivatives (**38a**, **b**; **39a**, **b**; **40a-h**; and **41a-h**).

azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction. Antimicrobial (antifungal and antibacterial) and antioxidant screening revealed that the compounds **38b**, **39b**, and **41e-g** with MIC of 25 mg/ml were found to be most active antifungal agents; furthermore, rest compounds showed moderate antimicrobial properties. Compounds **38a**, **b**; **39a**, **b**; **40e-h**; **41a-h** exhibited flexible antibacterial properties toward both Gram-positive and Gram-negative bacteria. Compound **38a** displayed a ZOI of 19 mm toward *S. aureus* ATCC 29213 and compounds **38a**; **40b-d**; **41a**, **b** displayed ZOI of 19-20 mm against *S. lutea*. The other compounds exhibited poor to moderate inhibition toward Gram-positive and Gram-negative bacteria. Antifungal screening was carried against *A. niger*, where the compounds exhibited brilliant antifungal profiles toward the selected fungal strain in comparison to Clotrimazole. Compounds **38b**, **39b**, **40d**, **40e**, **40h**, **41b**, and **41d-g** displayed the highest ZOI against *A. niger* in the range 32–34 mm. Compounds **38b**, **39b**, and **41e-g** were found to be most active toward the fungal strain (MIC of 25 mg/ml), similar to that of Clotrimazole [37].

A library of 1,2,3-triazole hybrids (**42a-y**) (Figure 9.23) has been prepared by using click reaction of substituted benzyl azide and 5-(4-ethynyl-1-phenyl-1Hpyrazol-3-yl)-4-methyl-2-aryl-1,3-thiazole. Antimicrobial screening of all prepared thiazolyl-pyrazolyl-1,2,3-triazole derivatives was carried in a Gram positive strain *Staphylococcus albus* (NCIM 2178), two Gram negative, *Escherichia coli* (NCIM 2574), *Proteus mirabilis* (NCIM 2388), and for *in vitro* antifungal activity in fungal species like *Aspergillus niger* (ATCC 504), *Rhodotorula glutinis* (NCIM 3168), and *Candida albicans*



Comp	R	R1	Comp	R	R1	Comp	R	R1
42a	H	H	**42k**	F	H	**42u**	Br	H
42b	H	CH_3	**42l**	F	CH_3	**42v**	Br	CH_3
42c	H	F	**42m**	F	F	**42w**	Br	F
42d	H	Cl	**42n**	F	Cl	**42x**	Br	Cl
42e	H	Br	**42o**	F	Br	**42y**	Br	Br
42f	CH_3	H	**42p**	Cl	H			
42g	CH_3	CH_3	**42q**	Cl	CH_3			
42h	CH_3	F	**42r**	Cl	F			
42i	CH_3	Cl	**42s**	Cl	Cl			
42j	CH_3	Br	**42t**	Cl	Br			

Figure 9.23 Molecular structures of 1,2,3-triazole derivatives (**42a-y**).

(NCIM 3100). Ten thiazolyl-pyrazolyl-1,2,3-triazole derivatives, **42b**, **42g**, **42i**, **42j**, **42k**, **42l**, **42m**, **42n**, **42p**, and **42v**, exhibited good antifungal activity toward *A. niger* with MIC of 31.5 mg/ml. Compounds **42g**, **42i**, **42k**, **42l** and **42m** were also assessed for ergosterol biosynthesis inhibition in *A. niger* cells at a concentration of 31.5 mg/ml. Sterol inhibition assay exposed that there was a decrease in ergosterol biosynthesis in fungal samples on treating with azole derivatives. Enhanced antifungal results suggest that, these such compounds could be further optimized and used as potential agents to treat fungal infection [38].

A series of four sets of phenothiazine bearing 1,2,3-triazole derivatives **43a-g**, **44a-g**, **45a-h** and **46a-n** (Figure 9.24) were synthesized and screened for their *in vitro* growth inhibition profiles in M.tb H37Rv strain (ATCC-27294). Compounds (**43c**, **43e** and **43g**) having ether linker exhibited moderate antitubercular potential (MIC of 12.5 mg/ml). Modifications with an oxime linker at ether linkage in the **43a-g** derivatives didn't affect the activity of the oxime derivatives (**44a-g**). All the phenothiazine-N-substituted 1,2,3-triazole analogs **45a-g** showed higher activity in comparison to the ether (**43a-g**) and oxime (**44a-g**) derivatives. Compounds **45b**, **45c**, **45e** and **45f** revealed strong anti-tubercular potential with MIC of 1.6 mg/ml, that was twofold greater than the activity of standard first-line tuberculosis drug Pyrazinamide (MIC of 3.125 mg/ml) and the compound **45g** exhibited outstanding activity (MIC of 3.12 mg/ml). The activity of the molecule did not alter with the introduction of a triflouromethyl substituent at position-2 of the phenothiazine ring in compound **45e**. Furthermore, no further improvement in the anti-TB activity of derivatives was observed on clubbing of TB-active pharmacophores with active compounds **45e** and **45h**. However, a library of effective molecules obtained by the molecular hybridization (**46a-n**) exhibited MIC of 1.6 mg/ml. The cytotoxicity profiles of the active compounds (MIC ¼ 1.6 mg/ml) exposed their nontoxic nature toward normal VERO cell lines. Moreover, compounds **45e**, **46i**, and **46m** exhibited noteworthy antibacterial properties toward the selected

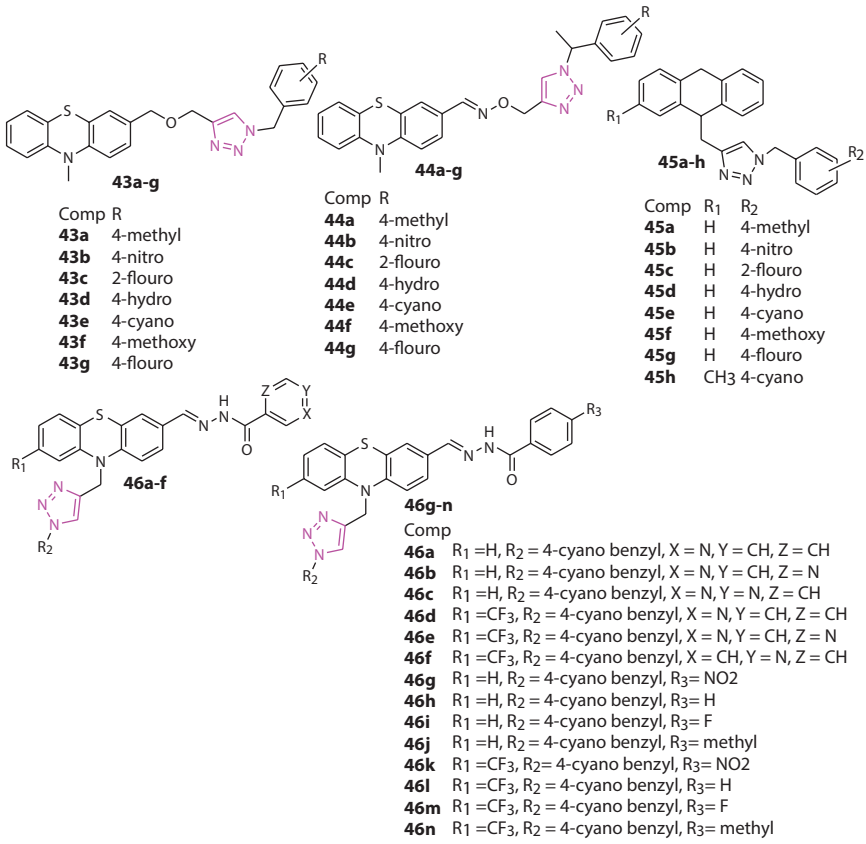
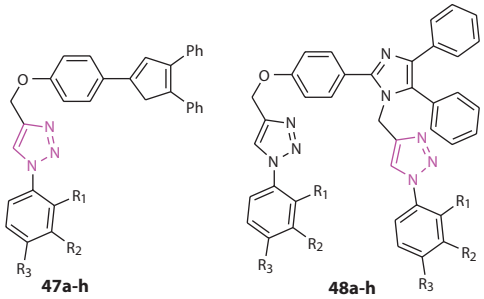


Figure 9.24 Structures of 1,2,3-triazole substituted compounds (**43a-g**, **44a-g**, **45a-h** and **46a-n**).

bacteria strains. The structure activity relationship studies exposed that the presence of electron withdrawing groups like F, NO_2 and CN on 1,2,3-triazole ring play significant part in improving the pharmacological activities of the compounds. The phenothiazine tethered 1,2,3-triazole derivatives could be strong candidates for the development and identification of novel anti-TB drugs [39].

Several imidazole-based 1,2,3-triazole hybrids starting from 4-hydroxy benzaldehyde were prepared by using click reaction followed by multi component reaction and *vice versa*. The antimicrobial potential of all the resulting imidazole-1,2,3-triazole hybrids (**47a-h** and **48a-h**) (Figure 9.25) were evaluated against six microbial strains: *C. albicans, S. aureus*, *E. coli*, *P. vulgari, B. cereus*, and *A. fumigates*. The results were presented as MIC and

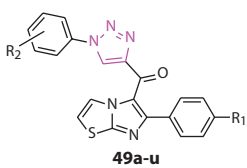


Comp	R_1	R_2	R_3
47a, 48a	H	Cl	H
47b, 48b	H	H	Cl
47c, 48c	H	H	Me
47d, 48d	OMe	H	H
47e, 48e	OMe	H	NO_2
47f, 48f	NO_2	H	OMe
47g, 48g	NO_2	H	H
47h, 48h	H	H	NO_2

Figure 9.25 Structures of imidazole-based 1,2,3-triazole hybrids (**47a-4** and **48a-h**).

all the evaluated compounds exhibited good MIC values (1 mg/ml), toward gram positive and gram-negative bacteria in comparison to ampicillin (100 µg/ml). The screened compounds exhibited remarkable antimicrobial activity toward the selected strains, like the reference drug Ampicillin and fluconazole. Among the various tested compounds, compounds **47c**, **47h**, **48d**, **48e,** and **48h** inhibited microbial growth very efficiently compared to others in the series with MIC values ranging from 8.86 to 33.25 µg/ml. Compounds **48d** (R_1=OCH_3), and **48e** (R_1=OCH_3, R_3=NO_2) were found to be more potential anti-bacterial and anti-fungal agents [40].

A library of triazolo fused Imidazo[2,1-b]thiazole derivatives (**49a–u**) (Figure 9.26) were prepared and assessed for their *in vitro* antibacterial, minimum bactericidal concentration (MBC), antifungal, minimum fungicidal concentration (MFC) and inhibition of biofilm formation activities. Of the compounds, **49c**, **49d**, **49e**, **49j** and **49l** exhibited excellent antibacterial profiles toward all the tested pathogens with MICs ranging between 1.9 and 7.8 µg/ml and showed moderate antifungal strengths (MIC of 7.8 and 15.6 µg/ml). In addition, compounds **49c**, **49d**, **49e**, **49j** and **49l** also exhibited promising antibacterial activities with MBC values of 3.9–15.6 µg/ml. Biofilm inhibition assay exposed that compounds **49c**, **49d**, **49e**, **49j**, and **49l** exhibited strong inhibition of biofilm formation in the selected bacterial biofilms, specifically compound **49c** showed huge anti-biofilm profiles



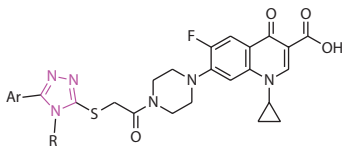
Comp	R_1	R_2	Comp	R_1	R_2	Comp	R_1	R_2
49a	H	3,4,5-Tome	**49h**	Cl	3,4,5-Tome	**49o**	OCH_3	3,4,5-Tome
49b	H	4-OCH_3	**49i**	Cl	4-OCH_3	**49p**	OCH_3	4-OCH_3
49c	H	4-Cl	**49j**	Cl	4-Cl	**49q**	OCH_3	4-Cl
49d	H	2-Br 4-F	**49k**	Cl	2-Br 4-F	**49r**	OCH_3	2-Br 4-F
49e	H	4-Br	**49l**	Cl	4-Br	**49s**	OCH_3	4-Br
49f	H	4-CF_3	**49m**	Cl	4-CF_3	**49t**	OCH_3	4-CF_3
49g	H	4-F	**49n**	Cl	4-F	**49u**	OCH_3	4-F

Figure 9.26 Structures of triazolo fused Imidazo[2,1-b]thiazole derivatives (**49a–u**).

toward all the selected bacterial biofilms with IC_{50} values of 9.8 and 10.3 μg/ml in *S. aureus* MTCC 96 and *E. coli* MTCC 739 strains, respectively. Compound **49c** caused biofilm disruption indicated by FE-SEM micrographs. Moreover, biofilm assay exposed that the biofilms were successfully disrupted by the compounds with biofilm IC_{50} of 14.3 μg/ml for compound **49c** [41].

New N-4-piperazinyl ciprofloxacin triazole derivatives (**50a-o**) (Figure 9.27) were tested against different microbial pathogens. Biological evaluation results against *M. smegmatis* exposed that compounds **50a** and **50c** showed promising antimycobacterial profile in comparison to the reference drug isoniazid. Also, compound **50a** exhibited a wide range of antibacterial properties in selected Gram-positive or Gram-negative bacteria. Additionally, **50g** and **50l** displayed decent antifungal activity in comparison to the selected drug ketoconazole. Evidently, the unsubstituted phenyl moiety on the triazole ring enhanced the antibacterial as well as antimycobacterial properties like compound **50a**. Substutions with ethyl and phenyl groups on the triazole ring decreases the antimycobacterial activity as in compounds **50f**, **50h**, **50n**, and **50o**, respectively. Also, the occurrence of p-chlorophenyl group on the triazole ring improves the antifungal activity. Compounds **50a**, **50i**, and **50o** displayed broad-spectrum activity toward the tested strains. However, the phenyl triazole derived compounds **50g** and **50l** exhibited substantial antifungal activity than the reference drug ketoconazole (MICs of 3.8, 6.13, and 2.60 μM). The results revealed that there is no exact substituent on the N-4-piperazine moiety that could govern the activity and the improved activity of some derivatives might be due to enhancement of the physicochemical properties and subsequently higher permeability to microbial cells [42].

Several ferrocene–oxime ether benzyl 1H-1,2,3 triazole derivatives **51a–h** (Figure 9.28) were prepared by Cu(I) catalyzed azide–alkyne [3+2] cycloaddition. These triazole derivatives were assessed for antimicrobial actions in selected bacterial and fungal strains. *In vitro* antibacterial and antifungal estimations disclosed modest inhibitory activity of compounds



50a-o

Comp	R_1	R_2	Comp	R_1	R_2
50a	Allyl	C_6H_5	**50i**	Et	3,4-di-OCH_3-C_6H_3
50b	Allyl	4-Cl-C_6H_4	**50j**	Et	3,4,5-tri-OCH_3-C_6H_2
50c	Allyl	4-OCH_3-C_6H_4	**50k**	Ph	C_6H_5
50d	Allyl	3,4-di-OCH_3-C_6H_3	**50l**	Ph	4-Cl-C_6H_4
50e	Allyl	3,4,5-tri-OCH_3-C_6H_2	**50m**	Ph	4-OCH_3-C_6H_4
50f	Et	C_6H_5	**50n**	Ph	3,4-di-OCH_3-C_6H_3
50g	Et	4-Cl-C_6H_4	**50o**	Ph	3,4,5-tri-OCH_3-C_6H_2
50h	Et	4-OCH_3-C_6H_4			

Figure 9.27 Structures of N-4-piperazinyl ciprofloxacin triazole hybrids (**50a-o**).

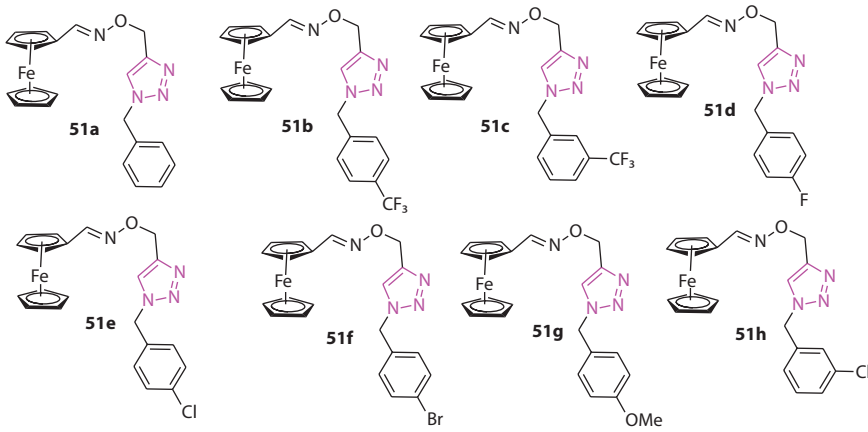


Figure 9.28 Structures of 1,2,3 triazole derivatives (**51a–h**).

51a–h toward the selected microbial strains, compared to the standard drugs. Compound **51d** revealed strong activity against all selected strains, similar to or even better than the standard drugs. Furthermore, **51d** revealed two-fold activity against *S. aureus* than the standard drug streptomycin sulphate. The ZOI (in mm) and MIC values showed that substitution in the benzyl group attached to the ferrocenyl triazole moiety controlled the antimicrobial activity of such compounds [43].

9.4 Conclusion

Designing a potential drug candidate is a cumbersome procedure entailing substantial risks with high returns. In the last few years, the research and development cost per new drug has escalated by over a hundred-fold. The academic studies offer a vast ground for the industrial counterpart to work and develop novel drug candidates for reasonable clinical applications. In this process, pharmacophores play a prominent role and luckily their properties can be adjusted and augmented with diverse chemical groups resulting in expected pharmacological efficiency. The effectiveness of the triazole scaffolds is clear from the clinically available triazole-based drug candidates. In spite of the occurrence of the triazole moieties in innumerable clinically significant medicinal agents, there is still further scope in this promising moiety as a number of different molecular targets are accessible for several 1,2,3 and 1,2,4-triazole derivatives. We have tried to give a thorough review of the varied and promising triazole derivatives as

antimicrobial and anticancer agents in this chapter so that the information presented can be valuable for further study of this promising ring structure to assess their biological profile in a better way and for development of further pharmacologically important medicinal candidates for evading various debilitating diseases, particularly microbial infections and cancer.

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10

Fused Triazolo Isoquinoline Derivatives—Design, Synthesis, and Biological Evaluation

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Abstract

Fused 1, 2, 4-triazolo isoquinoline derivatives (**56a-56p**) were synthesized in a 7-step synthetic sequence. The key step in the scheme involves 1, 3-dipolar [3+2] cycloaddition of azomethine imine and ethyl cyanoformate. The hydrolysis of both ester and benzoyl groups in a single step was demonstrated and the corresponding carboxylic acid derivatives formed were coupled with various amines to generate unknown 1, 2, 4-triazolo isoquinoline derivatives. The structures of the compounds (**56a-56p**) were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS analysis. The compounds were tested against ten different fungal strains and compounds **56b**, **56g**, **56h**, **56i**, and **56m** were found to possess promising antifungal activity against *C. albicans* MTCC 227, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, and *C. krusei* MTCC 3020 with MIC value of 3.9 μg mL^{-1}. Compounds **56g**, **56h**, **56i**, and **56m** were highly promising against *C. albicans* MTCC 227, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, and *C. krusei* MTCC 3020 exhibiting MFC value of 7.8 μg mL^{-1} which was equipotent to miconazole drug. Molecular modelling studies carried out to study the binding modes of **56b**, **56g** and **56h** correlates well with the antifungal activity and supported by ergosterol biosynthesis inhibition assay data.

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***Keywords*:** Fused 1,2,4-triazolo isoquinoline, azomethine imine, antifungal activity, ergosterol biosynthesis inhibition, cytotoxicity, molecular docking studies

10.1 Introduction

Infections caused by many microorganisms are creating a great threat to human survival. There has been a battle between human beings and disease-causing microorganisms for decades of history. Fungal infections like candidiasis are responsible for causing a precarious situation both in humans as well as in plants. The tremendous growth in the incidence of life-threatening fungal infections has become a significant cause of mortality in immune-compromised individuals such as patients suffering from cancer, organ transplants, or undergoing AIDS.

Hence, many antifungal azole drugs Fluconazole (FCZ), Ravuconazole, and Miconazole, etc., developed resistance toward most of the fungal pathogens, each of them still exhibits limitations such as resistance, the spectrum of activity, side effects, drug-drug interactions, or pharmacokinetic profile [1] and growing emergence of antifungal resistance. Therefore, present antifungal treatments are limited for the treatment of systemic infections so that considerable growth in antifungal therapies has been observed recently and became a major concern to public health and scientific communities worldwide, especially in the field of multi-drug-resistant fungi.

Researchers aim to discover new antifungal drugs either by extraction from natural sources such as plants, microorganisms, or testing previously existing medical compounds or by systematic screens of chemical compound libraries. However, the development of novel antifungals is challenging because fungi are eukaryotic organisms like mammals. Fungal cells share many similarities with the host cell except the cell wall. Hence, human and fungal cells are eukaryotic; it is not very easy to design new molecules with antifungal activity without causing any side effects.

Triazoles, isoquinolines, and fused triazole isoquinoline derivatives are a very fascinating class of compounds due to their versatile pharmacological and biological activities; especially triazole-containing drugs are known for their antifungal activity. As a part of our on-going program to discover and develop a new class of antifungal molecules, we are interested to develop a new synthetic methodology to synthesize

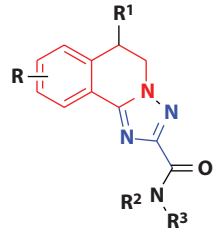


Figure 10.1 Fused [1,2,4]-triazolo isoquinoline derivatives.

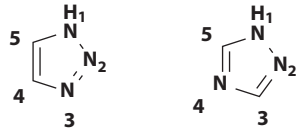
novel [1,2,4]-triazolo isoquinoline derivatives (Figure 10.1) by employing 1,3-dipolar [3+2] cycloaddition, to elicit antifungal activities against different fungal pathogens.

10.2 Literature Review on 1,2,4 Triazoles

10.2.1 Chemistry

The chemistry of heterocycles has continuous to be a field for exploration in synthetic and medicinal chemistry. The chemistry of heterocycles has continuous to be and explore the field in organic or pharmaceutical chemistry. There is a significant and continuous concern in the chemistry of 5-member N-heterocycle compounds, mainly tetrazole, triazoles, and their substituted derivatives [2]. The triazole scaffold is versatile and its importance in several clinically used drugs highlighted the significance of this nucleus. The most relevant studies have revealed that triazole derivatives have a broad spectrum of pharmacological importance such as antibacterial, antifungal, and anticancer properties. In 1885, Bladin was the first scion who gave the name of triazole to the carbon-nitrogen ring system ($C_2N_3H_3$) and described triazole derivatives [3].

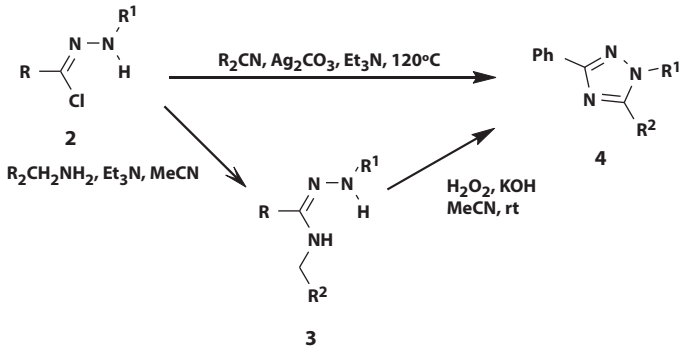
Triazoles are five-membered heterocyclic aromatic ring compounds similar to pyrazole and imidazole with an additional nitrogen atom in the ring structure. The success of imidazole as an important functional unit of many medicinal agents led to the introduction of the triazoles. The triazoles are also called the isosters of imidazoles in which the carbon atom of imidazole is isosterically replaced by nitrogen. It occurs as a pair of isomeric forms 1,2,3-triazole **1a**, and 1,2,4-triazole, **1b** [4]. The two isomers are as follows:



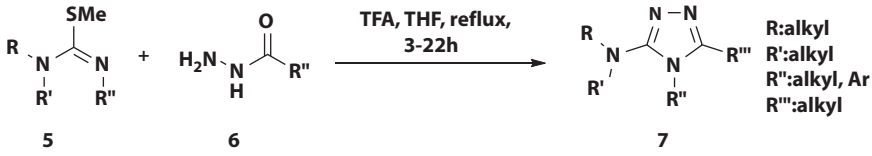
Aromaticity is the main reason for the stability of the triazole nucleus. An aromatic sextet is formed by the donation of one π electron from each atom connected by double bonds, in addition to the remaining two electrons from a nitrogen atom. Also, the triazole nucleus is stabilized by the resonance that it can be represented by tautomeric forms 1,2,4-Triazoles exists in two tautomeric forms 1H-1,2,4 triazole and 4H-1,2,4-triazole. Many studies have indicated that tautomer 1H-1,2,4 triazole is more stable than tautomer 4H-1,2,4-triazole [5].

10.2.2 Synthetic Approach

Paulvannan *et al.* [6] reported an improved synthetic route for 1,3,5-trisubstituted 1,2,4-triazoles **4** *via* 1,3-dipolar cycloaddition of nitrile imine, generated *in situ* from oxime chloride in the presence of Ag_2CO_3 and Et_3N. In an alternative two-step approach [7] first prepared intermediate **3** from the reaction of **2** with a primary amine and Et_3N, which was then treated with a solution of 30% H_2O_2/aqueous KOH to yield **4**.

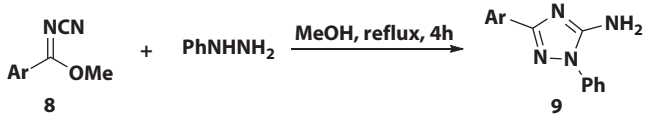


Batchelor *et al.* [8] reported mild and chemoselective synthesis 3-*N*,*N*-Dialkylamino-1,2,4-triazoles **7** from *S*-methyl isothioureas **5** and acyl hydrazides **6** in good yields.



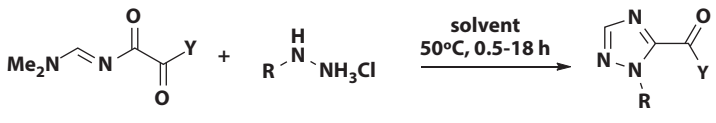
5 6 7

Yin *et al.* [9] developed a mild, catalyst-free one-pot cyanoimidation of aldehydes in high yields. Cyanamide is used as a nitrogen source and NBS as an oxidant. Subsequently, the substituted *N*-cyano benzimidate **8** undergoes a cyclization reaction to give 1,2,4-triazole derivatives **9** in high yields.



8 9

Xu *et al.* [10] reported the synthesis of 1,5-disubstituted-1,2,4-triazole **12** from a series of new oxamide-derived amidine reagents in excellent yield. Aromatic and aliphatic hydrazines **11** both react with the amidine **10** reagents under very mild reaction conditions.

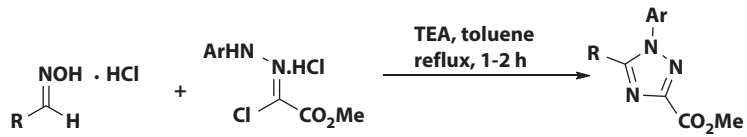


10 11 12

Y: NH-benzyl,NH-tBu'
OtBu, N (allyl)2
R: Ar, alkyl

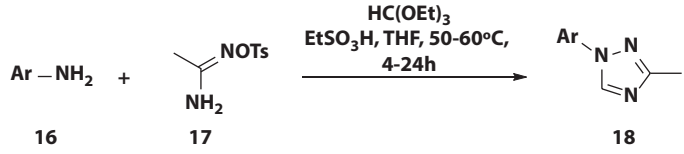
Solvents: AcOH, 2-PrOH
2 eq. AcOH in MeOH

Wang *et al.* [11] reported the synthesis of 1,3,5-trisubstituted 1,2,4-triazole derivatives **15** *via* an effective 1,3-dipolar cycloaddition. The synthetic sequence involves the reaction of oximes **13** with hydrazonoyl hydrochlorides **14** using TEA in good yields.

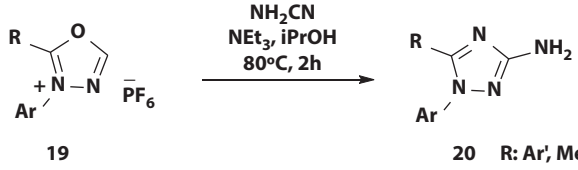


13 14 15

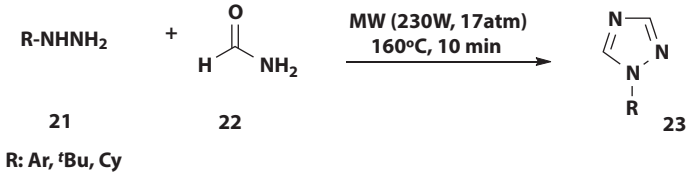
Tam *et al.* [12] developed a multicomponent process for the synthesis of 1-aryl 1,2,4-triazoles **18** from anilines **16** with (E)-N'-(tosyloxy)acetimidamide, **17**. The reaction scope was explored with 21 different substrates.



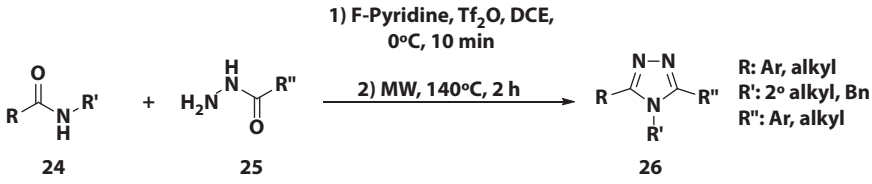
Wong *et al.* [13] synthesized 1,5-disubstituted 3-amino-1*H*-1,2,4-triazoles **20** by using hexafluorophosphoric acid to promote the formation of 1,3,4-oxadiazolium hexafluorophosphate salts **19** from *N*′-acyl-*N*-aroyl-*N*-aryl hydrazides or *N*′-acyl-*N*-acyl-*N*-aryl hydrazides under mild conditions. A subsequent reaction with cyanamide in propan-2-ol in the presence of TEA generates 1,5-disubstituted 3-amino-1*H*-1,2,4-triazoles **20** in good yields.



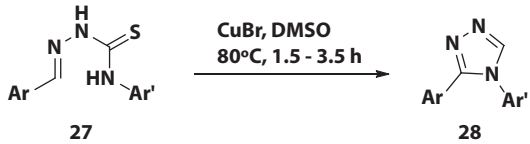
A simple, efficient, mild, and catalyst-free method was used by Shelke *et al.* [14] for the synthesis of substituted 1,2,4-triazoles **23** from hydrazines **21** and formamide **22** under microwave irradiation with excellent functional group tolerance in good yields.



