Progress in the Chemistry of Organic Natural Products

A. Douglas Kinghorn · Heinz Falk
Simon Gibbons · Jun'ichi Kobayashi
Yoshinori Asakawa · Ji-Kai Liu *Editors*

107

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Progress in the Chemistry of Organic Natural Products

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Volume 107

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Preface

The editors of *Progress in the Chemistry of Organic Natural Products* are pleased to announce that with the publication of Volume 107 of the book series in 2018, this marks the 80th anniversary of its founding in 1938 by Professor László Zechmeister. This monograph series was established under the title "Fortschritte der Chemie Organischer Naturstoffe", but for many years was simply known as "Zechmeister". From its inception, and continuing up to the present time, the major aim of the editors of this book series has been to convey to the reader significant new developments in the chemistry of natural product molecules from organisms. In Volume 100 of "Progress", published in 2015, a detailed profile of the coverage of the book series in the 75 years from 1938 to 2013 was provided [1].

As of Volume 107, we are delighted to welcome two highly esteemed natural product scientists as new Series Editors, namely, Professor Yoshinori Asakawa, of Tokushima Bunri University, Tokushima, Japan, and Professor Ji-Kai Liu, of South-Central University for Nationalities, Wuhan, People's Republic of China. Professor Asakawa is a world expert on the chemistry and biology of liverworts and is a distinguished and esteemed member of the natural products community who has provided three extensive monographs in the book series to date [2–4]. Professor Liu has both academic and industrial experience and is highly accomplished in the isolation chemistry, total synthesis and biosynthesis of secondary metabolites purified from higher fungi. With a colleague, he provided an excellent chapter on this topic in Volume 106 of our book series [5]. He is the founding Editor-in-Chief of the journal *Natural Products and Bioprospecting* (Springer Open journal).

The year 2018 also marks the 20th year in succession that Emeritus Professor Heinz Falk has served as Series Editor of *Progress in the Chemistry of Organic Natural Products*. He began in this capacity for Volume 75, and since then has played a major role in overseeing the many changes that have occurred, and has also ensured that the high standards established earlier for our book series have been maintained. Together with his colleague, Dr. Klaus Wolkenstein, of the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, Professor Falk

contributed an illuminating chapter on molecular fossils and biomarkers for Volume 104 of the series [6].

The first of the two chapters in Volume 107 delves into the logistics of tropical plant collecting and chemical and biological work-up in the search for new cancer chemotherapeutic agents. This was written by the Series Editor Professor Douglas Kinghorn, along with colleagues from the Ohio State University and the University of Illinois at Chicago. In the second chapter, Professor Runner Majinda, of the University of Botswana, Gaborone, Botswana, offers an updated treatise on the fascinating topic of the *Erythrina* alkaloids.

Columbus, OH — Douglas Kinghorn
Linz, Austria — Heinz Falk
London, UK — Simon Gibbons
Sapporo, Japan — Jun'ichi Kobayashi

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The Search for Anticancer Agents from Tropical Plants

Joshua M. Henkin, Yulin Ren, Djaja Djendoel Soejarto, and A. Douglas Kinghorn

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1 Introduction

There is a long and sustained history of use of secondary metabolite constituents of terrestrial and marine organisms as sources of small-molecule therapeutic agents [1–6]. Even as the end of the second decade of the twenty-first century nears, new examples of natural products and their derivatives are still being introduced into Western medicine as therapeutic agents. The screening of chemically complex natural product extracts for the discovery of new drugs in a timely manner presents a number of logistical challenges, but various modern technological approaches may be applied to enhance this process [6–9]. It is considered that when innovative methods of discovery are applied, natural products will continue to offer a vast resource to yield structurally novel compounds with promising biological activities [6–10].

Medicinal plants have a long history of use by humans, and originally were employed as crude drugs in forms such as teas, tinctures, poultices, and powders [11, 12]. Beginning with the purification of morphine from the opium poppy at the beginning of the nineteenth century, a number of important plant-derived drugs have been obtained in pure form in the intervening period, including artemisinin, atropine, colchicine, cocaine, digoxin, galanthamine, quinine, paclitaxel, and vinblastine [11–16]. There are a large number of secondary metabolites known already from plants, with about 170,000 unique compounds of this type having been characterized [9], of which the largest groups are isoprenoids, phenolics, and terpenoids [14]. Given that a high proportion of the estimated 270,000 species of plants in existence has not yet been subjected to any phytochemical or biological activity investigation, there is a good chance that additional new lead compounds for use in drug discovery programs will continue to be elucidated for the foreseeable future.

Cancer remains a major public health problem in countries all over the world, in developed and developing countries alike. According to recent figures on global

cancer, there were over eight million deaths due to cancer in all countries in 2012, with about 65% of all deaths occurring in developing countries [16]. When the United States is taken specifically, while there has been an overall drop of about 25% in cancer mortalities over the last 20 years, still approximately 600,000 deaths from this cause are expected in 2018 [17]. As one approach to alleviating the burden of cancer, natural products have been used for many decades as cancer chemotherapeutic agents, with approximately 50% of the small-molecular-weight molecules approved for this purpose in western medicine from the 1940s to 2014 being either structurally unmodified natural products or derivatives of natural product lead compounds produced by semi-synthesis [6]. Of the anticancer compounds used clinically, these may be obtained from higher plants, terrestrial microbes, and marine organisms, with the number of such compounds available commercially continuing to expand [6, 18]. These compounds act via a wide variety of different cellular mechanisms [19].

When higher plants are considered specifically as sources of cancer chemotherapeutic agents, initial success occurred in the late 1950s and early 1960s through the isolation and development of the *Catharanthus* (vinca) alkaloids that are now known as vinblastine and vincristine [20]. The U.S. National Cancer Institute launched an extensive diverse plant collection program in the early 1960s that led to the discovery of camptothecin and taxol (later renamed paclitaxel), which together afforded four approved anticancer agents by the mid-1990s [20, 21]. A number of review articles have appeared specifically on the topic of plant-derived anticancer agents, e.g. [20–25].

2 Clinically Used Plant-derived Anticancer Agents and Compounds in Clinical Trials

2.1 Plant-derived Compounds Used Clinically as Cancer Chemotherapeutic Agents

We have previously reviewed the history of the initial discovery of the two earliest classes of plant-derived anticancer introduced clinically, namely, the vinca (*Catharanthus*) bisindole alkaloids and the podophyllotoxin lignan derivatives [25]. Currently, there are five bisindole alkaloids that are used clinically for anticancer therapy in either or both the United States and Europe, namely, the natural products vinblastine (**1**) and vincristine (**2**), and the semisynthetic derivatives vindesine (**3**), vinorelbine (**4**), and vinflunine (**5**) (Fig. 1) [26]. These compounds are used to treat a wide variety of different types of solid tumors, leukemias, and lymphomas [25, 26]. In 2012, vincristine sulfate liposome injection was approved clinically for advanced Philadelphia chromosome-negative acute lymphocytic leukemia in adults [27]. This is a cholesterol and sphingomyelin-based nanoparticle

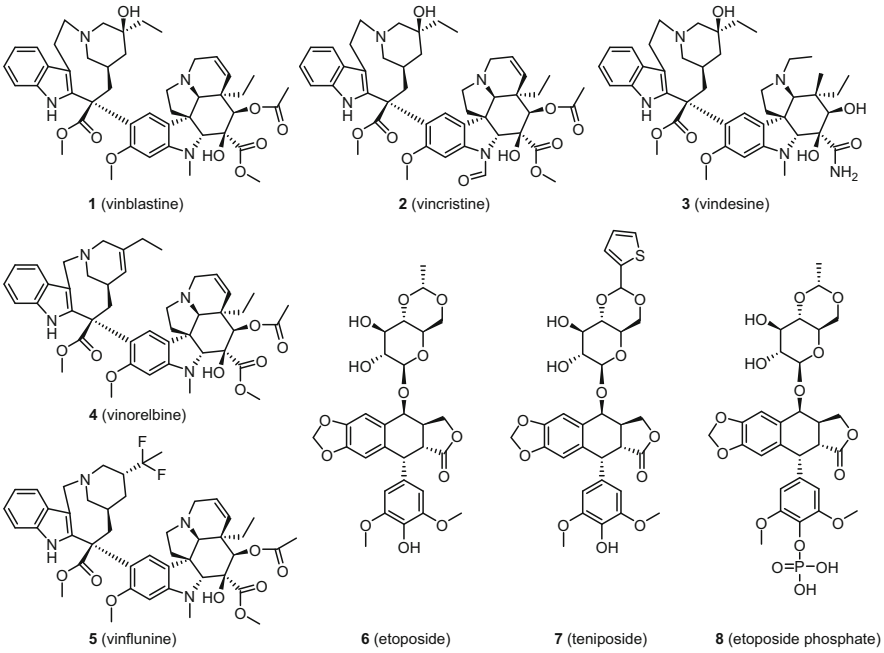


Fig. 1 Structures of clinically used bisindole alkaloid (**1**–**5**) and epipodophyllotoxin (**6**–**8**) anticancer agents

formulation that is intended to overcome dosing and pharmacokinetic limitations of unmodified vincristine sulfate [27, 28].

Summaries of the early development of the plant lignan podophyllotoxin at the U.S. National Cancer Institute as a potential anticancer agent, leading to the later introduction into therapy of the epipodophyllotoxin analogs etoposide (**6**), teniposide (**7**), and etoposide phosphate (**8**) (Fig. 1), along with their clinical uses, have been published [25, 29]. There remains a strong interest in developing new anticancer agents based on the podophyllotoxin molecule, although no new compounds of this type have been approved in recent years [29].

The seminal work of the late Monroe Wall and Mansukh Wani of the Research Triangle Institute, Research Triangle Park, NC in the discovery of the key antineoplastic compounds camptothecin and taxol (paclitaxel) has been documented by others [22, 30]. These compounds are important not only for their respective mechanisms of action but also in serving as lead compounds for a wide range of analogs with potential anticancer activity. Camptothecin has been found to stabilize the DNA-topoisomerase I complex, and while this alkaloid is not used as a drug in its natural form due to its insolubility, its derivatives topotecan (**9**) and irinotecan (**10**) (Fig. 2) both have approved clinical use, and may be used to treat a range of different types of human cancers [31]. A nanoliposomal formulation of **10**, intended to provide enhanced pharmacotherapeutic parameters compared with the parent

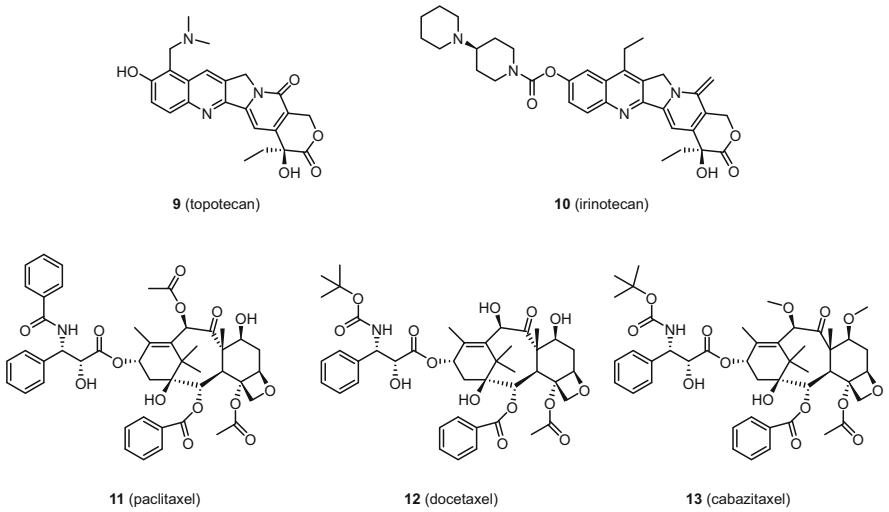


Fig. 2 Structures of clinically used camptothecin (**9** and **10**) and taxane (**11**–**13**) anticancer agents

compound, was approved recently by the U.S. Food and Drug Administration (FDA) for use in combination chemotherapy for gemitabine-resistant metastatic pancreatic cancer [28, 32].

Paclitaxel (**11**) (Fig. 2), a nitrogen-containing diterpenoid used clinically in its native form, has become a major resource in cancer chemotherapy [33], and its cellular mechanism action was shown by Susan Band Horwitz and her co-workers to involve the promotion and stabilization of tubulin polymerization and the inhibition of microtubule depolymerization, producing cell cycle arrest [34]. Two semi-synthetic analogs of paclitaxel are also currently used in oncology, namely, docetaxel (**12**) (Fig. 2), developed by the late Pierre Potier and colleagues in France [33], and cabazitaxel (**13**) (Fig. 2) [33, 35]. The latter compound was approved for patients with hormone-refractory prostate cancer showing resistance to treatment with docetaxel [35]. Due to the poor water solubility of paclitaxel and other disadvantages as an anticancer agent, some new polymer formulations have been developed. A nanoparticle albumen-bound form with the tradename Abraxane® that can be used as an injectable suspension gained marketing approval by FDA initially over a decade ago, and was later approved for advanced forms of breast cancer, non-small cell lung cancer, and pancreatic cancer [28]. A micellar form of paclitaxel (Genoxol®-PM), consisting of polyethylene glycol and poly(DL-lactic acid), has been FDA-approved for use in patients with breast cancer [28, 36]. According to Cragg et al., in 2013 there were several thousand clinical trials ongoing at that time on the already approved taxane derivatives [37].

In 2014, the plant alkaloid derivative omacetaxine mepesuccinate (**14**) (Fig. 3) was given full FDA approval, having earlier been granted accelerated approval for the treatment of adults with certain forms of chronic myeloid leukemia that are resistant or show intolerance to selected tyrosine kinase inhibitors [38, 39]. This

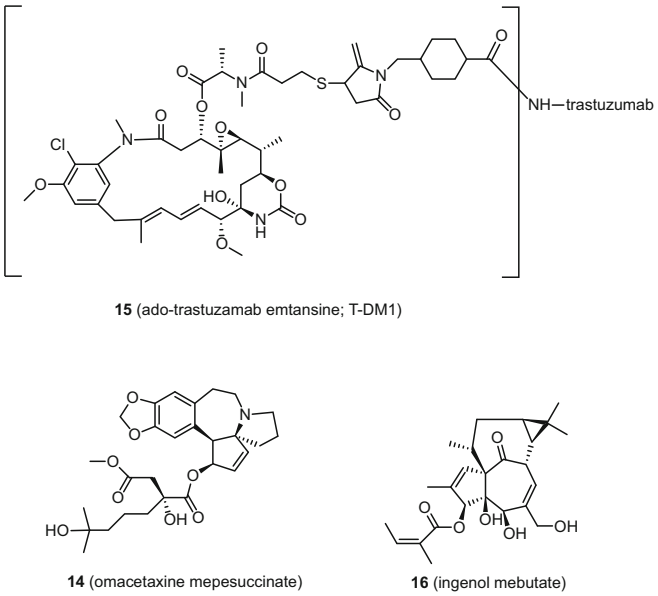


Fig. 3 Structures of omecetaxine mepesuccinate (**14**), ado-tratuzamab emrtansine (**15**), and ingenol mebutate (**16**)

Cephalotaxus alkaloid was first isolated from a Chinese plant as homoharringtonine by Richard Powell and the late Cecil Smith and their colleagues from the USDA laboratory at Peoria, Illinois in 1970 [40], and a review of the early relevant chemical work performed has been published [41]. Mechanistically, **14** interferes with protein biosynthesis in the cancer cell by binding to the A-cleft of ribosomes [42]. Omacetaxine mepesuccinate (**14**) is of importance in being the first member of an entirely new structural class of plant-derived anticancer agents utilized for cancer treatment, and also in being the initial protein translation inhibitor approved by the U.S. FDA for this purpose [42].

Two further compounds are considered relevant for inclusion in this section, namely, ado-trastuzamab emtansine (T-DM1) (**15**) (Fig. 3) and ingenol mebutate (**16**) (Fig. 3). In 1972, the lead compound maytansine, a chlorinated ansamycin derivative, was reported by the late S. Morris Kupchan and associates as an anti-leukemic constituent of the East African plant, *Maytenus ovatus* [43, 44]. Excellent review contributions on the development of the maytansinoids have appeared in the literature [44, 45], and it is now considered that such compounds are actually of microbial endophyte origin rather than being biosynthesized by plants [46]. Ado-trastuzamab emtansine (**15**), an antibody-drug conjugate [47], was registered for the chemotherapy of patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer [48]. In turn, ingenol mebutate (ingenol-3-angelate) (**16**) was approved by the FDA in 2012 as a topical treatment for actinic keratosis, which is a skin ailment that can lead to the formation of

squamous cell carcinoma [49]. The history of the development of this compound as a biologically active constituent of the plant *Euphorbia peplus* was described in a recent volume of this book series [50].

2.2 *Examples of Derivatives of Plant Secondary Metabolites Undergoing Clinical Trials as Potential Anticancer Agents*

As may be expected, there is a considerable interest in finding new anticancer agents of plant origin to supplement those used already clinically. Accordingly, there are a large number of such compounds undergoing clinical trials (e.g. [8, 9, 24, 37, 51]). In the following paragraphs, 11 anticancer leads of plant origin that are in current clinical trials have been selected as representative examples, including several derivatives of camptothecin and paclitaxel.

As mentioned earlier, the naturally occurring pentacyclic quinoline alkaloid, camptothecin, is the parent compound of topotecan (**9**) and irinotecan (**10**) (Fig. 2) [31, 51]. Structure-activity relationship studies have shown that the (20*S*)-hydroxy group, the pyridone (D-ring) and lactone (E-ring) units, and a planar A, B, C, D, and E ring system are essential for camptothecin to mediate its cytotoxicity. Also, an increase of water solubility and substitution at the C-7, C-9, C-10, and/or the C-11 position can increase the resultant cytotoxic activity of camptothecin, but substitution at the C- and D-rings reduces the potency [31, 51, 52]. Following these observations, several derivatives showing an improved efficacy, including exatecan (**17**) and afeletecan (**18**) (Fig. 4), have been prepared and evaluated in cancer clinical trials.

Exatecan (**17**), used as its mesylate salt (DX-8951f), is a water-soluble synthetic derivative of camptothecin, with potentially enhanced therapeutic parameters, when compared with topotecan (**9**) or irinotecan (**10**) (Fig. 2). The safety, dosing limits, and pharmacokinetics of **17** were evaluated in several phase I clinical trials [53]. In a phase II study, exatecan mesylate showed a modest activity against metastatic gastric cancer, and was considered worthy of further development [54]. However, in a randomized phase III study, **17** plus gemcitabine were not found to be superior to gemcitabine alone in the first-line treatment of advanced pancreatic cancer, hence, further work has been discontinued [31, 55]. Recently, a new antibody-drug conjugate (ADC) comprising an anti-HER2 antibody, a cleavable peptide linker, and an exatecan-derived topoisomerase inhibitor (DXd), namely, DS-8201a, has been developed. It has a higher drug-to-antibody-ratio than that of T-DM1 (**15**) (Fig. 3), a HER2-targeting ADC comprising the antibody trastuzumab and maytansine, and is effective against cancers with low HER2 expression. Also, DS-8201a was found to inhibit T-DM1-resistant cancer cell growth, and it appears promising to overcome HER2-positive gastric cancer T-DM1 resistance [56]. Afeletecan (BAY 38-3441) (**18**), a 20-*O*-linked CPT glycoconjugate, is a camptothecin prodrug. It showed improved

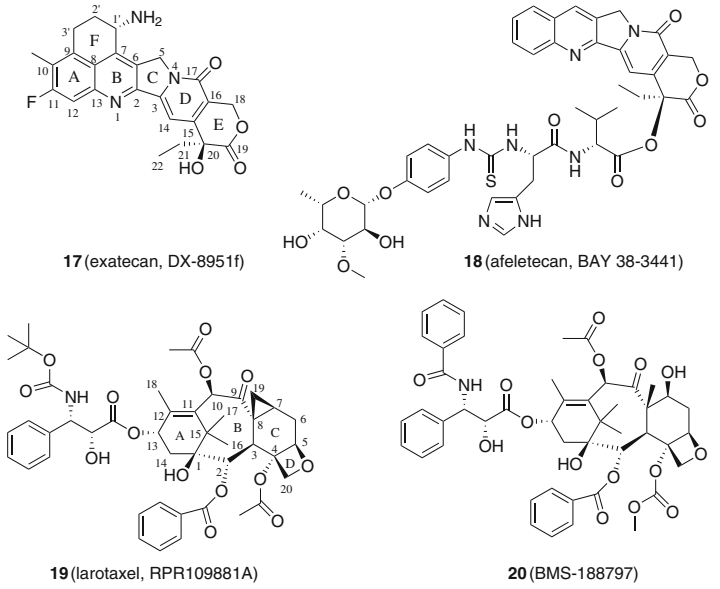


Fig. 4 Structures of some camptothecin and paclitaxel derivatives **17**–**20** that have reached cancer clinical trials

anticancer activity and has been selected for evaluation in cancer clinical trials [57, 58]. A phase I study for the treatment of a wide variety of malignancies showed that **18** was tolerated [59], indicating that glycoconjugation is supportive of effective drug delivery. Other camptothecin derivatives that were undergoing oncology clinical trials recently included AR-67, cositecan, CZ48, diflomotecan, DRF-1042, elomotecan, gimatecan, lurtotecan, and namitecan, with several other camptothecin conjugates also being in clinical trials [8, 31, 58].

Paclitaxel (**11**) (Fig. 2) is one of the most important natural product anticancer drugs that is used in its native chemical form, as it occurs in the producing organism. Structure-activity relationship studies have shown that the taxane (A, B, C, and D) ring system (both its constitution and conformation) and the C-13 side chain are essential, with the C-2 benzyl and C-4 acetyl groups being critical, for **11** to bind to tubulin and to mediate its cytotoxicity. In addition, the substituents at the C-1, C-4, C-7, C-9, C-10, and C-14 positions can be modified to improve its cytotoxic potency [60]. Based on these conclusions, two promising leads, larotaxel (RPR 109881A) (**19**) and BMS-188797 (**20**) (Fig. 4), were produced through changing the C-7 and C-13 side chains and modification of the C-4 substituent of **11**, respectively. However, it should be pointed out that these are only two examples of over 20 paclitaxel derivatives that were in various stages of clinical trial recently [8, 33, 37].

Larotaxel (RPR 109881A) (**19**) is a semi-synthetic taxane analogue, which showed a broad spectrum of cytotoxicity and more potent activity than docetaxel (**12**) against murine P388 leukemia cells, and also effectiveness toward multidrug-resistant (MDR) cancer cell lines. Phase I and II studies showed that this agent has a

manageable toxicity profile and confirmed that it can potentially overcome the MDR mechanism [61]. However, a phase III study designed to compare overall survival of bladder cancer patients treated with larotaxel/cisplatin or gemcitabine/cisplatin did not show any difference between these two groups [62], indicating that further modification of larotaxel is required to improve on its anticancer potential. The paclitaxel derivative BMS-188797 (**20**) (Fig. 4) shows potential advantages over the parent compound. The maximum tolerated dose (MTD), dose-limiting toxicity (DLT), and preliminary activity of BMS-188797 were established in several phase I studies, after which a phase II study for this compound was suggested [63, 64]. While no recent development of this compound (4-desacetylpaclitaxel 4-methyl carbonate) appears to have taken place, this compound is of interest in that it shows only a minimal structural variation from the molecular structure of the parent compound, paclitaxel (**11**).