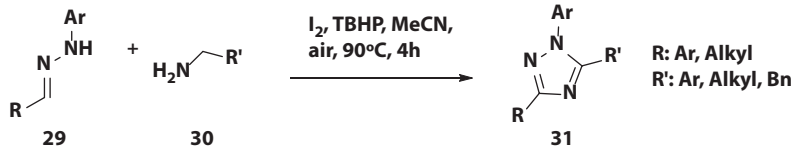
Bechara *et al.* [15] synthesized 3,4,5-trisubstituted 1,2,4-triazoles **26** by triflic anhydride activation followed by microwave-induced cyclodehydration in a one-pot synthesis from secondary amides **24** and hydrazides **25**. In addition, the 1,2,4-triazole moiety was shown to be a useful directing group for Ru-catalyzed C-H arylation. A Pd-catalyzed intramolecular C-H functionalization reaction allowed access to 1,2,4-triazolophenanthridine.



Gogoi *et al.* [16] carried out a Cu (II) catalyzed the construction of 4,5-disubstituted 1,2,4-triazole-3-thiones **28** from arylidenearylthiosemi carbazides **27** in good yields. Upon prolonging the reaction time, the *in situ* generated thiones were transformed to 4,5-disubstituted 1,2,4-triazoles *via* a desulfurization process.



Chen *et al.* [17] reported a general and metal-free synthesis of 1,3,5-trisubstituted 1,2,4-triazoles **31** from hydrazones **29** and aliphatic amines **30** under oxidative conditions *via* a cascade C-H functionalization, double C-N bonds formation, and oxidative aromatization sequence in the presence of iodine as a catalyst.



10.2.3 Biological Importance

1,2,4-Triazole derivatives are fascinated scientific and practical interest due to the wide variety of chemical properties, synthetic versatility, and pharmacological activities, such as antibacterial [18], antifungal [19], anti-inflammatory [20], anticancer [21], anticonvulsant [22], and antiviral [23] properties.

Currently, severe systemic fungal infections were associated with three major pathogens from the *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* in clinical practice [24]. Most of the fungal infections are involved with *Candida albicans* being an opportunistic pathogen and other *Candida* species such as *C. glabrata*, *C. krusei*, and *C. parapsilosis* are prominent and exhibit lifestyle changes from ubiquitous commensal to pathogenic triazole-containing drugs (Figure 10.2) such as FCZ itraconazole ravuconazole voriconazole ICI 153066 and posaconazole [25] are famous for their antifungal activity antifungal. They possess more activity and reduced toxicity compared to imidazole antifungals azoles function as inhibitors of the fungal cytochrome P450 enzyme, lanosterol

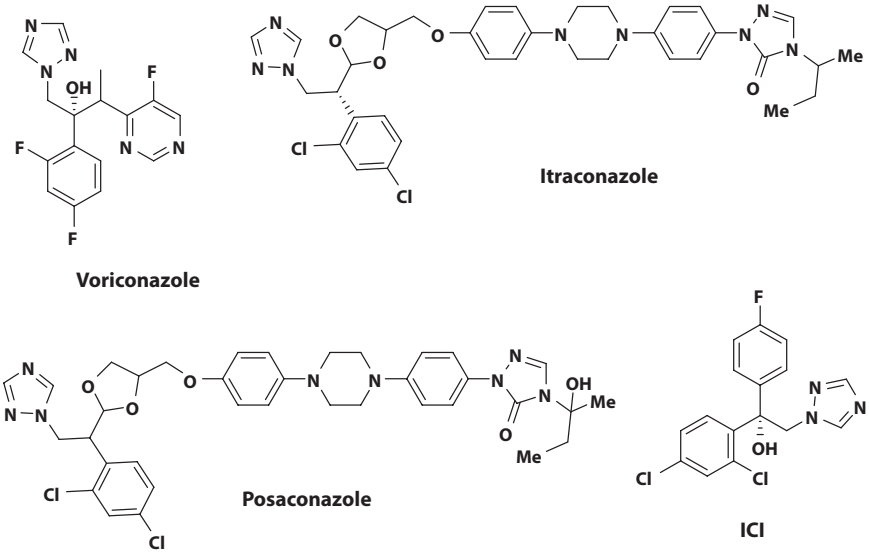
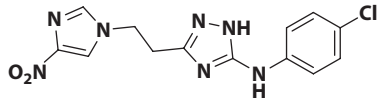


Figure 10.2 Antifungal drugs.

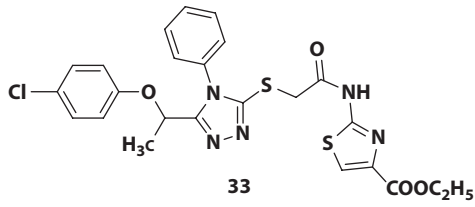
14α-demethylase (CYP51) which blocks the ergosterol biosynthesis pathway through the mechanism involving heterocyclic nitrogen atom (N-4 of triazole) which binds to the heme iron atom [25] and causing depletion of ergosterol source essential for the formation of intracellular regulatory sterols, which are required for cell cycle regulation, cell development, and multiplication CYP51 catalyzes the oxidative removal of the 14a-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis [26]. Selective inhibition of CYP51 would cause depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols leading to the growth inhibition of fungal cells.

Demirayak *et al.* [27] reported some 3-arylamino-5-[2-(substituted imidazole-1-yl or benzimidazole-1-yl)ethyl]-1,2,4-triazole derivatives **32** which were evaluated for antifungal activity against *Candida albicans* and *Candida glabrata* by using the tube dilution method. The *in vitro* antifungal activity results showed that *Candida glabrata* is the most sensitive microorganism to ketoconazole. Compounds tested showed ketoconazole equipotent activity against *Candida albicans* but *Candida glabrata* was more sensitive to both ketoconazole as compared to the compounds tested against *Candida albicans*. Hence, the tested compounds can be regarded as highly active antifungal substances against *Candida glabrata* and less active against *Candida albicans*.



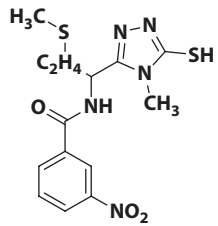
32

Collin *et al.* [28] have prepared 1,2,4-triazolo mercapto and aminonitriles as promising antifungal agents; 1,2,4-triazole bearing benzothiophene nucleus and studied their antimicrobial activities. Zitouni *et al.* [29] synthesized 1,2,4-triazole derivatives **33** and screened against *C. albicans*, *C. glabrata*, *E. coli*, *S. aureus*, and *P. aeruginosa*. The results indicated that some of the compounds exhibited significant antifungal activities.



33

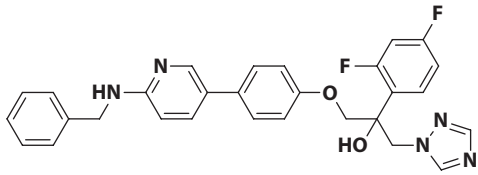
In another study, Pintilie *et al.* [30] synthesized new 1,2,4-triazole compounds containing a D, L-methionine moiety **34** and antimicrobial effects of the synthesized compounds were investigated against *Staphylococcus aureus* ATCC 25923, *Bacillus antracis* ATCC 8705, *Bacillus cereus* ATCC 10987, *Sarcina lutea* ATCC 9341, and *Escherichia coli* ATCC 25922 strains. The newly synthesized compounds exhibited promising activities against *Bacillus antracis* and *Bacillus cereus*.



34

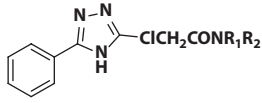
Liu *et al.* [31] synthesized a series of 1-(substituted biaryloxy)-2-(2,4-difluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl) propan-2-ol derivatives, **35** and their antifungal activity was evaluated against eight human pathogenic fungi *in vitro*. Seventeen compounds showed activity between 4- and 64-fold higher than voriconazole against *Candida albicans*. The structure-activity relationship suggested that the introduction of a biaryloxy

side-chain greatly enhanced the antifungal activity of triazole analogs against *Candida* species.



35

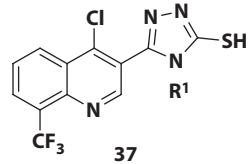
R.R. Somani *et al.* [32] synthesized 3,5-disubstituted-1,2,4-triazole derivatives **36** and tested for antifungal activity. All compounds were evaluated for antifungal activity against *C. albicans* and *A. niger* using FCZ as the reference standard. Among all the tested compounds **a–d**, **f** and **h** displayed good activity against C. albicans and A. niger while **a** and **d** exhibited promising activity against *C. albicans* and *A. niger*.



36

a-R^1:H, R^2- 4-methoxyphenyl,
b-R^1:H, R^2:4-bromophenyl,
c-R^1:H, R^2-4-Clphenyl,
d-R^1:H, R^2-4-NO_2phenyl,
e-R^1:H, R^2-3-NO_2phenyl,
f-R^1:H, R^2-Methylphenyl,
g-R^1:H, R^2-4-phenylenediamine,
h-R^1:H, R^2-2,6dichlorophenyl,
i-R^1:H, R^2-2,6-Dimethylphenyl,
j-R^1:H, R^2-*n*-Butylamine,
k-R^1:H, R^2-Morpholinyl,
l-R^1:H, R^2-Butylamine

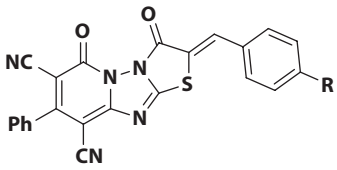
Compounds like **b**, **c**, **f**, **g**, **h**, **I**, and **l** exhibit similar antifungal activity even at a lower concentration. The antifungal activity due to the triazole ring as it contains the toxophoric moiety-(N-C-N-). This activity is mainly enhanced due to the coupling of various chloroacetanilides at 3-position. Hence, it can be concluded that 3,5-disubstitution on 1,2,4-triazole can lead to potential bioactivity. Quinoline derivative carrying 1,2,4-triazole **37** synthesized by Eswaran and co-worker [33] and investigated for their antimicrobial activity.



37

Suresh *et al.* [34] found that (Z)-2-(4-substituted benzylidene)-7isocyano-3,6-dioxo-8-phenyl-3,6-dihydro-2H-thiazolo[3',2':2,3]

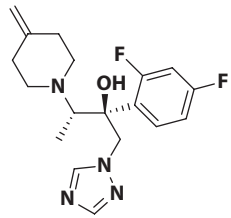
[1,2,4]-triazolo[1,5-a]pyridine-9-carbon nitrile **38** exhibit good antifungal activity against *A. flavus*, *A. fumigatus*, *C. albicans*, *P. marneffei*, and *Trichophyton mentagrophytes*.



38

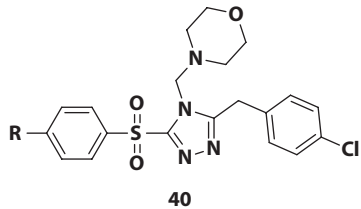
a:R=H **f:R=Br**
b:R=Cl **g:R=OCOCH$_3$**
c:R=NO$_2$ **h:R=CH$_3$**
d:R=OCH$_3$ **i:R=CH(CH$_3$)$_2$**
e:R=N(CH$_3$)$_2$ **j:R=C(CH$_3$)$_3$**

The mechanism of action of triazole antifungal was investigated with *Trichophyton mentagrophytes* and *Candida albicans*. Explained Efinaconazole **39** dose-dependently decreased ergosterol production and accumulated 4,4-dimethylsterols and 4α methyl sterols. Efinaconazole induced morphological and ultrastructural changes in *T. mentagrophytes* hyphae that became more prominent with increasing drug concentrations. In conclusion, the primary mechanism of action of efinaconazole is blockage of ergosterol biosynthesis, presumably through sterol 14α-demethylase inhibition, leading to secondary degenerative changes.



39

Narayana Rao *et al.* [35] have been synthesized a new class of 1,2,4-triazole analogs and tested their antimicrobial biological activity. 4-[(3-(4substituted-phenoxymethyl)-5-benzylsulfonyl)-1,2,4-triazol4-yl) methyl]-morpholine **40** and all the title compounds showed good antibacterial and antifungal activities.

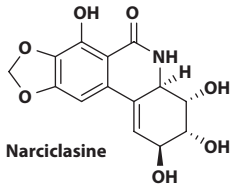


40

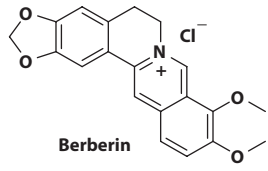
a:R=H
b:R=CH$_3$
c:R=OCH$_3$
d:R=NO$_2$
e:R=Cl
d:R=Br
e:R=F

10.3 Review on Isoquinoline and Fused Triazolo Isoquinolines

Isoquinoline is an important aromatic heterocyclic template performing a significant role in organic chemistry as a key structural unit in many natural products [36] and building blocks in pharmaceuticals. Narciclasine is an isoquinoline fused powerful antitumor agent [37] and Papaverine is a known drug for the treatment of visceral spasm and vasospasm [38]. Berberine has been used as an antibacterial drug [39]. Fused isoquinoline derivatives are a very interesting class of compounds due to their significant pharmacological and biological activities [40].

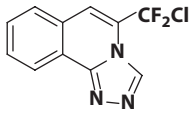


Narciclasine

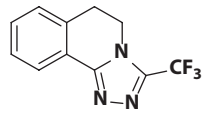


Berberin

[1,2,4]-Triazoles are also called s-triazoles, whereas [1,2,3]-triazoles are also called v-triazoles. s-Triazolo-[3,4-a]-isoquinolines are active against various forms of arthritis, edemas, etc., in man and mammals as anti-inflammatory agents [41]. 3-Chlorodiflurомethyl-s-triazolo-[3,4-a]-isoquinoline and 3-trifluoromethyl-5,6-dihydro-s-triazolo-[3,4-a] isoquinoline was useful as anti-inflammatory agents and also possessed anti-secretory activity [42]. s-Triazolo-[3,4-a]-isoquinoline and its 3-substituent derivatives are also useful as coronary dilating agents [43]. They also possess good cardiovascular [44], s-Triazolo-[5,1-a]-isoquinolines, and their derivatives are useful as antioxidants and corrosion inhibitors [45]. Various 1,2,4-triazole derivatives have resulted in many potential drugs and are known to exhibit a broad spectrum of biological activities. 3-Amino-1,2,4-triazole was the first triazole derivative manufactured on large scale from aminoguanidine format was useful as neutral [46]. The 3-chlorodifluoromethyl-s-triazolo-[3,4-a]-isoquinoline and 3-trifluoromethyl-5,6-dihydro-s-triazolo-[3,4-a]isoquinoline were useful as anti-inflammatory drugs and also possessed anti-secretory activity [47].

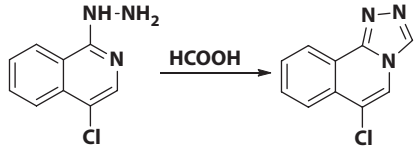


3-Chlorodifluoromethyl-s-triazolo-[3,4-a]-isoquinoline



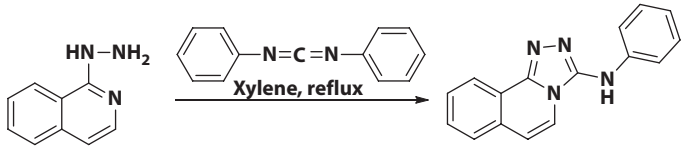
3-trifluoromethyl-5,6-dihydro-s-triazolo-[3,4-a]isoquinoline

There are few previously reported triazolo isoquinoline derivatives, such as Sidhu *et al.* [48] reported the synthesis of s-triazolo-[3,4-a]-isoquinoline **42** from 1-hydrazinoisoquinoline **41** with formic acid or acetic acid.



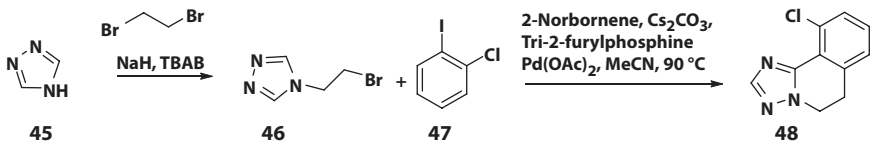
41 **42**

The reaction of 1-hydrazinoisoquinoline with an acid chloride, Ac_2O, CF_3COOH, $CH_2(CO_2Et)_2$, $HC(OEt)_3$, and $ClCH_2COOH$ in the presence of an organic base also gave s-triazolo-[3,4-a]-isoquinolines **42**. Reimlinger *et al.* [49] prepared 3-Alkylamino or 3-arylamino-s-triazolo-[3,4-a]-isoquinoline by treating a solution of 1-hydrazinoisoquinoline **43** in xylene or $C_6H_4Cl_2$ with carboiimides **44**.



43 **44**

Laleu *et al.* [50] synthesized 5,6-dihydro [1,2,4]triazolo[5, 1a]isoquinoline derivatives **48** in a two-step process. Step 1 involves n-alkylation of 1,2,41H triazole **45** with 1,2-dibromoethane at 0°C under an argon atmosphere by using NaH and dry tetrabutylammonium bromide. Step 2 is the 1,2,4-Triazole annulation. The Ary iodide **47** was treated with alkylated triazole **46**, 2-Norbornene, Cs_2CO_3, Tri-2-furyl phosphine, and $Pd(OAc)_2$, in MeCN at room temperature (rt) → 90°C.

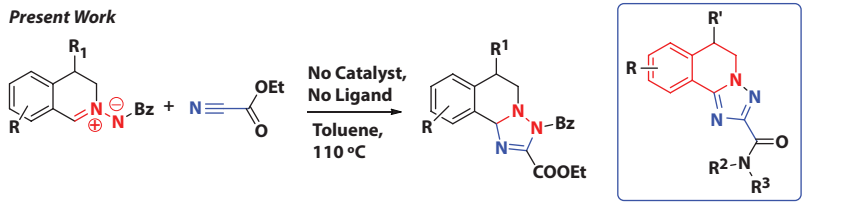


45 **46** **47** **48**

Fused [1,2,4]-Triazoles isoquinolines are active against various forms of arthritis, edemas, etc., in man and mammals as anti-inflammatory agents. Various 1,2,4-triazole derivatives have resulted in many potential drugs and are known to exhibit a broad spectrum of biological activities [51].

10.4 Present Work

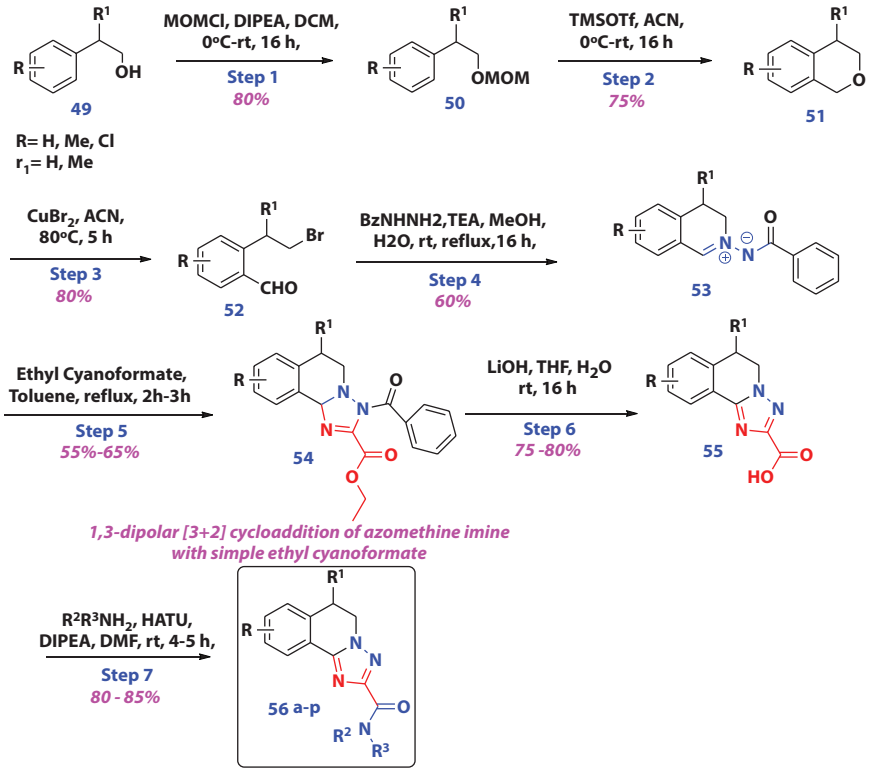
The resistance developed in many existing antibacterial agents has increased the emergence of innovation of new molecules possessing antimicrobial activity. As a part of our on-going program to discover a new class of active molecules by the conjugation of 1,2,4 triazoles and isoquinolines, we have developed a new and mild, catalyst and ligand-free synthetic methodology to synthesize novel [1,2,4]-triazolo isoquinoline derivatives in excellent yields by employing 1,3-dipolar [3+2] cycloaddition as unknown protocol, to elicit antifungal activities against different fungal pathogens. Then, all synthesized compounds were a screed against different candida pathogens for antifungal activity.



10.5 Results and Discussion

10.5.1 Synthetic Studies

The synthesis of the target compounds **56a–p** was accomplished in seven-step synthetic sequence and the route is described in Scheme 10.1. The 2(2-bromoethyl) benzaldehyde derivative **52**, was synthesized by standard procedures the reaction of 2-phenylethan-1-ol derivative **49** with methoxymethyl chloride resulted (2-(methoxymethoxy)-ethyl)benzene derivative **50** and which on further treatment with trimethylsilyl triflate afforded isochromane derivative **51**. The compound **51** on subsequent reaction with copper (II) bromide gave 2(2-bromoethyl) benzaldehyde



Scheme 10.1 Synthetic strategy of fused [1,2,4] triazolo isoquinoline derivatives.

derivative **52** in very good yield (**80%**). The azomethine imine derivative **53** was prepared as per the reported procedure. The reaction of benzoyl hydrazine with compound **52** afforded azomethine imine of N-(3,4-dihydroisoquinolin-2-yl) benzamide derivative **53** in good yield (**60%**), where the benzoyl group stabilized the azomethine imine.

The cyclized compound **54** was prepared by a metal-free synthetic protocol developed under neutral and milder reaction conditions using an alternative approach of azomethine imine 1,3-dipolar [3+2] cycloaddition reaction. The compound **53** was treated with ethyl cyanoformate in toluene at 120°C for 2-3 h to afforded the ethyl ester of benzoyl tetrahydro triazolo isoquinoline derivative **54** in good yield (**60%**) as a single regioisomer. It followed the unidirectional formal 1,3-dipolar [3+2] cycloaddition pathways with the azomethine imine **53** involving the lone pair to afford the 1,2,4-triazolo isoquinoline derivative **54** and another regioisomer formation in this reaction was not observed. Relative to the metal-catalyzed

[52], 1,3-dipolar cycloaddition reaction has the advantage of the rapid construction of the double bond to access the 1,2,4-triazole ring system. This new reaction involving ethyl cyanoformate is very reliable, selective, biocompatible, and exhibited a broad scope.

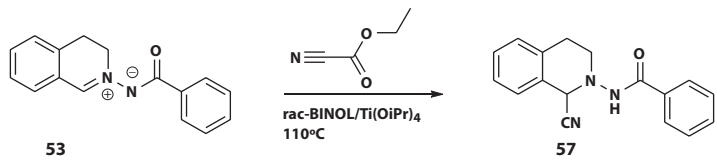
To get better reaction conditions, the effects of different solvents including acetonitrile, 1,2-dichloroethane, and toluene were screened. Cyclization did not occur when the reaction was carried at reflux with and without using TEA in acetonitrile and 1,2-dichloroethane in longer reaction time (Table 10.1). However, the formation of cyclized compound **54** was observed in toluene at reflux temperature without using any catalyst, ligand, and base. Surprisingly, when the reaction was done in toluene using *rac*-BINOL/Ti(O*i*Pr)$_4$ (Scheme 10.2) at 110°C, gave an undesired compound **57**. The cyclized compound **54** was characterized by ^{1}H NMR. The peak observed at δ 6.0 ppm as a singlet in ^{1}H NMR corresponds to the proton of the 1,2,4-triazole ring system. The formation of completely aromatized 1,2,4-triazolo isoquinoline carboxylic acid derivative **55** of compound **54** was observed on hydrolysis with lithium hydroxide as a base. Later, compound **54** was observed to be unstable at rt and it rapidly aromatizes to give completely aromatized 1,2,4-triazole ring system. The carboxylic acid derivative **55** was further treated with various amines to give 1,2,4-triazolo isoquinoline amide derivatives **56a-p** (Table 10.2) and these were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS data.

The reagent and substrate addition was done at rt (25°C) and stirred for the next 2–3 h at reflux temperature.

Table 10.1 Optimization of reaction conditions for [1,2,4] triazolo isoquinoline[a].

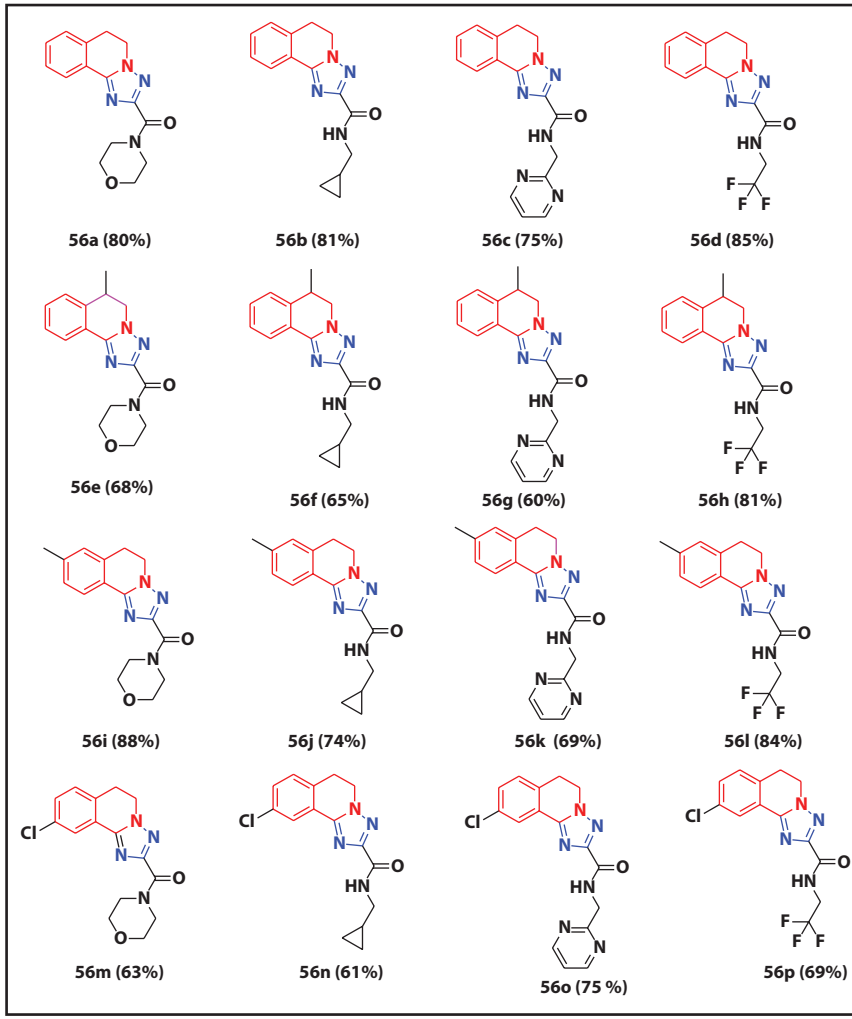
S. No.	Solvent	Conditions	Yield
1	Acetonitrile	85–90°C, 14 h	No reaction
2	Acetonitrile	TEA, 85–90°C, 16 h	No reaction
3	1,2-Dichloroethane	85–90°C, 16 h	No reaction
4	1,2-Dichloroethane	TEA, 85–90°C, 16 h	No reaction
5	Toluene	110°C, 2–3 h	60%
6	Toluene	TEA, 110°C, 2–3 h	38%
7	Toluene	rac-BINOL, Ti(OiPr)$_4$/ Toluene,110°C, 1–3 h	Compound 150 with 52%

[a]All reactions were carried out on approximately 8 mmol scales at 0.4 M concentration.



Scheme 10.2 Synthetic approach with ligand and catalyst.

Table 10.2 Substituted fused [1,2,4] triazolo isoquinoline derivatives.



10.5.1.1 Confirmation of Regioisomer

The [3+2] cycloaddition of a 1,3-dipole to a dipolarophile involves 4π-electrons from the dipole and 2π-electrons from the dipolarophile (Figure 10.3). The 4π-electron component is of such nature that the stabilized all octet structure can only be represented by zwitterionic forms in which the positive charge is located on the central hetero atom and the negative charge is distributed over the two terminal atoms. There exist two principal types of dipoles. The bent allyl type 1,3-dipoles have their four π-electrons in three parallel atomic pz-orbitals perpendiculars to the plane of the dipole.

The reaction between a 1,3-dipole and a dipolarophile leads to a 5-membered heterocycle and proceeds usually *via* a concerted mechanism [53]. This means that the three pz-orbitals of the 1,3-dipole and the two pz-orbitals of the dipolarophile (cyanide) both combined suprafacially. It is thermally allowed with the description [π4s + π2s] according to the Woodward-Hoffmann rules [54]. Unsymmetrically substituted azomethine imine can form regioisomers with unsymmetrical dipolarophiles, but the regiochemistry will be induced by the asymmetry in the dipole frontier orbitals caused by the substituents. Presumably, the azomethine imine will react readily with both electron-deficient and electron-rich dipolarophiles due to the narrow frontier orbital separation. The presence of metals, such as a Lewis acid, can alter both the orbital coefficients of the reacting atoms and the energy of the frontier orbitals of the 1,3-dipole, or the triple bond of cyanide. Thus, Lewis acids may have an influence not only on the reactivity but also on the selectivity of the 1,3-dipolar cycloaddition reaction, which leads to the formation of two regioisomers but in our case, the reaction is involved without any catalyst which gave a single isomer.