In addition to the diterpenoid ester, paclitaxel (**11**) (Fig. 2), other plant-derived terpenoids, including three sesquiterpene lactones **21**–**23** (Fig. 5) and a triterpenoid **24** (Fig. 5) [65], have reached oncology clinical trials. For example, L12ADT (**21**), a prodrug of the sesquiterpene lactone thapsargin (from *Thapsia garganica*) conjugated with a peptide moiety, was found to be selectively toxic to PSA-producing prostate cancer cells, with the mechanism of action proposed as depletion of androgen receptor protein via protein synthesis inhibition [66]. Thus far, several clinical trials for the treatment of metastatic prostate cancer have been reported for **21** [65, 66]. The very well-known sesquiterpene lactone artemisinin from *Artemisia annua* has been used in either its structurally unmodified or derivatized forms to treat malaria for several decades [65, 67]. Recently, artemisinin was found to exhibit selective cytotoxicity in vitro and antitumor potential in vivo, with intracellular heme proposed as the molecular target. Several new analogues of

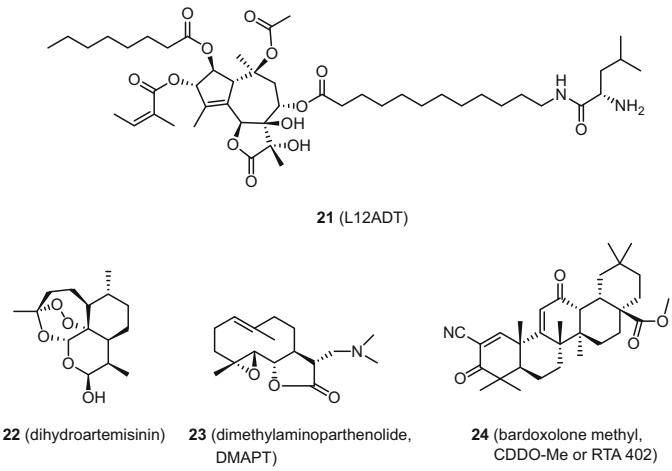


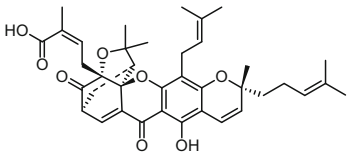
Fig. 5 Structures of some plant-derived terpenoid derivatives **21**–**24** that have reached cancer clinical trials

this compound with improved anticancer efficacy have been prepared, and some of these have been evaluated in cancer clinical trials, such as dihydroartemisinin (**22**) [65, 67]. Dimethylaminoparthenolide (DMAPT) (**23**) is a water-soluble derivative of a sesquiterpene lactone anticancer lead, parthenolide, a constituent of *Tanacetum parthenium* (feverfew). It showed enhanced antitumor efficacy toward several tumor models, targeting NF-κB inhibition, DNA binding, and/or p53 tumor protein activation. This compound has been evaluated in a phase I clinical trial for the treatment of acute lymphoblastic and myeloid leukemia and other hematological tumors [65, 68].

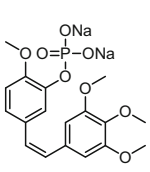
Bardoxolone methyl (CDDO-Me or RTA 402; Fig. 5) (**24**), a synthetic pentacyclic oleanane-type triterpenoid produced from oleanolic acid [69], has been tested in phase I/II trials for cancers and in phase III trials for chronic kidney disease [70]. It was found to be able to neutralize myeloid-derived suppressor cell activity, indicating an ability to enhance the cancer immunotherapy [71]. A phase I study for **24** in patients with advanced solid tumors or lymphomas has been reported, with its MTD, DLT, and appropriate dose for phase II studies established [72]. Some adverse effects have been recorded, however, in some clinical trials with **24** [37].

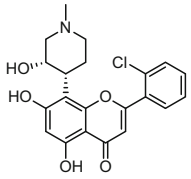
A further general group of plant-derived compounds to reach clinical trials as potential anticancer agents are phenolic substances, inclusive of gambogic acid (**25**) (Fig. 6), combretastatin A-4 phosphate (CA4P; Fig. 6) (**26**), and flavopiridol or alvocidib (HMR-1275; Fig. 6) (**27**). These compounds will be discussed in turn.

Gambogic acid (GA) (**25**) is a naturally occurring caged xanthone isolated originally from the dried resin of *Garcinia hanburyi*, with the structure established completely by analysis of its single-crystal X-ray diffraction data and electronic circular dichroism [73, 74]. It shows potent antitumor potential through inhibition of human topoisomerase and telomerase activity [74]. A phase I study has been



25 (gambogic acid)





26 (combretastatin A-4 phosphate, CA4P) **27** (flavopiridol or alvocidib, HMR-1275)

Fig. 6 Structures of some plant-derived phenolic derivatives **25**–**27** that have reached cancer clinical trials

completed for **25**, with the MTD, DLT, and a dose regimen developed for a Phase II trial established [75]. Recently, a human serum albumin (HSA)-based delivery system for gambogic acid (HSA-GA) has been developed, and the antitumor efficacy of HSA-GA was found to be improved over the unmodified parent compound, along with having enhanced water solubility and diminished potential for toxicity [76]. In 1982, George R. Pettit and colleagues isolated the phenol (−)-combretastatin from the South African plant, *Combretum caffrum*, and this compound was shown to have substantial astrocyte reversal effects and potent cytotoxicity against the P388 murine lymphocytic leukemia cell line [77]. Many structurally related compounds subsequently have been isolated from this plant, including a series of (*Z*)-stilbenoids, with some of these compounds shown to bind potently to tubulin [78]. Combretastatin A-4 phosphate (CA4P, fosbretubulin) (**26**), is a disodium phosphate salt of the natural stilbenoid phenol, combretastatin A-4, and a vascular-disrupting agent, with potential for use also in ophthalmology [78]. The MTD and DLT data have been characterized for **26** in a phase I trial, and it has been tested in phase II/III trials for the treatment of acute myelogenous leukemia and relapsed ovarian cancer [79]. Finally, among this group of phenolic compounds, a semisynthetic flavonoid alkaloid, flavopiridol (alvocidib, HMR-1275) (**27**), has been characterized as the first cyclin-dependent kinase (CDK) inhibitor to be tested in cancer clinical trials [80]. This compound is based structurally on the lead compound, rohitukine, an alkaloid from the Indian medicinal plant, *Dysoxylum binectariferum* [81]. Several phase I and II trials have been completed on flavopiridol, using this compound both as a single agent or in combination with standard chemotherapeutic regimens [80]. In a more recent small phase study, flavopiridol was found effective for the treatment of refractory or relapsed chronic lymphocytic leukemia in older patients [82].

3 Approaches to the Discovery of Cancer Chemotherapeutic Agents from Higher Plants

3.1 Development of Plant Collection Agreements Inclusive of Equitable Benefit Sharing

All plant materials for research in the discovery of new bioactive compounds initially must be collected from the field through conscientious fieldwork activities. Such efforts, whether spurred by ethnobotanical information or simply proceeding through general floristic explorations, often take place in locations distant from the laboratories where discoveries of biologically active compounds occur. Whether field work is conducted within the same country as the subsequent phytochemical and biological research or in regions well beyond the confines of that country, some type of permit is required to enter a natural area for the purposes of plant collection and procurement of samples and voucher specimens underpinning an integrated "bioprospecting" research program. A permit from a land owner can normally be

obtained expeditiously, but the process of gaining a permit to enter and collect plants in a foreign country is considerably more complex. In fact, it has never been more difficult to pursue this type of research endeavor than is the case at the present time.

By 1992, gaining a permit to collect plants in a foreign country became a complex issue due to the adoption of the resolution of the "Rio Summit" in Rio de Janeiro, now known as the United Nations CBD (Convention on Biological Diversity). The CBD acknowledges that plant resources of a country are under the sovereign rights of that country (CBD Article 3 [83]), and a permit to enter, collect, and carry out research on the plants from that country must be obtained under the condition that the results and potential benefits to be derived from the research should be shared if permission is given (CBD Article 1 [83]). This is a situation today known as Access and Benefit Sharing (ABS) and is the focal point of the Nagoya Protocol [84]. Hence, for an individual or a research group wishing to carry out a plant collection expedition in a foreign country, a collection permit must be obtained, through a tortuous and exhaustively negotiated process, from the proper government authority in the country of collection. The development of a collaborative research agreement between an institution in a country where the research is performed and an institution in a country where the plants to be collected are found, will normally expedite the collection permit issue considerably. Others have written on the impact of conforming to international agreements on obtaining plant samples for drug discovery programs, e.g. [85, 86].

In our plant-derived drug discovery research work to be discussed, the University of Illinois at Chicago (UIC) has been the component responsible in the acquisition of the plant materials to be used in the discovery process. Hence, once a contact has been made with a potential partner and once an agreement has been reached to carry out joint research, a Memorandum of Agreement (MOA) was set down to be negotiated between the UIC and the potential partner.

In the two sequential projects that are described in Sect. 3.2.1, the collaborative research agreements executed have the following parts (components): (1) introduction, (2) statement of purpose or scope of cooperation, (3) statement of objectives (areas of cooperation/goals), (4) statement on the responsibilities of the UIC, (5) statement on the responsibilities of the partner, (6) statement of joint responsibilities, (7) statement on IPR (Intellectual Property Rights) and benefit-sharing, (8) statement on the transfer of biological materials, (9) statement on dispute resolution, (10) statement on renewal and amendment, (11) statement on term and termination, (12) statement on confidentiality and the binding nature of the agreement, and (13) the signature page.

Beyond the core components of the agreement, appendices are attached that cover the: (1) research objective, (2) research plan that defines the performance of each party, (3) funding and budget, and (4) record on biological material transfer.

3.2 *Logistics of Conducting Plant Collections in Tropical Countries*

3.2.1 Countries of Origin for Tropical Plant Samples Investigated in Two Research Projects Directed Toward the Discovery of New Anticancer Agents

The first phase (Phase I) of our multidisciplinary, multi-institutional search for anticancer agents from plants ran from September 1, 1990 to April 30, 2005, supported via funding from the U.S. National Cancer Institute, National Institutes of Health (NCI, NIH), through a NCDDG (National Cooperative Natural Products Drug Discovery Groups) U01/U19 cooperative research program project award. This project was entitled "Novel Strategies for Plant-derived Anticancer Agents", and involved the participation of a university (University of Illinois at Chicago, Chicago, IL, USA), a private research institute (Research Triangle Institute, Research Triangle Park, North Carolina, USA), and a large pharmaceutical company (initially Glaxo-Wellcome Medicines Research Center, Stevenage, UK and then Bristol-Myers Squibb, Princeton, NJ, USA). Periodic reviews of the research group composition, strategies, and technical progress made in this initial project have been published [87–91]. The second phase (Phase II), inheriting many of the insights, research aims, and personnel present in Phase I, has run from 2007 up through the present day with funding through the program project (P01) grant mechanism, again from the U.S. NCI, NIH, with the title of this project being "Discovery of Anticancer Agents of Diverse Natural Origin". The range of organisms being studied in this current project now extends to aquatic and terrestrial cyanobacteria and filamentous fungi, and additional universities and industrial companies other than those involved previously are now included. However, for this Phase II project, tropical plants are collected and authenticated taxonomically by the University of Illinois at Chicago, with the laboratory work-up occurring at The Ohio State University. Progress in this project has been subjected to periodic review [92–95].

During the initial phase of our collaborative work mentioned above (1990–2005), the majority of the plant materials investigated were sourced primarily from Indonesia, Thailand, Zimbabwe, the USA, the Philippines, the Dominican Republic, Costa Rica, Peru, Ecuador, and Papua New Guinea, through a network of botanist collaborators. In each case, initial contact was made with a scientific colleague, and, following an expression of willingness to collaborate in the project, further negotiations ensued. A letter of invitation to collaborate was sent to botanists or to directors of prospective partner institutions together with full details of the policies for collection developed by the University of Illinois at Chicago (UIC) [96], including a new protocol for royalty sharing. Confirmation of willingness to collect and to supply materials for the NCDDG consortium program from each of the collaborators in a potential country of collection was followed by the drafting of an agreement to be negotiated and signed.

By 2005, 6370 primary screening plant samples had been acquired and tested that originated from 34 countries (see below and the map shown as Fig. 7 for major and significant collections acquired), usually in a range of 300–500 g of dried weight of a given acquisition. Some of these samples were acquired prior to the enactment of regulations on access to genetic resources in many of the source countries concerned. In fact, several plant samples had been procured even prior to the signing of the United Nations Convention on Biological Diversity (UN CBD), in June, 1992 [83]. After 1997, when the University of Illinois established its current policy on access to plant genetic resources in natural product drug discovery [96, 97], samples were acquired in compliance with a given source country's access law, strictly through the framework of the United Nations CBD. The largest number of samples came from Indonesia (2249), followed by Zimbabwe (985), Thailand (904), USA (396), Philippines (297), Dominican Republic (290), Peru (191), Costa Rica (189), Ecuador (162), Japan (157), and Papua New Guinea (100), while significant numbers were received from Guatemala (91), Brazil (71), Ghana (70), Madagascar (69), Panama (36), Cameroon (30), Australia (23), Lesotho (16), India (13), South Africa (12), and Turkey (11) (Fig. 7). Small numbers of samples were received from Sri Lanka (5), Colombia (3), Puerto Rico (3), Saudi Arabia (3), UK (3), Argentina (2), Kenya (2), Mexico (2), Fiji (1), Jamaica (1), Nigeria (1), and Taiwan (1).

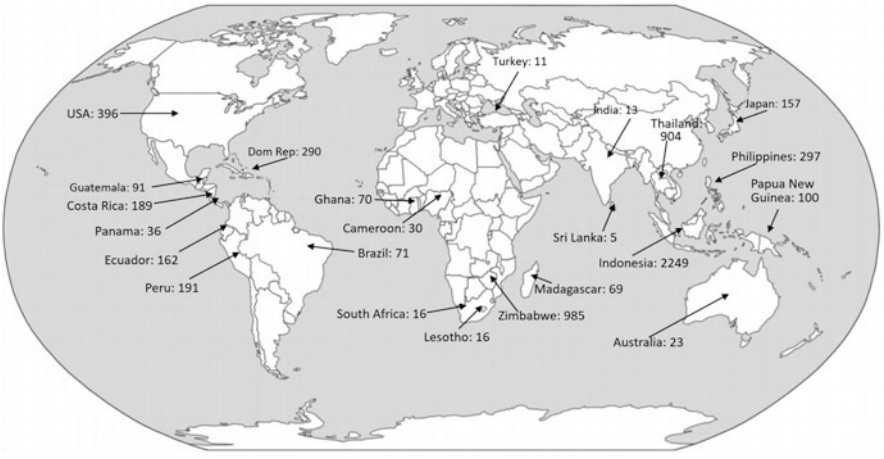


Fig. 7 World map showing the countries of origin of primary plant samples collected for the U.S. National Cancer Institute-funded National Cooperative Drug Discovery Groups (NCDDG) project entitled "Novel Strategies for Plant-Derived Anticancer Agents" (1990–2005). Countries with less than five primary samples collected are excluded from being named. Base map from: WaterproofPaper.com at https://www.waterproofpaper.com/printable-maps/world.shtml

During the second phase (Phase II) of the program, primary screening plant samples were acquired primarily from Vietnam and Laos. Formal ABS (Access and Benefit Sharing) agreements that fulfilled the requirements of the United Nations CBD [83] and the Nagoya Protocol [84] were established between the University of Illinois at Chicago (UIC) and the Institute of Ecology and Biological Resources (IEBR) of the Vietnam Academy of Science and Technology, Hanoi, Vietnam, as well as between UIC and the Institute of Traditional Medicine (ITM), Ministry of Health, Lao People's Democratic Republic (Lao PDR). The stipulations and detailed institutional responsibilities entailed within these agreements (see Sects. 3.1 and 3.2.1 above) guided the acquisition of Phase II plant materials. During the plant acquisition process, joint fieldwork expeditions were conducted between a UIC botanist-pharmacognosist and a botanist of the host country institution (IEBR). In Vietnam, plant collection fieldwork has been carried out in the Hon Ba mountains (2004, 2006, 2008, 2011; 441 samples), the Kego Nature Reserve (2007; 216 samples), the Hoang Lien mountains (2009, 2010; 299 samples), the Nui Chua mountains (2010, 2011; 300 samples), and Xuan Son National Park (2015; 125 samples) (map shown as Fig. 8). In Laos, joint plant collection expeditions have been undertaken in 2015 (Xieng, Khouang, and Bolikhamsai provinces; 201 samples) and in 2017 (Bokeo, Savannhaket, and Attapeu provinces; 300 samples) (Fig. 8).

In cases where initial cytotoxicity tests of a particular sample demonstrated activity, a recollection of the active species in a larger quantity (several kilograms dried weight) was made for the isolation of the active compounds, and for scale-up and more detailed biological evaluation. In the NCDDG project (Phase I), altogether 177 plant recollections were made, while in the P01 project (Phase II), 65 recollections were made in Vietnam, and 6 in Laos. Altogether, 248 plant recollections have been made in our joint Phase I and Phase II projects. It should be noted, however, that in a recollection effort, it may be necessary to renegotiate an earlier enacted plant collection with different agencies in the source countries than previously, as was the case when the plant *Dichapetalum gelonioides* was recollected for our anticancer project from the Philippines in 2003 [98].

3.2.2 Selection of Plants for Collection

During Phase I of the collaborative project referred to in Sect. 3.2.1, the approach to selecting plants for collection was primarily biodiversity-based, which is known widely as a "random" approach. As the project moved forward and an increasingly large number of plants was collected, a gradual change of collection strategy was

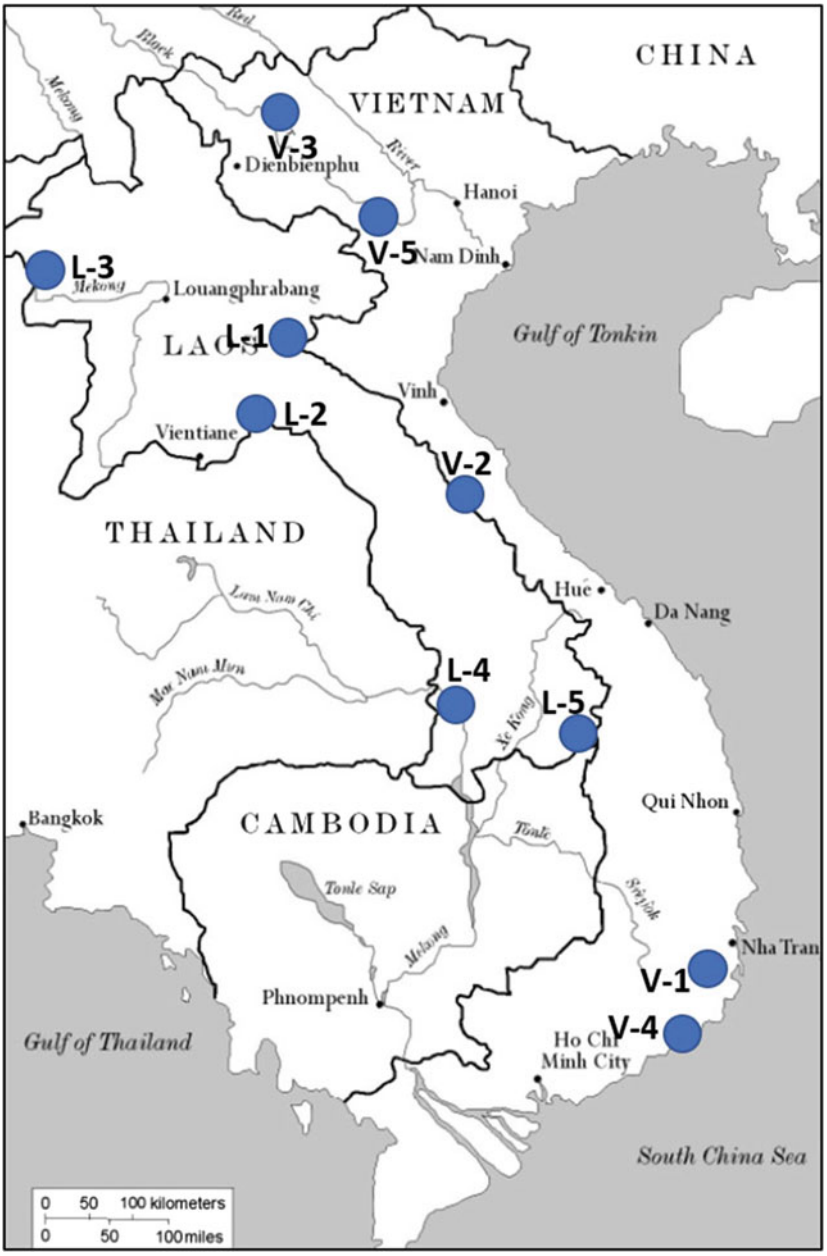


Fig. 8 Indo-China map showing the locations of the origin of primary samples collected for the U.S. National Cancer Institute program project entitled "Discovery of Anticancer Agents of Diverse Natural Origin" (2007-to date). **L-1:** Xieng Khouang Biodiversity Preserve (Xieng Khouang province, Laos, 100); **L-2:** Somsavath Medicinal Plants Preserve (Bolikhamsai province, Laos, 101); **L-3:** Bokeo Biodiversity Preserve (Bokeo province, Laos, 83); **L-4:** Svannhakhet Biodiversity Preserve (Savannhaket province, Laos, 110); **L-5:** Attapeu Medicinal Plants Preserve (Attapeu province, Laos,

implemented. An innovation was the introduction of a "Collection Manual" provided to plant collectors to serve as a guide in the collection process. Among the selection criteria introduced in the Collection Manual were: (1) avoidance of the collection of any species and genera that had previously been obtained and studied in the project, (2) avoidance of the collection of genera of no further interest based on literature review on the chemistry and experimental literature using the NAPRALERT database [99], and (3) collection of taxa native to the geographic locality, especially those with ethnomedical information pertinent to cancer, following a literature review (via the NAPRALERT database) of the species, unless the Collection Manual had listed them as being of no further interest. These collection criteria have continued through the running of the current Phase II project to this day. There has been one additional and innovative collection criterion added recently, namely, the collection of taxa that have traditional medicinal use in a broad sense (and not only cancer), based on previously available information from literature surveillance and previous ethnomedical fieldwork [100].

3.2.3 Logistics of Plant Collection Expeditions

Field methods to collect tropical plants for biomedical studies, specifically for the discovery of anticancer drugs during Phase I of our work, have previously been published [101, 102]. These same techniques have been used during the Phase II of our current anticancer drug discovery endeavor, in Vietnam and Laos [100]. During Phase I, in cases where a scientist based at the University of Illinois at Chicago (UIC) undertook plant collections, fieldwork was implemented jointly with appropriate institutional personnel of a source country institution. During Phase II, this modus operandi has remained same. Thus, in the Phase II portion of the work, joint fieldwork has been implemented between a UIC-based scientist/botanist and a botanist representing the institution bound by the Memorandum of Agreement, in this case, either the Institute of Ecology and Biological Resources in Vietnam or the Institute of Traditional Medicine in Laos.

In each of these fieldwork operations, diverse activities were planned and coordinated by the expedition leader (UIC-based) and co-leader (host institution-based). In all cases, the location of the targeted area for the expedition was planned and agreed upon well ahead of the field work conducted, based on floristic and other considerations. The field personnel normally consisted of the U.S.-based scientist, the host-based scientist, and one or more herbarium assistants. Communications

Fig. 8 (continued) 107). **V-1:** Hon Ba Forest Reserve (Khan Hoa province, Vietnam, 441); **V-2:** Kego Nature Reserve (Ha Tinh province, Vietnam, 216); **V-3:** Hoang Lien National Park (Lao Cai province, Vietnam, 299); **V-4:** Nui Chua National Park (Ninh Thuan province, Vietnam, 308); **V-5:** Xuan Son National Park (Phu Tho province, Vietnam, 125). Base map from: WaterproofPaper.com at https://www.waterproofpaper.com/printable-maps/world.shtml

between the host institution officer and the local authorities at the expedition areas normally ensued once an expedition plan, detailing the time frame, the scope, and personnel of the fieldwork had been agreed on.

During the implementation of the fieldwork, a series of activities need to be executed: travels and transportation from the host institution to the expedition area; a search to locate a base station in the expedition area; consultation with local authorities; recruitment of workers (including tree climbers) and carriers (in remote expedition locations); purchase of supplies and other provisions for fieldwork; purchase of materials for plant sample collecting and drying; purchase of materials for herbarium collection and processing; arranging for the shipment of collected materials (plant samples and herbarium specimens) and for the return transportation of the expedition personnel from the expedition base station to the host institution. In a major expedition, with a goal to collect 100–300 primary plant samples for initial screening, normally a large expedition staff (6–12 people; Fig. 9),

Fig. 9 Plant collection expedition at Kego Nature Reserve ("Kego"), Cam Xuyen District, Ha Tinh Province, Vietnam (November 29–December 12, 2008). An expedition team made up of 14 persons (designated Expedition Leader; 2 Expedition Co-leaders; 4 staff members of the Kego Nature Reserve; a motorboat driver; 6 workers and tree climbers) when in the process of departing from a collection location at the end of a day's foray. Names of expedition personnel: Vuong Tan Tu, M. Sc., IEBR—Designated Expedition Leader by IEBR; Dr. Tran Ngoc Ninh, IEBR—Expedition Co-Leader; Dr. D.D. Soejarto, UIC—Expedition Co-Leader; Mr. Tran Dinh Duc, staff member of Kego Nature Reserve; Mr. Nguyen Thaai Son, staff member of Kego Nature Reserve; Mr. Nguyen Van Duc, staff of Kego Nature Reserve; Mr. Vo Ta Nam, motorboat driver; Mr. Bui Duc Hui, Kego Nature Reserve—a ranger assigned to accompany expedition team; six workers and tree climbers (one guide, three workers, two tree climbers); photograph: D. D. Soejarto

that includes workers and tree climbers and one or more observers delegated by local authorities, was assembled.

Of the multiple activities to be conducted, the *most challenging part of the expedition* is the drying of the primary samples collected and the drying and processing of recollected plant samples. The main concern is to prevent contamination by fungal overgrowths, if the plant material is not fully dried. Several different types of procedures have been used for this purpose, but the most satisfactory method is to place plant samples at the time of collecting inside the containment of nylon mesh bags, and placing the bagged samples on a concrete floor during clear, sunny weather (Figs. 10 and 11). Sample bags must be spread out close to a door of a

Fig. 10 Primary plant samples contained in nylon mesh bags that are being dried on a concrete floor in a sunny day. Top: Blue-colored mesh bags were manufactured and used. Bottom: Colorless nylon mesh bags were manufactured and used; photographs: D.D. Soejarto

Fig. 11 During the rainy season, primary plant samples may be dried indoors by using electric heat (top) and by using a combination of electric heat and charcoal heat (bottom); photographs: D. D. Soejarto

building, so that it will be a relatively quick process to store samples in a dry place indoor in the event of any sudden downpour. If no concrete floor is available, a short-grass field or corrugated aluminum roofing may be used. During rainy days, indoor drying using artificial heat (charcoal or electric heat) may serve as an alternative (Fig. 11). Importantly, a worker may be hired to monitor the drying process and ensure its successful conclusion. Most plant samples will normally be fully dry in 3–4 days under good sunlight.

3.2.4 The Conservation Challenge

Another challenge to be faced during fieldwork operations to collect plants for anticancer or other biomedical investigations is to perform the collection work without endangering the environment and/or the local population of the species to be collected. Toward this end, our plant collecting practices have always integrated this vital consideration. This has been achieved primarily through the use of tree climbers (Fig. 12) to collect the aerial parts of a tree; by making a narrow longitudinal strip (Fig. 13) cut on one side of a tree trunk in sampling the stembark; by tracing the root to a distance (1–3 m) from the base of the tree in sampling the root (Fig. 14); by leaving the collection site in the same condition as it was found; and by completely extinguishing all campfires. In cases of the collection of rare species and small herbaceous plants, measures should be taken not to harvest all plants of a species from a single population.

Fig. 12 Tree climbers recruited and employed from a village near the collection locality to acquire the aerial parts of primary samples (LF + TW; FL; FR) from tall trees. Left: A tree climber in Nui Chua National Park, Ninh Thuan Province, Ninh Hai District, Vietnam, January 2010. Right: Hoang Lien National Park, Lao Cai Province, Sapa District, Sin Chai, Laos, May 2009; photographs: D. D. Soejarto

Fig. 13 Sampling of the trunk bark of a tree, by removing a narrow longitudinal strip. Top: Kego Nature Reserve, Ha Tinh Province, Vietnam, December 2008. Bottom: Nui Chua National Park, Vietnam, January 2010; photographs: D. D. Soejarto

Fig. 14 Collection of a root sample, with the root traced to a distance of 1–2 m from the base of the tree, and then cut. Top: Nui Chua National Park, Vietnam, January 2010. Bottom: Kego Nature Reserve, Vietnam, December 2008; photographs: D. D. Soejarto

3.3 Documentation Involved in Conducting Plant Collections in Tropical Countries

3.3.1 Plant Samples and Their Herbarium Vouchers

It is through the use of appropriate documentation that makes plant collection fieldwork a science. In the context of the present chapter, the goal of fieldwork is to collect documented raw plant materials to be submitted for anticancer testing.

Two types of plant material are to be collected. The first is a plant sample, referred to as the "primary sample", consisting of the various anatomical parts of the plant targeted. In case of a tree or a shrub, normally three parts, the leaf and twigs (LF +TW), the stem bark (SB), and the roots (RT) are collected (Fig. 15). This practice is based on theoretical considerations that bioactive phytochemicals are distributed and stored at different concentrations in different parts of the plant. Thus, by collecting samples from the three different primary plant parts, the most interesting compounds should not be missed. Each fresh sample collected is placed inside a zippered nylon mesh bag (Figs. 10 and 16), to allow confinement and prevent contamination with other samples being collected; this containment facilitates drying and handling. The porous mesh bag admits sunlight into the sample and allows moisture to escape during the drying process. Most plant-derived plant secondary metabolite constituents of potential medicinal value seem to be relatively stable to heat, so that the sun drying of primary or recollected samples at temperatures below 40°C is the preferred method of field drying (Fig. 10).

If abundant flowers (FL) and/or fruits (FR) are available for the desired plant, these also may be collected. For herbaceous species, one (whole plant including the underground part; WP) or two samples (aerial parts—PX; and underground parts roots/RT or rhizomes/RZ) may be collected. Each of these primary samples is collected at a minimum of 300–500 g projected dried weight, enough to generate sufficient quantities of extracts for initial biological testing. To acquire such a quantity, fresh plant material in the amount of 1–3 kg may need to be collected, depending on the type and nature of the plant material. For each sample, a plastic tag bearing the collection field number, the sequential sample number for that particular expedition, and the plant part, written using a permanent marker, is assigned (Fig. 16).

The second type of materials to be collected are the voucher herbarium specimens, consisting of an intact twig, bearing leaves and flowers and/or fruits (referred to as a "fertile" specimen) (Figs. 17–20). This should be pressed from the same plant (namely, from the same tree or shrub, or from the same population, in the case of herbs) from which plant samples are gathered. The presence of flowers and/or fruits enables the botanists participating in the expedition to narrow down the taxonomic identity of the plant collected. A voucher specimen that has no flowers and/or fruits (referred to as "sterile") is to be rejected. Each voucher herbarium specimen from the collection of a given organism bears a tag with the same number of collection written on it. For the purposes of plant anticancer bioprospecting, these voucher specimens

Fig. 15 Workers in action to chop leaf and twig (top), stem bark (center), and root samples (bottom) (Nui Chua National Park, Vietnam, January 2010); photographs: D. D. Soejarto

Fig. 16 Zippered bags made of nylon mesh as an ideal containment for the various plant parts collected. Top: Blue-colored bags were manufactured and used. Bottom: A white plastic slip bearing the collection number (DDS_13997), the plant part (LF + TW + FR), with the sequential number of collection of this primary sample being clearly visible, indicating that this primary sample is number 114 during this particular expedition; photographs: D. D. Soejarto

first and foremost serve to document the taxonomic identity of the plant species collected, and they can be consulted at any time as reference information to assist with future peer-reviewed publications and additional preclinical and clinical research.

Fig. 17 Two herbarium specimens as vouchers to document the primary samples DDS_14671, *Breynia vitis-idea* (Burm. f.) C.E.C. Fisch. (Euphorbiaceae) (top) and DDS_14662, *Hibiscus gagnepainii* Boiss. (Malvaceae) (bottom); photographs; D. D. Soejarto

As previously mentioned, in the event that a plant sample exhibits significant activity in an initial in vitro assay or assays, a recollection of the same plant part of this species is then requested. A recollection is made to acquire a larger quantity of plant material, ideally 3–5 kg dry weight, to generate an adequate amount of extract for scale-up isolation and more advanced biological testing. It is at this point that the

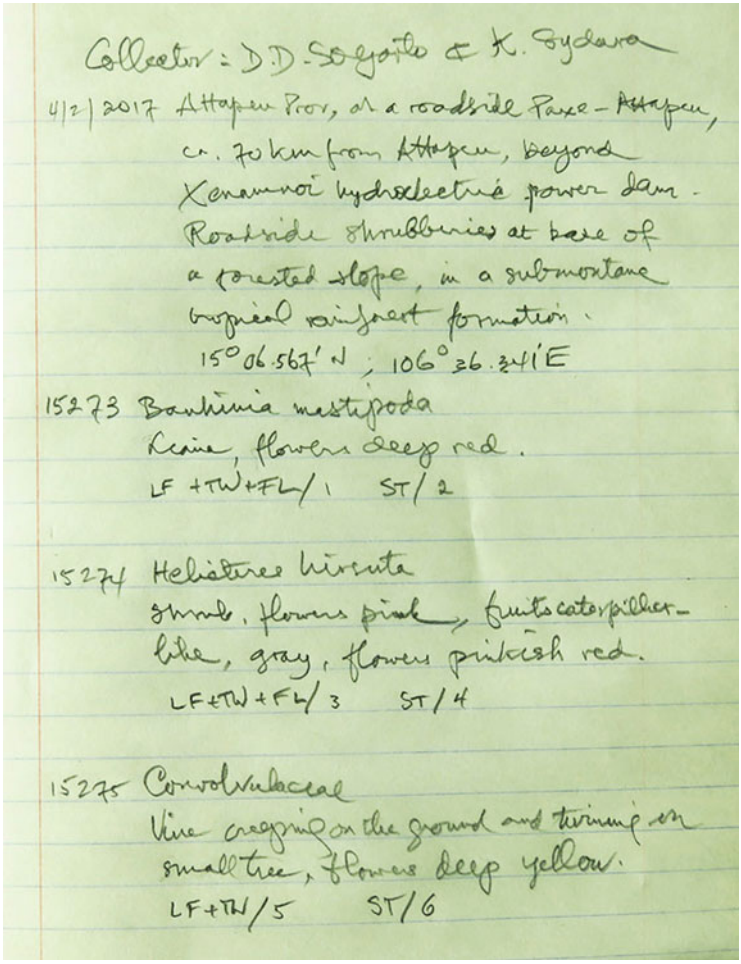


Fig. 18 An example of a page of field notes included in a Field Notebook at the time of collection to document sample collection numbers DDS_15273 (*Bauhinia mastipoda*) to DDS_15275 (a species of Convolvulaceae), made during a collection foray in Attapeu Province, Laos, April 2, 2017; photograph: D. D. Soejarto

voucher herbarium specimen of this active species becomes crucial to pinpoint the date and the geographic location of the original collection. In this way, recollection work can be undertaken such that it is assured the very same species, and ideally where possible the same individual organisms, form the basis for the new collection of the primary sample harvested earlier, but now in greater quantity. This leads to the greatest level of confidence possible that the initial test results can be reproducible and thereby facilitate bioactive compound re-isolation.

Fig. 19 A voucher herbarium specimen when pressed at the time of the collection of primary screening samples (Nui Chua National Park, Vietnam, January 2010); photograph: D. D. Soejarto

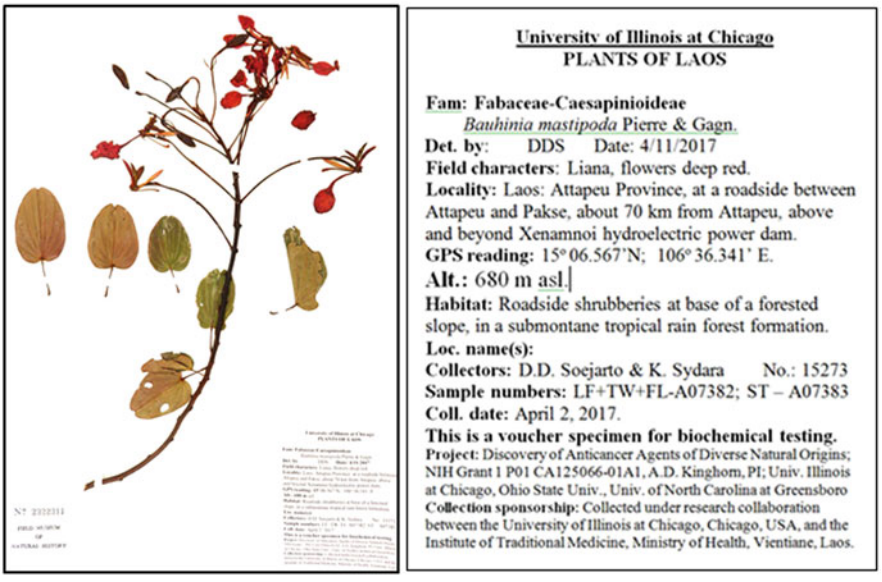


Fig. 20 A fully mounted voucher herbarium specimen of *Bauhinia mastipoda*. Left: Mounted specimen bearing a field label at the lower right hand corner. Right: A field label that is attached on the lower right hand corner of the mounted specimen, magnified; photographs: D. D. Soejarto

3.3.2 Field Notebook

As mentioned above, a voucher herbarium specimen must be pressed between newspapers to document the plant samples collected, while the field data are recorded in a field notebook (Fig. 18). Primary *botanical field data* include the number of collection, the date of collection, the name(s) of the collector, the collection location (country, province, municipality, pinpointed landmark), habitat, elevation above sea level, geographic coordinates (GPS reading), description or field characters of the plant collected, local name (if known), and the taxonomy (family, genus, species, if known). In terms of the taxonomy, this information is optional or provisional during the time of recording, since the exact taxonomic identity of the plant collected can only be ascertained by consultation with herbarium reference materials and guidebooks in a laboratory setting, following the conclusion of the expedition. It is imperative that the plant family be discerned either in the field or in the herbarium so that the taxonomic identity of the plant may be narrowed down further. The lack of a species identification precludes publication of a subsequent phytochemical-focused research article describing the chemical characterization and the biologically active isolates present.

As described previously, for every primary individual plant part sample or group of plant part samples gathered (for example, LF+TW, SB, RT from a tree), a series of herbarium specimens must be pressed (Fig. 20) and given the same number of collection as the primary sample. As a rule, at least three vouchers are pressed, for upkeep in different institutions, per the following guidelines: one specimen is to be deposited in the host country institution; the first duplicate specimen is to be deposited in the institution of the U.S.-based researcher; and the second duplicate is to be exchanged or gifted, in return for taxonomic identification or confirmation of identification. Often, however, collectors press more than three voucher herbarium specimens, for distribution to other major herbarium institutions, especially to those with collection foci in the country and/or floristic region where the expedition was undertaken. For all of our recent collection programs, one set of voucher specimens has been deposited at the herbarium institution of the Institute of Ecology and Biological Resources (IEBR) in Vietnam (located at IEBR; code: HN), and at the herbarium of the Institute of Traditional Medicine (ITM) in Vientiane, Lao PDR. A second set of collections from each country has been deposited at the John G. Searle Herbarium of the Field Museum (code: F) in Chicago, IL, USA.

3.3.3 Field Label Data

Following the conclusion of a field expedition, the botanical field data that were annotated in the field notebook (Fig. 18) during the course of the fieldwork are

converted into or transferred to a field label data form. The field label data form, or simply "field label", is compulsory and must be completed and assigned to each voucher herbarium specimen collected. Once the herbarium specimen has been mounted on a standard, acid-free herbarium sheet (42 × 29 cm), the field label is attached to the sheet (Fig. 20). In that condition, a mounted specimen with the field label attached, normally in the lower right hand corner of the sheet, serves in perpetuity as the record of the plant collected. Such a specimen is then stored and curated in the permanent collection of the institution. A herbarium specimen without a field label is useless and is to be discarded.

3.4 Processing of Collected Materials and Considerations Concerning the Export and Import of Plant Materials

3.4.1 Processing of Collected Plant Samples

As described previously, in our fieldwork operations, zippered nylon mesh bags of various sizes are used as containment of the plant samples collected for biological testing. For leaf and twig samples (LF+TW), in order to achieve the weight required (up to 500 g dry weight), the amount of fresh leaf and twig to be collected is large and bulky, for which a large bag is required. For solid materials such as stems (ST), stem bark (SB), and roots (RT), a smaller bag is adequate. It may be reiterated that adequate sample drying on any dry, exposed, and sunlit surface, such as a concrete floor, is most ideal.

Once the plant materials are fully dry, they are transferred into cloth bags, in order to maintain sample aeration and to prevent molding. Each cloth bag is labeled with the collector-collection number and the plant part. A plastic bag containment is not desirable, due to the sample molding that may develop under tropical conditions, leading to damage of the plant material. If the fieldwork operation continues in the same general area for several more days, such packed samples may be stored by arranging them on a bench (Fig. 21) or by hanging them along wires in a ventilated location. At the completion of the expedition, these packed samples are transferred by groups into several large rice sacks for shipment (by air or by land) to the host institution. On arrival at the host institution, the rice sacks containing the packed samples are opened and the cloth-packed samples may be stored in a ventilated location or may continue to be dried in sunlit areas.

Fig. 21 Packed primary samples arranged on a bench to ventilate, before being packed in boxes or rice sacks for dispatch from the field base to the laboratories of the host-country institution; photograph: D. D. Soejarto

3.4.2 Processing of Voucher Herbarium Specimens

In casual, day-to-day plant collection work, one or more voucher herbarium specimens, each with a collection number tag affixed, are placed between newspapers and pressed in the plant presses. Then, the plant presses containing the pressed specimens are placed in the sun to dry.

Such a method, however, is not workable during a large-scale collection expedition, where dozens of voucher specimens may be gathered daily. Therefore, in our expedition we use the so-called "wet method" of collection. In this process, herbarium vouchers inside their newspapers, upon arrival at the collection base after a day's work, are packed into bundles and placed inside a heavy-duty plastic bag. Then, diluted alcohol (50 parts industrial alcohol + 50 parts water, *v/v*) is poured into the bag until all parts of the bundled specimens are soaked (Fig. 22). When this is performed immediately after arrival from the field at the field base, the alcohol penetrates into and fixes the live tissues, and leaves the specimens intact. The plastic bag containing the bundles of herbarium specimens is then closed and shaken to achieve an even penetration of the alcohol. Once accomplished, the bag is fully closed and tightly sealed. Under these conditions the preserved specimens may stay in good condition for a month or longer.

Fig. 22 Images displaying the so called "wet method" in collecting voucher herbarium specimens, in order to preserve the specimens in the field, at the conclusion of a day's foray. Bundles of preserved specimens are opened and the specimens re-arranged and re-pressed upon arrival at the host institution's laboratories. Top: Soaking bundled specimens with 50% alcohol. Center: Condition of a fixed voucher specimen upon opening the package of the treated specimens; in this condition the specimen can be manipulated the same way as in the fresh condition. Bottom: Changing the soaked newspaper with a new, dry one and re-arranging the specimens for drying. See text for details; photographs: D. D. Soejarto

Upon arrival at the host institution, the plastic bags containing the fixed herbarium specimens may be opened and the specimens removed one by one. Then, each specimen is straightened out and re-arranged as desired, after the wet newspaper has been replaced with a dry one. Each specimen inside the newspaper is "sandwiched" between carton sheets or between corrugated aluminum sheets, and stacked up, to be dried inside a drying chamber (Fig. 23). In the absence of a drying chamber, packets of specimens with newly replaced newspapers may be dried in the sun, inside the plant presses. Once the herbarium specimens are fully dry, they are sorted, labeled, and distributed to designated institutions.

3.4.3 Exporting and Importing Collected Plant Materials

During our fieldwork in Vietnam, for each expedition, dried plant samples collected were packed inside cloth bags, and in turn, 20–30 such bagged samples were packed inside a large carton box for shipment from Hanoi to Chicago. A shipment consisting of 5–10 carton boxes of primary samples and their herbarium vouchers was dispatched by air freight, addressed to the following location with appropriate facilities: The University of Illinois Pharmacognosy Field Station, 21W042 Finley Road, Downer's Grove, IL 60515-1767, USA. Documents that accompanied the shipment included: (1) a statement from our host country institution in Vietnam (the Institute of Ecology and Biological Resources of the Vietnam Academy of Science and Technology, Hanoi) that the plant samples and the herbarium specimens in the shipment had been heat sterilized, (2) a list of the plants contained in the shipment (species, if known, otherwise genus-family or family only), (3) a United States Department of Agriculture (USDA) plant import permit number P587-081020-008, and (4) a phytosanitary certificate issued by the Vietnam Customs and Plant Inspection [103]. When each shipment arrived at the port of entry (Seattle or Los Angeles), U.S. Border Protection (USDA and U.S. Customs officers) examined the shipment and, finding all of these documents in the shipment, would then release the shipment for forwarding to O'Hare International Airport in Chicago. The airline company that forwarded the shipment would place a telephone call to the University of Illinois (UIC), and either a university broker or a staff member of the UIC Field Station would go to the airport cargo bay to pick up the carton boxes. Upon arrival at the UIC Field Station, the boxes were opened, and the samples and voucher herbarium specimens were subsequently inventoried (Fig. 24).

Fig. 23 Drying voucher herbarium specimens. Top: After the wet and often torn newspaper has been replaced with new and dry sheets, the voucher herbarium specimens are then bundled between cardboard sheets and dried in a ventilated drying box heated by several light bulbs at the host institution's laboratories. Bottom: When an electric drying box is not available, voucher specimens may be dried in the sun; photographs: D. D. Soejarto

Fig. 24 On arrival in Chicago, at the University of Illinois Pharmacognosy Field Station, boxes containing the primary samples and their voucher herbarium specimens are opened and the contents removed and inventoried; photograph: D. D. Soejarto

3.5 Specific Phytochemical Considerations

Once a plant that has been collected in a tropical country is received in the phytochemical laboratory in a dried and powdered form, it is necessary for this processed material to be extracted with solvent in a standardized and reproducible manner. This is the first step toward the subsequent purification of given compound or compounds responsible for any biological activity germane to cancer that may be associated with a particular plant part. Of the various major organism types that commonly are utilized for anticancer and other drug discovery studies (e.g. terrestrial microbes, higher plants, and marine organisms), however, the chemical work-up of higher plants is complicated by the occurrence in many species of plant polyphenols, which are also known colloquially as "vegetable tannins". These are constituted by two major groups, condensed proanthocyanidins and galloyl and hexahydroxydiphenoyl esters and their derivatives [104, 105]. These compounds are water soluble, with molecular weights of between 500 and 3000, and are able to precipitate gelatin and other proteins [104, 105]. Plant polyphenols do have value as drug substances, as exemplified by the oligomeric proanthocyanidin mixture, crofelemer, from the South American plant, *Croton lechleri,* which was approved by the U.S. FDA recently under the "botanical drug category" for the symptomatic relief of non-infectious diarrhea in adult patients with HIV/AIDS who are taking antiretroviral therapy [106]. However, in the laboratory, plant polyphenols are difficult to separate and are complex molecules that require considerable time and effort to characterize structurally (e.g. [107, 108]). Moreover, these compounds tend to provide false positive results in enzyme-inhibition assays, so procedures have been developed to remove plant polyphenols from plant extracts to be screened in bioassays related to anticancer drug discovery [109, 110]. Examples of the types of laboratory processing that may be applied to plant extracts to remove tannins include passage over polyamide [111], Sephadex LH-20 [109], and polyvinylpyrrolidone [109], as well as the use of caffeine [109] or a saline wash (1% NaCl solution) in the plant solvent partitioning process [110].