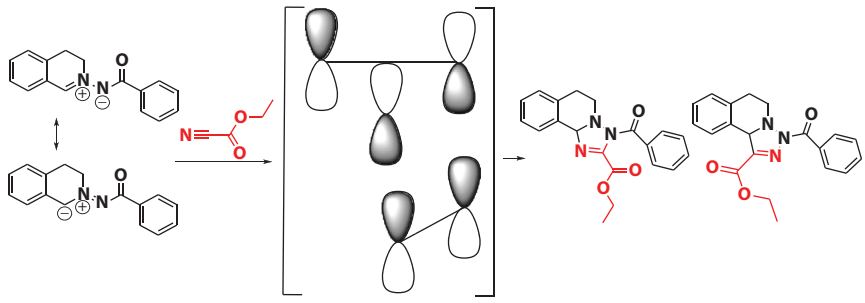
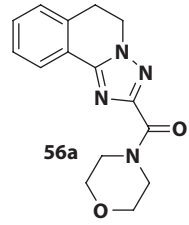


Figure 10.3 Proposed Huisgen's concerted mechanism of azomethine imine and ethyl cyanoformate to form a 1,2,4-triazolo isoquinoline ring system.

10.5.2 Spectral Analysis

The structures of all the synthesized compounds were predicted by ^{1}H NMR, ^{13}C NMR, and mass spectral analyses. To assign the ^{1}H and ^{13}C NMR signals compound, **56a** has been chosen as a representative compound.



10.5.2.1 ^{1}H NMR Spectral and Mass Analysis

The ^{1}H NMR spectra of compounds **56a-p** have been recorded in $CDCl_3$ and analyzed. The signals were assigned based on their position, multiplicity, and integral values. Compound **56a** was chosen as a representative compound.

In ^{1}H NMR spectrum of compound **56a**, a doublet of a doublet at 8.05 integral value with J = 1.5 and 7 Hz, multiplet at 7.43–7.40 (m, 2H), and a doublet at 7.32 with *J* = 7 Hz four protons assigned for aromatic phenyl ring protons. Four protons as triplets at 4.47 with *J* = 7 Hz and 3.32 with *J* = 7.5 Hz are assigned as isoquinoline two-CH_2 protons. Morpholine 12 protons appeared as four triplets at 4.0 with *J* = 4.5 Hz, 3.85 with *J* = 5 Hz, 3.80 with *J* = 5 Hz, and 3.75 with *J* = 4.5 Hz. The observed m/z values 285 (M+H) for **56a** is good agreement with the proposed molecular formula and the structure of the synthesized compound.

10.5.2.2 ^{13}C NMR Spectral Analysis

In the ^{13}C NMR spectrum of compound **56a**, the less intense signal observed in the far downfield region at 160.5 ppm was assigned to the carbonyl carbon of amide. Two less intense signals observed at 156.7 and 151.41 ppm are ascribed as quaternary carbons of the triazole ring. Six aromatic carbons of the phenyl group are observed between 133.4 and 123.96 ppm. Signals observed in the upfield in the range of 67.1–28.2 ppm are assigned as aliphatic carbons of morpholine and isoquinoline.

10.5.3 Biological Studies

All the synthesized compounds were evaluated for different biological activities such as antifungal, minimum fungicidal concentration (MFC), ergosterol biosynthesis inhibition, and cytotoxicity.

10.5.3.1 Antifungal Activity

Based on the good diffusion assay results depicted in Table 10.4 of the present study, it was found that only compounds **56b**, **56g**, **56h**, **56i**, and **56m** exhibited promising anti-fungal activity against *C. albicans* MTCC 227, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, and *C. krusei* MTCC 3020 with MIC value of 3.9 μg ml^{-1} which was comparable to the reference standard miconazole drug (MIC = 3.9 μg ml^{-1}). The results indicated that the promising compounds showed clear halo formation suggesting potential fungicidal activity. While all the other tested compounds showed MIC values of >125 μg ml^{-1}. Based on the structure-activity relationship, it was observed that the activity of all tested compounds varied based on the amide substituent attached to the basic triazolo isoquinoline skeleton.

Few compounds **56b**, **56g**, **56h**, **56i**, and **56m** only exhibited antifungal activity among the 16 tested compounds, which indicated that the amide linkage was responsible for the activity of the respective compound. Compound **56b** has a methyl cyclopropane substituent attached to the basic triazolo isoquinoline scaffold, while compound **56g** has a methyl pyrimidine substituent which probably may be contributing to the antifungal activity. It was observed that the potency of the compound **56h** may be increased by the presence of trifluoromethyl substituent. Based on the structure-activity relationship, it was observed that compounds **212i** and **56m** with a morpholine substituent, two electronegative atoms in the ring may be contributed to the antifungal activity. The antifungal activity results in this regard are tabulated in Table 10.4.

10.5.3.2 Minimum Fungicidal Concentration

Considering antifungal activity results, the selected compounds **56b**, **56g**, **56h**, **56i**, and **56m** were further evaluated for MFC against different *Candida* strains in comparison to the miconazole drug. These tested compounds **56b**, **56g**, **56h**, **56i**, and **56m** showed MFC values ranging between 7.8 and 15.6 μg ml^{-1}. While the standard miconazole drug exhibited a MFC value of 7.8 μg ml^{-1} for all the tested *Candida* strains. Among them, the compounds **56g**, **56h**, **56i**, and **56m** were highly promising against *C. albicans* MTCC 227, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, and *C. krusei* MTCC 3020 exhibiting MFC value of 7.8 μg ml^{-1} which was equipotent to miconazole drug. The MFC activity data to this regard is represented in Table 10.5.

Table 10.4 Antifungal activity of fused [1,2,4] triazolo isoquinoline derivatives (**56b**, **56g**, **56h**, **56i**, and **56m**).

Compounds	Minimum Inhibitory Concentration (μg/ml)									
	[a]*C.a*	[b]*C.a*	[c]*C.a*	[d]*C.a*	[e]*C.a*	[f]*C.a*	[g]*C.p*	[h]*C.g*	[i]*C.k*	[j]*I.h*
56b	7.8	>125	7.8	7.8	7.8	>125	>125	>125	7.8	>125
56g	7.8	>125	3.9	7.8	7.8	>125	>125	>125	3.9	>125
56h	3.9	>125	3.9	7.8	7.8	>125	>125	>125	3.9	>125
56i	7.8	>125	3.9	7.8	7.8	>125	>125	>125	7.8	>125
56m	7.8	>125	3.9	3.9	7.8	>125	>125	>125	7.8	>125
Miconazole	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*Candida glabrata* MTCC 3019
[i]*Candida krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

Table 10.5 Minimum fungicidal concentration (MFC) of fused [1,2,4] triazolo isoquinoline derivatives (**56b**, **56g**, **56h**, **56i**, and **56m**).

Compounds	Minimum fungicidal concentration (μg/ml)									
	[a]*C.a*	[b]*C.a*	[c]*C.a*	[d]*C. a*	[e]*C..a*	[f]*C.a*	[g]*C.p*	[h]*C.g*	[i]*C.k*	[j]*I.h*
56b	15.6	>125	15.6	15.6	15.6	>125	>125	>125	15.6	>125
56g	15.6	>125	7.8	15.6	15.6	>125	>125	>125	7.8	>125
56h	7.8	>125	7.8	15.6	15.6	>125	>125	>125	7.8	>125
56i	15.6	>125	7.8	15.6	15.6	>125	>125	>125	15.6	>125
56m	15.6	>125	7.8	7.8	15.6	>125	>125	>125	15.6	>125
Miconazole	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*Candida glabrata* MTCC 3019
[i]*Candida krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

10.5.3.3 Ergosterol Biosynthesis Inhibition

Candida albicans is a major causative agent of candidiasis and nosocomial infections. The currently available antifungal drugs (e.g., azoles) for the treatment of infections caused by *Candida*, target the ergosterol biosynthetic pathway. Ergosterol is an important component of the fungal cell wall. Considering this fact, we further investigated the effect of some representative test compounds **56b**, **56g**, and **56h** in comparison to the reference drug miconazole to outline its mode of action in the ergosterol biosynthetic pathway in various *Candida* strains such as *C. albicans* MTCC 227, *C. albicans* MTCC 3017, and *C. krusei* MTCC 3020. In this regard, the UV spectral scans of the sterol profiles for these *Candida* strains treated with various concentrations of the test compounds was determined by the sterol quantification assay. It was noticed that ergosterol content was 100% in the untreated *Candida* strains, while a significant dose-dependent decrease in the ergosterol production was observed in the case of *Candida* strains treated with both the test compounds and miconazole standard. As the dose of test compounds increased from 0 to 16 μg ml^{-1}, the concentration of ergosterol and dehydroergosterol decreased as indicated by the decline in the peak heights.

The UV-visible scans showed a flat line at the effective dose of test compounds in various *Candida* strains suggesting that no detectable levels of sterols were observed. The results in this regard are shown in (Figures 10.4 to 10.6). Our findings indicated that the test compounds altered the sterol profile and thus exerted its antifungal activity by inhibiting the synthesis of ergosterol biosynthesis. The selective cytotoxic behavior of the test compounds suggested that they have an affinity target for the specific site in the pathway of ergosterol biosynthesis. The fungicidal activity of the test

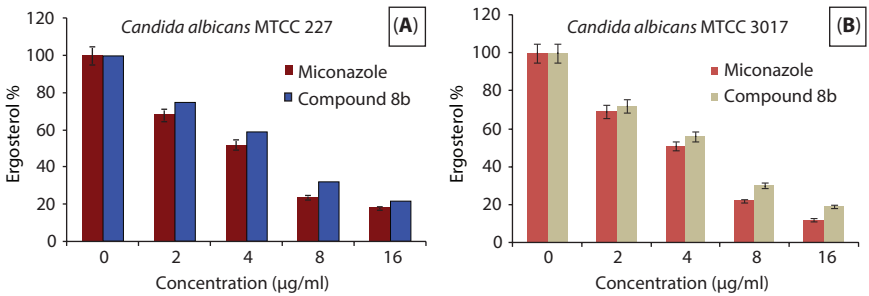


Figure 10.4 Effect of compound **56b** and miconazole (standard control) at various concentrations on inhibition of ergosterol biosynthesis in (A) *C. albicans* MTCC 227 and (B) *C. albicans* MTCC 3017.

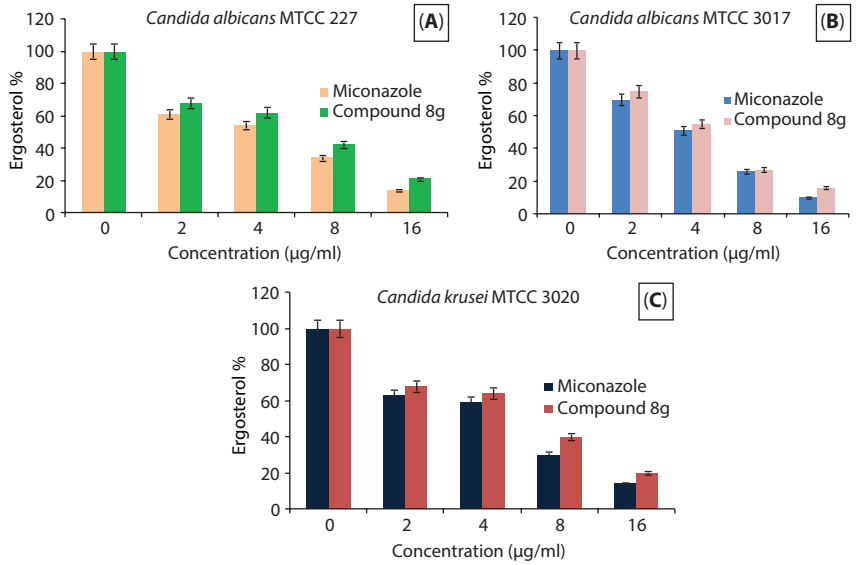


Figure 10.5 Effect of compound **56g** and miconazole (standard control) at various concentrations on inhibition of ergosterol biosynthesis in (A) *C. albicans* MTCC 227, (B) *C. albicans* MTCC 3017, and (C) *C. krusei* MTCC 3020.

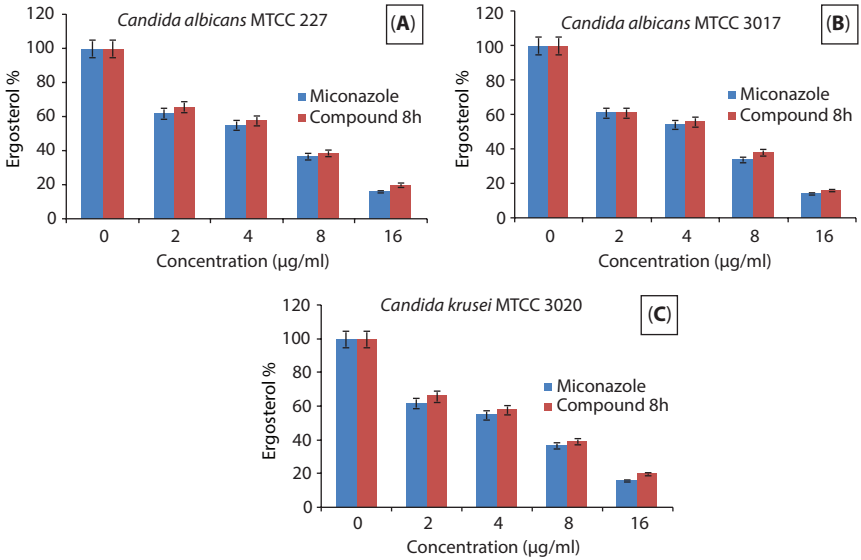


Figure 10.6 Effect of compound **56h** and miconazole (standard control) at various concentrations on inhibition of ergosterol biosynthesis in (A) *C. albicans* MTCC 227, (B) *C. albicans* MTCC 3017, and (C) *C. krusei* MTCC 3020.

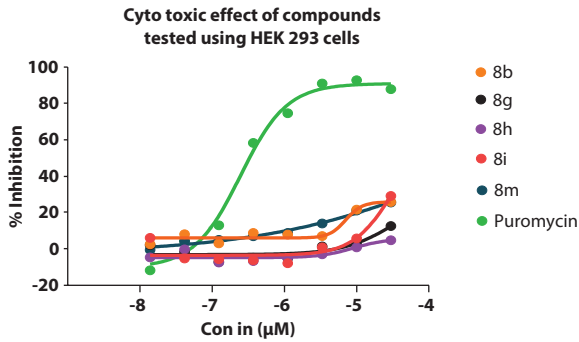
compounds may be due to the cell membrane permeability, disruption of the cell integrity, and subsequent cell death.

10.5.3.4 Cytotoxic Activity

For cytotoxic activity against HEK293 (normal human embryonic kidney cells; ATCC No. CRL-1573), the results are presented in Figure 10.7. The data indicated that the tested compounds were not cytotoxic. These results suggest that the compounds are lower cytotoxic toward the normal cells than the antifungal activity and thus considered to possess immense therapeutic potential.

10.5.4 Molecular Docking Studies

The ergosterol biosynthesis inhibition for the compounds **56b**, **56g**, and **56h** prompted us to study the binding modes of these compounds with the enzyme lanosterol 14α-demethylase (CYP 51) using computational techniques. *Candida albicans* lanosterol 14-alpha-demethylase (CYP51) protein structure complexed with VT1161 was downloaded from the PDB



Compound ID	IC50, µM	pIC50	IC50_Lower 95% Confidence interval	IC50_Upper 95% Confidence interval	Max Response	Max response concentration, µM	HillSlope	No. of points used to derive DRC
8b	>30	<4.5	-	-	26	30	-	8
8g	>30	<4.5	-	-	12	30	-	8
8h	>30	<4.5	-	-	5	30	-	8
8i	>30	<4.5	-	-	29	30	-	8
8m	>30	<4.5	-	-	25	30	-	8
Puromycin	0.246	6.6	0.14	0.42	92	30	1.3	8

Figure 10.7 Cytotoxicity of compounds **56b**, **56g**, **56h**, **56i**, and **56m** against HEK293 (normal human embryonic kidney cells; IC_{50} values in µM).

database (PDB ID: 5TZ1) having a resolution of 2.0Å and was imported into Accelrys Discovery Studio 2.1. The best ligand conformation is chosen based on the LibDock score and highly interacting amino acid residues. Of the 10 conformations generated for each compound, the compound with the highest LibDock score was taken for interaction analysis of the hydrogen bonding. LibDock scores of all the compounds along with their hydrogen bond interactions and bond lengths are depicted in Table 10.6. From the overall docking and interaction analysis, the best conformation of the compound **56b** docked complex showed a LibDock score of 135.956 kcal/mol with binding energy 325.85096 kcal/mol and formed single hydrogen bond interactions. The first hydrogen bonds were formed between the HG1 of THR311 interacting with the oxygen 20 atoms of the compound **56b** (A: THR311: HG1 - Comp2: O20) with a hydrogen bond distance of 2.453000Å, and the other non-bonded interactions were formed between HEM 601 moieties of Fe (Figure 10.8). However, the interaction of HG1 of THR311 with the oxygen 20 atoms could be responsible for the higher energy of the compound **56b**. The amino acids in the proximity of compound **56b** include Thr311, Gly308, Ile304, Leu139, Gly472, His466, Ile471, Lys143, Leu139, Tyr132, Pro375, Pro462, Thr315, Met34, Ile379, Tyr118, and Met974. The compounds **56g** and **56h** binds efficiently to enzyme binding pocket with a binding energy of 229.90330 and 288.85327 kcal/mol and showed non-bonded interactions between HEM 601 moieties of Fe (Figure 10.8). Besides, the amide side chains are the pharmacophores, and the spatial orientations of the pharmacophores were just oriented in the hydrophobic pocket. The side chains are very important in adjusting the physicochemical properties of the complete molecule to avoid some negative side effects and enhanced their pharmacodynamics and pharmacokinetic activities. Interestingly, the molecular modeling studies of compounds **56b**, **56g**, and **56h** comply with the antifungal activities and data ergosterol biosynthesis inhibition assay. In summary, compound **56b** efficiently interacted with lanosterol 14α-demethylase among the tested compounds.

All synthesized 1,2,4-triazolo isoquinoline derivatives were for antifungal and MFC against 10 different fungal strains and compounds **56a**, **56g**, **56h**, **56i**, and **56m** were found to possess promising antifungal activity against different candida pathogens. The compounds **56g**, **56h**, **56i**, and **56m** were highly promising against *C. albicans* exhibiting MFC value of 7.8 μg ml^{-1} which was equipotent to miconazole drug. A brief mechanistic study was done on the effective conjugates **56b**, **56g**, and **56h** indicated that

Table 10.6 Docking results of compound **212b**, **212g**, and **212h**.

Target	Libdock score	Interacting amino acids	Interacting atoms	H-Distance	Binding energy (K.Cal/Mol)
56b	135.956	Thr311, Gly308 Ile304, Leu139 Gly472, His466 Ile471, Lys143 Leu139, Tyr132 Pro375, Pro462 Thr315, Met34 Ile379, Tyr118, Met974	A:THR311: HG1 – **56b**: O20 A:HEM601: CHB – **56b**: H32 A:HEM601: C1D - **56b**: H26 A:LEU376:HN - **56b**:H36	2.453000 2.143000 2.117000 1.647000	325.85096
56g	13.016	Thr311, Gly308 Ile304, Leu139 Gly472, His466 Ile471, Lys143 Leu139, Tyr132 Pro375, Pro462 Thr315, Met34 Ile379, Tyr118, Met974	**56g**:H39 - A:HEM601:H5	1.528000	229.90330
56h	119.345	Thr311, Gly308 Ile304, Leu139 Gly472, His466 Ile471, Lys143 Leu139, Tyr132 Pro375, Pro462 Thr315, Met34 Ile379, Tyr118, Met974	**56h**:H35 - A:HEM601:C4D **56h**:H32 - A:THR311:CG2	2.114000 1.934000	288.85327

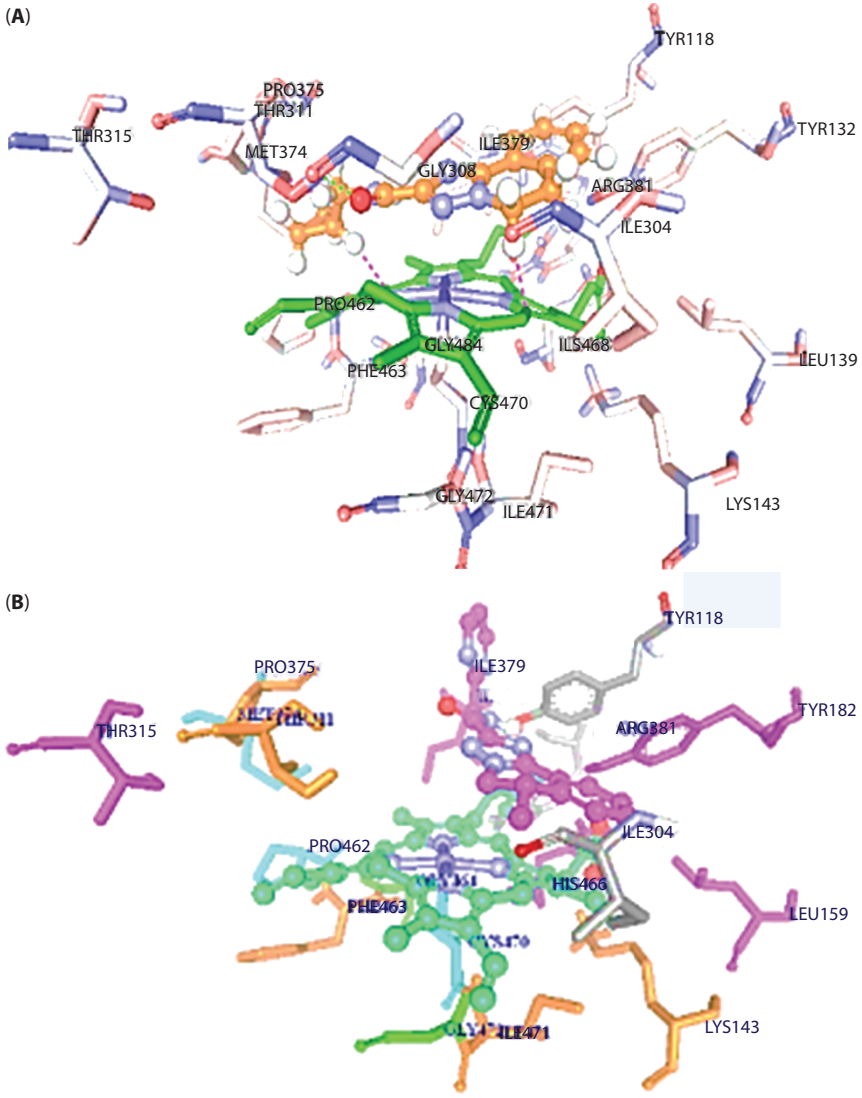


Figure 10.8 (A) Binding poses of compound **56b**; (B) Compound **56g**; (C) Compound **56h**; (D) Miconazole in the binding site of lanosterol 14α-demethylase.

they exhibited antifungal activity by inhibition of ergosterol biosynthesis. Molecular modeling studies carried on **56b**, **56g**, and **56h** correlates well with the antifungal activity and supported by ergosterol biosynthesis inhibition assay data. The work reported herein provides an intuition for the development of newer antifungal agents.

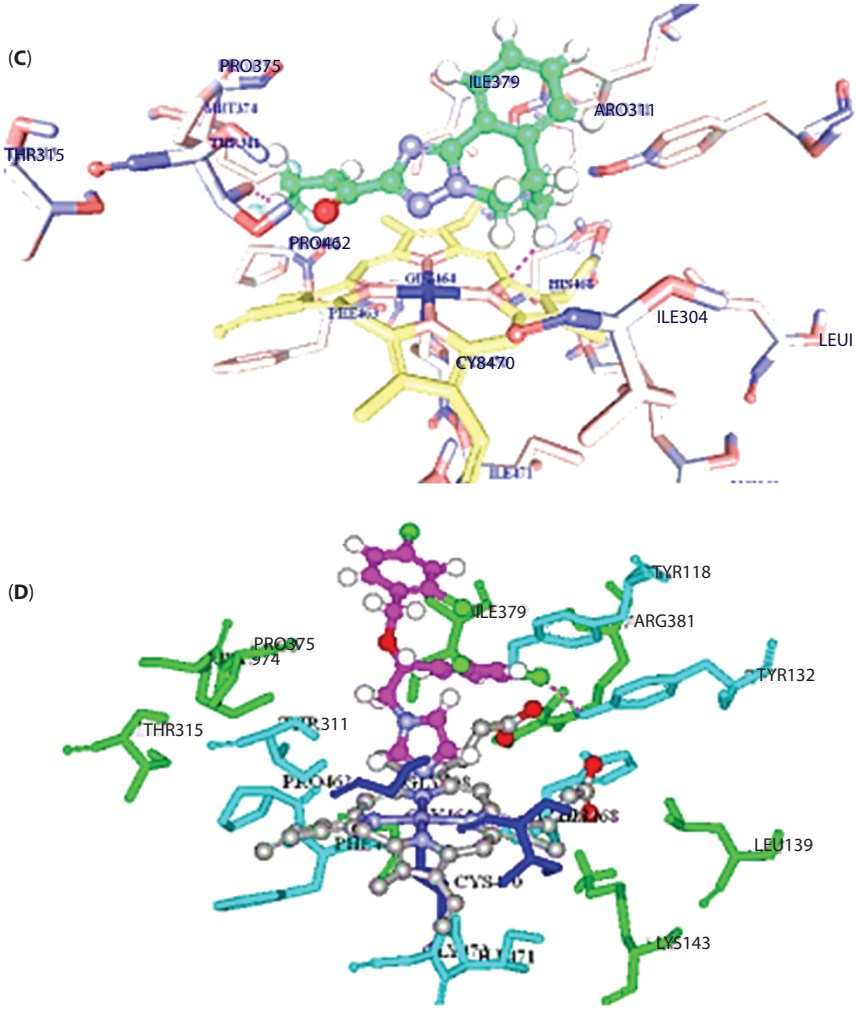


Figure 10.8 (Continued) (A) Binding poses of compound **56b**; (B) Compound **56g**; (C) Compound **56h**; (D) Miconazole in the binding site of lanosterol 14α-demethylase.

10.5.5 Experimental Section

10.5.5.1 Chemistry

Novel 1,2,4-triazolo isoquinoline derivatives **(56a-p)** were synthesized starting from 2-phenylethan-1-ol derivative **49** in a seven-step synthetic sequence. The key step in the scheme involves 1,3-dipolar [3+2] cycloaddition of azomethine imine and ethyl cyanoformate as an unknown reaction

protocol. We have also demonstrated the hydrolysis of both ester and benzoyl groups in a single step and the corresponding carboxylic acid derivatives were coupled with various amines to generate unknown 1,2,4-triazolo isoquinoline derivatives.

10.5.5.1.1 General Procedure for the Synthesis of Isochromanes (51)

A mixture of the substituted phenyl ethyl alcohol **49** (81.96 mmol), chloromethyl methyl ether (122 mmol), and N,N-diisopropylethylamine (163.9 mmol) in dry DCM (100 mL) was stirred under nitrogen atmosphere for 16 h at rt. The reaction mixture was then washed with H_2O (2 × 250 ml), dried over anhydrous $MgSO_4$, and the solvent was removed in a vacuum. The crude MOM acetal **50** was dissolved in dried acetonitrile (120 ml) and cooled (0°C), a solution of trimethylsilyl trifluoromethanesulfonate (TMSOTf) (10 mmol) was added slowly. The reaction was carried out under an N_2 atmosphere for 3 h, quenched by the addition of l M aqueous $NaHCO_3$ solution (200 ml), and extracted with EtOAc. The organic layer was washed with brine (2 × 300 ml), dried over $MgSO_4$, and evaporated under reduced pressure. Purification done by flash column chromatography afforded relevant substituted isochromane **51** in 75%–80% yield as a liquid.

10.5.5.1.2 General Procedure for the Synthesis of Benzaldehyde Derivatives (52)

To a solution, the substituted isochromane derivatives **51** (29.8 mmol) in acetonitrile (60 ml) was added $CuBr_2$ (35.7 mmol) under a nitrogen atmosphere. The solution was refluxed for 3 h and then cooled to rt. The reaction mixture was added to water, extracted with EtOAc (2 × 200 ml). The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Then, the crude compound was purified by flash column chromatography to give relevant substituted benzaldehyde **52** in **70%–80%** yield as a liquid.

10.5.5.1.3 General Procedure for the Synthesis of *N-B*benzoylimini-3, 4-dihydroisoqunolineum Betaine (53)

To a 0.5 M solution of the corresponding 2-(2-bromoethyl) benzaldehyde **52** (4.7 mmol) in MeOH (10 mL) was added benzoyl hydrazone (4.95 mmol) at rt and heated to reflux for 1 h. Then, the reaction was cooled to rt, the reaction solution was treated with triethylamine (7.05 mmol), poured into water, and stirred for 30 min to give a white precipitate. This solid material was washed with cold ether and then dissolved in dichloromethane to give

a yellow solution. This yellow-colored solution was dried over $MgSO_4$ and evaporated under reduced pressure afforded *N*-benzoylimino-3, 4-dihydroisoquinolium betaine **53** in **55%–60**% yield as a yellow solid.

10.5.5.1.4 General Procedure for the Synthesis of the Ethyl Ester of 1,2,4-Triazolo Isoquinoline Carboxylic Acid Derivative (54)

To a solution of *N*-benzoylimino-3, 4-dihydroisoquinolinium betaine **53** (1.6 mmol) in toluene (10 ml) was added ethyl cyanoformate (2.5 mmol) under a nitrogen atmosphere. The solution was refluxed for 2-3 h and then cooled to rt. Then, the reaction was concentrated and the crude compound was purified by flash column chromatography to give triazole derivative **54a-d** in **50%–55%** yield as semisolid.

10.5.5.1.5 General Procedure for the Synthesis of 1,2,4-Triazolo Isoquinoline Carboxylic Acid Derivative (55)

To a solution of the ethyl ester of benzoyl tetrahydro, triazolo isoquinoline **54** (1.56 mmol) in THF (10 mL) and water (2 ml) was added lithium hydroxide (3.12 mmol) at rt. The reaction was stirred at rt for 16 h. Later, THF was evaporated and water (2 ml) was added. Acidified with a 1N HCl, solid precipitated was filtered and then dried afforded carboxylic acid derivatives **55a-d** in **75%–80%** yield as a white solid.

10.5.5.1.6 General Procedure for the Synthesis of 1,2,4-Triazolo Isoquinoline Amide Derivatives (56a-p)

To a solution of 1,2,4-triazolo isoquinoline carboxylic acid derivative **55** (0.465 mmol), in dry DMF (3 ml) was added coupling reagent HATU (0.69 mmol). Cooled to 0°C, DIPEA (1.39 mmol) was added followed by a corresponding amine (1 eq). The reaction was for stirred for 4 h at rt. The reaction mixture was added to ice water, extracted with EtOAc (2 × 50 ml). The organic extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Then, the crude compound was purified by flash column chromatography to give relevant amide derivative **56a-p** in **80%–85%** yield as a solid.