There are many different approaches that may be involved in the operation of an efficient solvent extraction procedure for a powdered plant, and this may be accomplished using various procedures such as maceration, percolation, or extraction using a Soxhlet apparatus [112]. With the judicious selection of solvents it is possible to produce extracts that are enriched in compounds such as alkaloids or saponins [112]. A basic extraction procedure developed in the laboratory of the late Morris Kupchan is used widely in natural product drug discovery projects, using a minimal number of solvents of different polarities, and is referred to widely as a "Kupchan" partition scheme (e.g. [113]). The late Monroe Wall and colleagues developed a modification of this process in which the final chloroform partition is treated with 1% sodium chloride solution in water, which partially removes any vegetable tannins present [110], which is currently used in our laboratory and is summarized in Fig. 25. The Kupchan partition scheme and the above modification are advantageous for anticancer drug screening purposes, since only a relatively few partitioned dried

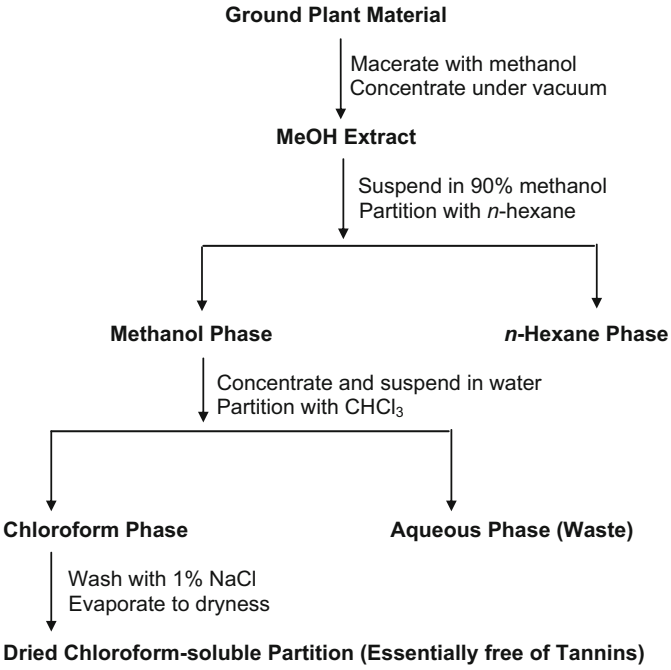


Fig. 25 Modified initial solvent extraction and partition scheme suitable for the preparation of small-scale samples of powdered plant materials for initial biological screening

extracts are obtained for subsequent bioassay, which reduces the workload for a given project involving multiple plant acquisitions. Other methods for the initial processing of powdered plants include steam distillation, ultrasound-assisted and pressurized extraction methods, and supercritical fluid extraction [114, 115].

Of the various major classes of organisms that can be used for anticancer drug discovery, plants have the largest metabolome, having about 25% of their genes encoding specialized secondary metabolites [116, 117]. It has been estimated that as many as 40,000 different compounds may be present in a single plant leaf [117]. When this point is considered along with the large number of known plant-derived small-molecule organic compounds mentioned earlier, it is normally necessary to perform several sequential chromatographic separation steps to purify one or more bioactive compounds present with potential anticancer activity. To facilitate this process, the technique of "bioactivity-guided fractionation" is most often applied, which has been defined as the "process of isolating active constituents from some type of biomass (plants, microbes, marine invertebrates, etc.) using a decision tree that is dictated solely by bioactivity" [118]. Several very early pioneers of this technique in the United States who focused on anticancer agent discovery from plants included the late Monroe Wall and Mansukh Wani (Research Triangle Institute, North Carolina) [22, 119, 120], the late Morris Kupchan (University of Virginia and earlier the University of Wisconsin-Madison) [43, 113], Richard

Powell and the late Cecil Smith, Jr. (U.S. Department of Agriculture, Peoria, Illinois) [40], George R. Pettit (Arizona State University) [77], and the late Jonathan Hartwell (National Cancer Institute, Bethesda, MD [121, 122]. Later on, several additional prominent groups in the U.S. applied this separation strategy to plant constituents with potential antitumor activity. Typically, the purification of plant-derived potential antitumor agents required also the initial large-scale gravity-column chromatography of a bioactive dried solvent partition over silica gel, followed by further separation by additional chromatography, leading to ultimate compound purification by crystallization or preparative thin-layer chromatography [123–128]. More recently, a more varied selection of stationary phase materials has become available for the chromatographic purification of potential oncolytic agents from plants, and semi-preparative high-performance liquid chromatography may be used for final compound purification (e.g. [129–131]).

The landscape in terms of natural products separation from extracts is changing rapidly, and many efforts have been made to conduct this process in a quicker and more efficient manner than previously. Some of these new approaches may be mentioned briefly. A key concern is "dereplication" [132], which has been defined as the "process of determining whether an observed biological effect of an extract or specimen is due to a known compound" [118]. Descriptions on natural product dereplication procedures for crude extracts have appeared in which constituents of plants with known or potential anticancer activity have been included, with mass spectrometry data acquisition coupled to standard compound databases being a major strategy for this purpose (e.g. [133, 134]). "Molecular networking", a process in which MS/MS data are organized on the basis of chemical similarity, has been found to be a very useful addition to more conventional natural products dereplication procedures [135], and may be applied to plant constituents [136]. Additional approaches to identify already known plant constituents occurring in crude extracts also have focused on NMR spectroscopy (e.g. [137, 138]).

Refinements in spectroscopic and spectrometric methods [139–142] have permitted the structure elucidation of natural products in ever-lower quantities. For example, using NMR microcryoprobes, useable spectra to assist in compound molecular characterization may be obtained with as little as 20 μg of material [9]. Other advances in the application of NMR spectroscopy for natural products structure elucidation include the availability of automated databases, inclusive of the "CSEARCH" database that contains numerous ^{13}C NMR spectra [143]. Also, there have been enhancements in the number and types of chiral derivatizing reagents that can be used to assign the absolute configuration of plant-derived and natural products that contain secondary alcohol functionalities [144, 145]. For many years, single-crystal X-ray crystallography has been applied as a confirmatory tool for the structure elucidation of many plant-derived natural products, including those with potential anticancer activity [e.g. 119, 120]. While there are X-ray crystallographic methods that may lead to the assignment of the absolute configuration of a new natural product, over the last 15 years electronic circular dichroism (ECD) is a chiroptical method that has become widely utilized for this purpose [144]. Using this method, time-dependent density functional theory calculations are produced, and

conclusions on the absolute configuration of a given compound may be made by comparison of experimental and calculated ECD spectra [144]. Unlike ECD, vibrational circular dichroism requires no chromophore to occur in the UV-visible region, and exhibits high resolution and conformational stability, and is applicable to a wide range of plant secondary metabolite structural types [144, 146].

Owing to the molecular complexity of plants and their extracts noted earlier, it is usual for investigators to report several new potential anticancer agents simultaneously in the same scientific communication [e.g. 147–149]. However, it is quite rare for an investigator group to report a totally new chemotype from a higher plant sources with in vivo activity germane to cancer. Three examples of such important lead compounds include the quinoline alkaloid, camptothecin (**28**) (1966; Fig. 26) [119], the acetogenin, uvaricin (**29**) (1982; Fig. 26) [126], and the cyclopenta[*b*]benzofuran, rocaglamide (**30**) (1982; Fig. 26) [150]. The enormous impact of camptothecin on oncology drug development has been noted earlier in this chapter [22, 30–32, 35, 51–59], and its discovery also had a major positive influence on the field of natural products chemistry. Although no analogs of uvaricin or rocaglamide have gained market approval as cancer chemotherapeutic agents to date, extensive efforts have been made to develop derivatives of these major lead compounds for the potential treatment of cancer [151–156].

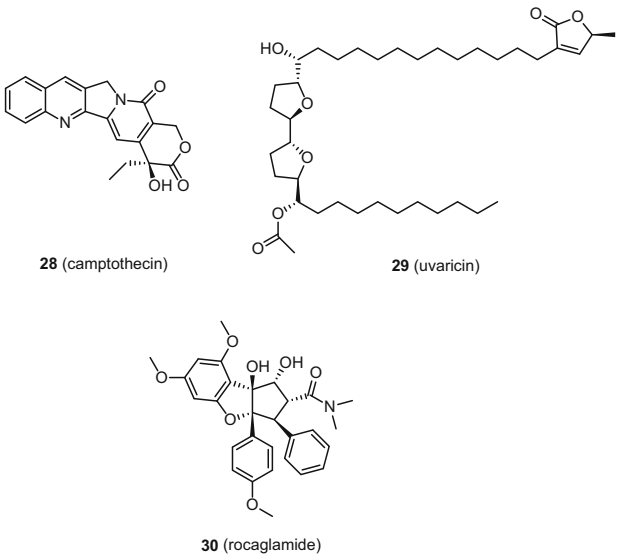


Fig. 26 Structures of three examples of plant-derived secondary metabolites (**28**–**30**) with antineoplastic activity each representing a new chemotype

3.6 Biological Testing of Initial Extracts, Chromatographic Fractions, and Purified Constituents and Their Analogs

In the early years of their investigation, purified plant-derived natural products with potential anticancer activity were tested in vitro against a restricted number of human and murine cancer cell lines, followed by in vivo testing. For example, when it was first isolated, camptothecin (**28**) (Fig. 26) was evaluated against the L1210 murine leukemia cell line in vitro, in addition to this same murine cell line in vivo coupled with the Walker 256 mammary carcinoma in vivo assay using rats [119]. Such testing was performed through the auspices of the U.S. National Cancer Institute (NCI), and this workflow was incorporated into the in vivo prescreening of crude extracts. Later on, NCI replaced the Walker 256 assay since it proved to be overly susceptible to the presence of plant tannins, and this was substituted by the P388 murine leukemia cell line in vivo [157]. In an insightful review article, the late Matthew Suffness and the late John Douros provided useful terminology in relation to the outcome of various types of in vitro and in vivo tests of this type, and their definitions still hold true today. Thus, "cytotoxicity" was defined as "toxicity to tumor cells in culture", with the terms "antitumor", "anticancer", and "antineoplastic" not being regarded as applicable to in vitro results. After in vivo evaluation in tumor-bearing experimental animal models, Suffness and Douros applied the terms "antitumor" or "antineoplastic" to pure active compounds [157]. These authors preferred to reserve the term "anticancer" for compounds that tested successfully in clinical trials in man, in order to avoid imbuing potentially false hopes that cancer patients might have in otherwise incompletely tested drug candidates [157].

In the late 1980s, a large-scale in vitro approach was adopted at the U.S. NCI to replace the transplantable murine models used hitherto, involving 60 difference cell lines representative of nine distinct tumor types: breast, CNS, colon, leukemia, lung, melanoma, ovarian, and prostate [158]. By 2010, this "NCI60" screening procedure had been applied to some 100,000 pure compounds and 50,000 natural product extracts [159]. In addition to providing screening data for crude natural product extracts and compounds of interest, use of the COMPARE algorithm with the NCI60 assay also permits some mechanistic information to be obtained, since patterns of sensitivity and expression within the panel may be compared with those of standard cancer chemotherapeutic agents [158–160]. As an example of the use of this method, the cytotoxic compound camptothecin (**28**) (Fig. 26) and a methoxylated derivative were discovered to occur in a new botanical source that was housed in a plant extract repository at the U.S. NCI, Frederick, MD, namely, *Pyrenacantha klaineana* (Icacinaceae) [161].

There have been efforts to use mechanism-based screens rather than cancer cell lines to enhance the discovery process of plant-derived natural product lead compounds. For example, a microbiological screen used for its simplicity and speed of use involved a differential response of DNA repair-deficient and repair-proficient yeast (*Saccharomyces cerevisiae*) strains to natural product test samples [162]. Also, based on an earlier procedure developed for several bleomycin derivatives [163], a

DNA stand-scission assay was modified for potential plant-derived anticancer agents by the Wall and Wani group at Research Triangle Institute as a companion procedure to the determination of cytotoxicity using a cancer cell line [164]. In previous collaborative work between our own laboratories with two major pharmaceutical companies, bioactive principles of tropical plants were determined using farnesyl transferase (FTP) [165], recombinant human ligase I [166], and proteasome [167] inhibitory activity activities. More recent target-based bioassays that have been utilized for plant compounds in our natural anticancer drug discovery program include those involving disruption of the mitochondrial membrane potential (MMP) [168], inhibition of nuclear factor-kappa B (NF-κB) (p65) in an enzyme-linked immunosorbent assay (ELISA) [169], the induction of semaphorin B [170], and the stimulation of natural killer (NK) cells [171]. It is considered that in an effective program for the discovery of potential new cancer chemotherapeutic agents from plants, the use of as broad a range of cell-based and target-based in vitro bioassays as possible is advantageous.

Much has been written about how the established chemical diversity of plant and other natural product extracts can be harnessed using "high throughput screening" (HTS), in which large numbers of samples can be evaluated in an automated manner for biological activity or other endpoint (e.g. [7, 9, 139, 172, 173]). Of the in vitro HTS assays used widely, these are mainly either cell-based or biochemical in their design [172]. One way of enhancing the chances of gaining positive results with HTS assays is by "prefractionation" of a crude extract, in which a small number of fractions is produced, usually with different polarities, with separation by a technique such as high-performance liquid chromatography [172, 173]. This preliminary procedure has the advantages of concentrating less abundant biologically active substances and removing compounds that might interfere with an assay that depends on fluorescence detection, if used [173].

A particular challenge is to demonstrate that a promising in vitro-active plant secondary metabolite also has activity in a cancer related in vivo model, such as a human tumor xenograft bioassay in mice [174, 175]. However, a traditional xenograft assay may be expensive to perform in terms of the purchase and upkeep of the test animals involved. To partially offset this, an in vivo hollow fiber assay was developed by investigators at the NCI Developmental Therapeutics Program as an intermediate step to help predict which compounds found active in the NCI60-panel might also be active in a subsequent xenograft system [176, 177]. The in vivo hollow fiber assay has now been adopted in laboratories in many countries around the world for this purpose [178]. This procedure involves the propagation of human cancer cells in inert hollow polyvinylidene fluoride fibers with pores that are small enough to retain the cancer cells but large enough to permit entry of potential chemotherapeutic drugs. Fibers containing proliferating cancer cells are transplanted into the peritoneum (intraperitoneal implantation) or under the skin (subcutaneous implantation), with the immunodeficient host mice treated with a test agent, and the fibers are subsequently retrieved for analysis of viable cell mass [178]. In order to assist with enhancing the solubility of some natural products, it is possible to perform co-precipitation with polyvinylpyrrolidone (PVP). This assay has advantages in that

different types of cancer cells can be included in the hollow fibers used, and it is relatively rapid and inexpensive to perform and, crucially for rare natural products, is economical in terms of the compound quantities required [178]. The in vivo hollow fiber assay has been performed successfully on a wide spectrum of natural product structural types in our research efforts directed toward the discovery of anticancer agents from plants, using a standard protocol transferred from the U.S. NCI, Frederick, Maryland [178, 179]. Among the compounds from our laboratories shown to be active (with more than 50% growth inhibition) for one or more types of cancer cells using the in vivo hollow fiber assay are 3-chlorodeoxylapachol (**31**) (Fig. 27) [180], goyazensolide (**32**) (Fig. 27) [181, 182], 13-hydroxy-15-oxozoapatlin (**33**) (Fig. 27) [179, 183], and (+)-strebloside (**34**) (Fig. 27) [184]. However, for various reasons, including a lack of structural novelty, or in having a well-established mechanism of action evident, or as a result of the paucity of substance available for additional laboratory work, these four compounds have not been developed any further in our project. Additional examples of compounds from our program found to be active in hollow fiber and/or murine xenograft in vivo assays that have been subjected to additional development efforts will be mentioned in Sect. 4 of this chapter.

To assist with the design of the experimental protocols for the in vivo testing of a potential antitumor agent of plant origin, preliminary pharmacokinetics experiments may prove useful. As an example of this, taccalonolides AF (**35**) and AJ (**36**) (Fig. 28) were compared for their pharmacokinetic parameters in the serum of 6–7 week-old female athymic mice, after tail vein injection [185]. They showed quite different elimination half lives, with values of 44 and 8.1 min found for **35** and **36**, respectively. The two compounds were also tested in a murine xenograft model using SCC-4 oral cancer cells, and, for this experiment, these substances were formulated in phosphate buffered saline (PBS) in 8% ethanol. It was concluded

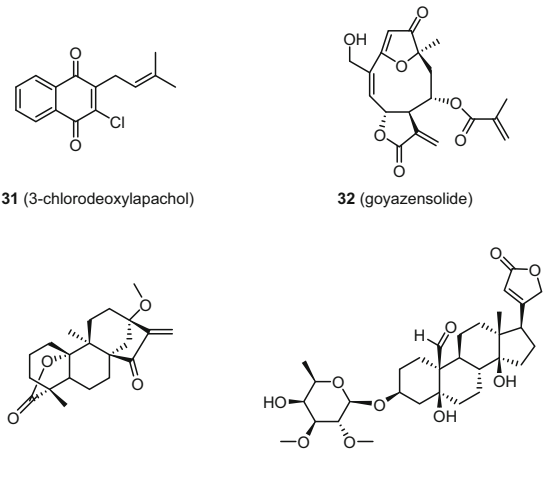


Fig. 27 Structures of four plant constituents (**31**–**34**), while showing positive test data in an in vivo hollow fiber assay used, but were not progressed further

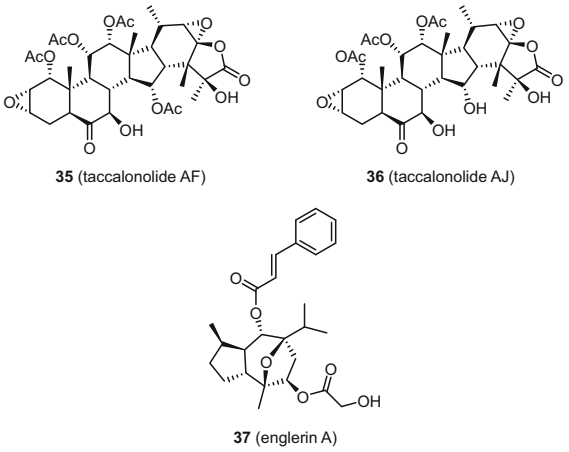


Fig. 28 Structures of taccalonolide AF (**35**), taccalonolide AJ (**36**), and englerin A (**37**)

that the superior efficacy in vivo of **36** relative to that of **35** was due to its substantially longer half-life in vivo [185].

It is highly informative if the exact mechanism of action of a potential plant-derived anticancer agent can be established in the cancer cell early in the drug discovery process. However, this may not be a simple task for a compound that does not exhibit a pattern of selectivity in the NCI60 cell panel consistent with one or more standard anticancer agents with known mechanisms [160]. A good example of this is englerin A (**37**) (Fig. 28), a guaiane sesquiterpene ester with selective inhibition of renal cancer cell lines obtained at the U.S. National Cancer Institute-Frederick, Maryland as a result of isolation work on the Tanzanian plant, *Phyllanthus engleri* [186]. From detailed cellular observations in sensitive cancer cell lines in the decade since its isolation and characterization, the inhibitory of activity of **37** has been correlated with the modulation of the transforming growth factor (TGF)-β1-induced epithelial-mesenchymal transition, protein kinase C θ, and transient receptor potential canonical 4/5 (TRPC4/TRPC5), among other possible modes of action [187].

By conducting a specific mechanism of action study, it is practical to probe in some detail the effects on cellular signaling by cytotoxic natural products. To take one example of such a compound investigated in this manner by our group, the *C*-benzylated flavanone, dichamanetin (**38**) (Fig. 29) may be discussed. In a phytochemical study on a sample of the aerial parts of *Piper sarmentosum* collected in Vietnam, dichamanetin was isolated in a very high yield (0.29% *w*/*w*), and found to exhibit an IC_{50} value of 3.6 μ*M* in a JC-1 mitochondrial transmembrane potential (MTP) bioassay [168]. This compound was then studied using a combination of biological methods to better comprehend how it exerts its antiproliferative effects, partly due to its availability in quantity and also because of the limited information on this plant natural product in the literature [188]. Initially, **38** was of interest as an antimicrobial agent, and, when isolated from *Uvaria chamae*, demonstrated inhibitory effects on the growth of three bacterial strains [189]. The racemic form of

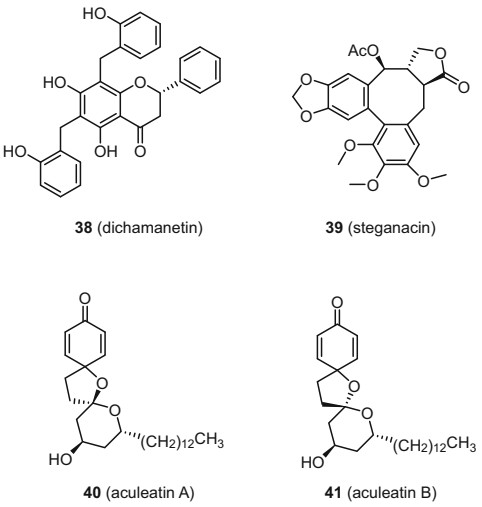


Fig. 29 Structures of dichamnetin (**38**), steganacin (**39**), and aculeatins A (**40**) and B (**41**)

dichamanetin was synthesized, and this compound was found to target *Escherichia coli* FtsZ GPTase activity, with an IC_{50} value of 12.5 μ*M* [190]. McLaughlin and associates isolated dichamanetin (**38**) from *Melodorum fruticosum*, and showed this phenolic substance to be cytotoxic when tested against HT-29 human colon cancer cells (ED_{50} 5.1 μg/cm^3), in addition to showing lethality to brine shrimp (*Artemia salina*) larvae (LD_{50} 3 ppm) [191]. It was found in the mechanistic study by our collaborative project that **38** reduced the viability of human HT-29 colon, DU145 prostate, and MDA-MB-231 breast cancer cells, in a time- and dose-dependent manner, and induced G_1 arrest in the cell cycle. It was also determined that the selective cytotoxic effect of this compound in the cancer cells used is mediated by the induction of oxidative stress, by using a colorimetric assay to measure reactive oxygen species levels in HT-29 cells. This work involved several different types of evaluation, which were performed using enzyme-linked immunosorbent (ELISA) assays, as well as cell sorting and western blot procedures [188].

It is important to determine if a newly isolated biologically active plant compound acts mechanistically in one of the same major ways as established anticancer agents. For example, the vinca (bisindole) alkaloids and the combretastatins act as tubulin polymerization inhibitors, while the taxanes are tubulin polymerization promoters, also known as microtubulin stabilizers [26, 33, 192]. The taccalonolides, which were referred to earlier in this chapter, are a relatively new class of plant-derived microtubule stabilizers, and are a group of oxygenated pentacyclic steroids that are found in the genus *Tacca* [131, 185, 193]. These compounds were first described in 1987 from the Chinese plant, *Tacca plantaginea* [194], and two representative members of this series when isolated from *Tacca chantrieri* were demonstrated as being able to increase the density of cellular microtubules in interphase cells and to form thick bundles of microtubules [195]. Among the methods that may be used to

demonstrate that a pure plant compound or extract interacts with tubulin is a turbidimetric tubulin polymerization assay, in which purified porcine brain tubulin in a buffer containing GTP is polymerized in the presence of the test compound, with the resultant absorbance determined on a spectrophotometer [196].

Another example of a screening procedure for a major cancer target that can be applied to plant constituents of interest involves the enzyme DNA topoisomerase II (topo II), which is an established molecular target of the plant-derived anticancer drug, etoposide (**6**) (Fig. 1) [29]. Etoposide causes DNA damage through "poisoning" or interfacial inhibition of topo II [29, 171, 197]. Using a standard procedure [197], this phenomenon may be evaluated for a pure plant natural product molecule by trapping topo II-plasmid DNA covalent complexes with sodium docyl sulfate, digesting the enzyme using proteinase K, and releasing cleaved DNA, which is then examined by fluorescence using gel electrophoresis on an agarose gel [171].

One of the advantages of performing activity-guided fractionation procedures on a plant extract is that it is common to purify in the same investigation several less abundant structural analogs of a major bioactive compound with the cancer-related bioassay used. By testing all structural analogs obtained by isolation from a given plant, it is possible to conduct a preliminary structure-activity relationship study, normally in relation to the differential cytotoxic potencies of compounds when evaluated against a panel of cancer cell lines. For example, in an early study performed in our collaborative project to discover anticancer agents from plants, six dibenzocyclo-octadiene lignans were isolated from the stem bark of *Steganotaenia araliacea*, collected in Zimbabwe. Using ASK astrocytoma cells to monitor purification [157], three previously known and three new lignans were isolated [198]. These isolates were evaluated as pure entities not only in the ASK assay, but also for cytotoxicity against a panel of 11 cancer cell lines, and for in vitro inhibition of microtubule assembly. The previously known compound steganacin (**39**) (Fig. 29) proved to be the most active among the substances purified in all three of these assays. As a result of having a number of dibenzocyclo-octadiene lignan derivatives tested simultaneously, it was found that the resultant biological activity decreased among the members of this compound group when a C-5 α-methyl group was present or if the C-6, C-7 lactone ring was cleaved [198].

When a biologically active plant natural product lead compound is isolated in a significant amounts, it may be suitable for analog development in order to increase structure-cytotoxicity relationship information. The known dioxodispiroketal derivatives aculeatins A (**40**) (Fig. 29) and B (**41**) (Fig. 29) were isolated from the leaves of *Amomum aculeatum* collected in West Java, Indonesia, along with several new compounds [199, 200]. Aculeatin A (**40**) was isolated as an antimalarial agent from the same plant by the Sticher group that was formerly at ETH, Zurich [201], and, along with several analogues, has been subjected to more than 20 total synthesis procedures to date (e.g. [202–204]). In our work on *A. aculeatum*, **40** was obtained in over a 300 mg quantity from about 800 g of the dried plant leaves. A photograph of a voucher specimen representing this collection is shown in Fig. 30. In addition to being shown as cytotoxic against three cancer cell lines, **40** inhibited the growth of

Fig. 30 Photograph of a voucher specimen of *Amomum aculeatum* Roxb. (Zingiberaceae). This specimen is presently in deposit at the John G. Searle Herbarium of the Field Museum of Natural History (FM), Chicago, IL. Top: Photograph of the herbarium specimen (FM accession number: 2285577). Bottom: Field label magnified; photographs: D. D. Soejarto

MCF-7 breast cancer cells (10–60%) over the dose range 6.25–50 mg/kg, when admistered intraperitoneally. Using the residual amounts available of **40** several semisynthetic analogs were prepared by standard methods of preparation. However, none of the new dioxodispiroketal natural products nor any of the analogs generated exhibited greater potency as a cytotoxic agent when compared with **40** [199, 200]. This compound has not been further developed as a potential anticancer agent, however, since it was found not to be active when evaluated in two different xenograft assays in tumor-bearing mice [200].

There is a very large literature on the biological testing of derivatives of plant natural products produced by chemical synthesis methods, for their potential anticancer activity. While a detailed discussion of these reports is beyond the scope of the present chapter, the range of structural types of plant secondary metabolites deemed worthy for development as potential oncology agents is quite diverse and shows considerable promise for the future. Examples of compound types for which synthetic analogs have been generated and evaluated biologically, and are representative of structural classes not previously mentioned in the present contribution, include the sesquiterpenoid nortrilobolide (**42**) (Fig. 31) [205], the *ent*-kaurene diterpenoid, oridonin (**43**) (Fig. 31), [206], derivatives of the stilbenoid, schweinfurthin A (**44**) (Fig. 31) [207], analogs of neo-tanshinlactone (**45**) (Fig. 31) [208] and of the *Tylophora* phenanthrene-based alkaloid, tylophorine (**46**) (Fig. 31) [209], and the phthalideisoquinoline alkaloid, noscapine (**47**) (Fig. 31) [210].

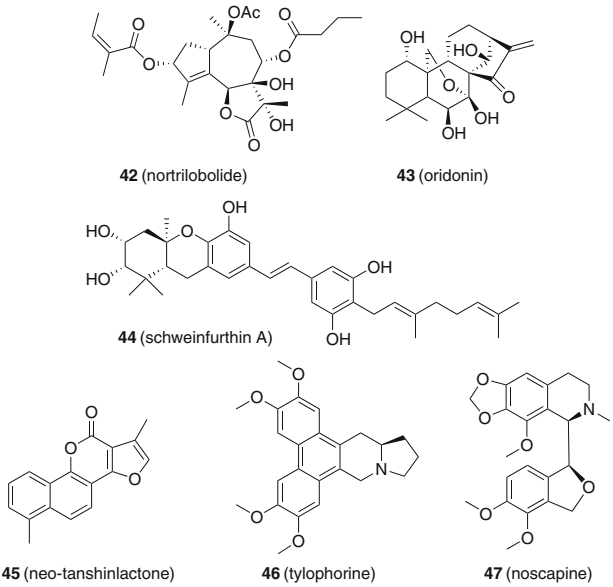


Fig. 31 Structures of nortilobolide (**42**), oridonin (**43**), schweinfurthin A (**44**), neo-tashinlactone (**45**), tylophorine (**46**), and noscapine (**47**)

4 Examples of Lead Bioactive Compounds Obtained from Tropical Plants

Since 1990, which marked the beginning of our collaborative multi-disciplinary investigations directed towards the discovery of new anticancer agents from tropical plants and more recently other organisms, hundreds of biologically active small organic molecules of potential interest have been purified and structurally characterized in our joint work. The secondary metabolites that have been obtained from plants are representative of a wide chemical diversity, and many were obtained as new molecular entities when first isolated. However, it has been found that a molecule does not need to be a new composition of matter to be of further interest for more advanced evaluation. Several of the compounds isolated in our work have been evaluated using in vivo biological test systems and in some depth in terms of their mechanism of cellular action. Some of these have been synthesized chemically in order to afford sufficient material for more detailed biological testing. In previous scientific reviews and book chapters that were cited in Sect. 3.2.1 [87–95], a considerable number of compounds that were promising at the time of their laboratory investigation were presented. In the remaining part of this section, four tropical plant-derived compounds or compound groups of the greatest interest to our group effort as potential lead anticancer compounds are described in detail. These compounds are arranged in alphabetical order, but it should be noted that for some of these leads considerably more development work has been performed to date than for others.

4.1 Betulinic Acid

The pentacyclic triterpenoid, betulinic acid (lup-20(30)-en-28-oic acid) (**48**) (Fig. 32) is a common compound found in plants of numerous different families [211, 212]. Early reports of the occurrence of this compound included those from the seeds of *Zizyphus vulgaris* var. *spinosus* [213] and the bark of *Platanus acerifolia* [214]. In our initial work in the search for plant-derived anticancer agents, betulinic acid would quite often occur in biologically active plant crude extracts selected for activity-guided fractionation. However, this compound attracted little attention, since it was found to be inactive against the cancer cell types used. For example, typical results of this type were obtained in our collaborative work as a constituent of the stem bark of *Dichapetalum gelonioides* collected in the Philippines, wherein **48** was found to be inactive ($ED_{50} > 20$ μg/cm^3) for all representatives of a panel of 11 solid-tumor cancer cell lines and also for human umbilical vein endothelial cells [98]. However, when **48** as a constituent of *Zizyphus mauritiana* Lam. (Rhamnaceae) stem bark was tested against three different patient-derived melanoma cells, designated as MEL-1, MEL-2, and MEL-4, obtained through the Department of Surgical Oncology, College of Medicine, University of Illinois at

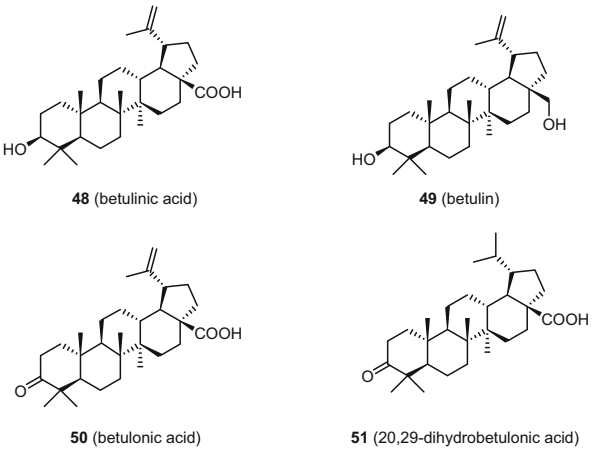


Fig. 32 Structures of betulinic acid (**48**), betulin (**49**), betulonic acid (**50**), and a C-20–C-29 hydrogenated derivative (**51**) of betulonic acid (**50**)

Chicago, the ED_{50} values obtained were in the range 1.1–4.8 μg/cm^3 [215]. In contrast, the ED_{50} value of **48** was >20 μg/cm^3 for each of the following human cancer cell lines: A431 (squamous), BC-1 (breast), COL-2 (colon), HT-1080 (sarcoma), KB (nasopharyngeal), LNCaP (prostate), LU-1 (lung), and U373 (glioma). Betulinic acid (**48**) was compared directly against a panel of standard natural product-derived anticancer agents, which all showed broad and potent cytotoxicity for the 11 cell lines used [215]. The plant of origin of **48** for this initial work was collected by Tangai Chagwedera, of the University of Zimbabwe, Harare, Zimbabwe [215], and a photograph of a voucher specimen is shown in Fig. 33.

At the time this first study on **48** was conducted by our group [215], the concept of using relatively large numbers of cell types in a single panel reflective of different types of human cancer, in the hope of finding selectivity of activity, was still a relatively new idea [160]. In our project on screening tropical plants for their potential anticancer activity, although several thousand extracts of different polarities were evaluated, selective cytotoxic activity for only one cell line type (e.g. melanoma) was demonstrated very infrequently indeed. Following this initial in vitro evidence of the selective inhibition of **48** for human melanoma, follow-up in vivo and preliminary mechanism of cellular action studies were conducted. Using MEL-2 cells, injected subcutaneously into 4-week-old athymic mice, statistically significant tumor growth inhibition was evident for each of three doses tested (50, 250, and 500 mg/kg body weight), with the highest of these three doses being the most effective. In a second in vivo experiment using MEL-1 cells, a low dose of 5 mg/kg body weight of **48** was found to completely inhibit tumor growth. Furthermore, when treatment with betulinic acid was delayed until day 41 after the start of the experiment in MEL-1 tumor-bearing mice, a decrease in tumor size resulted, corresponding to 80% regression [215]. It was noted that no toxicity was evident for any of the in vivo experiments carried out in this initial study on betulinic acid, as evaluated by the healthy condition of the treated mice [215]. In a preliminary mechanism of action study carried out on betulinic acid, the antineoplastic activity

Fig. 33 Photograph of a voucher specimen of *Ziziphus mauritiana* Lam. (Rhamnaceae) collected in Zimbabwe (Chagwadera_956); photograph: D. D. Soejarto

was modulated by apoptosis induction, and this test compound was found also to induce 50 kilobase pair DNA fragments [215].

To carry out the above-mentioned study at such high dose levels of betulinic acid as 500 mg/kg body weight, the bioavailability and solubility of this triterpenoid were enhanced by co-precipitation with polyvinylpyrrolidone (PVP) [215]. In a systematic study of the pharmacokinetics and tissue distribution of **48** in CD-1 mice, it was shown that after 50 and 250 mg/kg intraperitoneal injections the serum concentrations reached peaks at 9.0 and 13.8 min, respectively. The major organs and tissues in which betulinic acid was distributed were the perirenal fat, and the ovaries, spleen, and mammary glands [216].

In order to obtain larger quantities of betulinic acid (**48**) (Fig. 32) in pure form for additional laboratory work, it is convenient to derivatize by oxidation the more abundant lupane-type triterpene, betulin (**49**) (Fig. 32) [217–220]. Thus, over 15 betulinic acid derivatives were generated from **49** as a starting product by modifying the functional group of this parent compound at the C-3, C-20, and C-28 positions, and then testing them against both the KB (human epidermoid oral carcinoma) and MEL2 (melanoma) cell lines [220]. One of the key observations made was that for betulonic acid (**50**) (Fig. 32), a natural product with a C-3 carbonyl group, the cytotoxic selectivity for melanoma cells was lost. Betulin (**49**) was inactive ($ED_{50} > 20$ μg/cm^3) for both KB and MEL2 cells, thus indicating the importance of the carboxylic acid unit of betulinic acid attached to C-17, rather than when a primary alcoholic moiety occurs at this position [220]. No derivative was produced with a greater inhibitory potency than betulinic acid (**48**) against Mel2 cells while maintaining a lack of cytotoxicity for KB cells, with the exception of 20,29-dihydrobetulonic acid (**51**) (Fig. 32) (ED_{50} values, Mel2 cells 0.7 μg/cm^3; KB cells >20 μg/cm^{3}l) [220].

Although the possibility of betulinic acid being selectively active against only melanoma cells was regarded as being of great interest after this was reported in 1995 [215], shortly thereafter this triterpenoid was reported as being cytotoxic for neuroblastoma [221], malignant brain tumor [222], ovarian carcinoma [223], and other solid tumor [223] cells. However, it has been found that non-neoplastic cells and normal tissues remain relatively resistant to the effects of betulinic acid (**48**) [223, 224]. There have been a number of reports on the elucidation of the molecular mechanism of the antitumor action of betulinic acid, with a common effect being to trigger in cancer cells the mitochondrial pathway of apoptosis, and this topic has been subjected to review [e.g. 211, 212, 224, 225].

Melanoma of the skin is a common form of cancer in both males and females that is increasing in incidence [17]. This develops from benign nevocellular nevi in a multistep manner, leading in turn to preneoplastic nevi, primary melanoma, and finally malignant melanoma [226]. Betulinic acid (**48**) was found to reduce DNA breakage induced by ultraviolet light in congenital melanocytic cells of nevi, so it was suggested that this compound might have a role as a chemopreventive agent to prevent melanoma formation [227]. Accordingly, preclinical development of betulinic acid at the Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois was conducted through the former

RAID (Rapid Access to Intervention Development) program of the Developmental Therapeutics Program of the U.S. National Cancer Institute [228, 229]. The developmental steps involved performing preclinical toxicology studies, preparation of GMP/GLP grade of **48**, formulation of a topical preparation (a 20% cream), and submission of an IND (number 56,839) and clinical plan to the U.S. FDA [229]. Accordingly, **48** reached phase I/II trials for the treatment of dysplastic melanocytic nevus, in which it was applied topically as a 20% ointment, with the work carried out at the College of Medicine, University of Illinois at Chicago [230].

Currently, the overall topic of anticancer drug development based on the lupane triterpenoid betulinic acid (**48**) (Fig. 32) remains of considerable interest [e.g. 212, 231]. Moreover, several groups from all over the world are continuing to develop new derivatives of this compound and its close structural analog, betulin (**49**), as improved anticancer agents [e.g., 232–236].

4.2 Pervilleines A–C and F

From the roots of the southern Madagascan plant, *Erythroxylum pervillei* Baill. (Erythoxylaceae) a series of new tropane alkaloid aromatic esters was isolated, namely, pervilleines A–F (**52**–**57**) (Fig. 34) and pervilleine A *N*-oxide (**58**) (Fig. 34) [237]. The plant is used locally in its region of collection as a fish poison and to treat abdominal pain and tumors, and it was collected for our initial investigation by the late Philippe Rasoanaivo, of the Institut Malgache de Recherches Appliquées (IMRA), Antananarivo, Madagascar [237]. These compounds were isolated using activity-guided fractionation by following their potential to reverse multidrug resistance

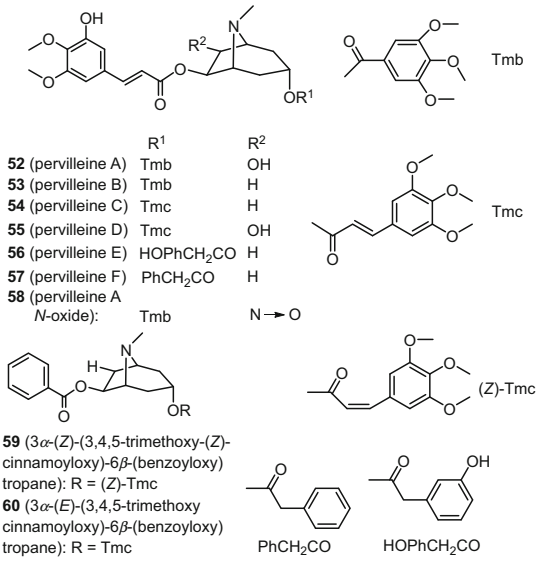


Fig. 34 Structures of pervilleines A–F (**52**–**57**) and pervilleine A *N*-oxide (**58**) from *Erythroxylum pervillei* and of the related tropane alkaloid aromatic esters **59** and **60** from *Erythroxylum rotundifolium*

using KB-V1 vinblastine-resistant oral epidermoid carcinoma cells. Pervillines A–C and F were each obtained in approximately the same percentage yield, and occurred in the range 0.0035–0.0043% *w/w*. Of the alkaloids isolated, pervilleine A (**52**) (3*α*-(3,4,5-trimethoxybenzoyloxy)-6*β*-(*E*)-(3,4,5-trimethoxycinnamoyloxy)-7*β*-one) (Fig. 34) was particularly notable, since it selectively inhibited KB-V1$^+$ cells (drug-resistant KB cells assessed in the presence of 1 μg/cm^3 vinblastine; ED_{50} 0.3 μg/cm^3), while being inactive for KB-V1$^-$ cells (drug-resistant KB cells assessed in the absence of 1 μg/ml vinblastine; $ED_{50} > 20$ μg/cm^3) [237]. Moreover, the selectivity of pervilleine A for KB-V1$^+$ cells was emphasized, since it was also inactive ($ED_{50} > 20$ μg/cm^3) against the BC1 breast, Lu1 lung, Col2 colon, KB, LNCaP prostate, and SW626 ovarian carcinoma human cell lines. In contrast, pervilleine A *N*-oxide was inactive for all cell lines in the panel, including KB-V1$^+$ cells. Pervilleines B–F (**53**–**57**) were also found to be inhibitory to KB-V1$^+$ cells (ED_{50} range, 0.1–1.9 μg/cm^3), but did not show selectivity to the entire panel of eight cell lines in the same manner as pervilleine A. As a result of comparing the activities of pervilleines A–F with two known alkaloids that were also isolated and tested, it was concluded that within the pervilleine tropane alkaloid class, the presence of a C-6 trimethoxycinnamoyl ester unit is required for resultant growth inhibitory activity of KB-V1$^+$ cells. However, when the C-7 hydroxy group substituent of pervilleine A is absent, as in pervilleines B and C, this led to reduced cell line selectivity, although the potency against KB-V1$^+$ cells was maintained [237]. A second phytochemical study on tropane alkaloid aromatic esters was conducted on *Erythroxylum rotundiflorum* Lunan (Erythroxylaceae), collected in the Dominican Republic, and led to the isolation and characterization of three new and three known analogs. However, none of these compounds showed cancer cell panel selectivity for only KB-V1$^+$ cells in the same manner as pervilleine A (**52**). This study did provide additional information on the structure-cytotoxicity relationships of the tropane alkaloid aromatic esters, however, since the (*Z*) and (*E*) diastereomers as evident in the C-6 trimethoxycinnamoyl unit in compounds **59** (Fig. 34) and **60** (Fig. 34), respectively, had little or no influence on their quite similar in vitro biological profiles [238].

The very promising cancer cell line cytotoxicity profile of pervilleine A (**52**) (Fig. 34) was tested further in a collaborative study with colleagues at the U.S. National Cancer Institute, Frederick, Maryland. This compound was found to restore the vinblastine sensitivity to CEM/VLB$_{100}$ cells, and the chemosensitivity of KB-8-5 cells to colchicine also was restored (IC_{50} values of 0.02 and 0.61 μ*M*, respectively). Mechanistically, when multidrug-resistant KB-V1 and CEM/VLB$_{100}$ cells were incubated with pervilleine A for 72 h at doses of up to 45 μ*M*, this did not affect either the transcription of *MDR1* or levels of P-glycoprotein. Accordingly, pervilleine A (**52**) was determined as an inhibitor of P-glycoprotein. This was confirmed when KB-V1 or KB-8-5 cells were tested using Cr *nu/nu* mice in the in vivo hollow fiber assay [178, 179], and an inhibition of up to 75% growth was demonstrated when pervilleine A and vinblastine were used in combination. Equimolar doses of verapamil (Fig. 35, **61**), a positive control, were less effective, and when either pervilleine A or vinblastine was used as a single agent, no significant cell growth was apparent [239]. Further in vitro work and analogous in vivo hollow fiber

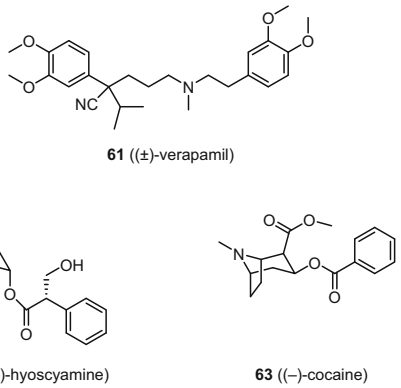


Fig. 35 Structures of verapamil (**61**), (−)-hyoscyamine (**62**), and (−)-cocaine (**63**), showing their similarity to the structures of the pervilleine tropane alkaloid aromatic esters

studies were performed on pervilleines B, C, and F, respectively, with these three additional analogues showing similar inhibitory potencies to pervilleine A in the various evaluations performed [240–242].

The pervilleine aromatic ester tropane alkaloids are similar structurally to (−)-hyoscyamine (**62**) (Fig. 35) and (−)-cocaine (**63**) (Fig. 35). These two compounds are competitive antagonists of acetylcholine and other muscarinic receptors, and a blocker of nerve impulses and local norepinephrine intake. When tested at 30 μ*M* in the guinea pig ileum, pervilleine A hydrochloride salt exhibited non-competitive inhibition of the cholinergic response, but it did not affect the carbachol-induced contraction of the rat anococcygeus smooth muscle. This compound also did not affect the contractile response of rat vas deferens to norepinephrine at a contraction level of 100 μ*M*. Accordingly, pervilleine A showed a lack of significant cholinergic and adrenergic receptor-related activities [243].