10.5.5.2 Biological Studies

10.5.5.2.1 Antifungal Activity

The antifungal activity of the synthesized fused [1,2,4]-triazolo isoquinoline derivatives was determined using Muller-Hinton agar well diffusion method against different *Candida* strains, namely, *Candida albicans*

MTCC 227, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020, and *Issatchenkia hanoiensis* MTCC 4755 procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the media Petri plates, containing Muller-Hinton agar with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard). Wells of 6.0 mm diameter were prepared using a cork borer. The solution of synthesized compounds in 10% DMSO at a dose range of 125–0.97 μg was added in each well under sterile conditions in a laminar airflow chamber. The standard antibiotic solution of miconazole at a dose range of 125–0.97 μg $well^{-1}$ served as positive control and the well containing DMSO served as a negative control. The plates were incubated for 24 h at 30°C and the well containing the least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in duplicates and mean values are represented.

10.5.5.2.2 Minimum Fungicidal Concentration Assay

MFC was performed as a microtitre assay on different *Candida* strains such as *Candida albicans* MTCC 227, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020, and *Issatchenkia hanoiensis* MTCC 4755, based on Clinical and Laboratory Standards Institute (CLSI), approved standard document M27-A4 (Serial dilutions of test compounds were prepared in Mueller-Hinton broth with different concentrations ranging from 125 to 0.97 μg ml^{-1}). To the test compounds, 100 μl of overnight cultured *Candida* suspensions were added to reach a final concentration of 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard) and incubated at 37°C for 24 h. After 24 h of incubation, the MFC was determined by sampling 10 μl of suspension from the tubes onto Mueller-Hinton agar plates and were incubated for 24 h at 37°C to observe the growth of test organisms. All the experiments were carried in duplicates and mean values are represented, where MFC is the lowest concentration of compound required to kill a particular *Candida* strain.

10.5.5.2.3 Ergosterol Quantification Assay

A panel of *Candida* strains including *C. albicans* MTCC 227, *C. albicans* MTCC 3017, and *C. krusei* MTCC 3020 were used for testing the effect of some representative compounds **56b**, **56g**, and **56h** on their total sterol content. The

sterols from the test *Candida* strains were isolated by the total sterol extraction method with minor modifications. Initially, Muller-Hinton broth (50 ml) was spiked with various concentrations of the test compounds (0, 2, 4, 8, and 16 μg ml^{-1}) and these medium samples were inoculated with single colonies of the test *Candida* strains. The treated culture medium samples were further incubated at 30°C for 20 h with agitation at 150 rpm. The cells were collected at the stationary phase by centrifugation at 8,200 ×*g* for 6 min followed by washing the pellet twice with sterile distilled water. The net wet weight of each *Candida* pellet was weighed and recorded. To each *Candida* pellet, 5 ml of 25% alcoholic KOH solution was added and mixed vigorously. Each of these *Candida* suspensions was collected in sterile glass vials and incubated at 85°C for 60 min in a water bath. After cooling to rt, a mixture of sterile water and n-heptane (1:3) was added to each *Candida* suspension and vortexed vigorously for 5 min for the extraction of intracellular sterols into the heptane layer. From each *Candida* strain bilayer, the heptane layer with sterols was collected carefully and stored at −20°C for 24 h. The sterol suspension (30 μl) of each *Candida* strain was diluted with 100% ethanol in 1:5 ratio and scanned from 230 to 310 nm using UV/Vis double beam spectrophotometer (Lambda 25, PerkinElmer, Shelton, CT, USA) to observe the four characteristic peaks corresponding to ergosterol and dehydroergosterol.

The ergosterol content was calculated as a percentage of the wet weight of the cells using the following equations:

$$\% \text{ ergosterol} + \% \text{ 24(28) DHE} = [(A281.5/290) \times F]/\text{pellet weight}$$

$$\% \text{ 24(28) DHE} = [(A230/518) \times F]/\text{pellet weight,}$$

and

$$\% \text{ ergosterol} = [\% \text{ ergosterol} + \% \text{ 24(28) DHE}] - \% \text{ 24(28) DHE}$$

where *F* is the factor for dilution in ethanol and 290 and 518 are the E values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively. All the experiments were performed in triplicates and mean values were determined.

10.5.5.2.4 Cytotoxicity Evaluation

The cytotoxic activities of the compounds **56g**, **56h**, **56i**, and **56m** were tested using an XTT based colorimetric cellular cytotoxicity assay against HEK293 (normal human embryonic kidney cells; ATCC No. CRL-1573). The second-generation tetrazolium dye, XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), is bioreduced to

form an orange colored formazan derivative which is quantified by measuring the color intensity at 450 nm. The cell lines were cultured in EMEM medium, supplemented with 10% heat-inactivated FBS, in a humidified 5% CO_2 atmosphere. Cells when reached to 80%–90% confluence were detached from the flasks by trypsinization, neutralized, counted, and then seeded 5,000 cells/well in 96 well clear bottom tissue culture plates. After 24 h, the cells were treated with compounds and incubated at 37°C for 72 h in a 5% CO_2 incubator. After incubation, the XTT reagent was added and incubated for 2 h. The absorbance was measured at 450 nm using a SpectraMax384 plate reader. The IC_{50} values (50% inhibitory concentration) were calculated from the plotted absorbance data for the dose-response curves. The cytotoxic potency (IC_{50} values in μM) of the compounds was determined by analyzing the data using GraphPad Prism statistical tool.

10.5.6 Molecular Modeling Procedure

For accurate docking of the compounds **56b**, **56g**, and **56h** into the active site of the protein, molecular docking was carried out through the LibDock module in Accelrys Discovery Studio 2.1. LibDock is a high-throughput site-featured docking algorithm. The binding site features are called as "hotspots" in which these site spheres were resolved with a grid fixed in the active site. It counts the hotspot map for the polar and a polar cluster in the active site of the protein which is further used for the alignment of the ligand conformations to the interaction sites of the protein for the formation of considerable interaction. Finally, it returns all the minimized ligand poses and their rankings based on the ligands score. Following the LibDock score, each pose is assessed which uses a simple pair-wise method. The ligands with high LibDock scores were preferred for estimating binding energies of the protein-ligand complex. The complex pose with the best binding energy was used for further binding mode analysis. For the docking validation, the co-crystallized ligand VT1161 in the lanosterol 14α-demethylase *C. albicans* binding site is redocked. The binding affinities of the synthetic compounds were compared and analyzed regarding VT1161 to identify structural characteristics of the complexes formed by these compounds and the protein.

10.6 Conclusion

We have developed a catalyst and ligand-free 1,3-dipolar [3+2] cycloaddition of azomethine imine with ethyl cyanoformate. Various triazolo

isoquinoline analogs could be prepared under a thermal condition in excellent yields (up to 90%). This process has shown significant potential for inexpensive, rapid, and easy access to pharmacologically relevant molecules and could be easily scaled up for further biomedical research.

All synthesized 1,2,4-triazolo isoquinoline derivatives were for antifungal and MFC against 10 different fungal strains and compounds **56a**, **56g**, **56h**, **56i**, and **56m** were found to possess promising antifungal activity against different candida pathogens. The compounds **56g**, **56h**, **56i**, and **56m** were highly promising against *C. albicans* exhibiting MFC value of 7.8 μg ml^{-1} which was equipotent to miconazole drug. The mechanistic study was carried out on effective conjugates **56b**, **56g**, and **56h** which indicated that they inhibited the ergosterol biosynthesis. Molecular modeling studies on binding modes of **56b**, **56g**, and **56h** showed correlation well with the antifungal activity. The data of ergosterol biosynthesis inhibition assay also supported. The work reported herein provides insight for innovation of newer antifungal agents.

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11

Amide as a Potential Pharmacophore for Drug Designing of Novel Anticonvulsant Compounds

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Abstract

Amide pharmacophore has gained significant consideration in the field of current medicinal chemistry. Literature indicates that compounds having amide pharmacophore have wide range of pharmacological activities, *viz*., anticonvulsant, antitubercular, antimicrobial, analgesic and anti-inflammatory, insecticidal, antitumor, fibrinolytic, and antiplatelet aggregatory. This chapter focuses on the anticonvulsant activity of substituted amides. The well-known and recent data on the preparation of amides used in the synthesis of these biologically active anticonvulsant compounds are summarized. The favorable amide derivatives possessing anticonvulsant activity will reignite the interest of medicinal chemists in amide and its derivatives.

Keywords: Review, amide, pharmacophore, chemistry, anticonvulsant activity

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11.1 Introduction

Epilepsy is a diverse group of disorders characterized by the neuronal hyper excitability and hyper synchronous neuronal discharge existing with episodes of sensory, motor, or autonomic phenomenon with or without loss of perception [1]. Epilepsy is one of the most common neurological disorders and affects about 1% of the world's population. The presently available anticonvulsants/anti-epileptic drugs (AEDs) are effective in decreasing the number of seizures and its severity in less than 70% of patients. Still, more than 30% patients are there where these available AEDs are ineffective and not able to provide symptomatic relief. Moreover, their practice is associated with detrimental side effects fluctuating from cosmetic (gingival hyperplasia) to life threatening conditions such as megaloblastic anemia [2–4]. Therefore, the continuous search for more effective and safer AEDs is urgently needed.

Dimmock *et al.* proposed anticonvulsant pharmacophore model which includes four binding sites for *in vivo* interaction with a macromolecular complex [5] (Figure 11.1).

a. An aryl hydrophobic binding site (A),
b. A hydrogen bonding domain (HBD),
c. An electron donor acceptor system (D), and
d. Another hydrophobic aryl ring (C) responsible for metabolism.

Amides provide a hydrogen bond donor group which is the essential feature of the pharmacophore for the molecules to display potential anticonvulsant activity as suggested by Dimmock (Figure 11.2).

Amides are commonly used in biologically active compounds with a broad variety of biotechnological, agricultural, and medical applications. Amides and its derivatives are associated with broad range of biological activities including anticonvulsant [6], antitubercular [7], antimicrobial [8], analgesic and anti-inflammatory [9], insecticidal [10], antitumor [11],

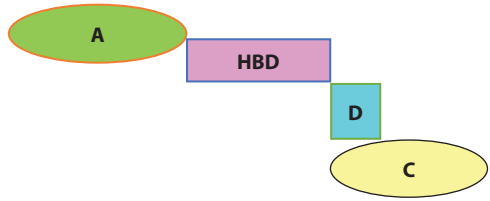


Figure 11.1 Suggested pharmacophore model for anticonvulsant activity.

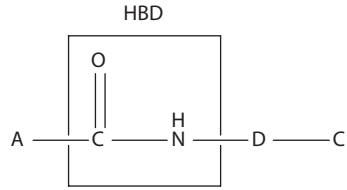


Figure 11.2 Amides as a pharmacophore.

fibrinolytic [12], and antiplatelet aggregator activities [13]. Amide bonds are much known in biological and natural pathways. Amides are one of the most essential functionalities of anticonvulsant drugs in chemical building blocks [14].

11.2 Chemistry of Amides

Amides are derived from carboxylic acid derivatives. When amides are added to several aromatic, aliphatic, and heterocyclic ring produces various kind of biological activity. General chemical structure of amide is given below [15] (Figure 11.3).

Where R_1 and R_2 may be H, aromatic group, aliphatic group, heterocyclic group, or cycloaliphatic group like piperidine and morpholine.

11.2.1 Synthesized Methods Utilized for Amide Bond Formation

The simplest method for making amides is by coupling a carboxylic acid, acid chloride, or acid anhydrides with 1° or 2° amine (Scheme 11.1).

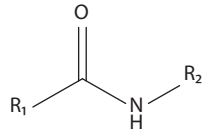
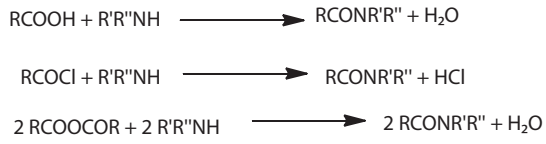


Figure 11.3 Molecular structure of amide.



Scheme 11.1 Synthetic route of amides formation.

11.2.2 Amide Pharmacophore Containing Anticonvulsant Drug

Many amide-derived drugs have been reported for the treatment of epilepsy. In recent decades, several demonstrations have attempted to recognize the structural features of compounds that are essential for anticonvulsant activity. Thus, the amide bond is one of the essential primary parts of anticonvulsants that can typically appear as lactam or imide in the form of a chain or as a part of the heterocyclic ring. The initial drug carbamazepine is a drug of choice for the treatment of generalized tonic-clonic seizure. Modification in the structure of carbamazepine has resulted in oxacarbazepine and eslicarbamazepine: two clinically most effective drugs for the management of epilepsy. The first γ-aminobutyric acid (GABA) agonist progabide is effective in all three types of epilepsies, *viz.*, Grand mal, partial, and myoclonic seizures (Figure 11.4) [16, 17].

11.2.3 Anticonvulsant Activity

Rapacz *et al.* synthesized five new amide compounds derived from 3-phenyl-2,5-dioxo-pyrrolidine-1-yl-acetic acid and evaluated for anticonvulsant activity in maximal electroshock (MES) test, the subcu-

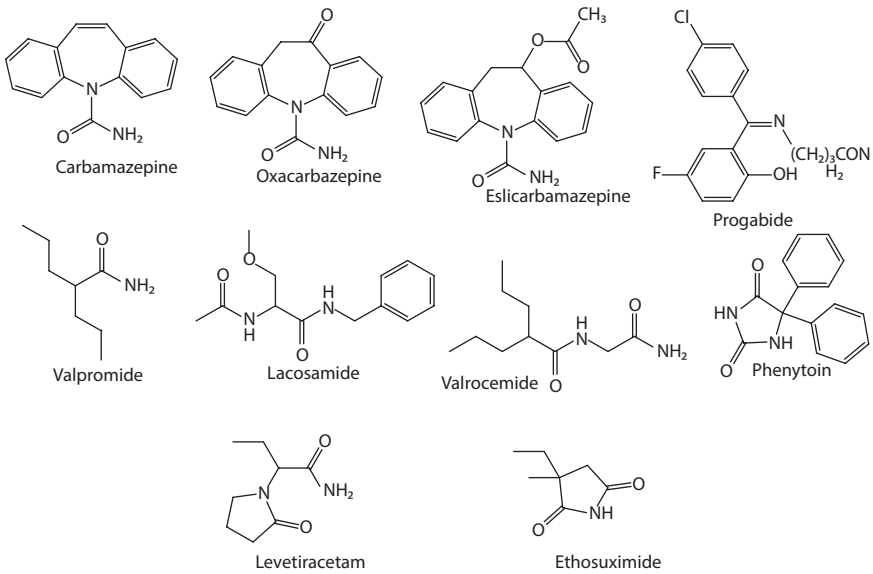
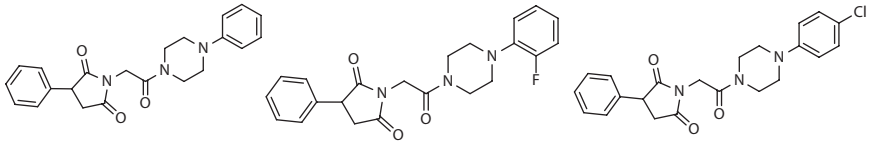


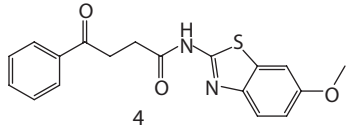
Figure 11.4 Molecular structure of amide-derived anticonvulsants.

taneous pentylenetetrazole (*sc*PTZ) test, and six-Hertz (6 Hz) model in mice. Three tested compounds (**1, 2,** and **3**) showed a wide range of anticonvulsant activity [18].



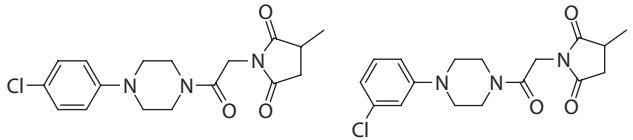
1 2 3

Hassan *et al.* synthesized a series of *N*-(substituted benzothiazol-2-yl) amide derivatives by the EDC coupling reactions of substituted-benzothiazol-2-amine with 4-oxo-4-phenylbutanoic acid/2-benzoyl benzoic acid and evaluated for their anticonvulsant activity against MES and *sc*PTZ. *N*-(6-methoxybenzothiazol-2-yl)-4-oxo-4-phenylbutanamide (**4**) (ED_{50} = 40.96 mg/kg, MES test) and (ED_{50} = 85.16 mg/kg, *sc*PTZ test) demonstrated the highest potency [19].



4

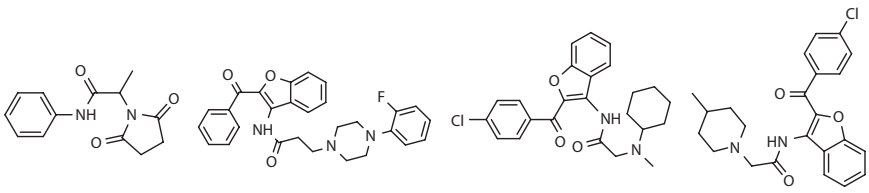
Obniska *et al.* synthesized 22 new 3-methyl- and 3-ethyl-3-methyl-2,5-dioxo-pyrrolidin-1-yl-acetamides and examined for their anticonvulsant activity against MES and *sc*PTZ. Compounds **5** and **6** were found to be the most effective anticonvulsant with median effective doses of 32.08 mg/kg (MES ED_{50}) and 40.34 mg/kg (*sc*PTZ ED_{50}), respectively [20].



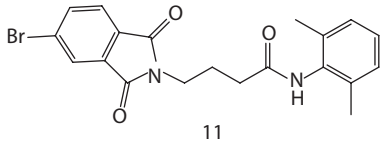
5 6

Kaminski *et al.* synthesized 21 new *N*-phenyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamide, and 2-(2,5-dioxopyrrolidin-1-yl)butanamide derivatives and evaluated for their anticonvulsant activity against MES and *sc*PTZ tests, and six-Hertz (6 Hz) model. Compound **7** was found to be the most effective anticonvulsant with median effective doses of 96.9 mg/kg (MES ED_{50}), 75.4 mg/kg (*sc*PTZ ED_{50}), 44.3 mg/kg (6 Hz ED_{50}), and median toxic dose of 335.8 mg/kg in the

rotarod test that generated adequate protective indexes (PI = TD_{50}/ED_{50}) of 3.5 (MES), 4.4 (*sc*PTZ), and 7.6 (6 Hz) [21]. Kamal *et al.* synthesized a series of 12 *N*-(2-benzoylbenzofuran-3-yl)-3-(substituted)-propanamides and assessed for their anticonvulsant activity against MES and *sc*MET tests. *N*-(2-benzoylbenzofuran-3-yl)-3-(4-(2-fluorophenyl)piperazin-1-yl) propanamide, **(8)** was found to be the most active compound in both MES and *sc*MET test at 30 and 100 mg/kg, respectively, after 0.5 and 4.0 h of dose [22]. Shakya *et al.* synthesized a series of *N*-(2-(benzoyl/4-chlorobenzoyl)-benzofuran-3-yl)-2-(substituted)-acetamides and evaluated for anticonvulsant activity against MES test in mice and GABA level in brain was estimated. The ED_{50} of the prepared compounds ranged from 0.055 to 0.259 mmol kg^{-1}. The majority of compounds exhibited anticonvulsant activity at a dose of 30 mg/kg during 0.5–4 h. [*N*-(2-(4-chlorobenzoyl) benzofuran-3-yl)-2 (cyclohexyl(methyl)amino)-acetamide] **(9)** and [*N*-(2-(4-chlorobenzoyl)benzofuran-3-yl)-2-(4-methylpiperidin-1-yl)-acetamide] **(10)** confirmed comparable anticonvulsant potency of 0.74 and 0.72, respectively, relative to phenytoin [23].

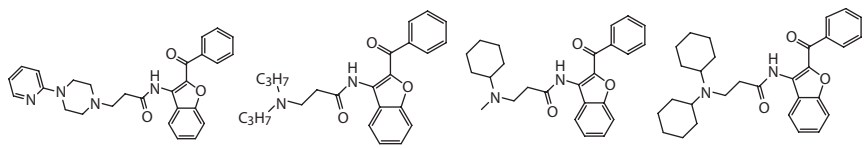


Khan *et al.* synthesized a series of 4-(5-bromo-1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-butyryl-*N*-(substituted phenyl)amides and examined for their anticonvulsant activity at three intraperitoneal doses of 30, 100, and 300 mg/kg in mice using MES model. Compound **11** showed potent anticonvulsant activity at the dose of 30 mg/kg, after 0.5 h and 4 h of administration [24].



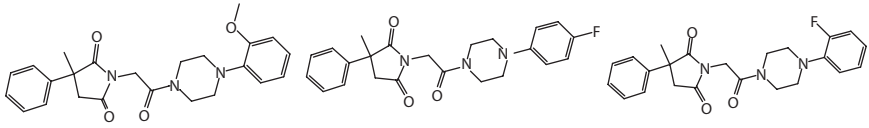
11

Kamal *et al.* synthesized a series of *N*-(2-(benzoyl)-benzofuran-3-yl)-2-(substituted)-butanamides and evaluated for their anticonvulsant effect against MES test. Compounds **12**, **13**, **14**, and **15** were found to have good anticonvulsant activity at the intraperitoneal dose of 30 mg/kg [25].



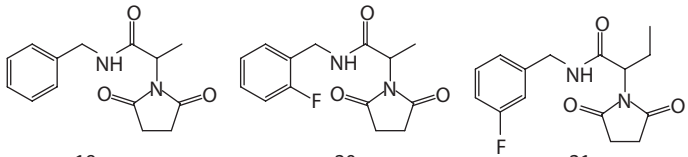
12 13 14 15

Obniska *et al.* synthesized a series of new 3-methyl-3-phenyl-2,5-dioxo-pyrrolidin-1-yl-acetamides and evaluated for their anticonvulsant effect in MES and *sc*PTZ seizure tests after intraperitoneal administration in mice. Results showed that 1-{2-[4-(2-methoxyphenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**16**), 1-{2-[4-(4-fluorophenyl)-piperazin-1-yl]-2-oxo-ethyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (**17**), and its 2-fluorophenyl analog (**18**) was found to be the most effective anticonvulsant with median effective doses of 97.51 mg/kg (ED_{50}), 104.11 mg/kg (ED_{50}), and 114.68 mg/kg (ED_{50}) respectively in MES test [26].



16 17 18

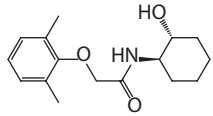
Kaminski *et al.* synthesized a series of 33 new *N*-benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide analogs, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl) propanamides analogs, and 2-(2,5-dioxopyrrolidin-1-yl)butanamide analogs and their anticonvulsant screening was performed using MES and *sc*PTZ model after intraperitoneal administration in mice. Compounds **19**, **20**, and **21** were found to be the most effective anticonvulsants with median effective doses of 67.65 mg/kg (MES ED_{50}), 42.83 mg/kg (*sc*PTZ ED_{50}); 54.90 mg/kg (MES ED_{50}), 50.29 mg/kg (*sc*PTZ ED_{50}); and 47.39 mg/kg (*sc*PTZ ED_{50}), respectively [27].



19 20 21

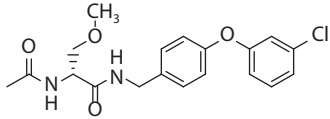
Pekala *et al.* synthesized geometric isomeric 2-(2,6-dimethylphenoxy)-*N*-(2-hydroxycyclohexyl)acetamides and evaluated for their anticonvulsant

activity against MES test after intraperitoneal administration in mice. The *trans*-2-(2,6-dimethylphenoxy)-*N*-(2-hydroxycyclohexyl)acetamide (**22**) was found to be the most potent exhibiting ED_{50} of 42.97 mg/kg and TD_{50} of 105.67 mg/kg [28].

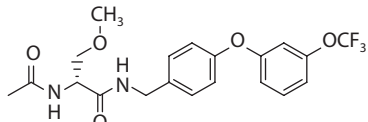


(22)

Torregrosa *et al.* synthesized six new 3"-substituted (*R*)-*N*-(phenoxybenzyl)-2-*N*-acetamido-3-methoxypropionamide derivatives and evaluated for their anticonvulsant effects in MES test intraperitoneally in mice and intraperitoneally and orally in rats. (*R*)-*N*-4′-((3″-chloro)phenoxy)benzyl2-*N*-acetamido-3-methoxypropionamide (**23**) and (*R*)-*N*-4′-((3″-trifluoromethoxy)phenoxy)benzyl 2-*N*-acetamido-3-methoxypropionamide (**24**) displayed high PI comparable with many anticonvulsant drugs [29].

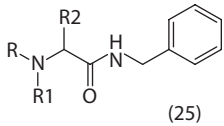


(23)



(24)

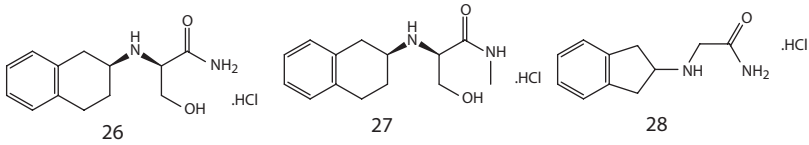
Beguin *et al*. synthesized amino acid amides (**25**) and evaluated for their anticonvulsant effects against MES and *sc*Met model. The amino acid amides showed moderate to excellent activity in MES test and were devoid of activity in *sc*Met test [30].



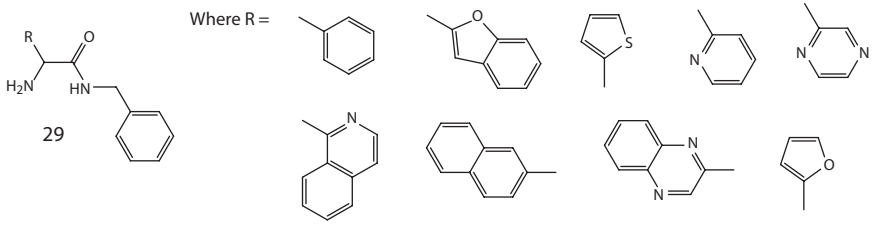
R = -H, -CH3, -CH2CH2OCH3
R1= -OCH3, -CH2CH2OCH3
R2 = -CH3,-C6H5

(25)

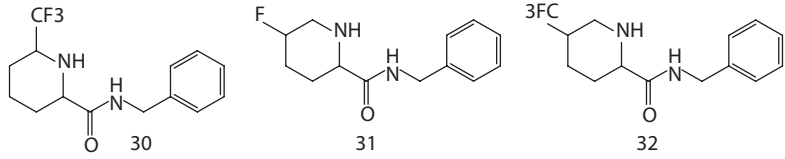
Ghidini *et al*. synthesized a series of amino acid amides and examined for anticonvulsant effect against MES, bicuculline and picrotoxin tests after oral administration in mice. Compounds **26**, **27**, and **28** containing a bicyclic (tetralinyl, indanyl) group linked to the aminoacetamide chain were found to be most effective anticonvulsants (ED_{50} between 10 and 100 mg/kg) against MES, bicuculline and picrotoxin tests at doses devoid of neurological toxicity [31].



Baruah *et al.* synthesized 16 new primary amino acid derivatives (PAADs) (**29**) from *N*-Benzyl 2-acetamido-2-substituted acetamide and their anticonvulsant effect was assessed using the MES test and the 6 Hz test. The PAADs exhibited prominent anticonvulsant activities. Several racemic C(2)-six-membered, C(2)-five-membered (hetero)aromatic, and benzannulated (hetero)aromatic PAADs presented significant effect in the MES [32].

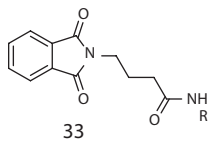


Ho *et al.* synthesized a series of 2-piperidinecarboxamide derivatives and examined for anticonvulsant effect using the MES and *sc*PTZ tests in mice and rats. Compounds **30**, **31**, and **32** were found to be the most effective anticonvulsant with median effective doses of 29, 31, 24 mg/kg, respectively, in MES test [33].



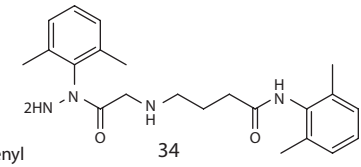
Ragavendran *et al.* synthesized a variety of *N*,*N*-phthaloyl GABA amides (**33**) and investigated for their anticonvulsant effect against MES, *sc*PTZ, subcutaneous strychnine (*sc*STY), and intraperitoneal picrotoxin (*ip*PIC)-induced seizure threshold tests. All of the compounds were devoid of anticonvulsant effect in the MES test. Most of the compounds were found to be effective in the *sc*STY and *ip*PIC models and very few compounds showed protection in the *sc*PTZ model [34]. Yogeeswari *et al.* synthesized

a series of ameltolide-GABA-amides and investigated for their anticonvulsant activity by using intraperitoneal MES, *sc*PTZ, and *sc*PIC models. The titled compounds showed promising activity in *sc*PTZ test demonstrating the connection of GABA-mediation. Compound 4-(2-(2,6-dimethylaminophenylamino)-2-oxoethylamino)-*N*-(2,6-dimethylphenyl)butanamide (**34**) was found to be the most potent derivative effective in MES, *sc*PTZ, and *sc*PIC tests with no neurotoxic side effects at the effective anticonvulsant dose [35].



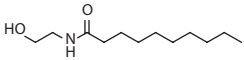
33

Where R = Substituted phenyl

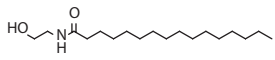


34

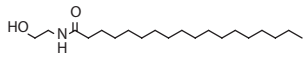
Guan *et al.* synthesized a variety of novel N-(2-hydroxyethyl) amides and evaluated for their anticonvulsant effects against MES test, and their neurotoxicity was assessed by the rotarod test. Results showed that N-(2-hydroxyethyl)decanamide (**35**), N-(2-hydroxyethyl)palmitamide (**36**), and N-(2-hydroxyeth-yl)stearamide (**37**) were found to be effective anticonvulsants in MES test [36].



35

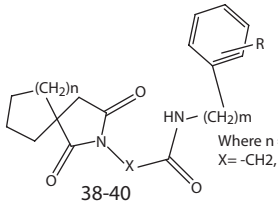


36



37

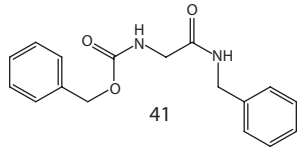
Obniska *et al.* synthesized a novel series of N-benzyl and N-phenyl amides of 2-(3-spirocyclohexanepyrrolidine-2,5-dione) acetic acid (**38**), 4-(3-spirocyclohexanepyrrolidine-2, 5-dione) benzoic acid (**39**), and 4-(3-spirocyclopentanepyrrolidine-2, 5-dione) benzoic acid (**40**) and investigated for their anticonvulsant effect by using intraperitoneal MES and *sc*MET tests. All the synthesized amides were ineffective but their LogP value ranged from 1 to 3.13 [37].