Due to the promising multidrug-resistance (MDR) activities of pervilleines A–C (**52**–**54**) and F(**57**) (Fig. 34), which were found to be comparable to that of a standard MDR inhibitory agent, (±)-verapamil (**61**) (Fig. 35), these compounds were selected for additional developmental studies through the former NCI RAID (Rapid Access to Intervention Development) program [228]. As a result, additional funding was provided to recollect a large quantity of *E. pervillei* plant material in Madagascar, with the objective of producing substantial amounts of each of **52**–**54** and **57** for further laboratory study [244]. This large-scale plant collection of *E. pervillei* was facilitated through the Madagascar Office of the Missouri Botanical Garden, St. Louis, MO, USA. When 50 kg of each of the roots and stem bark of this plant were collected from the original collection site in 2003, it was possible to isolate gram quantities of each of **52**–**54** and **57**. A photograph of the voucher specimen representing this collection is shown in Fig. 36. As a result of further testing of these four compounds, pervilleine A proved to exhibit the most promising properties as a MDR inhibitor, so the further testing of this compound in the form of its HCl salt was conducted. Following further successful in vivo hollow fiber evaluation, pervilleine A hydrochloride salt was tested at NCI-Frederick in a HCT-15 colon cancer murine xenograft model in combination with vincristine. Unfortunately, although there was

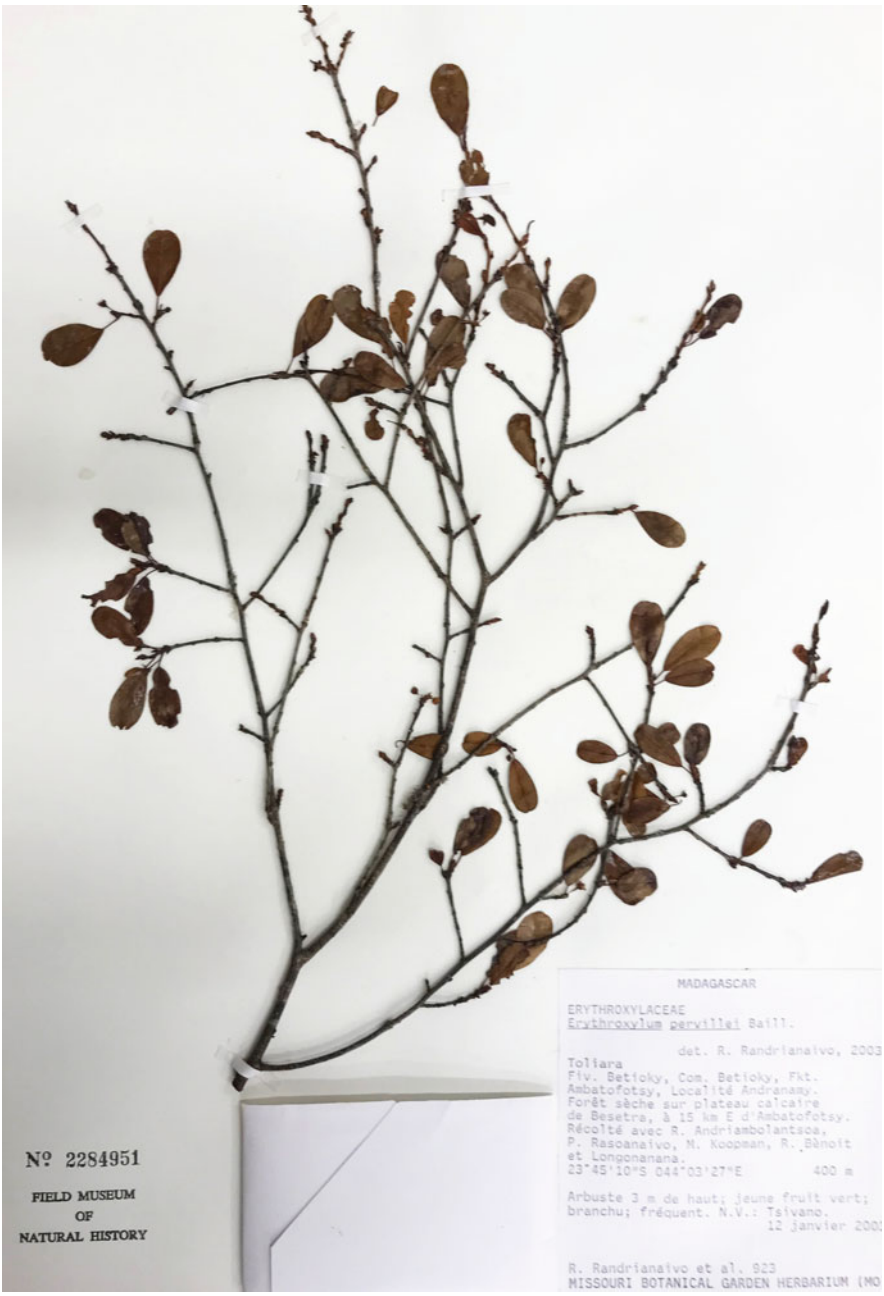


Fig. 36 Photograph of a voucher specimen of *Erythroxylum pervillei* Beille (Erythroxylaceae) collected in Madagascar on January 12, 2003 by Dr. Randrianaivo (Randrianaivo_923). Mounted specimen with field label (Field Museum accession number: 2284951); photographs: D. D. Soejarto

a perceptible inhibition of the tumor growth with this compound mixture, when compared with the same dose of vincristine alone, this was not regarded as being statistically significant at the doses used. As a result of this inconclusive in vivo testing, pervilleine A has not been further developed as a potential MDR inhibitor through our collaborative work [14]. Four new monoester tropane alkaloids were isolated as very minor constituents of the recollected *E. pervilleii* stem bark, but these were not subjected to any biological testing [244].

Two schemes have been proposed for the synthesis of two of the pervilleine alkaloids [245, 246]. In the first of these, a procedure was described for the enantiomeric synthesis of (+)-pervilleine C (**54**) (Fig. 34), which also provided confirmation of the absolute configuration of this molecule [245]. The process started with a mixture of methyl pyrrole *N*-carboxylate and 1,1,3,3-tetrabromopropanone to produce, after several intermediate steps, a trimethoxycinnamoyl monoester. The final stage to produce the target compound involved esterification of the relevant alcohol and acid chloride in toluene [245] (Chart 1). The second synthesis procedure did not produce the naturally occurring form of (–)-pervilleine B (**53**) (Fig. 34), but rather **54** instead. This involved the generation of hydroxylated 8-azabicyclo[3,2,1] octane and 9-azabicyclo[3,31]nonane ring systems from keto lactones. As a result of this work, the absolute configuration of natural (–)-pervilleine B was shown to be (1*R*,3*R*,5*S*,6*R*) [246].

The reports by our group on the pervilleine alkaloids as potential MDR inhibitors mentioned above, coupled with a knowledge of the structure of experimentally used MDR inhibitor, (±)-verapamil (**61**) (Fig. 35), stimulated a synthesis study that led to the development of a new class of MDR inhibitors, having one or two basic nitrogen

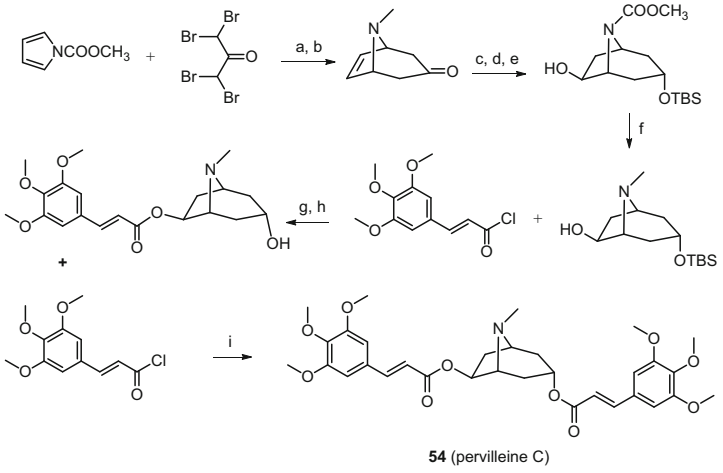


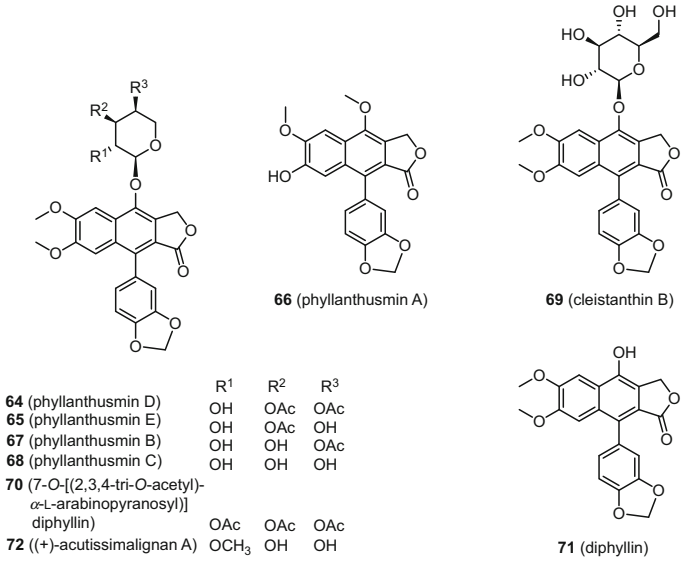
Chart 1 Synthesis scheme for (+)-pervilleine C (**54**) of Kulkani et al. [245]. Reactions and conditions: (a) Zn, Br_2, $B(Oet)_3$; (b) Zn/Cu, NH_4Cl, MeOH; c) L-selectride; (d) TBSCl, DMF; (e) (–)-$(lpc)_2BH$, MeOH, −25°C, MeOH, H_2O_2; (f) $LiAlH_4$, THF; (g) NaH, THF; (h) TBAF, THF; (i) Et_3N, toluene, heat

atoms flanked at the appropriate intramolecular distance by one or two aromatic units [247].

While no further work appears to have been performed in terms of developing the tropane aromatic ester alkaloids further as potential MDR inhibitors in recent years, other classes of plant-derived constituents have been evaluated for this purpose, including diterpenoids of the jatrophane [248] and lathyrol [249] types, as well as selected flavonoids [250] and some *Ipomoea* resin glycosides [251]. The problem of multidrug-resistance tumor cells developing during dosing with certain cancer chemotherapeutic agents is well-known. However, despite a number of synthetic compounds having undergone clinical trials as potential MDR inhibitors, none of these compounds has yet reached the market for this purpose.

4.3 Phyllanthusmins C and D

The large pantropical genus *Phyllanthus* (Phyllanthaceae) is represented by 800 species, and is the third largest in its plant family [252]. In 2006, Wu and Wu described three arylnaphthalene lignans from *Phyllanthus oligospermus*, collected in Taiwan, which they called phyllanthusmins A–C [253]. In our own work performed subsequently, two new (phyllanthusmins D (**64**) (Fig. 37) and E (**65**) (Fig. 37)) and four known glycosylated arylnaphthalene-type (diphyllin) lignan lactones (including



66 (phyllanthusmin A)

69 (cleistanthin B)

	R^1	R^2	R^3
64 (phyllanthusmin D)	OH	OAc	OAc
65 (phyllanthusmin E)	OH	OAc	OH
67 (phyllanthusmin B)	OH	OH	OAc
68 (phyllanthusmin C)	OH	OH	OH
70 (7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)] diphyllin)	OAc	OAc	OAc
72 ((+)-acutissimalignan A)	OCH_3	OH	OH

71 (diphyllin)

Fig. 37 Structures of the arylnaphthalene lignan lactone derivatives phyllanthusmins D (**64**), E (**65**), and A–C (**66–68**), cleistanthin B (**69**), 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)] diphyllin (**70**), diphyllin (**71**), and acutissimalignan A (**72**)

phyllanthusmins A–C (**66–68**) (Fig. 37) and cleistanthin B (**69**) (Fig. 37)) were isolated from various plant parts of *Phyllanthus poilanei* Beille (Fig. 38). The structure of phyllanthusmin D (**64**) was confirmed by single-crystal X-ray crystallography, and by acetylation to the semi-synthetic compound, 7-*O*-[(2,3,4-tri-*O*-acetyl)-*α*-L-arabinopyranosyl)]diphyllin (**70**) (Fig. 37). In addition, acid hydrolysis of **64** produced the known agyclone, diphyllin (**71**) (Fig. 37) [254]. While phyllanthusmin A (**66**) is not a glycoside, phyllanthusmins B–E all have an arabinose sugar unit in their respective molecule. The new compound phyllanthusmin D (**64**) was obtained from each of three collections of *P. poilanei* obtained at two locations in Vietnam, but only as a relatively minor constituent. Thus, initial collections of the stems and the combined flowers, fruits, leaves and twigs were made in 2004 in Khanh Hoa Province by Tran Noc Ninh (T.N.N.), Pham Huy Hoang, and one of the co-authors of this chapter (D.D.S.). In 2008, the same plant parts were collected again in Hatinh Province by D.D.S., T.N.N., and Vuong Tan Tu [254]. Finally, a larger sample of the combined leaves, twigs, and stems was obtained in 2011 from Khanh Hoa Province by D.D.S., T.N.N., and Bui Van Thanh [254].

As described in an initial report on *P. poilanei,* phyllanthusmin D (**64**) (Fig. 37) proved to be potently cytotoxic for HT-29 human colon cancer cells (IC_{50} 170 nM) when compared to phyllanthusmins B, C, and E, and the aglycone, diphyllin (IC_{50} values of 1.8, 3.2, 1.8, and 7.6 μM, respectively). In turn, phyllanthusmin A (**66**) was inactive for this cell line (IC_{50} value of >10 μM). While phyllanthusmins A, B, and E were not tested against normal (non-tumorigenic) CCD-112CoN human colon cells, both phyllanthusmin D (**64**) and C (**68**) were not cytotoxic for this additional cell line (IC_{50} values of >100 μM), demonstrating selectivity for the cancer cell line used [254]. In work by others, several diphyllin glycosides have been shown to be

Fig. 38 *Phyllanthus poilanei* Beille (Phyllanthaceae). Left: Natural habitat, showing the liana habit of the plant (Hon Ba Forest Reserve, Dien Khanh District, Khanh Hoa Province, Vietnam; 7/31/2011). Right: A voucher specimen (DDS_14886) ready to be pressed between newspapers; photographs: D. D. Soejarto

cytotoxic for small panels of cancer cells [e.g., 253, 255–259]. As a result of a phytochemical study by our group on *Phyllanthus songboiensis*, again collected in Vietnam, the known arylnaphthalene lignan, (+)-acutissimalignan A (**72**) (Fig. 37) was isolated as a cytotoxic constituent [171]. This compound, which contains a functionalized arabinose unit, was isolated originally from *Phyllanthus acutissima* by Tuchinda et al. in 2008 [259], and in our hands showed highly potent cytotoxicity against HT-29 human colon cancer cells, with an IC_{50} value of 19 n*M* [171]. Therefore, from an initial surveillance of the structural requirements for cytotoxicity of diphyllin lignans against cancer cells, based on the contributions of other groups and our own, it was apparent that such activity is dependent on both the type of sugar attached to the aglycone and the degree of sugar hydroxy group methoxylation.

Phyllanthusmin D (**64**) (Fig. 37) was shown to be active in the in vivo hollow fiber assay at UIC, using HT-29 human colon cancer cells implanted into immunodeficient NCr *nu/nu* mice, when injected intraperitoneally at a dose of 20 mg/kg. No gross toxicity was observed for the treated mice over the dose range of this compound used (5–20 mg/kg) [254]. Following this very promising result, **64** was investigated mechanistically. First, using an annexin V staining method and western blot analysis, this compound was found to induce HT-29 cell apoptosis through activation of caspase 3, a well-known anticancer compound target and key effector of programmed cell death. Furthermore, **64** did this in a more potent manner than the positive-control used, etoposide (**6**) (Fig. 1), an aryltetralin lignan derivative. Second, since some diphyllin glycosides produced by synthesis with potential anticancer activity have been shown to act by inhibiting DNA topoisomerase IIα [260, 261], compound **64** and several analogs were evaluated for such an effect. This enzyme is inhibited by **6**, which causes DNA double-strand breaks to result in cellular death [29]. Using a standard protocol [197], phyllanthusmins C (**68**), D (**64**), 7-*O*-[(2,3,4-tri-*O*-acetyl)-*α*-L-arabinopyranosyl)]diphyllin (**70**) (Fig. 37), and acutissimalignan A (**72**) were found not to be topoisomerase II poisons, despite their general similarity in structure to **6** [171, 254].

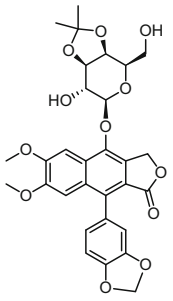
In an additional biological study, conducted at the Ohio State University College of Medicine and Comprehensive Cancer Center, selected compounds from *P. poilanei* were evaluated for their effects on human natural killer (NK) cells. It was found that phyllanthusmin C (**68**) enhanced interferon-gamma (IFN- γ) production by human NK cells through upregulation of toll-like receptor (TLR)-mediated NF-κB signaling. This lignan glycoside also synergized with the cytokine IL-12 and stimulated the production of IFN-γ production in both the human $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets [262]. Since **68** was only a relatively weakly potent cytotoxic agent against cancer cells, and human NK cells are a critical component of innate immunity and offer a first line of defense against tumor cells, the further investigation of the effects of this compound in the potential prevention of cancer seems warranted [254, 262].

The considerable promise of phyllanthusmins C (**68**) and D (**64**), as mentioned above, was supportive of a chemical synthesis program to produce an ensemble of glycosidic derivatives of diphyllin (**71**) (Fig. 37) varying in their sugar units and the functional groups present [263, 264]. For these studies, diphyllin was produced in

gram quantities based on the general method reported by Charlton et al. [265], and then this aglycone was reacted with prepared glycosyl bromides to produce variously functionalized sugar substituents, following known carbohydrate chemistry, and utilizing a procedure reported by Zhao and associates [261]. As a result, diphyllin glycosides derivatized with different sugar units, inclusive of L- and D-arabinose, D-galactose, D-glucose, and D-xylose, were prepared and evaluated against five different human cancer cell lines, namely, HT-29 colon, MDA-MB-231 melanoma, MDA-MB-435 breast, and OVCAR3 and OVCAR8 (both ovarian). A number of the compounds produced potent cytotoxicity data for one or more of the cell lines used. In addition, four of the most potent compounds that were synthesized were tested for their effects against topoisomerase IIα [197], but no inhibitory activity of this type was discerned [263, 264].

In a further study on a number of synthetic phyllanthusmin derivatives that has been conducted to date, the potential mechanism of action of these compounds was probed further. Glycosides based on the aglycone, diphyllin (**71**), have been determined to be vascular (V)-ATPase inhibitors that affect cellular lysosomal acidification [266, 267]. It is known that lysosomal acidification facilitated by V-ATP-ase plays a role in autophagy, in which a recycling of cellular components occurs. Accordingly, PHY-34 (**73**) (Fig. 39), the phyllanthusmin derivative synthesized with the highest (nanomolar) potency against the high-grade serous ovarian cancer cell lines used, was investigated for its effects on cancer cell autophagy [264, 268]. This compound was found to be bioavailable through intraperitoneal administration in vivo, and it was active both in an in vivo hollow fiber assay using HT-29 colon cancer cells and in a murine xenograft assay bearing implanted OVCAR8 ovarian cancer cells [268]. It was determined that **73** exerts its effects by inhibiting autophagy at a late stage in the pathway, leading to a disruption of lysosomal function [268].

Accordingly, synthesized derivatives of the phyllanthusmin lignan glycosides seem worthy of further investigation as potential novel cancer chemotherapeutic agents, owing to their biological potency, in vivo efficacy, and putative unusual mechanism of action.



73 (PHY-34)

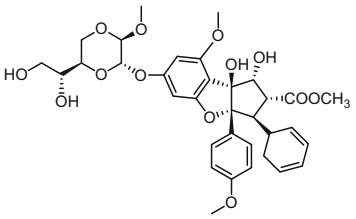
Fig. 39 Structure of the synthetic diphyllin lignan, PHY-34 (**73**)

4.4 Silvestrol

4.4.1 Backgound Phytochemical Studies on *Aglaia* Species (Meliaceae)

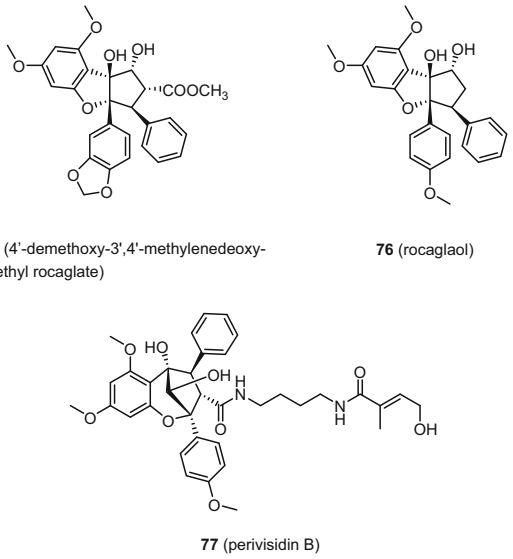
Of all the compounds isolated through our joint research endeavors to date, the cyclopenta[*b*]benzofuran, silvestrol (**74**) (Fig. 40), has been subjected to the greatest degree of laboratory follow-up work by others after our initial report of its isolation and full structure elucidation from an *Aglaia* species collected in Kalimantan, Indonesia [269]. This compound has been established as an inhibitor of protein synthesis, by acting on translation. As mentioned previously in Sect. 3.5, the cyclopenta[*b*]benzofuran (also known as "rocaglate" or "flavagline") compound group of plant secondary metabolites is of particular interest, since the initial representative in this series, rocaglamide (**30**) (Fig. 26), was described as recently as 1982 [150]. Prior to discussing the work performed on silvestrol, in the next few paragraphs our background work on related compounds isolated from the genus *Aglaia* will be described briefly.

The genus *Aglaia* is represented by about 120 species in the family Meliaceae that occur in tropical rainforests of the Indo-Australasian region [270]. Extracts of several of these species when collected in Indonesia, Vietnam, and Thailand were found to be active after initial screening in our collaborative project on the discovery of anticancer agents from plants. The first *Aglaia* species studied in our joint project was *Aglaia elliptica*, collected in Thailand, and fractionation of the fruits and stems of this species led to the isolation of five rocaglate derivatives, of which some were found to be potently cytotoxic to most members of a panel of 12 cancer cell lines used, with activities in the nanomolar level. The most abundant compound, 4′-demethoxy-3′,4′-methylenedeoxy-methyl rocaglate (**75**) (Fig. 41), was isolated from the fruits of *A. elliptica*, and was also the most active compound found in the study [271]. This isolation report was followed by one describing the in vivo testing and a mechanistic investigation on this compound. Thus, using with a xenograft model BC1 human breast cancer cells in athymic mice, the growth of tumors in the test animals used was delayed by treatment with **75** (administered at 10 mg/kg body weight, ip, three times per week). While the administration of **75** under these experimental conditions caused no overt signs of toxicity, tumor growth paralleled that of the control group at later time points [272]. Using [^{3}H]-leucine incorporation,



74 ((–)-silvestrol)

Fig. 40 Structure of silvestrol (**74**)



75 (4'-demethoxy-3',4'-methylenedeoxy-methyl rocaglate)

76 (rocaglaol)

77 (perivisidin B)

Fig. 41 Structures of the rocaglate derivatives 4′-demethylenedioxymethyl rocaglate (**75**), rocaglol (**76**), and pervirisidin B (**77**)

75 was found to strongly inhibit protein biosynthesis, while no effects were noted on nucleic acid biosynthesis [272].

Following these initial studies on *A. elliptica* by our group, the rocaglate derivatives of several other *Aglaia* species were investigated, namely, the Indonesian species *A. crassinerva* [147], *A. rubiginosa* [273], *A. edulis* [274], and *A. perviridis* from Vietnam [275]. While a number of cyclopenta[*b*]benzofuran analogs were obtained from these species, including several new compounds, among the most the potent cytotoxic agents for a small human cancer cell panel was found to the known compound, rocaglaol (Fig. 41, **76**). This compound was isolated from *Aglaia crassinerva* and exhibited IC_{50} values of 13.8, 23.0, and 9.2 n*M* against Lu1 (lung cancer), LNCaP (hormone-dependent prostate cancer), and MCF-7 (breast cancer) cells [147, 276]. In a follow-up mechanistic study using LNCaP cells, rocaglaol was demonstrated as being apoptotic through the mitochondrial pathway, and caused dose-dependent G2/M phase arrest. The latter effect was associated with down-regulation of protein phophatase Cdc25C and the dephosphorylation of Cdc2 [276]. Rocaglaol (**76**) was also isolated as a constituent of *Aglaia perviridis*, and in addition a new cyclopenta[*b*]benzofuran analog from this species called perivisidin B (**77**) (Fig. 41) exhibited inhibitory activity in a NF-κB (p65) ELISA assay (IC_{50} value of 2.2 μ*M*) [275]. Two journal review articles summarizing work performed on rocaglate derivatives from *Aglaia* species as potential anticancer agents have been published [156, 277].

4.4.2 Isolation of Silvestrol and Structurally Related Dioxanyl Ring-Containing Compounds from *Aglaia* Species

In 2004, our collaborative group first reported the structure and absolute configuration of the highly potent cytotoxic compound (−)-silvestrol (**74**) (Fig. 40), which was found to be a novel dioxanyl ring-containing cyclopenta[*b*]benzofuran constituent of both the fruits and twigs of an *Aglaia* species collected in Kalimantan, Indonesia [269]. The plant material was collected by the late Soedarsono Riswan, of the Herbarium Bogoriense, Bogor, Indonesia, in collaboration with the late Leonardus B.S. Kardono, Research and Development Chemistry, Indonesian Institute of Science, Tangerang, Indonesia. However, in our initial report, this species was misidentified taxonomically as *Aglaia sylvestris* (M. Roemer) Merrill (syn. *A. pyrimidata* Hance) [269]. The plant material collected required additional taxonomic scrutiny and was subsequently re-identified correctly as *Aglaia foveolata* Pannell [278]. A photograph of a voucher specimen of *A. foveolata* collected in Indonesia is shown as Fig. 42. The structure and absolute configuration of silvestrol were determined by detailed spectroscopic data interpretation and by X-ray crystallography [269]. Also obtained at the time of its initial isolation from *A. foveolata* by our group was episilvestrol (5‴-episilvestrol) (**78**) (Fig. 43). Further study of different plant parts of *A. foveolata* confirmed the presence of silvestrol in both the leaves and stem bark [279]. As a result of obtaining a large-scale recollection (ca. 45 kg) of *Aglaia foveolata* from Kalimantan, Indonesia, so that a larger quantity of pure silvestrol could be generated for additional biological testing, two further minor silvestrol derivatives were isolated and characterized, namely, 2-‴-episilvestrol (**79**) (Fig. 43) and 2‴,5‴-diepisilvestrol (**80**) (Fig. 43) [280]. This recollected plant material was again obtained by the late Drs. Riswan and Kardono, and part of the large-scale extraction procedure was conducted by SAIC-Frederick, Inc., with the cooperation of Dr. David Newman of the Natural Products Branch, National Cancer Institute, Frederick, Maryland [280]. Rocaglate derivatives containing a dioxanyl ring such as silvestrol appear to be quite rare among species in the genus *Aglaia.* However, silvestrol has been found also as a constituent of *Aglaia leptantha* [281], a Malaysian species shown subsequently to be *Aglaia stellatopilosa* [156, 282]. In recent work conducted on the leaves of *A. perviridis* collected in southern mainland China, silvestrol (**74**), episilvestrol (**78**), and a new acylated silvestrol derivative, aglapervirisin A (**81**) (Fig. 43), were all reported as rocaglate constituents of this species [283].

Thus far, silvestrol (**74**) and/or its other structural cyclopenta[*b*]benzofuran analogs containing a dioxanyl ring have been reported in only the three *Aglaia* species mentioned above, namely, *A. foveolata*, *A. perviridis*, and *A. stellatopilosa*.

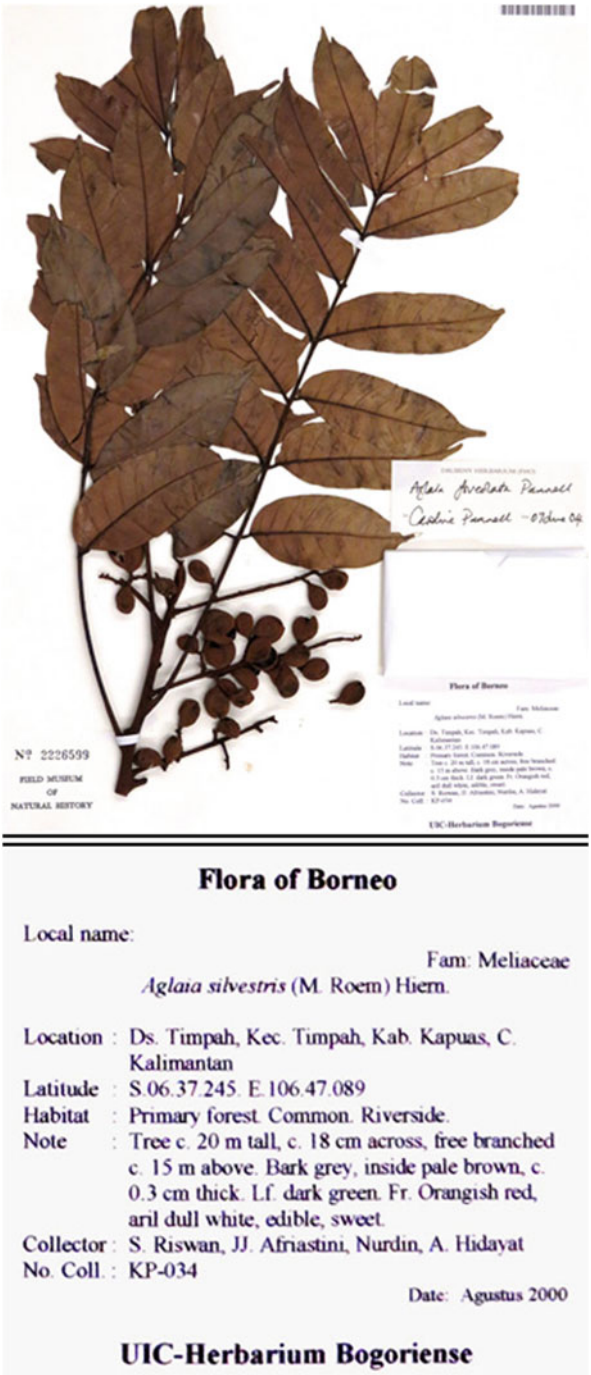
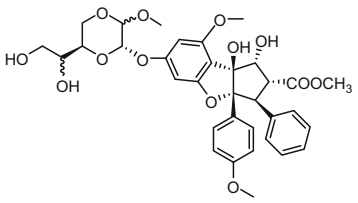
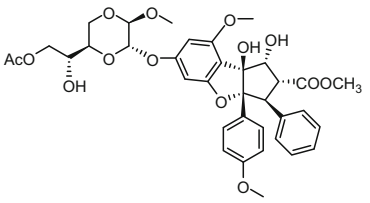


Fig. 42 Photograph of a voucher specimen of *Aglaia foveolata* Pannell (Meliaceae) collected in Indonesia by Soedarsono Riswan et al. in August 2000 (Riswan_KP-034; Field Museum accession number: 2226599); however, see Sect. 4.4.2. Photograph D. D. Soejarto



78 (5‴-episilvestrol): (2‴*R*,5‴*S*)
79 (2‴-episilvestrol): (2‴*S*,5‴*R*)
80 (2‴,5‴-diepisilvestrol): (2‴*S*,5‴*S*)



81 (aglapervirisin A)

Fig. 43 Structures of the naturally occurring dioxanyl ring-containing cyclopenta[*b*]benzofuran derivatives episilvestrol (5‴-episilvestrol) (**78**), 2‴-episilvestrol) (**79**), 2‴,5‴-episilvestrol) (**80**), and aglapervirisin A (**81**)

4.4.3 Chemical Synthesis Methods for Silvestrol and Related Dioxanyl Ring-Containing Compounds

Silvestrol has proved to be an attractive bioactive molecular target for chemical synthesis groups, due to the need to construct the highly functionalized cyclopenta[*b*]benzofuran (flavagline) core with five contiguous stereogenic centers and the unprecedented dioxanyl ring present. This topic along with methods of synthesis for the biogenetically related compound rocaglamide (**30**) (Fig. 26) [150] has been reviewed in detail by members of our natural product anticancer agent team previously [156], and so only the most salient details will be mentioned in this chapter. In 2007, in back-to-back papers in the international edition of the journal *Angewandte Chemie*, descriptions of the chemical enantioselective synthesis of (−)-silvestrol (**74**) (Fig. 40) were published by the Porco group at Boston University and the Rizzacasa group at the University of Melbourne [284, 285]. Both groups utilized a [3+2] photocycloaddition with a differently functionalized 3-hydroxyflavone to generate the cyclobenzo[*b*]furan core, having a free phenolic group at the C-6 position. A dioxanyloxy fragment was then attached onto this free phenol through a Mitsunobu reaction to produce (−)-silvestrol (**74**), which showed identical physical and spectroscopic parameters to the natural product [268, 284, 285]. In the case of the synthesis by the Porco group, the dioxanyl ring was conducted from (2*S*,2*S*)-1,2-di-*O*-benzylidinethreitol [284]. For the Rizzacasa synthesis, a commercially available brominated and acetylated sugar derivative was used as the starting material to produce the dioxanyl ring system of **74** and (−)-episilvestrol (5‴-episilvestrol) (**77**) (Fig. 43) [285]. Porco and colleagues also produced

1‴-episilvestrol (**82**) (Fig. 44), which was about five-fold less active than the parent compound in an in vitro assay used to assess protein synthesis inhibition [284]. A follow-up full paper by the Rizzacasa group appeared in 2009, in which the syntheses of (−)-silvestrol (**74**), (−)-episilvestrol (**77**), and (−)-4′-desmethylepisilvestrol (**83**) (Fig. 44) were described, with the latter compound determined to be about four times less potent than **74** when evaluated for cytotoxicity against A549 human lung cancer cells [286]. Silvestrol (**74**) was also synthesized from **77** using a Mitsunobu inversion procedure by Rizzacasa and his colleagues [286].

In 2012, the Rizzacasa group reported also the total synthesis of 1‴,2‴-diepisilvestrol (**80**) (Fig. 43) [287], a compound reported initially in our own work as a trace constituent of *Aglaia foveolata* stem bark, as mentioned earlier [280]. This compound was generated through a reaction sequence involving a Mitsunobu coupling between a cyclopenta[*b*]furan phenol and a dioxane lactol. In an in vitro protein translation assay **80** was much less active than episilvestrol (5‴-episilvestrol) (Fig. 43, **78**), prepared by this same group earlier [285, 286]. This is consistent with considerably weaker cytotoxic potency against HT-29 cell human colon cancer cells of this C-2‴ epimer (IC_{50} 1.1 μ*M*) compared with episilvestrol (**77**) (IC_{50} 0.001 μ*M*) [280]. Also synthesized was a further derivative that has not yet been found to occur naturally, namely, 1‴,2‴,5-‴-triepisilvestrol (**84**) (Fig. 44). Since the last-mentioned compound was inactive in the in vitro protein translation assay employed, this chemical synthesis study confirmed that the naturally occurring configurations at C-1‴ and C-2‴ of silvestrol (**74**) are both important for the mediation of its resultant biological activity [280, 285, 287].

(–)-Silvestrol (**74**) has been used as a lead compound in order to design synthetic analogs with activity as selectively and potently acting inhibitors of protein synthesis.

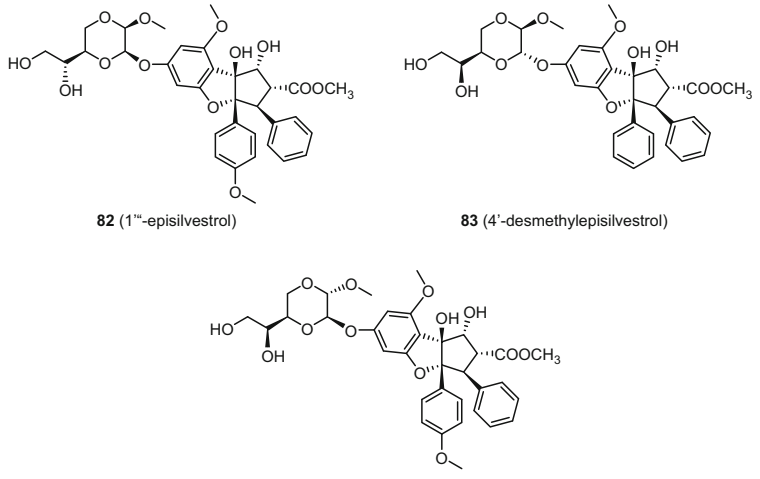
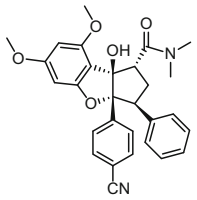


Fig. 44 Structures of the synthetic dioxanyl ring-containing cyclopenta[*b*]benzofuran derivatives 1‴-episilvestrol (**82**), (−)-4′-desmethylepisilvestrol (**83**), and 1‴,2‴,5‴-triepisilvestrol (**84**)

Fig. 45 Structure of a C-4′-cyano synthetic derivative **85** of silvestrol (**74**)



85

The target compounds were obtained using a similar strategy to that used earlier [284–286], in using in each case a late-stage Mitsonubu glycosylation reaction to combine a cyclopenta[*b*]benzofuran moiety with a dioxanyl ring. When a number of compounds were obtained having varied dioxanyl ring moieties, it was found that several of these had the type of activity desired. In addition, some simplified derivatives were generated with a similar cytotoxic potency to **74**, but without a dioxanyl ring, including the C-4′ cyano derivative **85** (Fig. 45) [288].

4.4.4 Biological Testing and Mechanism of Action Evaluation of Silvestrol

From the time of its initial isolation, (–)-silvestrol (**74**) (Fig. 40) has proven to be of great interest biologically in both in vitro and in vivo test systems. In the initial report published by our group, this compound was found to be highly cytotoxic against a panel of three solid tumor human cell lines (Lu1, LNCaP, MCF-7) with ED_{50} values of 1.2, 1.5, and 1.5 n*M*, respectively, similar to that observed for a positive control substance, paclitaxel (ED_{50} values of 2.3, 4.7, and 0.7 n*M*, respectively). However, while paclitaxel (**11**) (ED_{50} 105.5 n*M*) was somewhat less cytotoxic to a normal cell line (HUVEC, human umbilical vein endothelial cells), **74** showed no selectivity for this cell line (ED_{50} 4.6 n*M*) relative to the three cancer cells used [269]. It was observed that 5‴-episilvestrol (**78**) (episilvestrol; Fig. 43) was only slightly less potent as a cytotoxic agent, when compared with **74** (best activity; EC_{50} 2.7 n*M* against MCF-7 cells) [269]. Some structure-cytotoxicity relationship information for this compound class was afforded using HT-29 human colon cancer cells, when it was found that both 2‴-episilvestrol (**79**) (Fig. 43) and 2‴,5‴-diepisilvestrol (**80**) (Fig. 43) were much less cytotoxic than either **74** and 5‴-episilvestrol, and thereby showed the importance of the C-2‴ configuration in mediating such activity within this compound class for the first time [280]. The recently reported acylated silvestrol, aglapervirisin A (**81**) (Fig. 43), which retains the same configuration as silvestrol at all stereocenters in the molecule, was found to be approximately equipotent to the parent compound when evaluated against a small panel of human cancer cell lines [283].

(–)-Silvestrol (**74**) then was tested in the murine in vivo hollow fiber assay at the University of Illinois at Chicago. At the uppermost tested dose of 5 mg/kg body weight, silvestrol showed 63.2% inhibition of the growth of KB (human

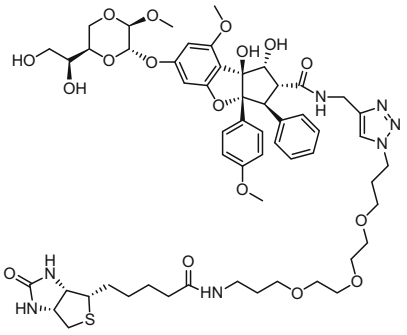
nasopharyngeal) cells when administered through the intraperitoneal route, but only 26.8% when given subcutaneously [269]. Similar results were obtained using both LNCaP cells and Col2 (human colon cancer) cells, with no significant weight loss occurring for the test mice in all cases. As a follow-up to this promising work, silvestrol was also tested in the P388 murine lymphocytic leukemia model and was determined as being active at a maximum tolerated dose of 2.5 mg/kg when administered as five daily intraperitoneal injections, corresponding to a maximum increase in lifespan with a treatment/control (T/C) ratio of 150%. In contrast, when **74** was injected intravenously twice daily for 5 days in the P388 model, a T/C ratio of only 129% was obtained at a cumulative dose of 2 mg/kg/day [269]. This mouse xenograft testing was conducted in the laboratories of a former industrial partner of our program project group, namely, Bristol-Myers Squibb, Princeton, NJ [269].

At the University of Illinois at Chicago, following the initial observations of its extremely potent cytotoxicity for cancer cells and the subsequent demonstration of in vivo activity in a murine xenograft model, preliminary studies were conducted to elucidate the cellular mechanism of action of (–)-silvestrol (**74**) in LNCaP cells [289, 290]. In a similar manner to rocaglaol (**76**) (Fig. 41) [276], on cell cycle analysis by flow cytometry, **74** induced a block in the cycle at the G_2/M checkpoint, and caused apoptosis through the mitochondrial pathway [290]. Also using LNCaP cells, silvestrol down-regulated the p53 protein within 30 min of exposure, and, after 6 h, no p53 could be detected. The down-regulation of p53 associated with that of MDM2 was also observed and was not prevented by lactacystin, which indicated that silvestrol-induced degradation of p53 is not mediated by the proteasome [289]. In a more recent mechanistic study using MDA-MB-435 melanoma cells, also performed at the University of Illinois at Chicago, **74** was found to enhance autophagosome formation and to induce cell death through induction of early autophagy and caspase-mediated apoptosis [291].

An initial study was reported in 2009 that showed the potential of (–)-silvestrol (**74**) for treating human B-cell malignancies, as conducted at the College of Medicine, of The Ohio State University, Columbus, Ohio. This work was supported by the profile obtained for this compound in the NCI60 cell-line panel [292], which suggested its increased sensitivity against five out of six leukemia cell lines used, and also an unusual mechanism of action [292]. The LC_{50} value of **74** against a patient-derived chronic lymphocytic leukemia (CLL) cell line was 6.9 n*M* after 72 h, with no difference in sensitivity for cells of patients either having or not having the del (17p113.1) abnormality. Using both isolated cells and whole blood, silvestrol was more cytotoxic for B cells than for T cells. Mechanistically, **74** resulted in an early reduction in MCl-1 in CLL cells, leading to subsequent mitochondrial damage [292]. When tested in vivo in a SCID mouse xenograft model of acute lymphoblastic leukemia (ALL), mice transplanted with 697 ALL cells that were injected intraperitoneally with silvestrol (1.5 mg/kg every other day) resulted in some of the test mice surviving for 6 weeks after engraftment, without any symptoms of disease or toxicity being observed [292].

Two pivotal papers that appeared in 2008 and 2009 concerning the further in vivo testing of (–)-silvestrol (**74**) and its mechanism of action were both published by the Pelletier group of McGill University in Montreal, Canada [293, 294]. In the first of these, **74** was demonstrated as being able to enhance sensitivity in a mouse lymphoma model, and to act mechanistically as a chemical inducer of dimerization that promotes the interaction of RNA and the RNA helicase enzyme, eukaryotic initiation factor (eIF) 4A [293]. (–)-Silvestrol (**74**) was further found to possess antineoplastic activity in two murine xenograft models using implanted MDA-MB-231 breast cancer and PC-3 prostate cancer cells, being well tolerated by the test animals. It was postulated that this compound acts by inhibiting specifically the translation of malignancy-related mRNAs [294]. The Rizzacasa group from the University of Melbourne prepared a biotinylated derivative of 5‴-episilvestrol (**86**) (Fig. 46) [295], and showed both this product and **74** to be highly selective in binding eIF4AI/II. No other binding proteins were detected by affinity purification, hence giving further support for this being a principal cellular target of **74** and 5‴-episilvestrol (**78**) [295]. In a further paper, the Pelletier group showed synergistic effects in terms of the protein synthesis inhibition of **74** in acute myelogenous leukemia (AML) cell lines with three standard antileukemic drugs, namely, cytarabine, daunorubicin, and etoposide [296]. More recently, this same group showed that the translation inhibition of a group of rocaglate derivatives inclusive of silvestrol operates through an eIF4A phosphorylation-independent mechanism [297].

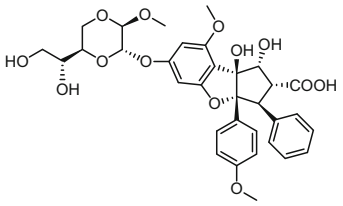
Several additional reports on the in vitro and in vivo testing of (–)-silvestrol (**74**) have appeared, based on work using specialized bioassay test systems carried out by colleagues at The Ohio State University College of Medicine. In a study to explore mechanisms of silvestrol resistance using a resistant 697 acute lymphoblastic leukemia cell line (ALL-R), it was demonstrated that **74** is a substrate of P-glycoprotein, although its re-sensitization could be accomplished by treatment with verapamil and cyclosporin A [298]. (–)-Silvestrol (**74**) was evaluated against mantle cell lymphoma cell lines (MCL) and primary tumor cells, and showed low nanomolar inhibitory potencies with the ability to cause cyclin D at a dose of 10 μ*M* after 16 h, leading also



86 (biotinylated episilvestrol derivative)

Fig. 46 Structure of a biotinylated derivative of 5‴-episilvestrol (**86**) used by Rizzacasa et al. to specifically probe the cellular proteins eIF4AI/II

to reductions in phosphorylated Rb, E2F1 protein, and E2F1 target description. A second line of inhibitory activity against MCL cells involved Mcl-1 depletion followed by mitochondrial depolarization and caspase-independent apoptosis. In a SCID-mouse xenograft model, engrafted with JeKo-1 MCL cells, median survival was prolonged in a significant manner by treatment with **74** (1.5 mg/kg, intraperitoneal administration, every 48 h) [299]. Silvestrol (**74**) was found to exhibit both potent in vitro and significant in vivo effects in laboratory models of acute myeloid leukemia (AML), and also to act through a novel mechanism leading to inhibition of *FLT3* and *miR-155* gene expression [300]. Using bioassays germane to hepatocellular carcinoma (HCC), inhibition of cell growth was demonstrated using four different HCC cell lines, with IC_{50} values in the range 12.5–86 n*M*, with increased apoptosis and caspase 3/7 activity occurring, concomitant with the loss of mitochondrial membrane potential and decreased expression of both Mcl-1 and Bcl-xL. Synergistic effects were shown in vitro when **74** was co-administered with both sorafenib and rapamycin. Subsequent in vivo testing with **74** did not alter liver structure in tumor-bearing mice, and an antineoplastic effect was demonstrated at a dose of 0.4 mg/kg [301]. When evaluated both in vitro and in vivo using Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines, **74** induced inhibition of growth and of the downstream AKT, STAT1, and STAT3 signaling pathways, in addition to decreased expression of the EBV oncogene latent membrane protein. Moreover, **74** promoted indirect antitumor effects in a potent manner by preserving expansion of innate and EBV antigen-specific adaptive immune components. Using an in vivo model, **74** demonstrated activity dependent upon the presence of CD8-positive T cells [302]. The pharmacokinetics of **74** was investigated using liquid chromatography-mass spectrometry, with ansamitocin P-3 as internal standard, as a supportive analytical procedure. When mice were dosed with silvestrol formulated in hydroxypropyl-β-cyclodextrin, there was 100% intraperitoneal systemic bioavailability, but only 1.7% oral bioavailability. Gradual degradation of **74** occurred in both mouse and human plasma, with about 60% of this substance remaining 6 h after dosing. However, in rat plasma, **74** was degraded completely to silvestric acid (**87**) (Fig. 47) within 10 min [303]. It was determined in our 2004 initial study that **87** was much less cytotoxic than **74** for the small panel of cancer cell lines used [269]. As mentioned in Sect. 4.4.3, Liu et al. have produced several simplified derivatives of **74** without a dioxanyl ring having potent antiproferative



87 (silvestric acid)

Fig. 47 Structure of the blood plasma hydrolysis product of silvestrol (**74**), silvestric acid (**87**)

and protein synthesis-inhibitory properties, in an attempt to reduce the potential metabolic sites in order to diminish the interactions with P-glycoprotein [288].