38-40

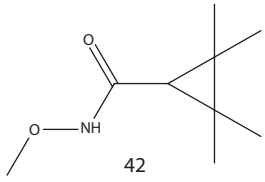
Where n = 1, 2; m= 0,1;
X= -CH2, -C6H4; R = H,4-F, 2-OCH3, 4-OCH3

Geurts *et al.* synthesized some novel ester and amide derivatives of *N*-(benzyloxycarbonyl)glycine and evaluated for their anticonvulsant effects by the MES test and by various chemically induced seizure models. *N*-(benzyloxycarbonyl)glycine benzylamide (**41**) was found to be the most effective anticonvulsant comparable to the standard drug phenytoin with median effective doses of 4.8 and 11.6 mg/kg at 30 min and 3 h after intraperitoneal administration in MES test, respectively. Compound **41** also effectively inhibited tonic seizures in different chemically induced models such as the strychnine, pentylenetetrazole, and 3-mercaptopropionic acid tests [38].



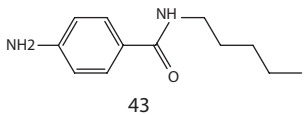
41

Sobol *et al.* prepared amides of 2,2,3,3-tetramethylcyclopropanecarboxylic acid and evaluated for their anticonvulsant effects against MES and *sc*PTZ models. *N*-methoxy-2,2,3,3-tetramethylcyclopropanecarboxamide (**42**) was found to be the most effective anticonvulsant with median effective doses of 35 mg/kg in rats and 74 mg/kg in mice in *sc*PTZ test. *N*-methoxy-2,2,3,3-tetramethylcyclopropanecarboxamide (**42**) median effective doses were 108 mg/kg in rats and 115 mg/kg in mice in MES test [39].

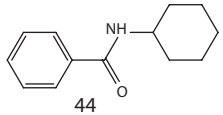


42

Clark *et al*. synthesized some novel 4-aminobenzamides and investigated for their anticonvulsant effects against MES and *sc*PTZ models. Among tested compounds, 4-amino-N-amylbenzamide (**43**) was found to be the most effective against MES with median effective dose of 42.98 mg/kg; however, the N-cyclohexylbenzamide (**44**) showed the greatest PI of 2.8 [40].

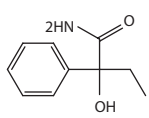


43



44

Meza-Toledo *et al.* synthesized a variety of phenyl alcohol amides. (±)-2-Hydroxy-2-phenylbutyramide (**45**), (±)-3-hydroxy-3-phenylpentanamide (**46**) and (±)-4-hydroxy-4-phenylhexanamide (**47**) and tested for their anticonvulsant and neurotoxicity effects. **45**, **46**, and **47** showed a wide range of anticonvulsant activity and a similar substantial activity in the MES test, Pentylenetetrazol, 4-aminopyridine, bicuculline, and thiosemicarbazide, but in the strychnine and picrotoxin tests, the protection was variable [41].



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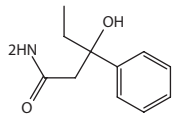

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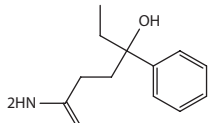
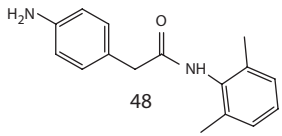


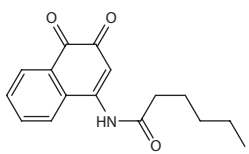
47

Clark *et al.* synthesized a series of 4-aminophenylacetamides and evaluated for anticonvulsant activity by MES and PTZ models. Unlike benzamides, there is a methylene group between the aromatic ring and amide carbonyl between these phenylacetamides. The more active and selective anticonvulsants prepared in this analysis were those with a supplementary aromatic ring as part of the amide nitrogen substituent. Compound 2-(4-aminophenyl)-*N*-(2,6-dimethylphenyl)acetamide (**48)** was found to be the most potent compound against MES (ED_{50} = 50.50 mg/kg) and PTZ-induced convulsions (ED_{50} = 93.20 mg/kg) [42].



48

Bansal *et al.* synthesized a series 4-amino-1,2-naphthoquinone analogs, and the synthesized compounds were evaluated for anticonvulsant activity by the MES test and *sc*PTZ test. The pharmacological results showed that majority of compounds were effective in MES and *sc*PTZ tests. Compounds *N*-(1,2-dihydro-1,2-dioxonaphthalen-4-yl) hexanamide (**49**) and 4-acetamido-*N*-(1,2-dihydro-1,2-dioxonaphthalen-4-yl)benzamide (**50**) were found active at the dose 10 mg/kg and emerged as the most active compounds [43].



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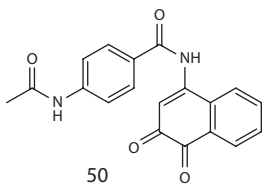
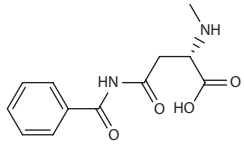


50

Paruszewski *et al.* synthesized a series of *N*-methyl benzyl-amides of *N*-methyl Asp (**51**) and *N*-methyl Glu (*R* and *S*) (**52**), benzylamides of some heterocyclic acids and their *N*-oxides and benzylamides of two heteroalicyclic acids. All the compounds were found active against MES test [44].



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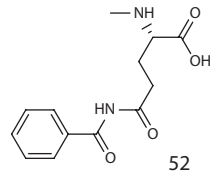
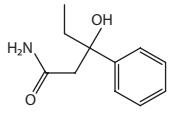


52

Garrido-Acosta *et al.* synthesized 3-hydroxy-3-ethyl-3-phenylproionamide (**53**), fluorinated 3-hydroxy-3-ethyl-3-phenylproionamide (**54**), and chlorinated 3-hydroxy-3-ethyl-3-phenylproionamide (**55**) and tested against MES and *ip*PTZ models. The ED_{50} of **53**, **54**, and **55** in the MES seizure model were 129.6, 87.1, and 62.0 mg/kg, respectively. The experimental potency was maximum for **55**, then **54** and was least for **53** [45].



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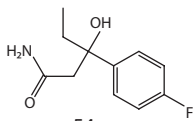

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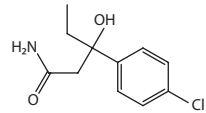
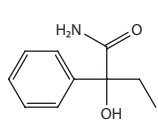


55

Sandoval *et al.* synthesized (±)-2-hydroxy-2-phenylbutyramide (**56**) and (±)-3-hydroxy-3-phenylpentamide (**57**). They were found to have anticonvulsant activity. This invention provides methods for the synthesis of (±)-3-hydroxy-3-phenylpentamide and (±)-2-hydroxy-2-phenylbutyramide [46].



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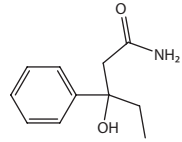
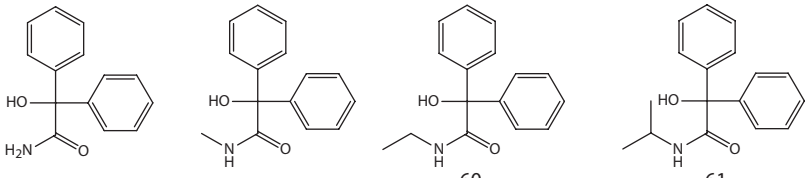


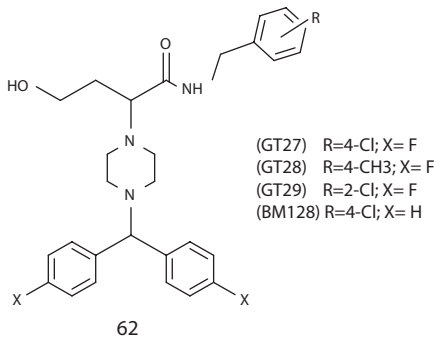
57

Albert *et al.* synthesized benzilic amide (**58**) and a series of its *N*-substituted derivatives and were tested for aniticonvulsant activities against MES and PTZ tests. The parent compound (**58**) and its methyl (**59**), ethyl (**60**), and isopropyl (**61**) analogs had marked activity in protecting against MES. Compounds **58**, **60**, and **61** also demonstrated similar activity against PTZ-induced convulsions. **58** was the most potent against both

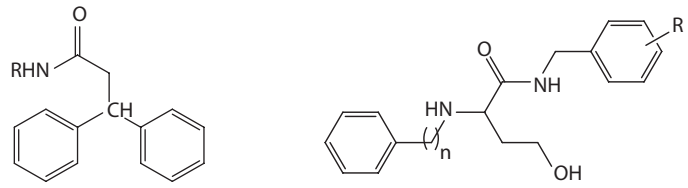
types of seizures, while **60** and **61**, due to their lower neurotoxicities, had optimal protective indices when measured against both convulsions [47].



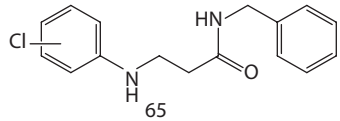
Salat *et al*. synthesized and investigated the anticonvulsant activity of four γ-hydroxybutyric acid amide derivatives (GT27, GT28, GT29, and BM128 (**62**). GT27, GT28, and BM128 at 100 mg/kg, increased the electroconvulsive threshold by approximately 4–11 mA as compared to the control mice [48].



Semwal synthesized a series of β,β-diphenyl propionic acid amides (**63**) and evaluated for their anticonvulsant activity against MES test. These compounds possessed significant level of anticonvulsants activity [49]. Malawska and Tabor synthesized a series of N-substituted amides of α-arylalkylamine-γ-hydroxybutyric acid (**64**) and evaluated for their anticonvulsant activity. These compounds possessed good anticonvulsant activity [50].

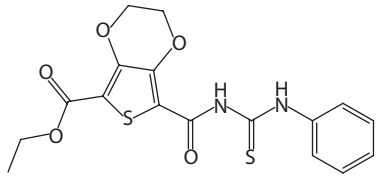


Idris *et al*. synthesized a series of isomeric *N*-benzyl-3-[(chlorophenyl) amino] propanamides (**65**) against MES and *sc*PTZ seizure test models. The isomer of *N*-benzyl-3-[(chlorophenyl)amino] propanamide were found to be active both in the MES and *sc*PTZ tests. The *ortho* and *para* isomers were found to be more potent than the standard drug phenytoin in the MES test, while all the 3 isomeric benzylated products were found to be far more potent than valproate in both the MES and the *sc*PTZ tests [51].

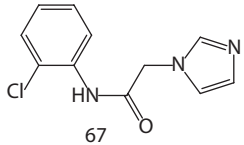


65

Kulandasamy *et al*. synthesized five new series of 3,4-ethylenedioxythiophene derivatives carrying important pharamacophores, *viz*., amide, ester, ether, and active secondary aryl moieties and evaluated for their anticonvulsant activity by using different models, *viz*., MES, *sc*MET, and 6 Hz screen and evaluated for their neurotoxicity in rotarod model. Compound **66** emerged as lead with no neurotoxicity [52]. Akturk *et al*. synthesized 5 ω-(1*H*-imidazol-1-yl)-*N*-phenylacetamide, propionamide, and butyramide derivatives having methoxy, methyl, nitro, and chloro in *ortho* position of *N*-phenyl ring or without any substituent and their anticonvulsant activity was determined against MES test. The most active compound in the series was 2-(1*H*-imidazol-1-yl)-*N*-(*o*-chlorophenyl)acetamide (**67**) [53].

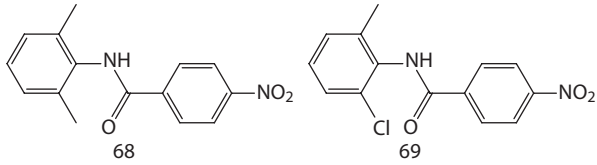


66

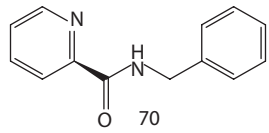


67

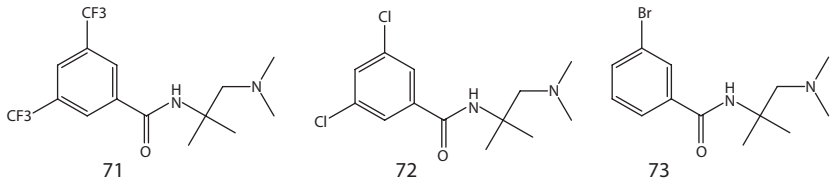
Bailleux *et al*. synthesized a series of 4-nitro-*N*-phenylbenzamides and evaluated for anticonvulsant activity and neurotoxicity study. Two out of four 4-nitro-*N*-phenylbenzamides were effective in the MES test, especially *N*-(2,6-dimethylphenyl)-4-nitrobenzamide (**68**) (ED_{50} = 31.8 μmol/kg, TD_{50} = 166.9 μmol/kg, PI = 5.2) and *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide (**69**) (ED_{50} = 90.3 μmol/kg, TD_{50} = 1.068 μmol/kg, PI = 11.8) [54].



Paruszewski *et al*. synthesized a series of benzylamides of *N*-alkylated, *N*-acylated, or free nine cyclic and one linear amino acids as potential anticonvulsants. Anticonvulsant activity of the compounds were examined in different seizure models. The respective ED_{50} and PI values of the compound **70** were as follows: against bicuculline, 73 and 1.4; against strychnine, 73 and 1.4; against PTZ, 47 and 2.2; against pilocarpine, 156 and 0.7; against AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), 10 and 10.3, against kainic acid (2-carboxy-4-isopropenyl-3-pyrrolidineacetic acid), 39 and 2.6; and against NMDA (*N*-methyl-D-aspartic acid), 114 and 0.9 [55].

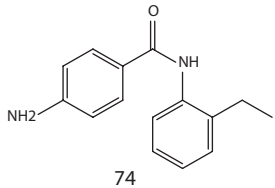


Mussoi *et al.* synthesized a series of benzamides containing *N,N*,2-trimethyl-1,2-propane diamine. The compounds were evaluated in the MES and *sc*MET screens for anticonvulsant activity. The 3,5-trifluoromethyl (**71**), 3,5-dichloro (**72**), and 3-bromo (**73**) derivatives were either equipotent or more effective than phenytoin [56].



Diouf *et al*. synthesized 4-amino-*N*-(2-ethylphenyl)benzamide (4-AEPB) (**74**) and evaluated for their anticonvulsant properties against MES and *sc*PTZ-induced seizures. This compound was found effective against MES but ineffective against *sc*PTZ-induced seizures [57]. Chan *et al*. synthesized a series of *N*-(tetrahydroisoquinolinyl)-2-methoxybenzamides and evaluated for their anticonvulsant properties. SAR studies have provided compound **75** with high affinity and good

anticonvulsant activity in animal models [58]. Ahsan and Amir *et al.* synthesized a series of *N*-(substituted phenyl) pyrrolidine-2-carboxamide derivatives and were evaluated for their anticonvulsant activity by using MES test in mice. Most of the compounds were found to be active in MES tests without any neurotoxicity. Compounds **76** and **77** were found the most active of the series without any neurotoxicity and less CNS depressant effect as compared to standard drug carbamazepine [59]. Pratap *et al*. designed and synthesized a series of new hybrid benzothiazole containing pyridazinones derivatives. *In silico* and *in vitro* studies revealed that some of these hybrid derivatives demonstrated excellent GABA aminotransferase (GABA AT) inhibitory activity. An attempt has also been made to validate the results of *in vitro* GABA AT inhibition of the most potent compound **78** (IC_{50} 9.10 μM) through *in vivo* anticonvulsant screening. Compound **78** administration significantly increases the whole brain GABA level, might be through the inhibition of GABA AT enzyme [60].



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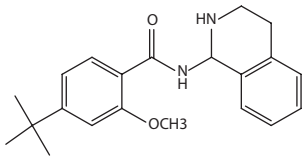

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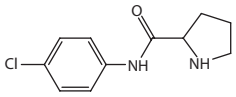

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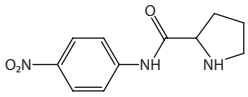

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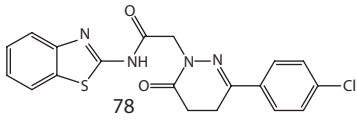
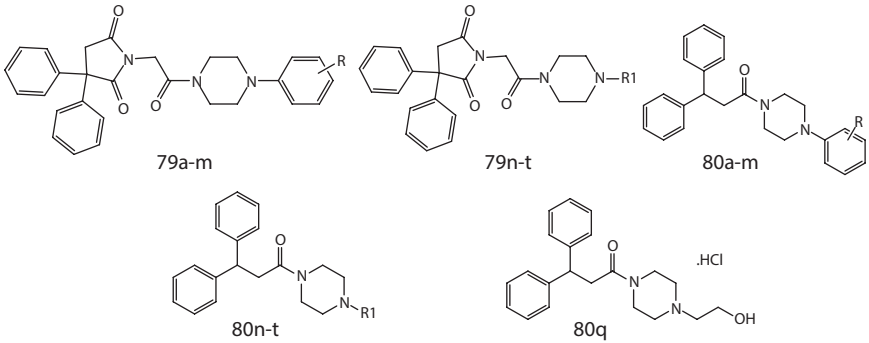


78

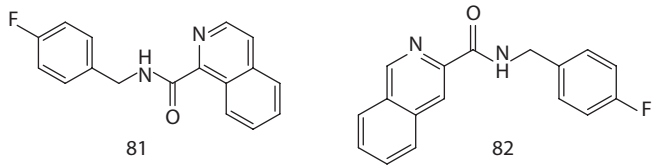
Obniska *et al*. synthesized new amides derived from 3,3-diphenyl-2,5-dioxo-pyrrolidin-1-yl-acetic acid (**79a–t**) and 3,3-diphenyl-propionic acid (**80a–t**) as potential anticonvulsant agents. The initial anticonvulsant activity was performed in mice intraperitoneally using the MES and *sc*PTZ seizure models, whereas the acute neurological toxicity was determined using the rotarod test. Furthermore, several compounds were studied also in the 6-Hz seizures recognized as the animal model of human pharmacoresistant epilepsy. In this series, compound **80q** displayed an extensive spectrum of activity across the preclinical seizure models (ED_{50} MES = 31.64 mg/kg; ED_{50} *sc*PTZ = 75.41 mg/kg; ED_{50} 6 Hz (32 mA) = 38.15 mg/kg). Consequently, compound **80q** revealed a wider variety of protection, higher activity, or/and a better safety profile than the commonly used antiepileptic drugs

such as phenytoin, valproic acid, ethosuximide, or/and levetiracetam. Other substances were active predominantly in the chemically induced seizures [17].



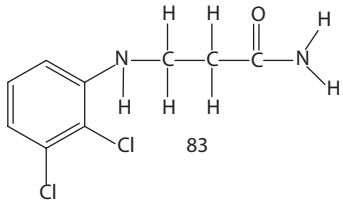
Where R= H, 2-F, 4-F, 2-Cl, 3-Cl, 4-Cl, 2,3-Cl, 3,4-Cl,
3-CF_3, 3-CH_3, 2-CH_3, 3-OCH_3, 2-OCH_3
R1= Benzyl, 2-pyrimidinyl, cyclohexyl, 4-methyl, 2-hydroxyethyl

Strupinska *et al.* synthesized a series of amides of isoquinoline-3- and isoquinoline-1-carboxylic acids and evaluated qualitatively for their anticonvulsant activity in the MES test, 6-Hz test and *sc*PTZ test and neurotoxicity was determined in the rotarod test. Among them, compounds **81** and **82** provided seizure protection in 6 Hz-test in mice at a dose of 300 mg/kg. On this basis, compound 1 was further tested quantitatively in 6Hz-test in mice after *i.p.* administration and showed activity ED_{50} = 385.69 mg/kg and TD_{50} > 600 mg/kg [61].



Malami *et al.* studied the anticonvulsant properties of three synthesized isomers of dichloro-substituted phenyl amino propanamides in mice and rats and determined their effects on votage-gated sodium channels ($Na_V1.6$) stably expressed in human embryonic kidney (HEK Cells 293). Each isomer was evaluated for anticonvulsant effects using MES and PTZ-induced seizure models in mice; tested against PTZ-induced kindling in rats and its synergistic effect with fluenamic acid in mice. Effects of **83** and **84** were studied on voltage-gated sodium channels at different

states of the channel, using electrophysiology techniques. The test compounds generally offered dose-dependent protection against MES and PTZ-induced seizure; confirmed synergistic effect when co-administered with flufenamic acid; and produced significant ($p < 0.05$) decrease in seizure progression in PTZ-kindled rats. In addition, **83** and **84** reduced sodium currents at different channel states in a concentration-dependent manner [62].



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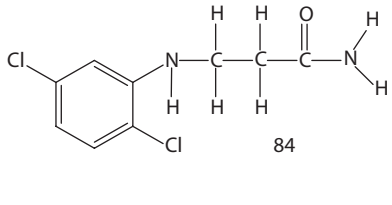

84

11.3 Conclusion

Amide is a unique pharmacophore that is associated with several biological activities. The article has outlined the anticonvulsant activities of the amide pharmacophore. The high degree of protection against seizures can be a positive sign for further investigation of amide derivatives as anticonvulsants. Further investigation of this pharmacophore could give some more encouraging results.

Acknowledgments

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12

Nitric Oxide, Carbon Monoxide, and Hydrogen Sulfide as Biologically Important Signaling Molecules With the Significance of Their Respective Donors in Ophthalmic Diseases

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Abstract

Nitric oxide (NO) along with carbon monoxide (CO) and hydrogen sulfide (H_2S) are biologically significant gaseous molecules generally called as "gasotransmitters". At a concentration higher or lower than optimum value may result in toxicity or malfunctioning of mammalian tissues. Soon after the acknowledgment of NO as multifunctional bio-signaling molecule in 1987, many interesting implications of this field emerged out. Meanwhile, several studies have proven the NO-biosynthetic pathway responsible for normal functioning of eye. High intraocular pressure (IOP) has been suggested as the main risk factor in this context, and collaborative approach with NO releasers is said to control IOP and hence the relation with glaucoma. Similar miracles were reflected from several other naturally produced gaseous molecules, *viz*., CO and H_2S after year 1990. The biological roles of both these molecules are now widely accepted and in the current era investigations focused mainly with development of efficient CO and H_2S releasing compounds. CO and H_2S donors are also said to help in normalising IOP like NO. Therefore, the trio-gasotransmitters have collective relation with the ophthalmic homeostasis in association with nervous control. On one hand, the antimicrobial

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efficiency of these three molecules is widely known, and on the other hand, their collaborative key role in ocular nerve functioning makes it remarkable to state here that their donors are supposed to act as a shield for both the infectious as well as the non-infectious eye defects.

***Keywords*:** Gasotransmitters, ophthalmic diseases, NO, CO, H_2S, NORMs, CORMs, H_2S-donors

12.1 Introduction

The scientific recognition of carbon monoxide (CO) and hydrogen sulfide (H_2S) as bio-conjugated molecules sharing similar functional role as nitric oxide (NO) resulted in coining the term "gasotransmitters" for these molecules based on size, lipophilic character, half-life, and several other features [1, 2]. Even though these gases share a number of common features, they also possess dissimilar characteristics and display noteworthy interactions, which complicate the interpretation of their physiological activities.

In the late 1990s, the scientific community saw a very unusual phenomenon, the conversion of NO from harmful gases into an important chemical messenger. The remarkable role of this molecule in signal transduction and cytotoxicity is considered to be one of the greatest marvels of biological chemistry in recent times. The biological diversity of this molecule is well documented in neuroscience, physiology, and immunology [3, 4]. Recommendations obtained from the chemical stress of NO included the "Molecule of the Year" vote in 1992 by the journal "Science", published by the American Association for the Advancement of Science (AAAS) [5]. At present, NO has been accepted to be linked to many physiological mechanisms including platelet aggregation and adhesion, neurotransmission, synaptic plasticity, vascular permeability, hepatic metabolism, senescence, and renal function [6–9]. In high concentration (μM), NO also plays a strategic role in the immune system [10] and in suppression of carcinogenic state [11, 12]. The prominence in the absence of biology and medicine was emphasized in 1998 when the Nobel Prize for Physiology and Medicine was awarded to Robert Furchgott, Louis Ignarro, and Ferid Murad for their role in transforming the role of NO in the nervous, cardiovascular and physiological systems. In microbial world, NO plays mediator role in denitrification [13–15] (Figure 12.1). In addition, the molecule is tailored in various ways due to its important therapeutic potential [16, 17].

$$NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$$

Figure 12.1 Denitrification involving NO as intermediate.

CO has long been known as a dangerous gas for mammals and is called as a "silent killer" [18]. CO, when inhaled, enters the bloodstream and forms carboxyhemoglobin (COHb) at a rate 240 times greater than oxygen [19]. This reduces the oxygen transport ability and results in hypoxia [20]. Biologically, CO is considered as a by-product of heme oxygenase (HO) metabolism [21], and in the early stage of its biological exploration, CO was found as a chronic neurotransmitting agent [22]. Therefore, the further studies have altered the general perception of CO as a harmful molecule [23]. CO has now become an important molecule in the physical monitoring of many organ systems. In the last few decades, investigations related to CO have shown this gaseous molecule as a major chemical messenger.

In the seventeenth century, Carl Wilhelm Scheele recognized H_2S through chemical analysis. However, it has long been speculated that the gas is derived from the sewage system and is linked to a series of special eye diseases that occur in sewage workers. The disease is associated with severe inflammation, secondary bacterial attacks, and even blindness [24]. Similar to NO and CO, internally generated H_2S is now considered a significant gasotransmitter [25] and within a neuromodulator, which draws a lot of attention in literature. Traditional neurotransmitters bind to and activate membrane receptors, whereas gasotransmitters are able to freely distribute to adjacent cells and directly bind to their target proteins to supplement biological functions by contributing to their short-term mutations [26].

Like NO which S-nitrosylates a variety of proteins, H_2S physically regulates the different protein functions by S-sulfhydration. However, S-nitrosylation inhibits enzymes, while S-sulfhydration stimulates them. Therefore, H_2S is an important physiologic gasotransmitter such as NO and CO [27–30].

The eye is one of the most sensitive parts of the brain. Any impairment in eye function requires high quality care. Among eye health problems intraocular pressure (IOP), cataract and retinal hypertension continue to remain as potential risk factors in treatment. Due to our growing interest in the synthesis of various chemicals tagged with NO, CO, and H_2S [31–39] and the many biological actions of H_2S and the more precise delivery of this flexible gas to target tissue in the form of H_2S sponsors [40], the current chapter focuses on in the applied interest of NO, CO, and H_2S compounds

on eye physiology. A historical view of the emergence of the term "gasotransmitter", within the production of NO, CO, and H_2S in mammals and to sought strong sponsors of NORMS, CORMS and H_2S-releasers (in the event of chronic biosynthesis and digestion) applicable in the most common eye defects are the main objectives of this literature update.

12.2 A Meaningful Introduction to Gasotransmitters

In general, gasotransmitters refer to the distinctive class of molecules like NO, CO, and H_2S, responsible for communication amongst body cells for a particular biological action. Albeit, these molecules exist in solvated form while in biological medium, the respective differences in size, action, shape and bio-membrane interactions stems their multitude biological roles reported so far. The signal transduction pathway among such carriers may range from short to long distances to transmit the required information [41]. The properties and functional diversity found in these bioessential signaling molecules, therefore gave rise to coin a new term in reference to their biological relevance as "gasotransmitters".

Several parameters may be found differentiating neurotransmitters from gasotransmitters. From the cellular biology it is clear that neurotransmitters stored in vesicles get released by the intervention of a suitable stimulus (Figure 12.2, top). These responses are receptor-specific in nature and depend on the molecular signaling to bring forth a physiological move. Hence, synaptic vesicles behave as a reservoir of information required at the time of safety or normal physiological functioning. Whereas, gasotransmitters are endogenously availed small molecules of signaling potentiality ("gaso" refers to their gaseous nature under normal conditions) [41, 42]. Gasotransmitters have the main characteristic feature of diffusing through cellular membrane without the aid of any receptor (Figure 12.2, bottom). No reservoir is required (like vesicles in neurotransmitters) but is rapidly produced in response to a stimulus when needed [1, 2]. Moreover, herein, gasotransmitters cell exocytosis fashion followed in neurotransmitters is not pronounced at all. Therefore, a separate term "gasotransmitter" coined by Wang in 2002 is suitable to distinguish them from neurotransmitters [25]. The vasorelaxant and gasotransmitter labeling of NO enhanced scientific vigor to an extraordinary fashion and activated to seek for other molecules of this class [25, 43, 44]. Gases other than these are also under interrogation to add further possible members to this group.

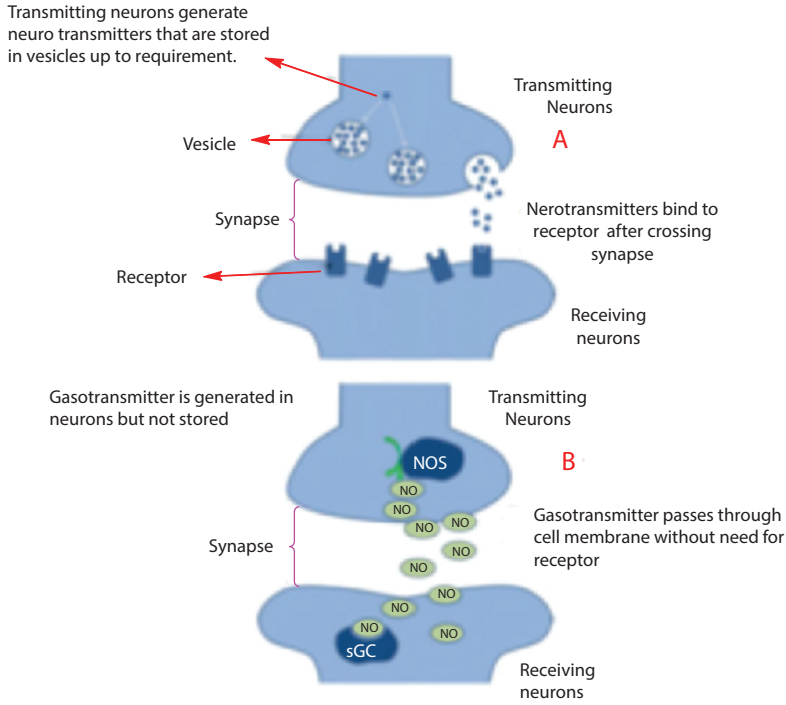


Figure 12.2 Diagram showing the mechanism of neurotransmitter (A, top) and gasotransmitter (B, bottom) action.

12.3 Biosynthesis and Target of NO, CO, and H_2S

12.3.1 Biological Synthesis and Target of NO

NO is biologically synthesized by the catalytic action (oxidation of the nitrogen colored red in Figure 12.3) of nitric oxide synthase (NOS) over L-arginine as substrate and resulting in the formation of L-citrulline [45] as shown in Figure 12.3. Since the NO production is involved in every system of a human body, there are three distinctive gene products and isoforms of NOS, producing NO in the presence of oxygen, flavins and NADPH [46]. Figures 12.4(i) and (ii) represent these three members as NOS-I, NOS-II, and NOS-II. These are also called as eNOS (endothelial), iNOS (inducible), and nNOS (neuronal), respectively, because of the specific target/production locus during their biosynthesis. NOS-I is central and peripheral nervous system linked [47], NOS-III is vaso-relaxation connected [46], and NOS-II is immunological directed. From the literature, it is found that NOS-II functions independent of the presence of calcium

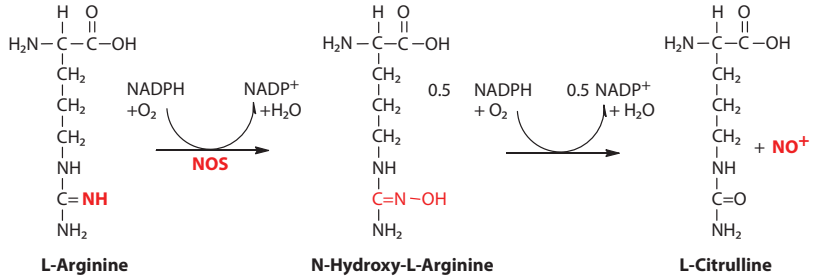


Figure 12.3 Reaction showing the biosynthesis of nitric oxide.

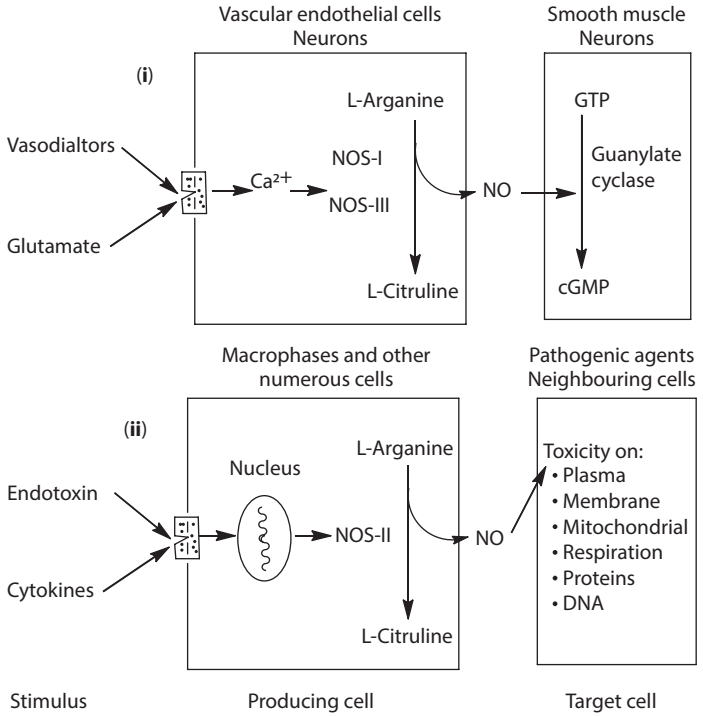


Figure 12.4 Interaction of three NOS isoforms.

and is a source of considerable amount of NO produced for a longer duration as compared to NOS-I and NOS-III [48]. Several examples L-arginine structural analogues have been found to prevent NOS from producing NO, *viz.*, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (LNA), N^G-nitro-L-arginine methyl ester (L-NAME), etc. [49]. However, by making instant and sufficient arginine availability, the action could get reversed

initially. The NO pathway has also been studied in the eye, and the inducible and constitutive isoforms have been documented by researchers [50–66].

12.3.2 Biological Production and Target of CO

As per the metabolic pathways concerned with the CO-biosynthesis, almost 14% of 500 μmol/day is obtained from lipid peroxidation and from photooxidation plus self-activation of cytochrome p-450. Bacteria and Xenobiotics also contribute the same minor percentage [67, 68]. Major contribution (almost 86%) is generated by the erythrocyte-breakdown, wherein the HO catalyzes this oxidation. Like NOS, HO also exists in two isoforms, *viz.*, HO-1 and HO-2. These are also called as inducible and constitutive, respectively. Both the isoforms show same rate-limiting step while catabolizing heme, the difference lies with the regulation, amino acid sequence, and distribution in the tissues. Another HO has been recently identified and named as HO-3. This form of HO was detected in the several organs of rats. Till date, no heme-degradation study has been reported for this newly detected HO member [69]. The metabolic pathway of HO-catalyzed heme oxidation involves several important stages as has been illustrated in Figure 12.5. In addition to CO, other intermediatory products like of α-meso-hydroxyheme, verdoheme, and biliverdin (converts to bilirubin as excretory product conjugated by glucoronic acid shown in Figure 12.6) are also involved [70, 71]. The bioaction of HO-1 under stressful situation gets enhanced and the CO-production gets increased than the optimal value [72]. Therefore, such an elevation in the concentration can be used as a sign convention medically to read the associated behavior. The similar correlation has been found in several diseases wherein a patient is expected to suffer from stress and strain conditions. For instance in bronchiectasis, asthma, cystic fibrosis, hyperglycemia, and other diseases CO level appears higher than the normal [73]. Hence, the detection level of CO because of inducible HO-1 can help in diagnosis of pathophysiological state.

CO in mammals has been found to show target specific action in two ways, *viz.*, soluble guanylyl cyclase (sGC) pathway and non-cGMP pathways. It is well-established fact that NO binds with heme by replacing one histidine unit to result in the activation of sGC [74]. Generally, NO binds to heme b to form a unstable short lived six-coordinate system to finally cleave the heme-His105 bond and forms a five coordinated heme complex with NO (Figure 12.7). Therefore, it is conformational change in other words that leads to sGC activation [75–78]. The resulting sGC catalyzes the conversion of GTP to cGMP in the fashion as given in Scheme 12.1. The

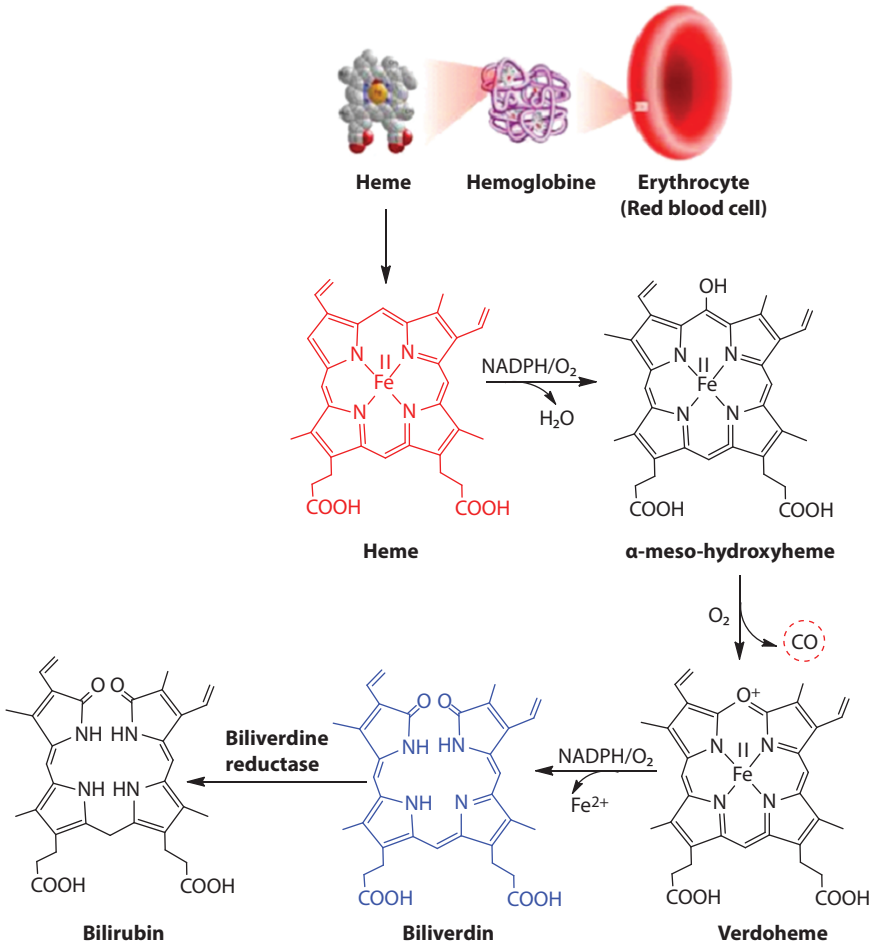


Figure 12.5 Oxidation of heme by heme oxygenase (HO) forming CO as a by-product.

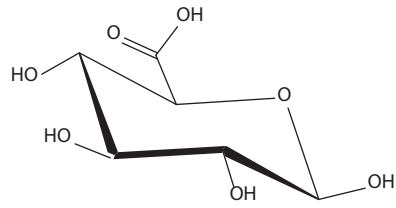


Figure 12.6 Chemical structure of glucuronic acid.

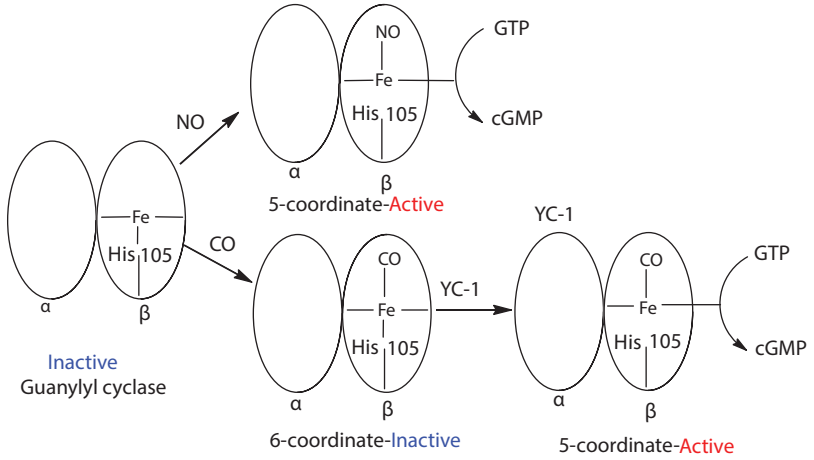
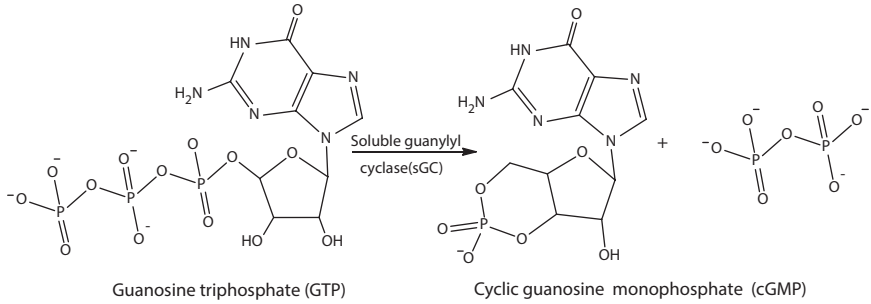


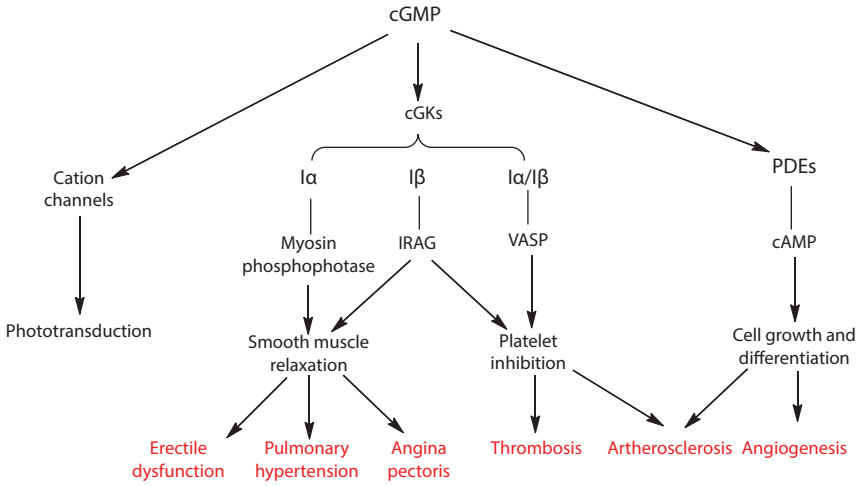
Figure 12.7 Diagram showing the activation of guanylyl cyclase by NO and CO/YC-1 (Taken from Ref. [79] E. Martin, K. Czarnecki, V. Jayaraman, F. Murad and J. Kincaid, *J. Am. Chem. Soc*., 127(2005) 4625–4631).



Scheme 12.1 sGC catalyzing the Conversion of GTP to cGMP.

cGMP formation is related to a number of sequential pathways entailed with several clinical implications as illustrated in Scheme 12.2 (red colored).

Therefore, in an analogous way like NO, this molecule (CO) also yields cGMP by activating guanylyl cyclase. However, it may be mentioned here that this activation is only 1/80th effective as NO. Some trials have been found to use synthetic compounds like YC-1 (Figure 12.8), when used in combination with CO can increase this effectiveness up to the level of NO. This indicates that there should be some naturally existing signaling molecule like YC-1 that shows the guanyl cyclase activation similar to NO. There are contrary observations reported for CO activity in the same activation study. CO while coordinating with guanylyl cyclase results in



Scheme 12.2 Cyclic guanosine monophosphate (cGMP) routes ameliorating medical conditions shown as red. cGKs, cGMP-dependent protein kinases, Iα and Iβ; IRAG, Inositol 1,4,5-triphosphate(IP_3)receptor-associated cGKIβ substrate; VASP, vasodilator-stimulated phosphoprotein; PDEs, phosphodiesterases; cAMP, cyclic adenosine monophosphate [Adopted from Ref. Brian E. Mann and Roberto Motterlini, Chem. Commun., (2007) 4197–4208].

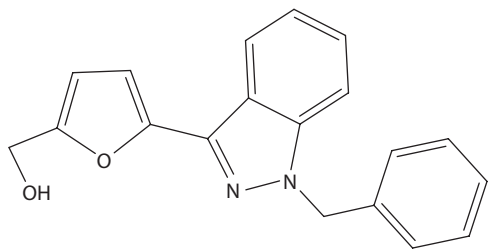


Figure 12.8 Structure of YC-1:1-Benzyl-3-(5′-hydroxymethyl-2furyl)indazole.

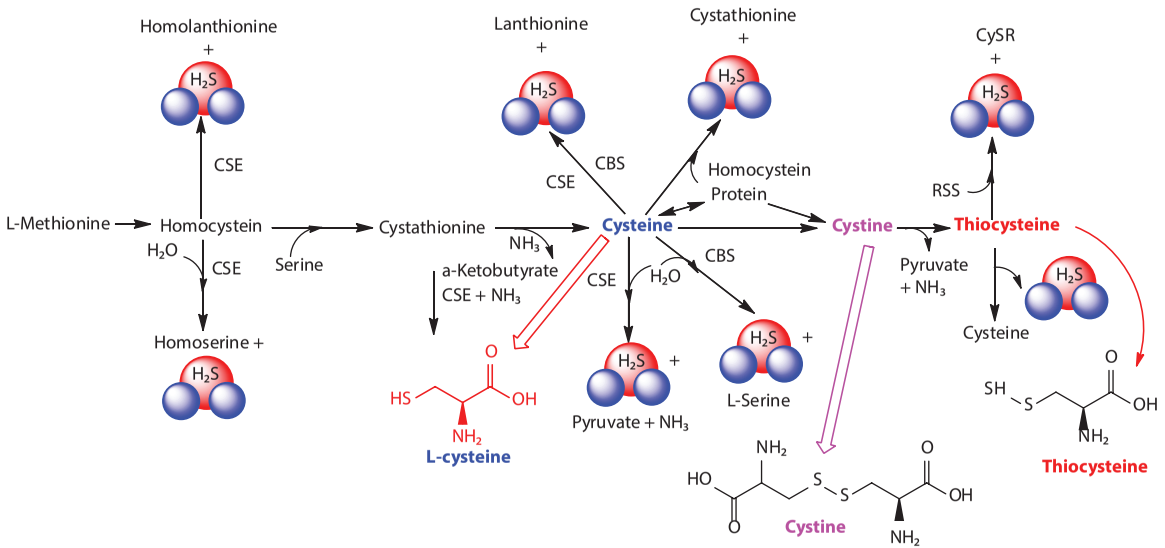
six-coordinated iron complex and central metal ion continues to remain as Fe^{II}. The retention of five-coordinated inability of CO with iron is due to the increased radii of the ion and decrease in the electronegativity. This shows CO-based vasorelaxation goes through a no similar pathway as in NO. On the other hand, considering YC-1 interaction with the displacement of His-105 produces a 5-coordinate complex with retaining CO as co-ligand [79] (Figure 12.8), there is another interpretation for the CO-dependent stimulation of sGC [80].

Non-cGMP CO-pathway is remarkably in other targets to lead vasorelaxation, incorporating the involvement of potassium channels (BK_{Ca}). The conductance BK_{Ca} channels are found distributed almost in every tissue and get affected by several modulators. Among contributing factors toward such channels, protein kinases, endogenous NO and CO along with heme are mentionable. Many physiological processes including neuronal excitability, contractility of muscles, and vascular tone maintenance are because of BK_{Ca} activity by involving the suitable yield of action potential [81].

12.3.3 Biosynthesis and Target Sites of H_2S

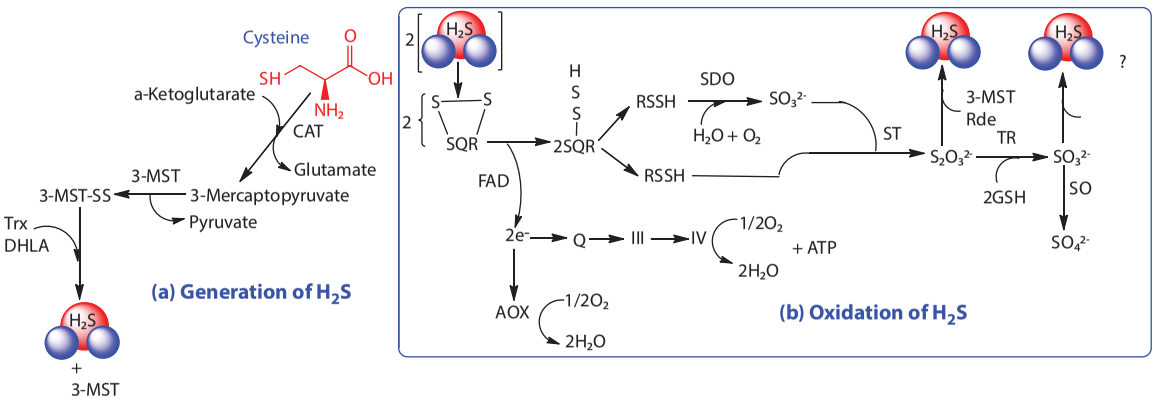
H_2S is considered as the recently recognized third member among gasotransmitters. This gas is also produced endogenously through enzymatic reactions (Figure 12.9) and is responsible for maintaining physiological balance. Recently, non-enzymatic pathways have also been reported to be responsible for biosynthesis of H_2S. The optimal concentration of H_2S under normal conditions is said to be present in micromoles (μM) [82]. L-cysteine is the important bioessential source of this gas involving the role of cystathionine β-synthase (CBS), cytosolic/pyridoxal-5′-phosphate (P5P)-dependent enzymes, and cystathionine γ-lyase (CSE) [83, 84] or the tandem enzymes, cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulphur transferase (3-MST), mainly confining the activity in mitochondria. Absolute H_2S is produced from CSE and CBS, whereas 3-mercaptopyruvate transfers sulphur to cysteine through the catalytic application of 3-MST resulting in the formation of persulfide [85, 86]. Thereafter, persulfide acts as a H_2S-releaser endogenously causing the reduction of disulfides, e.g., reduction of dihydrolipoic acid (DHLA) or thioredoxin (Trx) under physiological terms [87]. Strictly, therefore, it is thioredoxin reductase (TR) acting sequence wise in combination with glutathione (GSH) and sulfur oxidase (SO) in this phenomenon. The significance of H_2S (oxidized form) in respiratory chain is well documented acting as electron donor in Q, III, and IV steps of the chain that results in the generation of energy currency (ATP) and cellular oxygen consumption. Hence, biosynthesis and H_2S-mediated sensing of oxygen represent essential role of H_2S-biochemistry. CBS and CSE, the two H_2S-producing enzymes, are known to be present in ocular tissues (retina) [88–90]. 3MST and CAT being located in the retinal neurons, these pathways are the prominent way of H_2S production in mammalian retina [91–96].

The biological synthesis of H_2S as in the case of NO and CO must be followed by the consumption or target phenomenon. This gas has no color, flammable, and smells like that of rotten eggs. Its acid strength is weak



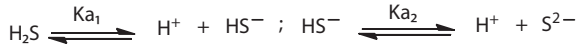
(i) Production of H_2S in tissues via cystosolic enzymes CBS and CSE

Figure 12.9 Biosynthesis of H_2S and its oxidation [Adopted from Ref. K. R. Olsona, J. A. Donald, R. A. Dombkowski and S. F. Perry, *Respiratory Physiology & Neurobiology*, 184 (2012) 117–129]. (*Continued*)



(ii) (a) Generation of H_2S via tandem enzymes, CAT, and (3-MST) and (b)

Figure 12.9 (Continued) Biosynthesis of H_2S and its oxidation [Adopted from Ref. K. R. Olsona, J. A. Donald, R. A. Dombkowski and S. F. Perry, *Respiratory Physiology & Neurobiology*, 184 (2012) 117–129].



Scheme 12.3 Reactions showing the dissociation of H_2S in aqueous solution.

(pKa value of 6.98 at 25°C and 6.76 at 37°C). The dissociation of this gas in water may be represented as shown in Scheme 12.3 ($Ka_1 = 1.3 \times 10^{-7}$ M, $Ka_2 = 1 \times 10^{-19}$) [39]. The H_2S that remains undissociated is volatile, while the dissociated form HS^- is not volatile. In physiological medium, these dissociation patterns are pH-dependant. At the 7.4 pH, one-third of H_2S remains undissociated. Physiological pH does not support the substantial presence of S^{2-} (because high pH is required for it). As per another scientific observation, H_2S mainly persists in HS^- form (82%) because of having weak acid strength (pKa_1: 6.76; pKa_2: 19.6) [97].

Therefore, both the H_2S as well as SH^- are contributory under biological activity of H_2S, despite the fact that SH^- represents more nucleophilic potential than cysteine (Cys) or reduced form of glutathione (GSH), that swiftly coordinates with bio-metallic centers or interacts with other compounds [98]. In mitochondrial chain reactions, H_2S displays sequential oxidation trend. Initially, it gets oxidized to thiosulfate, followed by conversion to sulfite and subsequently to sulfate. The first oxidation stage is non-enzymatic in nature, while the rest steps are carried out enzymatically using thiosulfate cyanide sulfurtransferase (TST). Despite the observation showing sulfate as major end product of the metabolic pathways followed by H_2S, urinary thiosulfate represents a non-specific indicator to sense the quantity of H_2S production within a body [99]. Overall, the target of H_2S is highly influenced by several factors especially the rapid oxidation disfavoring the long-distance transport. Therefore, the development of efficient storage system endogenously applicable is suggested. For instance, in NO-association, H_2S gets stored as nitrosothiols (RS-NO) implying the dual gas-combinatory fashion. This opens several areas of interest to seek answer for the unexplored queries regarding the isolated and combined gasotransmitters research. Meanwhile, the solvated fashion of H_2S reveals different solubility trend based on the nature of the solvent. Due to lipophilic feature of this gas, H_2S easily crosses cell membrane. The tested solubility experiments have shown it to be five times more soluble in lipophilic solvent as compared to water. The data furnished from its solubility behavior in various solvents other than water have been keenly recorded [100, 101]. The solution chemistry on further exploration depicts that concentration H_2S under varied physiological abnormalities within mammalian blood. Harmful implications of H_2S at the level of <100 ppm gets expressed in the form sore throat, eye irritation, dizziness, etc. [102–104].

The exposure at greater than 1,000 ppm affects CNS, respiratory chain, and may more even cause death [104].

12.4 Gasotransmitters in the Mission of Vision (Eye-Health Contribution)

Eye is one of the most important sense organs performing the function of vision through interacting with light, involving several physicochemical phenomena to memorize the surroundings and therefore acts as a natural perception mediator to translate the observations to the brain. So, ultimately light-phenomenon to nerve actions, so many tissues collaborate to let such a complex process to happen. The physiology of eye is not restricted to a simple conduction process only but is subjected to the role of the gasotransmitters introduced *vide supra*. This section details the role of NO, CO, and H_2S in maintaining a healthy eye, so is the title established as "the aim of gasotransmitters in the mission of vision".

12.4.1 NO News is Good News for Eyes: NO Donors for the Treatment of Eye Diseases

Since the dawn of NO-recognition as a key signaling molecule, the diversified biological role of this free radical met with its extended role in so many areas of physiological investigations. Keeping in view the combinatory functional status of NO and cGMP are entailed with a range of biological actions, there are numerous evidences supporting the fact that the NO-metabolic pathways are also involved in the normal functioning of an eye. The respective neurological role served as a motivational move for the researchers to find the possible responsiveness while studying eye functioning. These responsible roles include dynamics of aqueous humor (AqH) dynamics, retinal neurotransmission, and other light induced pathways. Any malfunctioning that results in the respective NO generation can cause eye abnormality [48]. As of now, the normal tissue functioning of eye in concern with eNOS and nNOS-based role has got wide acceptance [105]. This is because neural and immunologic expressive forms of NOS have been found in retina. Several reports are evidential in supporting nNOS responsible in photoreception via NO generation, and similar effect in bipolar cells. This, in turn, leads to stimulus for guanylate cyclase (GC) photosensitive rod cells and thereby increasing the calcium channel activation. By stopping NOS action in the retina of cats, results indicate impairment in photo-transduction [106]. Moreover, iNOS has also been found

responsible for keeping normal phagocytosis in the outer section of retina. Also, the role that NO plays in maintaining circulation of retina and links the molecule with ophthalmologic role.

The optimal concentration as discussed in the biosynthesis is always mainly eyed to confirm the normal functioning of any tissue. At the concentrations, other than optimum value results in so many eye diseases [105]. In case of low NO-concentration (eNOS or nNOS abnormal functioning) substances that could act as NO donors could be administered. On the other side, iNOS as pointed in the above sections is only intervening in pathological conditions expressive in terms of several cytokines (interleukin-1, interleukin-6, etc), inflammation, and endotoxins. Once initiated, iNOS continues to produce sufficient NO followed by conversion phenomenon free radicals, nitrogen dioxide, or nitrites as defensive way against pathogens. In hyperactivity of iNOS in several disorders like cataracts, age-related macular degeneration (AMD), myopia, and uveitis, iNOS inhibition could be suggested.

As it is widely known that blood pressure plays important role in keeping normal eye-sight, and on the other hand, NO is also considered as blood pressure regulator. Under such a stemming fact, some recent studies have found effective mutual relation of NO with hypertension based cataracts. The increase in lens nitrite (as NO metabolite) is suggested as one of the key regulator for to increase oxidative stress of lenticular part and hypertensive cataract formation [107]. Thus, NO involvement in retinal action continues to be of significant interest [108]. Let us specify this role by illustrating the role of NO in eye defects as elaborated in below figures (Figures 12.10 to 12.13) [Adopted from L. K. Wareham1, E. S. Buys and R. M. Sappington, The Nitric Oxide-Guanylate Cyclase Pathway and Glaucoma, *Nitric Oxide*, 77(2018) 75–87, Ref. 111].

The clinical investigations reveal that glaucoma is associated with increased IOP. In case of open-angle glaucoma (OAG), the finding of high IOP suggests imbalance between AqH generation and outflow. It is estimated that more than 60 million people suffer from primary OAG (POAG) at the world level, showing a possible graphic projection of about 79 million by the end of 2020 and more than hundred million by 2040 [109, 110]. The general form of glaucoma is indicative of high IOP and hence is known as ocular hypertension. From the available data, it is clear that 1/3rd of glaucomatous patients (vision loss) show normotensive IOP (normotensive glaucoma; NTG) and this disease have major impact of age factor, i.e., increases with age, with impendent of IOP. This shows that this mechanistic approach is not the sole explanation of the cause of this defect [111]. Thus, considering reduction in IOP is not the whole treatment for this disease. Hence, a number of evidences support

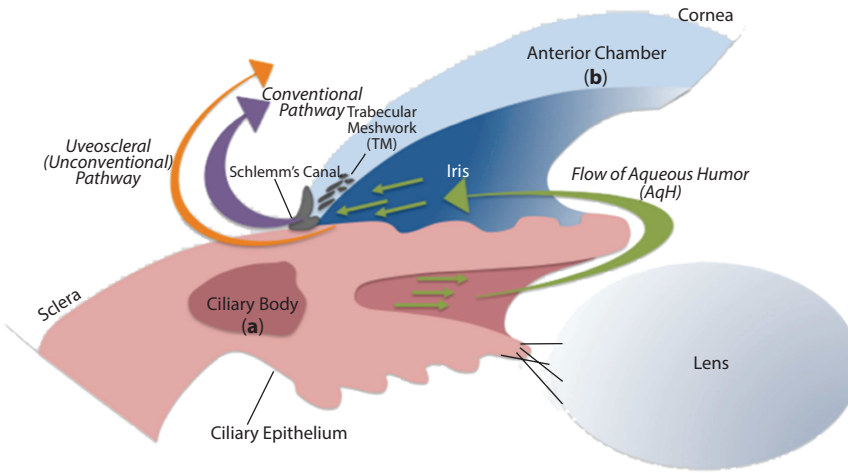


Figure 12.10 Diagrammatic representation of aqueous humor (AqH) equality. (a) AqH at the ciliary body in the eye flows (**green arrows**) through two routes independently that control AqH dynamics: (i) via the trabecular meshwork (TM) and Schlemm's canal (**purple arrow**) (conventional route) and (ii) via the uveoscleral tract (**orange arrow**) (non-conventional route). (b) The balance of (AqH) production at the ciliary body and elimination in the anterior chamber establishes intraocular pressure (IOP) in the eye.

NO as an efficient regulator of this type of hypertension in association with GC. NO as a therapeutic option for this treatment has shown positive results in decreasing IOP and stabilizes ocular blood pressure and confer neuroprotection. Therefore, current therapeutics considers both IOP-dependent and IOP-independent target mechanisms of the disease [112–114].

12.4.1.1 Nitric Oxide Releasing Molecules (NORMS) and the IOP

Several NORMS have been tested in animal models including mice, monkey, and rabbit to record the impact of these NO donors on IOP. In case of a normotensive rabbit animal model, the application of nitroglycerin, sodium nitroprusside (SNP), isosorbide dinitrate (ISDN), and sodium nitrite (Figure 12.14) showed a suitable decrease in IOP effective for 1 to 2 h. The concentration dependent analysis showed that SNP and nitroglycerin or glyceryl trinitrate (GTN) are active at lowering the IOP till 0.1% and 0.03%, respectively, is maintained, and on the other hand, doses higher than 0.1% and 0.03% of the two NO donors were found ineffective [115]. Similarly, other studies reported by Kotikoski *et al.* [116] in normotensive rabbits using SNP, spermine NONOate, and S-nitrosothiol (Figure 12.15), applied topically or intravitreal way showed similar effect of lowering IOP

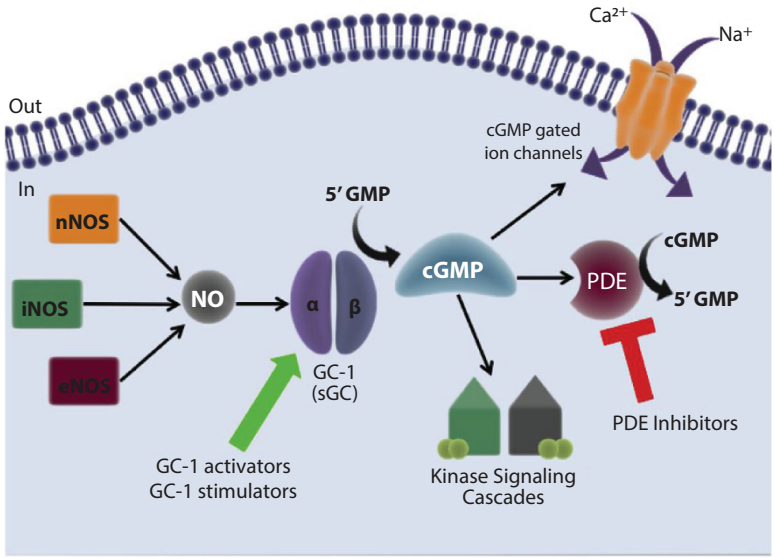


Figure 12.11 The NO-GC-1-cGMP pathway, steps in the way of IOP lowering. (a) NO is generated from L-arginine by nitric oxide synthase (NOS) available in three isoforms: (i) neuronal NOS-I (nNOS), (ii) endothelial NOS-III (eNOS), and (iii) inducible NOS-II (iNOS). (b) NO binds guanylate cyclase-1 (GC-1), a heterodimeric protein capable of converting guanosine 5′-monophosphate (5′GMP) to cyclic guanosine monophosphate (cGMP). The cGMP so produced can target cGMP-gated ion channels, and activate kinase signaling cascades. (c) Phosphodiesterase enzymes (PDEs) bind to cGMP and catalyze the decomposition of cGMP into 5'GMP. PDEs act as important regulators of signal transduction mediated by cGMP. (d) The cGMP bioavailability in the cell can be increased in two ways: (i) by the use of GC-1 stimulators and activators, which increase production of cGMP, or (ii) by the use of PDE inhibitors that prevent the decomposition of cGMP in the cell.

for 2- to 5-h duration. Behar-Cohen's group also reported the similar type of investigation using 3-morpholinosydnonimine (SIN-1) or S-nitro-N-acetylpenicillamine (SNAP) (Figure 12.16), and the results indicated a swift fall in IOP [117].

The studies reported by Sugiyama *et al.* [118] showing the hypotensive outcome of compounds shown in Figure 12.17 encompassing both the NO-releasing and NO-sequestering sensitivity. Kimura *et al.* [119] found that SNP and nipradilol reduce IOP, but latanoprost (Figure 12.18) was found not so effective IOP. However, the combinatory drug application of latanoprost with SNP or nipradilol showed considerable reduction in IOP than SNP or nipradilol when used separately. This proposes the use of synergistic effective compound like latanoprost for well pronounced IOP lowering results.

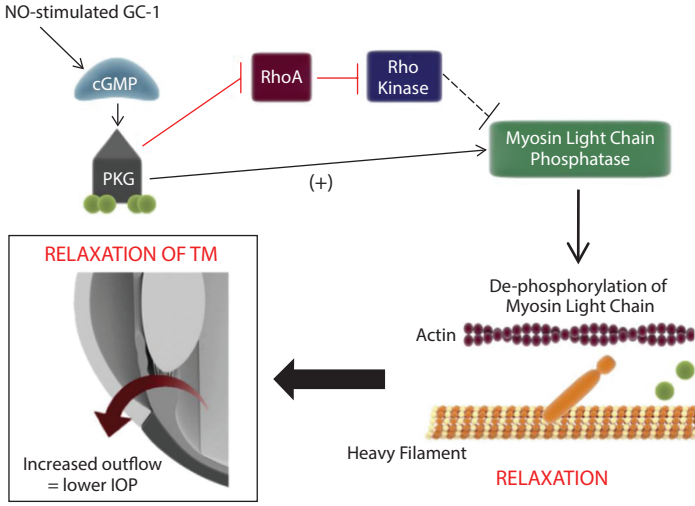


Figure 12.12 cGMP-assisted modification of IOP via increase in aqueous humor (AqH) outflow via three routes. (a) Nitric oxide activates generation of cGMP by GC-1. The cGMP activates protein kinase G (PKG). The PKG so activated can phosphorylate numerous targets with various downstream effects, including inhibition of Ras homolog family member A (RhoA). This prevents inhibition of myosin phosphatase by Rho Kinase. (b) Besides inhibition of RhoA, activated PKG can directly trigger myosin light chain phosphatase (MLCP). Thereafter, dephosphorylation of the regulatory light chain of myosin by MLCP prevents actin–myosin interaction, promoting cell relaxation. (c) This then leads to a widening of the intercellular spaces in the juxtacanalicular trabecular meshwork (TM) and Schlemm's canal. This facilitates conventional AqH outflow and thereby lowering IOP.

Non-arteritic anterior ischemic optic neuropathy (NAION), a common eye problem generally found middle-aged group (though no age group is safe) is linked with phosphodiesterase (PDE) inhibitors (such as Sildenafil) presumably due to hypotensive effect and vasorelaxation [120]. Hence, sildenafil (a well-known NORM) finds the application in lowering the blood pressure [121]. Several recent reports describe the use of erectile dysfunction (ED) drugs (Figure 12.19) questioning these drugs as responsible agents for NAION. Many factors have been elaborated to set this belief of contribution toward NAION. Therefore, among warning factors such possibilities of side effects must be highlighted [122]. As the same vision defects have been found among patients after sildenafil consumption [123]. It is established that PDE 5 (phosphodiesterase in the corpus cavernosum) along gets inhibited by using the ED drugs, escaping degradation of 3′-5′-cyclic guanosine monophosphate (cGMP) to guanosine 5′-monophosphate (5′GMP). The NO linkage with guanylyl cyclase

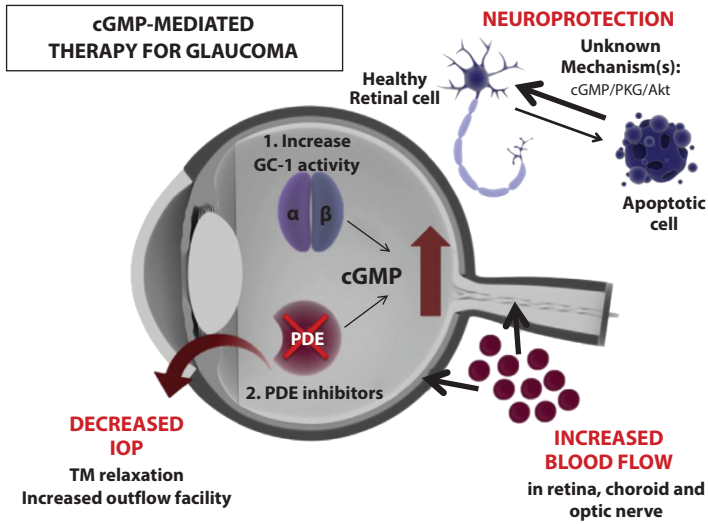


Figure 12.13 GC-1-directed therapy for glaucoma is pleiotropic in its action. Increased levels of cGMP are shown to have pleiotropic targets that are beneficial in the treatment of glaucoma in three ways. These are (a) relaxation of the trabecular meshwork to increase AqH outflow facility, which leads to lowering in IOP, (b) increasing blood flow to the retina, choroid and optic nerve head, (c) prevention of degeneration of retinal ganglion cells through mechanisms that may involve downstream kinase pathways. As shown in Figure 12.11, the cGMP concentrations in the eye can be increased in two ways: (i) by the use of GC-1 stimulators and activators, which aim to increase production of cGMP, or (ii) by the use of PDE inhibitors which prevent the decomposition of cGMP into 5'GMPin the cell to increase its bioavailability.

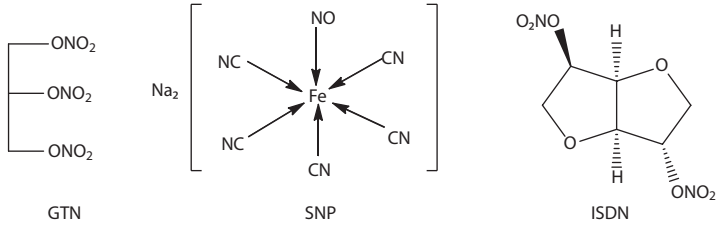


Figure 12.14 Structure of some nitric oxide donors.

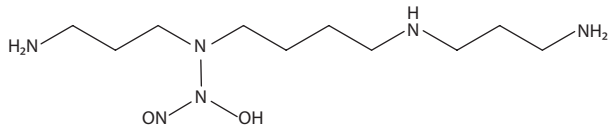


Figure 12.15 Chemical structure of spermine NONOate.

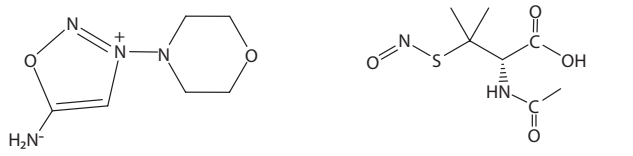


Figure 12.16 Chemical structure of SIN-1 and SNAP.

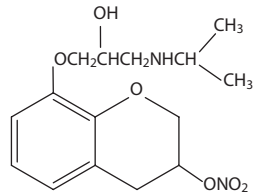


Figure 12.17 Chemical structure of nipradiol.

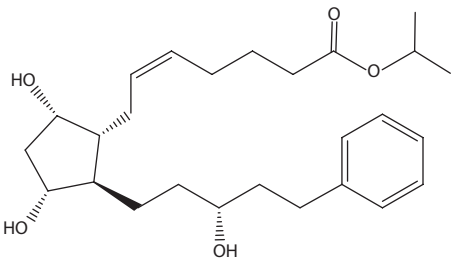


Figure 12.18 Chemical structure of latanoprost.

creates conformational modification in this enzyme, followed by catalytic cGMP generation from guanosine 5′-triphosphate (GTP), stimulating penis toward erection as has been displayed in Figure 12.20.

Another familiar example of NO-donor usable in lowering IOP is NO-bonded Latanoprost acid (LA) called as Latanoprostene bunod (LBN) and is generally referred for topical treatment, and its action of releasing NO is prostaglandin equivalent. The role of this compound in outflow of AqH has been described in Figure 12.10 and the mechanism of NO-release is given in Figure 12.21.

12.4.2 Carbon Monoxide, CORMS, and the Ocular System

Glaucoma as discussed earlier is an optic neuropathy and is considered as the major cause of eye defects in advanced countries [125–133].

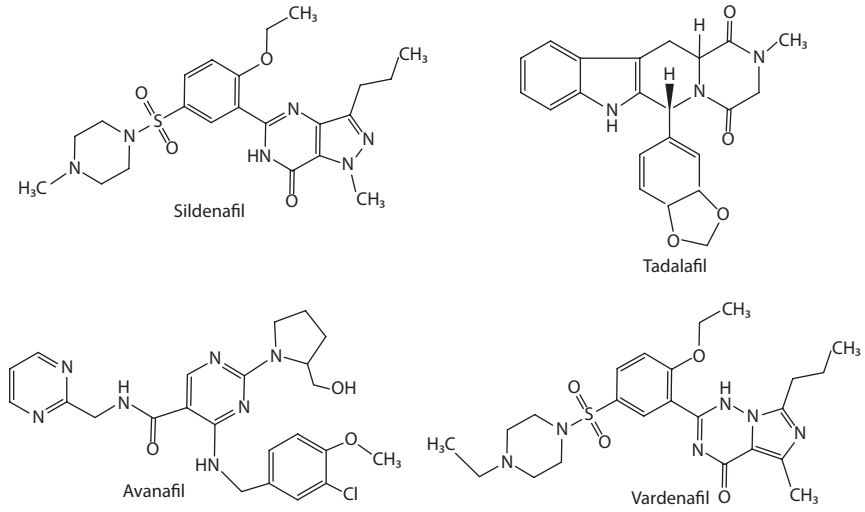


Figure 12.19 Structure of sildenafil and other similar ED drugs.

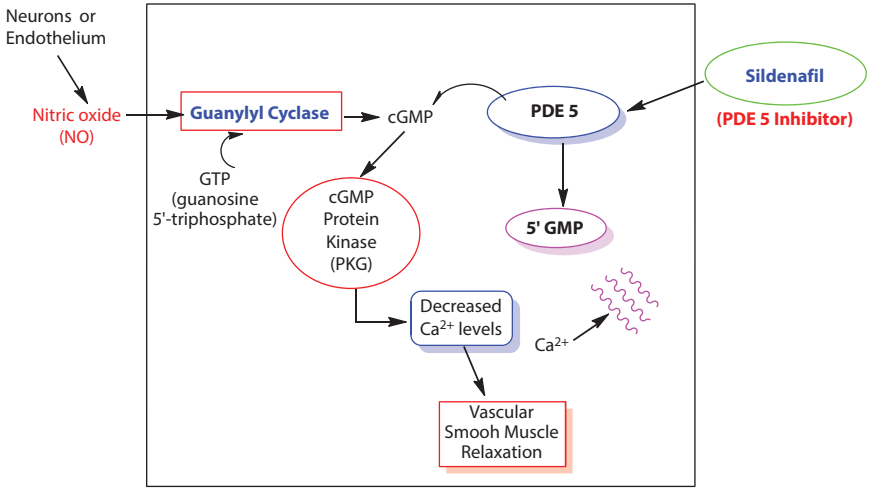


Figure 12.20 NO-cGMP routes for relaxation of arterial and trabecular smooth muscle.

A sequential treatment plan has been devised by the "European Glaucoma Society" suggesting the reduction of IOP as the first step, followed by medically supervised laser surgery of neural network called as "the trabecular meshwork" (TM) and filtering surgery of galucoma. As the main threat for glaucoma is elevated IOP, hence is the first target to be corrected in the treatment plan [134]. Meanwhile, CO is also expected to play a role in

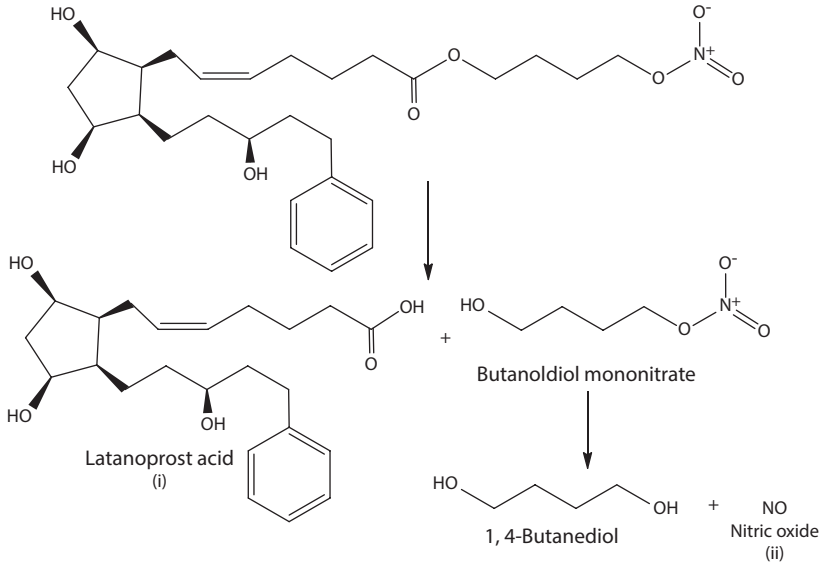


Figure 12.21 The release of nitric oxide from LBN [Adopted from Ref. 124; *J. Ocul. Pharma. and Therap*., 34(2018) 52–60].

lowering IOP like NO. Although very less literature reports are available justifying the use of CORMS in this context. However, some of the directions imposed for this view have been enlightened in the following.

Bucolo and Drago have recently updated that CO can furnish significant results of multiscale applications in treating eye impairments especially glaucoma [131]. CORM-3 as shown in Figure 12.22 is a famous CO releaser when studied by Stagni *et al*. To find the role of CO in treating ocular system defects found that the compound resulted in lowering IOP in the rabbit animal models they selected for the experiment [135]. The drug potency in the respective tests indicated that after 24 h of the consumption the IOP-lowering effect was seen for 30 min. Ingestion 1% dose was seen maximal 6 h duration.

From the results obtained by CO-based IOP-lowering, it is expected that the action is because of sGC enhancement. CO-dependent sGC activation

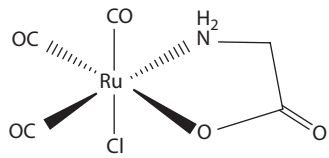


Figure 12.22 Chemical structure of CORM-3.

of sGC by CORM-3 imparts an increase in the outflow of AqH as given in Figure 12.23, linking the pathways, TM with Schlemm's canal. It is expected that CO exhibits this action by reducing the volume of TM cell [136].

The yearly rate of incidence of uveitis (a sight-threatening inflammatory disease of the eye) at the age between 20 and 60 years for both males and females is estimated to be with a frequency of 38–714 per 100,000 persons [137]. CORM-A1 is an example of CO-releasing compounds tested for its effect on uveoretinitis and is the first example of water soluble CO-releaser. Figure 12.24 and Scheme 12.4 may be referred for

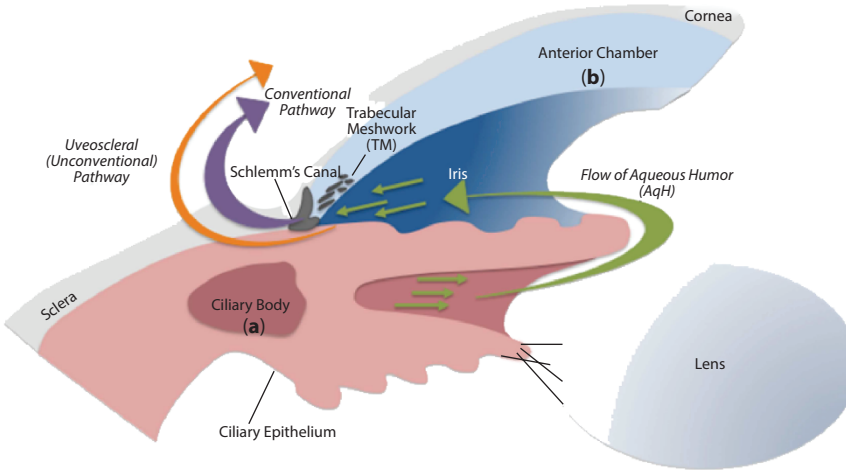


Figure 12.23 Diagram displaying the production and flow of aqueous humor (AqH). (a) AqH produced at the ciliary body in the eye flows (**green arrows**) through two routes independently that control AqH dynamics: (i) via the trabecular meshwork (TM) and Schlemm's canal (**purple arrow**) (conventional route) and (ii) via the uveoscleral tract (**orange arrow**) (non-conventional route). (b) The balance of (AqH) production at the ciliary body and elimination in the anterior chamber establishes intraocular pressure (IOP) in the eye.

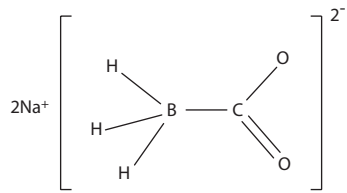
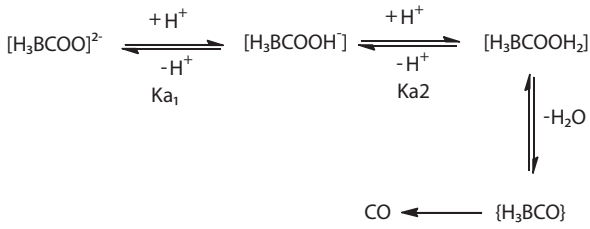


Figure 12.24 Chemical structure of CORM-A1.



Scheme 12.4 Mechanism of CO release from CORM-A1.

knowing the structural details and CO-releasing process. Nicoletti *et al.* [138] showed that CORM-A1is helpful in autoimmune responsive in uveoretinitis.

12.4.3 Hydrogen Sulfide and Ophthalmic Diseases

Being third latest member of gasotransmitters after NO and CO, H_2S has also been reported to exhibit numerous roles in maintaining normal physiological conditions [139]. The generation of H_2S as discussed in its biosynthesis section mainly involves the catalytic intervention of CBS, 3MST, CSE, and CAT. The same enzymatic actions have been found operational in all mammalian eyeballs confined to several locations. Any irregularity in such a distribution results in eye defects. So, many investigations have been reported detailing H_2S donors as IOP regulating compounds, retinal cell protection, antioxidative stress, and ocular protein modulation. Thus, H_2S donors represent promising drugs applicable in treating manifold ophthalmic diseases as discussed in the following.

Among different factors responsible for ocular defects as is known IOP is the main reason for glaucoma neuropathy [140], and finally, it is stability between AqH of ciliary body and outflow AqH that matters [141]. By facilitating cyclic adenosine monophosphate (cAMP) the outflow could be enhanced [142]. By allowing H_2S donors like L-Cysteine and sodium hydrosulfide (NaHS) to act on adenylyl cyclase and ATP-sensitive potassium channels (K_{ATP}) therefore could increase cAMP concentrations and could make the outflow of AqH easy [143]. Similar investigation conducted by Módis *et al.* reveals that H_2S inhibits phosphodiesterase (PDE) and enriches intra mitochondrial cAMP levels and results in the excitation of protein kinase A (PKA) to infuse bioenergetic consequences [144]. Similarly, another compound GYY4137 (Figure 12.25) has furnished positive results in stabilizing IOP [145, 146].

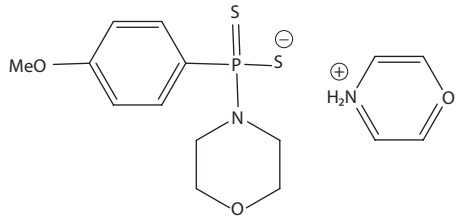


Figure 12.25 Chemical structure of GYY4137.

12.5 Concluding Remarks and Future Outlook

Gasotransmitters are, therefore, outstanding molecules having significant biological signaling role. Considering the fact that the scientific world is eager to design and develop molecular scaffolds in this context to be declared as medical or clinical relevant, so many questions are underway to be resolved. Half-life period, solubility, chemical environment effects, pH, thermodynamics, and kinetics all are among the queries being investigated in this field. The ocular diseases and the factors responsible for such impairments do contain mechanistic pathways half answered in relevance with gasotransmitters. Drug delivery challenges, transportation, combinatory implications of drugs, optoelectronic effects, etc., need to be explored in a more deepened way. Moreover, could synthetic chemists bring forth a molecular system of synergetic effect in a view to declare molecular designs having potentiality of releasing more than one "gasotransmitter" molecules?

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13

Influence of *rol* Genes for Enhanced Biosynthesis of Potent Natural Products

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Abstract

Owing to the low abundance of natural products accounting for less than 1% of total carbon, various strategies are adopted to enhance their concentration. A powerful mean of enhancing the productivity of important secondary metabolites is the genetic transformation especially by *Agrobacterium tumefacienes* and *Agrobacterium rhizogenes*. Secondary metabolites biosynthesis has been made possible by directly modifying the expression of genes using a technology named recombinant DNA technology. It is now possible to synthesize plant secondary compounds by directly modifying the pathways that lead to their synthesis. In light of several studies, it has been proven that in many plants' families' *rol* genes tend to be powerful inducers of secondary metabolism. These *rol* genes seem to have a potent effect on the morphology of plants as well as enhance the production of various plants secondary metabolites. The *rol* genes are the plant oncogenes, naturally present on the plasmids of the plant pathogen *Agrobacterium rhizogenes*. After infecting the plant with the *Agrobacterium*, these genes are transmitted into the plant genetic material resulting in formation of tumors and development of hairy root disease. These genes have remarkable effect on secondary metabolite production.

***Keywords*:** *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *rol* genes, natural products, secondary metabolites

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13.1 Introduction

The chemical substances that are isolated from living organisms are known as natural products [1]. In plants, a wide and diverse range of organic compounds are produced and masses of them do not have role in the process of development and growth of that particular plant. The term secondary metabolites or natural products that mostly used for such compounds and their distribution is different within taxonomic groups of plant kingdom. On contrarily, the primary metabolites are the substances that have role in direct growth and development of plant and play very important role in metabolism as well and are considered essential and are usually evident [2]. They can be found in stem, leaves, bark of plant, and root depending on the type of metabolite that particular plant is producing. Secondary metabolites that are considered to be most bioactive are phenolic acid, tannins, flavonoid, and alkaloids. The studies of these compounds were introduced by organic chemists of 19th and 20th centuries who were interested in these substances because of their importance as medicinal drugs, flavor, industrial material, and poison [3]. About 5% to 15% plant species have been analyzed so far among the known plant species [4].

Natural products are being used by human since the dawn of time as antidotes of health disorders and to cure different ailments having several other uses, as narcotics, dyes, and poison for warfare and hunting. Mostly, the crude forms of the important metabolites were used until 19th century when isolation of pure compounds started. Some of those natural products are still in use today, i.e., morphine and quinine. Morphine was isolated first time in 1803 from *Papaver somniferum* and *Papaver setigru*, having analgesics and narcotic effects [5]. Quinine is another example with antimalarial effects, isolated from Cinchona tree [6].

The secondary metabolites in past have been given very little attention and were considered the waste products of plant primary metabolism. Kossel was the first one who distinguished these compounds from those of primary ones, which were later named as secondary metabolites. These compounds have low abundance usually less than 1% of the total carbon [7]. They have significance in protection against herbivore and microbial infection: they are also important allelopathic agents and behave as attractants for pollinators and seed-dispersing animals. Besides this, these compounds have great utility as dyes, fibres, waxes, polymers, flavoring agents, and drugs [8].

Different strategies are in use for improving plant secondary metabolites but it is possible just because of recombinant DNA technology to alter the expression of biosynthetic genes and to manipulate metabolic pathways [7]. In literature, it was found that *rol* genes have their active role as inducer of secondary metabolism in several plants [9]. *rol* genes are plant oncogenes, which are cancer-inducing genes, most often mutated and expressed at high levels in tumor cells. A plant pathogen *Agrobacterium rhizogenes* carry these genes in plasmid. These *rol* genes can be transferred into plants genome where they can cause tumor and hairy root disease. Expression of *rol*A gene encodes protein that binds with DNA and triggers growth of plant, whereas that of the *rol*B gene has its role in the auxin signal transduction pathway regulation [10] and has effective role as inducer of secondary metabolism of plant. In *Vitis amurensis*, production of resveratrol increased and also production of anthraquinones increased in *Rubia cardifolia* [12]. The *rol*C gene encodes for cytokinin glucosidase and thus stimulates the production of many secondary compounds in variety of plants [13–17].

Out of these *rol* genes, the *rol*B and *rol*C genes are mostly studied for so many years as growth regulators. They also play vital role in cell differentiation. In addition, these are chief activators of plants secondary metabolism in transformed cells of many plant families [9]. Classically, these two genes *rol*B and *rol*C are considered to be closely related genes as they have same biological function. Sometimes, they often have different or opposite effect on death process of cell, balance of calcium in transformed cells [9], sensitivity to auxin, growth of transformed tissues [18], and secondary metabolism [12].

13.2 Secondary Metabolites or Natural Products

Plants secondary metabolite is a generic term that is employed for more than 30,000 different substances that are produced by plants and their importance has been discovered by the scientists [2]. Many of these secondary metabolites have shown important significance in protection against herbivores, attractants for pollinators, microbial infection, animals that help in seed dispersal, and as allelopathic agents that affect the competition among many plant species. All the mentioned ecological functions affect the existence of plant [19].

The ability of plants to survive and compete is therefore affected by the secondary metabolites of the plants. Secondary metabolites of plants are

also related to agriculture. The reproductive fitness of plant increases by the defensive component of plant that act by warding off bacteria, fungus and herbivores may also have role in making them undesirable food for humans. Many important plant crops have been chosen by artificial selection to produces low level of these substances (which of course make them more susceptible to insects and diseases).

13.2.1 Classes of Natural Products (Secondary Metabolites)

In nature plants are sessile organism so for their survival they need to protect themselves against exogenous biotic and abiotic factors, by adopting specific mechanisms of synthesizing the secondary metabolites which have important ecological and physiological effects. Plant secondary metabolites are categorized into three important categories

1. Terpenoids (including plant volatiles, glycosides, carotenoids, and sterols)
2. Phenolic compounds (includes phenolic acid, lignin, tannins, flavonoids, stilbenes, and coumarins)
3. Nitrogen-containing compounds (glucosinolates and alkaloids) [20].

These phytochemical compounds can be antimicrobial and act as attractant/repellant and detergent against many herbivores. The synthesis of such compounds can also be seen in undifferentiated plants as observed in laboratory conditions and they can be further induced with elicitors or by feeding precursor [21].

13.2.1.1 Terpenoids

The most diverse and largest class of natural product is terpenoids. There is variety in structure of these compounds ranging from linear to polycyclic molecules and size range from five-carbon hemiterpenes to natural rubber that consist of thousands of isoterpene units. Condensation of isoprene unit is a way to synthesize all the terpenes and is classified on the basis of presence of number of five carbon unit in their core structure. Most of flavor and aromatic compounds such as geraniol, linalool, menthol, and caryophyllene are consisting of monoterpenes that have two isoprene units and sesquiterpenes having three isoprene units. While bioactive

compounds other than these such as di, tri, and tetraterpenes have very special properties [22].

13.2.1.2 Phenolic Compounds

Distribution of phenolic compounds is very extensive in nature. The chemical structure of these compounds varies greatly including simple phenol such as hydrobenzoic acid derivatives and catechol as well as long-chain polymers that have high molecular weight like condensed tannins. Pharmacological and biological activities are presented by flavonoids and stilbenes because of their intermediate molecular weight. Flavanols, isoflavaoinds, and many others are composed of many multiple branches that originate from chalcone [23]. Using CC technique, phenolic compounds have been fractionated into aromatic, medicinal, and food plants. Different techniques such as toy pearl chromatography column and many more are being used for the process of fractionation [24, 25].

13.2.1.3 Alkaloids

Alkaloids are compounds that are synthesized by living organism and contain one or more nitrogen atoms in the heterocyclic ring that are derived from amino acids and are pharmacologically active compounds. The term alkaloid basically shows fact that all of these compounds are alkaline in nature. Among many groups of secondary metabolites, alkaloid is largest group that contains 12,000 isolated substances. The characteristic of this group is that it consists of immense variety among structural formulas that come from various biosynthetic pathways and presents distinctive pharmacological activities.

13.2.2 Strategies to Enhance Natural Products

Geographical location and environmental variations affect the production of uniform quality and quantity of plant secondary metabolites. In order to achieve increased production and extraction of plant secondary metabolites, various biotechnological approaches are applied because conventional methods are not cost-effective and sometimes lead to the extinction of some large plants. Biological techniques such as cell tissue culture have been considered a solution [26]. One of the ways of producing plant secondary metabolite such as flavors, food additives, colorants, fragrances, pharmaceutical, and agricultural can be produced by plant cell culture [27].

Numbers of strategies are present in plants that result in increased production of plant secondary metabolites. Obtaining fast-growing cell lines and metabolite of interest production.

1) Immobilization of cells to increase the production of metabolites.
2) Use of elicitors to increase productivity in a short time.
3) Permeability of membrane to facilitate diffusion of the metabolite.
4) Adsorption of inhibitory metabolites by feedback.
5) Scale-up of cell culture bioreactors.
6) Genetic engineering technique for enhanced production of specific metabolites.

Some of the strategies are described in the following.

13.2.2.1 Plant Cell Culture (Somaclonal Variation)

Somaclonal variation is a technique that is done in order to generate genetic modification in *in vitro* cultures that displayed as inheritable mutations in regenerated plantlets [28]. It is the term used globally for all variants of cell and tissue culture [29] and considered as one of the major problem in the regeneration of plants that are grown *in vitro*. Theoretically, it may be stated that plant cell growth in *in vitro*, i.e., via a sexual process, does not cause any mutation and involves only mitotic cell division [30]. Despite that, these mutational effects can be utilized in crop improvement by creating novel plant varieties which exhibit characteristics that increase their commercial value as disease-resistant, increased production of secondary metabolites, and improved biomass yield [31].

13.2.2.2 Genetic Transformation of Plant Cell

The basic principle of genetic transformation is to introduce and integrate the DNA in nuclear genome, chloroplast, and mitochondrial. The process of genetic transformation is classified as 1) direct transfer of DNA into the plant cells or tissues by using techniques such as imbibition, microinjection, electroporation, or biolistic and 2) indirect gene transfer use the soil organism named *Agrobacterium tumefaciens* for the purpose of foreign DNA transfer into plant genomic material [32]. Some of the ways to increase the production of plant secondary metabolite using genetic transformation are given as follows:

1. Increase the expression of precursors that form secondary metabolites.
2. Increased gene expression limited by the metabolic pathway of interest.
3. Fabrication of new metabolic pathways using the existing ones.
4. Inhibit competitive routes or catalytic steps of the metabolite of interest on the route using antisense DNA or interference RNA.
5. To handle regulatory genes that function as an activator or repressor of transcription.
6. To select mutant types for production of secondary metabolites.

13.2.2.3 Multiple Gene Transfer Through Improving Vectors

The transformation that is done by using multiple genes at the same time allows researchers to produce proteins of interest by studying and manipulating the entire metabolic pathway as well as production of plant secondary metabolites. However, there are several barriers to this process since first plant transformation methods were developed for introduction of only one or two genes. Due to this, as the more number of genes are introduced they lower the probability that all of them are integrated and expressed [33].

To solve the problem of genetic transformation by multiple genes, the transformation may be attained by using conventional methods such as sequential processing of the same transgenic line and crossing transgenic lines homozygous. Still, these methods are very time consuming and labor acquiring. Designing special vectors that help in insertion of multiple genes in a single transformation event and co-transformation mechanism allows to cope up with such problems [34].

13.2.3 Genetic Engineering/Metabolic Engineering

Term metabolic engineering is defined as one or more redirection enzymatic processes that involve the production of novel compounds in an organism, improving the production or prevents the degradation of existing compounds [35]. The aim of metabolic engineering is to overproduce specific compounds, but often, the interconnection of metabolic pathways is such that the number of possible ways to attach a substrate to a product

is enormous. For metabolic engineering process, it is necessary that the reactions are thermodynamically favorable [36].

To achieve this goal, molecular biology helps to modify metabolic pathways and use of statistical tools for data analysis and bioinformatics aims to design, analyze, and predict the behavior of metabolic fluxes in the newly developed route. By using these techniques, at least 100,000 secondary metabolites are produced by plants, which are used with medicinal value, fragrances, flavors, and pesticides, among others [37].

Despite mentioned potential usage, the plant extracts from which they obtained are insufficient to meet demand in the market, because they grow slowly or are difficult to grow. In early stages, chemical synthesis have potential to meet the demand of chemicals but the problems associated with this involve the complexity of some molecular structures and also stereochemical requirements of some secondary metabolites preclude the use of this technology [38].

By using the metabolic engineering massive secondary metabolites production with huge added value, in heterologous systems such as microorganisms, cell cultures, and intact plants, plant organs can be increased. The function of the gene transcript level can be determined by transcriptomics, genomics, metabolomics, and proteomics and are establishing novel metabolic pathways in organisms. By using genomic sequencing the code of proteins, enzymes, and transcription factors that are involved in the biosynthetic pathway of major secondary metabolite can also become possible to find out. The genome of 25 floras was published, of which just the genome of *Arabidopsis thaliana* and *Oryza sativa* are almost complete. The model plant's genetic information is to set related physiological processes in plants, such as *Medicago truncatula* to nitrogen fixation in legumes, tomato as a model of development of fruit and rice as a development model for grains [39]. The sequencing of model plants could allow establishing new metabolic pathways and complete existing ones. All these developments provide more effective ways and methods to increase yields of secondary metabolites with having high added value [40].

13.3 *rol* Genes

In the early 20th century, a bacterium named *Agrobacterium rhizogenes* firstly recognized as an important infectious agent because it infected the economically important crops like apples at that time [41]. It is a gram-negative bacterium that is living near plant roots. Extensive studies are done because it takes part in developing a plant disease called "hairy

root syndrome". This disease is mostly associated with numerous dicots and characterized by root overgrowth, i.e., ageotropic branching at plant area that is infected with pathogen. TDNA which is a Ri plasmid fragment is inserted to plant cells. This TDNA then stably fuse with the plant genetic material. This is an example of gene transfer between two kingdoms that is natural and result of interaction between two organisms. Several defense mechanisms operate in plant in response to bacterial invasion, which results in production of defensive proteins that suppress the bacterial growth. As a result of evolution, such mechanisms have evolved in bacterium that helps it to draw benefit of plant defensive proteins and in return get off defense pathways of plants [42]. The neoplastic tissue that is hairy roots of the plant synthesizes opines because of this complex and coordinated mechanism. As far as chemistry of these opines is concerned, they are the derivatives of sugar and amino acids. Plants eliminate these opines to the surrounding environment and do not utilize them instead they are utilized by bacterium as energy source. This condition results in a competition between *Agrobacterium* and other bacteria that are found in same environment. Plants that are revived with hairy roots display characteristic phenotypic traits which include reduction of apical dominance in both roots and stem, short internodes, wider leaves with wrinkled shape, and formation of adventitious roots, and along with such changes, there is alteration of flower morphology with reduced pollen seed [43]. Plants with such symptoms known to show "hairy root phenotype". The genes that are present in plasmid genome give products which are responsible for such transformation [44]. Using techniques like molecular and genetic mutational analysis, White *et al*. in 1985 identified the TDNA genes of bacterium and suggest that these genes are particularly responsible for hairy root syndrome and he named these as *rol* (rooting locus) A, B, C, and D genes. These *rol* genes thus have potential to alter the developmental programme of transformed cell as it takes place in animal tumor cell and led the scientists to call these genes "***rol* oncogenes**" [45].

13.3.1 Origin of *rol* Genes

As compared to bacteria, *rol* gene's functions are more associated with processes of plants that involve physiological and metabolic pathways. Evidence in support of their eukaryotic origin is supported by the fact that these genes have promoter whose structure is particularly that of the eukaryotic genes with reputed expression in plants using RNA polymerase II enzyme. In addition, the introns in *rol*A constitute a remnant eukaryotic feature and it is differently utilized depending on whether the gene is

active in the bacterium or in the plant. As *rol*A gene is rarely transcribed in bacterium, the splicing of intron does not occur; whereas in plants, intron has to be spliced for transcription of *rol* gene to proceed [46]. Thus, it's understood that the eukaryotic features and promoters of the TDNA were derived from plants and not the natural selection has produced *ex novo* eukaryotic DNA sequences in the bacterium plasmid DNA. Keeping in view these facts, it might be possible that TDNA genes are derived from ancient eukaryotic plant genes and cTDNA sequences represent their vestigial remains. As evolution proceeds, horizontal gene transfer of *rol* genes could have happened more than once and perhaps in both directions between two organisms [47]. Ancient transmission of DNA that happened between two organisms suggests that they have been found in proximity of each other. According to one hypothesis, RNA has mediated insertion of genes from plant to bacterial cell. Because of the action of reverse transcriptase that is present in plants after previous viral infection, plants gene transcripts have been inserted into the genome of bacterial cell.

13.3.2 Types of *rol* Genes

Production of transgenic plants by introducing a single *rol* gene or combination of *rol* genes permitted defining the main morphological and physiological alteration that is due to these genes. Pleiotropic changes that are mostly related to physiology, development, and morphology are all the result of the expression of these bacterial genes in transgenic plants [48]. These modifications can ultimately lead to hormonal imbalance in plants, which is considered as the most striking relationship between the function of *rol* gene as they interfere with these plant molecules. Despite the existence of these genes that are involved in hormone synthesis on the TDNA [49], these genes themselves are able to produce various physiological and morphological changes that lead to hormonal disequilibrium mostly the auxin and cytokinin and their association with each other [50]. These genes display effects that depend on distinct species or cultivars of plants and their physiological background. Four types of *rol* gene have been identified, i.e., *rol*A, *rol*B, *rol*C, and *rol*D.

13.3.2.1 The rolA Gene

Gene names "*rol*A" having a reading frame of 300 base pairs encodes a protein that is composed of 100 amino acids and exhibits an 11-kDa mass. This protein might belong to DNA binding proteins and shows similarity in structure with papillomavirus E2 DNA binding domain [51]. Simulatory

effect of gene is found in the production of nicotine [52]. As compared to the control calli, calli that were expressing the *rol*A gene like from *Rubia cordifolia* showed the 2.8-fold higher levels of anthraquinones production. The captivating biotechnological attribute of this gene is found that its expression in *Rubia cordifolia* calli ensured the stable level of anthraquinone and its expression provided efficient conditions for the growth of callus. Stability of this effect is proven by taking careful observation of *rol*A transformed callus line for 7 years and the effect remain the same during this time period [12]. To date, results of hormone quantifications in *rol*A expressing tobacco plants have been inconclusive [53]. The only significant difference detected between *rol*A expressing and wild-type plants was a reduced GA_1 content in the leaves of the *rol*A expressing plants, which could explain the dwarfed phenotype of these plants. These genes found to have a role in dwarfism.

The phenotype of this gene is associated with low levels of gibberellin hormone. The apical shoot of transgenic tobacco plants showed a low level of gibberellic acid as compared to control plants and also observed that growth in such plants is not restored by supplying gibberellic acid externally [50]. The *rol*A-induced transgenic tobacco plants showed phenotype like leaves that were wrinkled having low length to width ratio, overall plants were stunted as a result of shorter internodes and inflorescence pattern that is condensed having flowers that are larger in size but shows delayed onset and reduction of male fertility [54]. Also, the changes like smaller root system and long internodes with leaves that are small and wrinkled and flowers of small size having reduced pollen viability have been observed in some other *rol*A transformed tomato plants [55]. Studies have shown that this atypical wrinkled phenotype of leaves is perhaps a result of unequal growth of the tissues found in leaf blade [56]. It is also found that *rol*A genes does not lead to an improving rooting, and maybe due to this reason, thus far, none of ornamental flowering plant has been transformed with this gene. Hormone regulation and secondary metabolite synthesis is an important factor that is associated with the defense action or response of the pants [57]. The *rol* genes induce changes in phytohormone levels; thus, it might be predicted that they have a significant role in regulation of plant immunity. Evidence that supports this fact that transgenic tomato plants that are transformed with *rol*A gene have an increased tolerance to fungal pathogens with the lower levels of IAA and ABA as compared to plants in control group [58].

13.3.2.2 The rolB Gene

The gene *rol*B having a reading frame of 777 base pairs that encodes a protein comprised of 259 amino acids and exhibit a 30-kDa mass. The plasma

membrane is the major area of the cell where the protein is confined [10] and shows no typical motifs and has no similarity to any of the known protein outside *Agrobacterium*. This gene is evidently found to have a positive role in secondary metabolism of plant whereas acts negatively in cell growth. Strength of the expression of this gene defines its role as the inhibitor of callus growth. Largest effect of *rol*B gene has seen on accumulation of compound anthraquinone as compared to other genes like *rol*A and *rol*C genes. Culture with increase content of *rol*B gene expression shows 15-fold increase in anthraquinones. One important exemplar related to efficacy of transformation with *rol*B has been demonstrated for plant named *vitis amurensis* in which there have seen 100 times elevated levels of resveratrol as a result of transformation [11]. The compound resveratrol classified as chief stilbene whose promising role in prevention of tumor initiation; its progression and promotion have never been denied.

The transformed calli with *rol*B can produce approximately 3.15% dry weight of resveratrol and its production depends on the abundance of mRNA transcripts of *rol*B gene. The only limiting factor that reduces the application of *rol*B-transformed cell is their growth inhibition potential. The expression of this gene in transgenic tobacco plants from its endogenous promoter displays alteration in morphology of leaves and flowers along with increased adventitious root formation on the stem [59]. The gene expressed from the 35S promoter that results in root induction on *Agrobacterium* transformed tobacco leaf disc is less efficient than when the gene is expressed from its endogenous promoter [60]. This suggested that confined *rol*B activity is important in order to stimulate root initiation. Studies have shown that the biological consequences of *rol*B expression including leaf necrosis and initiation of roots seem similar to the effects as produced by the auxin [61]. However, in contrast to IAA overproducing plants, the adventitious root formation is not increased and apical dominance is normal. Also, the notion that leaf necrosis is atypical, auxin effect is questionable because transgenic IAA overproducing plants rarely display this characteristic [62].

13.3.2.3 The rolC Gene

The gene *rol*C having a reading frame of 540 base pairs encodes a protein, which is composed of 180 amino acids and exhibits a 20-kDa mass. The protein shows no typical motifs and shows no typical similarity to any known protein outside *Agrobacterium*. Phenotypic changes induced by this gene are stunted growth of the plant that results in small height of plant, leaves color appears as light green, and branching increases.

The mentioned changes lead scientists to make an idea that expression of this gene associated with the increase activity of the cytokinin [59]. Further, it was suggested that *rol*C gene is in fact β glucosidase the enzyme which has potential to convert inactive glucosidic conjugates to release the free cytokinin in its active form [61]. However, this hypothesis was not further supported by *in vivo* studies. One beneficial effect of the *rol*C gene is that it enhances the rooting in fruit trees that are transformed with the gene [63] and carnation plants thus specify that expression of *rol*C gene has activity that resembles with the axin. In rolC transformed tomato plants, it was observed that was an increase in the ratio of abscisic acid to indole 3 acetic acid [64]; thus, it might be reason for the dwarfed nature of these plants. The potential of *rol*C gene in enhancing the alkaloids called tropane, pyridine, indole, and other metabolites including ginsenosides and anthraquinones have been observed in plant cell cultures that are transformed with rolC gene [65]. In transgenic tobacco plants, the expression of gene from endogenous promoter has shown that these plants have an increased ratio of leaf length to leaf width. The plants are also and more highly branched. Flowers have developed earlier and have smaller flowers and reduced pollen production compared to wild type. Other alterations include reduced apical dominance, reduced production of seed and pollen viability, and if these genes are essentially expressed result in sterility in males and also result in change in leaves color to pale green. This gene thus having potential role in the transformation of several ornamental flowering species. During the expression of this gene from the strong 35S promoter, these characteristics are exaggerated, thus giving stunted plants with very short internodes and an increased number of lanceolate light green leaves. Apical dominance is also reduced resulting in bushy plants. These phenotypic alterations are also evident in 35S: *rol*C potato [66]. Studies have shown that *rol*C probably does not have a direct effect on the metabolism of plant hormones.

13.3.2.4 The rolD Gene

The gene *rol*D having a reading frame of 1032 base pairs that encode proteins made up of 344 amino acids and have no obvious motives or similarity to other proteins. Expression of *rol*D gene in plants like tobacco induces changes that include early flowering, rapid formation, and elongation of axillary inflorescence with an increased growth of adventitious roots meristem [67]. The gene is involved in the conversion of ornithine into proline that is done by an enzyme called ornithine cyclodeaminase and that enzyme is encoded by *rol*D gene [68]. It was suggested from the formation of proline that *rol*D gene might have its role in the process of flowering

[69]. It was supported by the fact that in *A. thaliana*, overexpression of a gene named AtP5CS1 that was actually involved in the production of an enzyme, which plays its role in synthesis of proline and overexpression of this gene results in accumulation of proline in plants and consequences in precocity in flowering. Thus, outcome of this is not observed in case of secondary metabolism. Changes like reduction of the levels of ornithine and elevation of levels of proline were observed in cells transformed with *rol*D. It is also known to play its role in assisting the effects of gene on morphology of plant like growth of hairy roots and their maintenance along with ample flowering in such plants [68]. The *rol*D gene in plants like tomato is known to increase the ability of cells to increase the defense response just as happens during the expression of PR1 genes [70]. The *rol*D gene thus plays its role in the defense mechanism of plants. Plants with *rol*D gene with a promoter activated by auxin are known to increase the ability for active defense and found that plants with gene show tolerance to toxins of *Fusarium oxysporum* [70].

13.3.3 The Combined Effect of Genes *rol* on Secondary Metabolism

On the basic level, the integrated effect of *rol* genes has been studied by utilizing cultures of hairy roots in which genes are expressed under control of their own promoters along with other TDNA genes. According to studies, it was found that in plant species that are transformed with these genes, there was an increase in the secondary metabolites production in hairy roots [71]. Hairy roots ability associated with production of secondary metabolites is a natural phenomenon that suggests that TDNA gene loci are responsible for such effects. Taking into account the TDNA *rol* gene loci, it is found that these genes have stilbenes role in the diverse biochemical processes in transformed plant cells, and thus, it is logical to state that major factors for such processes are *rol* genes of *A. rhizogenes*. It was observed that secondary metabolites that are accumulated in the roots of plants that are transformed with three *rol* genes that are *rol*A, *rol*B, and *rol*C and this effect was similar to that found in hairy roots of wild-type transformed plants [52]. A recent analysis of plant cells that are transformed with *rol* genes and pRiA4 gene shows that each *rol* gene has its own individual mechanism of anthraquinone activation [12]. Largely, the effects of these genes are assumed to be noteworthy in the pathways that result in production of higher levels of secondary metabolites in plants. It is also possible that TDNA genes other than *rol* genes might have their role on secondary metabolism in hairy roots but their influence found to be

less pronounced. This investigation put forward some findings that show that *rol*C gene and *rol*A gene have antagonistic effect on *rol*B gene induced increased production of anthraquinones. This observation was not astonishing, because antagonistic effect of these genes has already been shown at different levels. It was found that constitutive expression of *rol*B gene known to inhibit the growth of tobacco cells and *rol*C gene was to reverse this inhibition caused by *rol*B gene. In the same way, *rol*C declines sensitivity of transformed cells to auxin that is induced by rolB genes [72] and also declines the severity of phenotypes that are induced by this gene. It was also proposed that this antagonistic effect of *rol*C and B gene is mediated at calcium signaling level. Hence, it may be concluded that *rol*B gene plays its key role in activation of secondary metabolism as a result of transformation and *rol*C gene also has a unique role in secondary metabolism; thus, it provides a signal that tunes the action of *rol*B gene.

13.4 Mechanism of Action of *rol* Genes

The defense response in various plants can frequently be developed due to plant-microbe interactions. Reactive oxygen species (ROS) are important for the plant's defense mechanism regulation. To check the relationship between the production of secondary metabolism (production of phytoalexin) and the ROS production, there is a wide range of experiments. It is studied that the *rol*B and *rol*C genes' duty is not just the activation of the production of phytoalexin but also intracellular ROS level suppression. The defense response and the suppression of ROS show a special scenario in plant-microbe interactions. The result of the above-discussed studies shows that evading upstream defense control seems to be helping for the plant cell structure construction with stabilized secondary metabolites production [73]. In this section, we will be discussing the new verdicts related to the production of ROS and secondary metabolism under the supervision of combined expression along with individual expression of the *rol* genes in plant cells.

13.4.1 How *rol* Genes Regulate ROS Production and Mediate Secondary Metabolites Production

13.4.1.1 Agrobacterium (rol Gene) and ROS

In plant-pathogen relationship, ROS are very important. Various virulent and avirulent pathogens draw out the accumulation of ROS in plant cells

in varied dynamics, and these defense response elicitors are often cited as MAMPs which are microbe-associated molecular patterns. These virulent and avirulent pathogens also activate oxidative bursts [74]. ROS kills the host cells along with the pathogens by inducing a response called hypersensitive response; moreover, the ROS act as signaling molecules which trigger the mechanisms of defense. For the survival of their own, these pathogens usually inactivate the production of ROS through the interactions between plant and pathogen. A pathogen of plant named *Pseudomonas syringae* which is the highly studied pathogen model is being used for the purpose of demonstration of this outcome. The Pseudomonas syringae effector protein named HopAO1 (HopPtoD2) protein of the tomato strain DC300 will be transferred from bacteria into the plant cell for the purpose of supporting the bacterial growth through the process of suppressing the innate immunity in the host cells. The HopAO1 overwhelms the induction of ROS in plants [75] as well as a number of mechanisms of defense linked with MAMP-triggered innate immunity [76]. A plant pathogen *Agrobacterium tumefaciens* additionally employs this approach for the purpose of fighting against the mechanisms of defense of various plant species [77]. Hydrogen peroxide is an primary part of ROS of various plants and can decontaminate *A. tumefaciens* [78]. In plants, the induction of the hypersensitive response which is elicited by *P. syringae* pv. *phaseolicola* can also be suppressed by *A. tumefaciens* [79]. *A. tumefaciens* and *A. rhizogenes* are closely related plant pathogen. *A. rhizogenes* is being studied by various laboratories comprehensively, but it is very unexpected that *A. rhizogenes* effects on the metabolism of ROS in the cells of host plant have not been studied so often; many laboratories have studied this pathogen so it is unexpected that the in host cells have never been investigated.

13.4.1.2 Plants Secondary Metabolism and ROS

Plants secondary metabolites accumulation in some of the plant cell cultures carries out by ROS, but in a number of plants, ROS does not regulate the secondary metabolites [80]. Accumulation of different secondary metabolites is being mediated by ROS, for example, ROS mediate the accumulation of isoflavonoids in *Glycine max* and *Medicago sativa*, indole alkaloids in *C. roseus*, ginsenosides in *Panax ginseng*, thujaplicin in *Cupressus lusitanica*, momilactones in *Oryza sativa* cell cultures, furanocoumarin in *Petroselinum crispum* cell cultures, diterpene rishitin and acridone alkaloid p-coumaroyloctopamine in *Solanum tuberosum*, and capsidiol in *Nicotiana* [80]. In the process of the phytoalexin stimulation, NADPH oxidase is produced which generates the oxidative burst. There

are numerous examples of defense genes regulation which is dependent on Ca2+, but in this process, NADPH oxidase pathway is not involved all the time. There are different mechanisms which regulate the secondary metabolism of various plants. These regulation processes are poorly studied but it is generally postulated that important secondary metabolism inductors are ROS.

13.4.1.3 Stabilization of Secondary Metabolites Biosynthesis Through rol Genes

Stabilization of secondary metabolites activation through the *rol*B and C genes increases by time. Such as *R. cordifolia* transformed cells can produce a large amount of anthraquinones for an extended time period (more than 10 years) without any choice. It seems that the host cell regulatory control can be avoided by the *rol* genes. ROS basic and induced level suppression is specified by the data, which declare that the modulation of production of phytoalexin can be independent of secondary metabolism and NADPH oxidase pathway. These outcomes point toward bypassing of *rol* genes through host cells central mechanism. Due to this, a defense mechanism may be developed which will be independent of the central mechanism of the cell. This type of discussion is known to be a branch of system biology. A detail on secondary metabolites from protection is kind of investing in the field of knowledge. The *rol* genes complex mechanism can be untangled with this methodology [73].

13.5 Impact of *rol* Gene on Different Secondary Metabolites

13.5.1 Impact of *rol* Gene on Alkaliods

Transformation of the whole plant or plant tissue with *A.rizogenes* leads to the addition of hairy roots in plants and alteration of secondary metabolism. Some of these products which are synthesized from the roots of plants have great economic value in the pharmaceutical industry [81]. Scopolamine, the most important tropane alkaloids, is used in medicines and is produced in the roots of several medicinal plants species such as *Hyoscyamus muticus* and *Atropa belladonna*. The latter was altered with *A. rhizongenes* containing the desired gene that encode the enzymes that synthesized the desired metabolites, the result being the production of hairy roots and improved the level of scopolamine [82]. Another example in the

roots of *Valeriana wallichii* is an active compound valepotriate is presents. Its transformation with *A.rhizogenes* produced hairy roots with two- to three-fold increased valepotriate level [83]. Tobacco roots lines altered with *rol*A, *rol*B, and *rol*C, and alone *rol*C showed more the growing capacity; moreover, in transformed Tobacco roots, nicotine alkaloid synthesized in the roots, was more as compared to controls [84]. In ornamental plant like *lemon geranium*, when *rol* genes are inserted by Ri plasmid T-DNA, there is an increase in the essential oil and fragrance. *rol* genes are involved in improving fragrance in ornamental flowers. These insertions of *rol* genes in ornamental plant opened new research perspectives [85].

13.5.2 Impact of *rol* Genes on Flavonoids

Previously, there are several reports describing the impact of *rol* genes on flavonoids content of the plants. *A. carvifolia* showed the presence of flavonoids such as caffeic acid (30 μg/g DW), quercetin (10 μg/g DW), isoquercetin (400 μg/g DW), and rutin (300 μg/g DW). Compared to the untransformed plants, flavonoid levels increased 1.9- to 6-fold and 1.6- to 4-fold in *rol*B and *rol*C transgenics, respectively [86]. Similarly, *rol* genes transgenics of *Artemisia annua* were found with enhanced levels of flavonoids (rutin, quercetin, isoquercetin, and caffeic acid) when transformed with *rol*B (4- to 6-fold) and *rol*C gene (3.8- to 5.5-fold). Transformation of Artemisia dubia Wall with *rol*ABC gene construct resulted in increased production of total flavonoids and total phenolics. An increase in caffeic acid and catechin and a decrease in gallic acid content in the extracts of transformed plants compared to the untransformed control plants was detected [87].

In another report, *Lactuca sativa* L. (cv Grand Rapids) was transformed by *Agrobacterium*-mediated transformation with *rol*C gene. The transformed plants showed 53%–98% increase in total phenolic and 45%–58% increase in total flavonoid contents compared with untransformed plants [88]. Similarly, transformation of *Lactusa sativa* with *rol*ABC gene construct resulted in increased phenolic and flavonoids content [89].

13.5.3 Impact of *rol* Genes on Terpenoids

There are reports where transformation of various plant species resulted in enhanced secondary metabolites of the plant especially the steroids including phytoecdysteroids and artemisinin a sesquiterpenoid. *Ajuga bracteosa*, when transformed with *rol*ABC gene construct, showed a significant increase of phytoecdysteroids (14.5-fold higher than control plants) [90].

Artemisinin and its derivatives content were found significantly enhanced in *Artemisia annua*, i.e., two- to nine-fold increase in artemisinin, 4- to 12-fold increase in artesunate and 1.2- to 3-fold increase in dihydroartemisinin was observed when transformed with *rol*B gene. Whereas in the case of *rol*C gene transformants, a four-fold increase in artemisinin, four- to nine-fold increase in artesunate, and one- to two-fold increase in dihydroartemisinin concentration were observed [17]. Similarly in *Artemisia carvifolia*, artemisinin content increased 3- to 7-fold in transgenics bearing the *rol*B gene and 2.3- to 6-fold in those with the *rol*C gene. A similar pattern was observed for artemisinin analogs [16].

13.6 Conclusion

Collectively, the utilization of *rol* genes in plant industry for enhanced secondary metabolism is a trial-and-error process. This area required a more targeted research in order to proficiently employ *Agrobacterium*-mediated systems of transformation. More focused research is required, particularly on the effect and function of the individual genes and ORFs of *A. rhizogenes*. Different approaches can be adopted for this purpose such as the overexpression of a single gene, co-expression with fluorescent proteins, and endogenous promoter studies that will provide information for an improved understanding of natural transformation techniques. The elucidation of the copy numbers of inserted genes and the preferred integration sites of T-DNA transferred into foreign host cells along with gene expression analyses and protein characterizations could pave the way for augmenting the capabilities vital to the field of genetic engineering by *Agrobacterium* and its impact on plant secondary metabolism.

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Shahid-ul-Islam is currently working as Principal Project Scientist at the Indian Institute of Technology Delhi. He worked as DST-SERB National Postdoctoral Fellow at Indian Institute of Technology Delhi, from 2017 to 2019. Then he joined same Institute as Principal Project Scientist where he works on natural products and chemistry of metal based natural dyes using advanced technologies. He has to his credit several research publications, patents and books including several with the Wiley-Scrivener imprint.

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