Work has been conducted on the potential use of treating malignant peripheral sheath tumors (MPNSTs) and vestibular schwannomas (VSs) by collaborators at The Research Institute, Nationwide Children's Hospital, Columbus, OH. (–)-Silvestrol (**74**) showed nanomolar inhibitory potency for both MPNST and VS cells, and suppressed tumor growth for mouse models for *NF1*$^{-/-}$ MPNST and *Nf2*$^{-/-}$ schwannoma, with the effects on various cellular factors also being monitored. Using an RNA interference method, the role of eIF4A and eIF4E in cell growth was assessed, and it was concluded that the eIF4 complex is a potential therapeutic target in MPNSTs and VS [304]. In a further study, **74** was found to be growth inhibitory for both primary meningioma and *NF2*-deficient benign meningioma Ben-Men-1 cells, and resulted in G_2/M cellular arrest, and decreased the amounts of cyclins D1, E1, A, and B, PCNA, and Aurora A. Also, levels were reduced in silvestrol-treated Ben-Men-21 cells of three driving factors in meningioma cell proliferation, namely, phosphorylated AKT, ERK, and FAK. It was concluded that targeting protein translation could result in a potential treatment for meningiomas [305].

In a recent study conducted by Malaysian scientists, (–)-silvestrol (**74**) (Fig. 40) and 5‴-episilvestrol (**78**) (Fig. 43) were evaluated against nasopharyngeal carcinoma (NPC) cells. Treatment with both compounds showed cell cycle perturbation, an increase of cells in the G2/M phase, and synergistic effects in cell cultures were shown in combination with cisplatin, a standard agent used for the chemotherapy of NPC [306].

The Drug Development Group of the U.S. National Cancer Institute (NCI) selected **74** for preclinical evaluation at the IIA level (including additional sourcing of the plant of origin and preliminary formulation, pharmacokinetics, and toxicology), leading to its possible clinical development particularly to treat B-cell malignancies. The compound was later transferred to the NExT pipeline of NCI's Division of Experimental Therapeutics [307], where it was evaluated for preclinical toxicology. Eventually, further development work at NCI was ultimately suspended. However, depending on its availability, it may be expected that **74** in the future will be valuable both as a standard cytotoxic agent in the laboratory as well as a specific probe for the cellular eIF4A protein translation target. Furthermore, a recently patented alternative approach toward the potential clinical use of **74** is through the synthesis of antibody-drug conjugates incorporating this rocaglate derivative [308].

5 Conclusions

Since the early 1960s, as a result of the introduction of the bisindole alkaloids vinblastine (**1**) and vincristine (**2**) (Fig. 1) into oncology chemotherapy [26], plant secondary metabolites have been of major interest as sources of potential new anticancer agents. While representatives of only five different compound classes of

plant-derived cancer chemotherapeutic agents have been used clinically in the United States to date, this includes the very widely utilized taxanes, paclitaxel (**11**) and docetaxel (**12**) (Fig. 2) [33]. However, there are several additional plant-derived agents based on additional structural types that have reached clinical trials, inclusive of combretastatin A-4 (**26**) (Fig. 6) [78, 79] and flavopiridol (**27**) (Fig. 6) [80, 82].

In an important series of review articles that have appeared periodically, Gordon Gragg and David Newman [e.g. 1, 3, 4, 6, 19, 37], have documented convincingly the importance of organisms as sources of new drugs in western countries over the last 30 years, even in the current climate of alternative biological, biotechnological, and chemical approaches to drug discovery. Prominent among the types of new natural product-derived drugs introduced to the market in recent years are cancer chemotherapeutic agents. It is notable that natural product anticancer agents available for therapy have been obtained from all of plant, terrestrial microbial, and marine organism sources [6, 8, 18]. It has been pointed out that further drug discovery work on higher plants may not prove beneficial due to the limited prospects of discovering bioactive lead compounds based on new carbon skeletons. Also, this type of research is complicated by the overall chemical complexity of plant extracts, and the need to specifically remove vegetable tannins as complex phenolic substances that may provide false positives in bioassays used during laboratory work-up [14, 309]. Another complicating factor is a sourcing problem inherent in obtaining a resupply of a plant of interest, which may be complicated by the need to negotiate or renegotiate an international collection agreement [14, 309]. However, owing to the very solid track record of higher plants in affording new drugs since the dawn of organic chemistry at the turn of the nineteenth century [11–15], it would not seem prudent to suspend or de-emphasize further efforts to search for new bioactive compounds inclusive of new anticancer drugs from these important organisms.

Although a contrary viewpoint has been expressed that effective drug discovery from natural product sources can occur without seeking the acknowledged increased biodiversity of organisms from tropical countries [310], this has not been the experience of the present chapter authors. For example, using an experimental forest plot in southern Florida, the most promising lead compound obtained in our work was the compound, 3-chlorodeoxylapachol (**31**) (Fig. 27), which showed efficacy in the in vivo hollow fiber assay used [180]. However, this was deemed to be not of further interest for more advanced biological testing, since it is a simple derivative of lapachol, for which clinical trials had been initiated but then terminated due to a lack of promise [311]. In contrast, all of the plant compounds described in Sect. 4 of this chapter were obtained from source countries in tropical regions.

A major focus of this chapter has been a description of good practices in field work carried out towards obtaining primary collections and recollections of plants from tropical countries intended for use in the discovery of new anticancer agents. Detailed planning is required in advance of given field trip, and the work must be conducted in line with both the 1992 Convention on Biodiversity [83] and the 2010 Nagoya Protocol [84]. Efforts must be made to conserve the future supply of all

plants that are collected, and extensive use of herbarium resources are required to conduct this work in an expeditious and efficient manner. It is also important to communicate with colleagues in the source countries of plants collected. An example of this would be to convey the biological test results obtained of extracts of the species collected during a recent field trip expedition, and to document this information in the literature [e.g., 100].

Biologically active compounds that have been developed further in our plant-derived anticancer drug discovery program have shown considerable potency and/or selectivity in in vitro bioassays, as well as in vivo efficacy in hollow fiber [176, 177] and/or murine xenograft [174, 175] testing. However, while the representatives of the diphyllin glycoside [254], rocaglate [269], and tropane alkaloid ester [237] classes that have been further investigated were new compounds, even the very well known compound lupane-type triterpenoid, betulinic acid (**48**) (Fig. 32) has proven to be of outstanding interest for potential cancer therapy [215, 230]. If a plant antineoplastic compound is found to have an unusual cellular mechanism of action, this will greatly increase the overall scientific interest in the substance concerned. This has been exemplified by the compound (–)-silvestrol (**74**) (Fig. 40) [269], which was found to be a protein translation inhibitor acting on eukaryotic initiation factor (eIF) 4AI/II [293–295]. When developing further a potential anticancer agent from a plant, not only should a multidisciplinary collaborative group have access to as many relevant bioassays as possible, but efforts should be made to have compounds tested in more specialized assays available locally or nationally. Finally, it must be pointed out that the period between the initial publication of the structure of a plant natural product and the time of its first market approval may have taken many years. For example, in the case of paclitaxel (**11**) (Fig. 2), this was characterized structurally as taxol in 1971 [120], and only introduced clinically to treat breast cancer in 1994 [22]. An even more extreme example of this is omacetaxine mepesuccinate (**14**) (Fig. 3), which took 44 years to be given full approval for chronic myeloid leukemia by the U.S. FDA in 2014 [38, 39], after the initial isolation of this alkaloid as homoharringtonine from a plant source in 1970 [40].

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Joshua M. Henkin studied at the University of Pennsylvania, where he obtained his B.A. (2010) in Biochemistry. He performed undergraduate and post-baccalaureate research at the Monell Chemical Senses Center and at the University of Pennsylvania Museum of Archaeology and Anthropology, under the supervision of Dr. Patrick E. McGovern, and contributed to the characterization of almond volatiles and the phytochemistry of herbal wine residues from first millennium B.C.E. amphorae and other ceramic vessels discovered in archaeological contexts in southern France. This stimulated his burgeoning professional trajectory towards botany and pharmacognosy. He is currently pursuing his Ph.D. degree at the University of Illinois at Chicago, under the guidance of his major advisor, Prof. Djaja Djendoel Soejarto. He is investigating the medical ethnobotany and phytochemistry of plants collected from Laos and Vietnam, with a particular focus on the identification of taxa and potential lead compounds exhibiting cytotoxicity for anticancer drug discovery. His main academic interests include the botany, chemotaxonomy, and phytochemistry of plants employed medicinally and ritually, emphasizing the study of archaeological, ethnographic, and floristic accessions and collections at museums.

Yulin Ren received her Ph.D. degree in Medicinal Chemistry and Pharmacognosy in 2001 from Peking Union Medical College, Beijing, People's Republic of China, under the supervision of Professor Junshan Yang. After being trained as a postdoctoral researcher in both organic synthesis and biomedical sciences in the U.S., she joined the group of Prof. A. Douglas Kinghorn at the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University. Initially appointed as a Research Associate, she was promoted to Senior Scientist, and then to Research Scientist, with Principal Investigator status. Her research interests focus on plant natural product-based anticancer drug design and discovery, including the isolation of the major and minor components from higher plants, spectroscopy-aided structural determination of complex natural products, synthetic modification of bioactive lead compounds, single-crystal X-ray diffraction-supported determination of the conformation and configuration of compounds with multiple chiral centers, and bioassay design and development for the evaluation of their anticancer potential. Dr. Ren has co-authored over 50 peer-reviewed research and review articles and 2 book chapters, and has been named as a co-inventor on four U.S. and PCT worldwide patent publications. She received a Servier Young Investigator Award in Medicinal Chemistry in 2002, and an Outstanding Inventive Contributions Award from Ohio University (Athens, Ohio; 2002−2004). Dr. Ren has served as a referee for 13 scientific journals in her field.

Djaja Djendoel Soejarto obtained his Ph.D. degree in 1968 under Professor Richard E. Schultes at Harvard University. He served as a Latin American Teaching Fellow of Tufts University to teach at the Department of Biology of the University of Antioquia, Medellin, Colombia (1969–1972), and then as Associate Professor in Biology (1972–1976). He founded the Herbarium of the University of Antioquia in 1969 (now the second largest in Colombia), and carried out postdoctoral research (1972–1973) under the mentorship of Professor Schultes, on plants used for fertility regulation. He served as a Botanist Consultant to the Task Force on Plants for Fertility Regulation of the World Health Organization (1976–1979) and then became an Adjunct Associate Professor at the University of Illinois at Chicago (UIC) in 1979. He was promoted to Associate Professor in 1983, and to Professor in 1989. He retired in 2015 and currently has Emeritus status from UIC. Prof. Soejarto has also served as a Research Associate at the Field Museum (Botany), Chicago (1977–2013), and became an Adjunct Curator in 2013. His research interests embrace plant taxonomy, economic botany and ethnobotany, and pharmacognosy, with a focus on plant explorations in the tropics as part of multidisciplinary research endeavors in the discovery of biologically active compounds as potential candidates for pharmaceutical development.

Professor Soejarto has carried out extensive fieldwork in the context of plant drug discovery in Asia and the Americas. In 1994, he received an honorary plaque from the University of Antioquia (Colombia) for his contributions in the development of botanical science to the university during the dedication of the 100,000th specimen of the Herbarium of this institution; he also received a Recognition Plaque (2007) from the Colombian Association of Botany for his "Contribution to the Science of Botany in Colombia". He was recipient of the Senior University Scholar Award from the University of Illinois at Chicago (UIC) (1996), the Distinguished Economic Botany Award from the Society for Economic Botany (2011), and the Norman Farnsworth Excellence in Botanical Research from the American Botanical Council (2011). He served as the Principal Investigator of the plant collection contract in Southeast Asia with the U.S. National Cancer Institute (1986–2004), and as the Principal Investigator of an International Collaborative Biodiversity Groups (ICBG) National Institutes of Health-funded project entitled "Studies on Biodiversity of Vietnam and Laos" (1998–2012), focusing such research on plant drug discovery, biodiversity conservation, and economic development. He has authored or co-authored 313 publications (198 in peer-reviewed journals) and has served as Editorial Board member for ten scientific and professional journals. Prof. Soejarto was the Lead Editor of the "Journal of Ethnopharmacology" from 1988 to 2004. He has served as the mentor for 16 Ph.D. graduate students and 1 M.S. student at the Department of Medicinal Chemistry and Pharmacognosy, UIC. To his credit, 20 new plant species were discovered, and 4 new species carry his name as specific epithet.

A. Douglas Kinghorn has held the position of Professor and Jack L. Beal Chair in Natural Products Chemistry and Pharmacognosy at the College of Pharmacy, The Ohio State University (OSU) since 2004. He received Ph.D. (1975) and D.Sc. (1990) degrees from The School of Pharmacy, University of London (now the School of Pharmacy, University College London). He performed postdoctoral work at the University of Mississippi (1975–1976) and the University of Illinois at the Medical Center (1976–1977). From 1977–2004, he was a faculty member at the College of Pharmacy, University of Illinois at Chicago (UIC), where he rose through the academic ranks to become a Full Professor in 1986. At this institution, he was designated as the B. Kenneth West Senior Scholar (1993–1996) and was a recipient of the UIC 2002–2003 Award for Excellence in Teaching. In 2016, Prof. Kinghorn received the Distinguished Scholar Award of The Ohio State University. He is the Editor in Chief of the "Journal of Natural Products" (1994–present; co-published by the American Chemical Society and the American Society of Pharmacognosy) and of the book series "Progress in the Chemistry of Organic Natural Products" (2008–present; Springer International Publishing AG), and has served on the Editorial Boards of about 25 scientific journals. He is the Editor of "Toxic Plants" (1979; Columbia University Press, New York) and "Stevia: the Genus *Stevia*" (2002; Taylor & Francis, London), and Co-Editor of "Human Medicinal Agents from Plants" (1993; American Chemical Society Books, Washington, DC).

Prof. Kinghorn is a Fellow of five scientific and professional societies, and is also a Fellow of The School of Pharmacy, University of London. He is a former President of both the American Society of Pharmacognosy (1990–1991) and the Society for Economic Botany (1991–1992). He is an Honorary Member of the American Society of Pharmacognosy and the Phytochemical Society of Asia. Prof. Kinghorn received the 2010 Norman R. Farnsworth Research Achievement Award of the American Society of Pharmacognosy for lifetime contributions to natural products research. In 2011, he was awarded an honorary D.Sc. degree from the University of Bradford in the U.K. He has authored or co-authored over 550 peer-reviewed research articles, review articles, and book chapters. Since 1980, Prof. Kinghorn has served as Principal Investigator of projects supported by both the U.S. National Institutes of Health (NIH) and private industry. His research interests include the isolation and structural characterization of bioactive natural products from higher plants, particularly potential anticancer agents, antileishmanial compounds, cancer chemopreventives, and taste-modifying substances. Currently, he is Principal Investigator of a program project award from the U.S. National Cancer Institute, NIH, entitled "Discovery of Anticancer Agents of Diverse Natural Origin" (2007–2019). Previously, he was Principal Investigator of a National Cooperative Drug Discovery Groups (NCDDG) project entitled "Novel Strategies for Plant-derived Anticancer Agents", also funded by this same agency (1992–2005). Prof. Kinghorn has been Major and/or Thesis Advisor/Committee Chair to approximately 45 graduate students and has also directly supervised about 70 postdoctoral fellows and visiting scholars.

An Update of Erythrinan Alkaloids and Their Pharmacological Activities

Runner R. T. Majinda

Contents

1 Introduction

The genus *Erythrina* or the coral trees, is made of plants bearing bright red- ("erythros", meaning red in Greek) or yellow-colored flowers, and consists of trees, shrubs, and herbaceous plants that are distributed mainly in tropical and

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sub-tropical regions of the world. The genus *Erythrina* belongs to the family Leguminosae (syn: Fabaceae), subfamily Papilionoideae, tribe Phaseoleae (an alternative classification of family Fabaceae, subfamily Faboideae, and tribe Phaseoleae exists, but its use seems to have been replaced by the original classification). There is no general consensus as to the actual number of *Erythrina* species, with estimates during the past 4–6 decades putting these between 110 and 130 species [1]. In the 1970s to 1990s of the last century, work by Krukoff and Barneby 1974 [2] and Krukoff in 1982 [3] both put the estimate at 108 *Erythrina* species world-wide, while Mackinder in 1993 [4] and Bruneau in 1996 [5] estimated 130 and 112 species. In the past two decades, further estimates were made by Mackinder et al. in 2001 [6] and Bean in 2008 [7] who indicated the occurrence of 110 and 120 species of *Erythrina* world-wide. There are, however, further unreferenced estimates citing 200 species of *Erythrina* by Zhang et al. in 2017 [8] and Tan et al. in 2017 [9].

The genus *Erythrina* is known to elaborate, among other secondary metabolites, *Erythrina* alkaloids. The great majority of these alkaloids are characterized by a unique motif consisting of a tetracyclic spiroamine framework. These alkaloids possess a 6,5,6,6-membered indoloisoquinoline core [10, 11], specifically a 1,2,3,4,5,6,8,9-octahydroindolo[7*a*,1*a*]isoquinoline skeleton (Fig. 1). It soon became apparent that this core is not only confined to the *Erythrina* genus (Leguminosae, syn: Fabaceae) but also to the genera *Cocculus*, *Hyperbaena*, and *Pachygona* of the Menispermaceae family, thus necessitating a change to adopt the general name "erythrinan" alkaloids rather than the more specific name "erythrina" alkaloids. The alkaloids from *Erythrina* have C-15 and C-16 oxygenation patterns and are often referred to as "normal erythrinan alkaloids" while those from the Menispermaceae at some stage during their biosynthesis lose the C-16 oxygenation and thus are referred to as "abnormal erythrinan alkaloids". A group of alkaloids was identified with the *C*-homoerythrinan skeleton, which formerly was a small subgroup but is now clearly recognized as being more widespread in the plant kingdom than the erythrinan alkaloids. These alkaloids possess a 6,5,7,6-membered indolobenzazepine skeleton [12] (Fig. 1) first isolated from *Schelhammera pedunculata* (Liliaceae) [13]. Since these are more widespread than erythrinan alkaloids, the names "homoeythrinan" or "schelhammerans" are more appropriate than "homoerythrina" alkaloids. More than 70 homoerythrinan alkaloids have been reported to date [14].

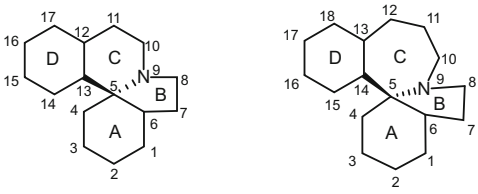


Fig. 1 Numbering system in erythrinan and homoerythrinan systems

The current contribution will not consider the homoerythrinan alkaloids but will concentrate only on the erythrinan alkaloids instead. Erythrinan alkaloids are classified according to the nature of the four rings forming the erythrinan nucleus (Fig. 1). The largest class comprises those in which the D ring is aromatic and hence referred to as aromatic erythrinan alkaloids, while the other group constitutes those in which the D ring is non-aromatic. The non-aromatic ring D could either be oxa- as in erythroidines (partial structures B-1 and B-2, Fig. 2), and cocculolidine (partial structure C in Fig. 2) or aza- as in erymelanthine (partial structure D in Fig. 2). The various appearances and substitution patterns in the four rings are shown in Fig. 2 and representative structures are illustrated in Fig. 4 and Table 10.

The first comprehensive review of known *Erythrina* alkaloids was arguably that by Hill [15], published in 1967, at which time only ten *Erythrina* alkaloids were known and the structures and configurations for most of them established. Subsequent minor reviews appeared between 1966 and 1980 by Kametani (1969) [16], Mondon (1970) [17], Kametani and Fukumoto (1972) [18], and Mathieson (1975) [19]. During this period, the number of *Erythrina* alkaloids rose sharply to over 60 [20] and the structures of these were deduced from mass spectrometric fragmentation analysis, ^{1}H-NMR spectroscopy, and by comparison with literature data for known compounds. It was during this period that some unusual (16-deoxy) erythrinan alkaloids were obtained from *Cocculus* species and also a group of the then nascent homoerythrina alkaloids were recognized. A review covering *Erythrina* and related alkaloids by Dyke and Quessy was published in 1981 [20]. This review covered *Erythrina* alkaloids, their occurrence, isolation and detection, structure determination, biosynthesis, and synthesis, with the homoerythrina and *Cephalotaxus* alkaloids included also, for the period from November 1966 to May 1979. In 1991, Amer and co-workers published a listing of 97 tetracyclic erythrina-type alkaloids originating mainly from *Erythrina* and *Cocculus* species together with their appropriate physical and spectroscopic data [21]. In 1996, Tsuda and Sano published a review of *Erythrina* and related alkaloids [14], which showed the number of erythrinan alkaloids to be 94 along with a substantial increase in homoerythrinan alkaloids. In their review, structure determination and synthesis aspects were covered. In 2007, a contribution by Reimann in this book series [12] summarized the literature relating to the erythrinan alkaloids isolated from the genera *Erythrina* and *Cocculus*, together with the homoerythrinan alkaloids, as obtained during the period 1996–2007. The search revealed that 12 more erythrinan alkaloids had been reported, bringing the total documented to over 105. This review also described the biosynthesis of *Erythrina* alkaloids in the light of the latest information, which led to a slight modification of the pathway suggested by Barton and co-workers [22–24]. The greater emphasis of this chapter was on the synthesis approaches towards *Erythrina* and related alkaloids. In this review, the results of a search were described for erythrinan alkaloids isolated from *Erythrina* species and Menispermaceae species. The most recent review on *Erythrina* and related alkaloids was published in 2010 by Parsons and Palframan [25]. This contribution summarized literature relating to the alkaloids isolated from the genus *Erythrina* since 1996, including work not covered in the 2007 review by Reimann [12], with particular

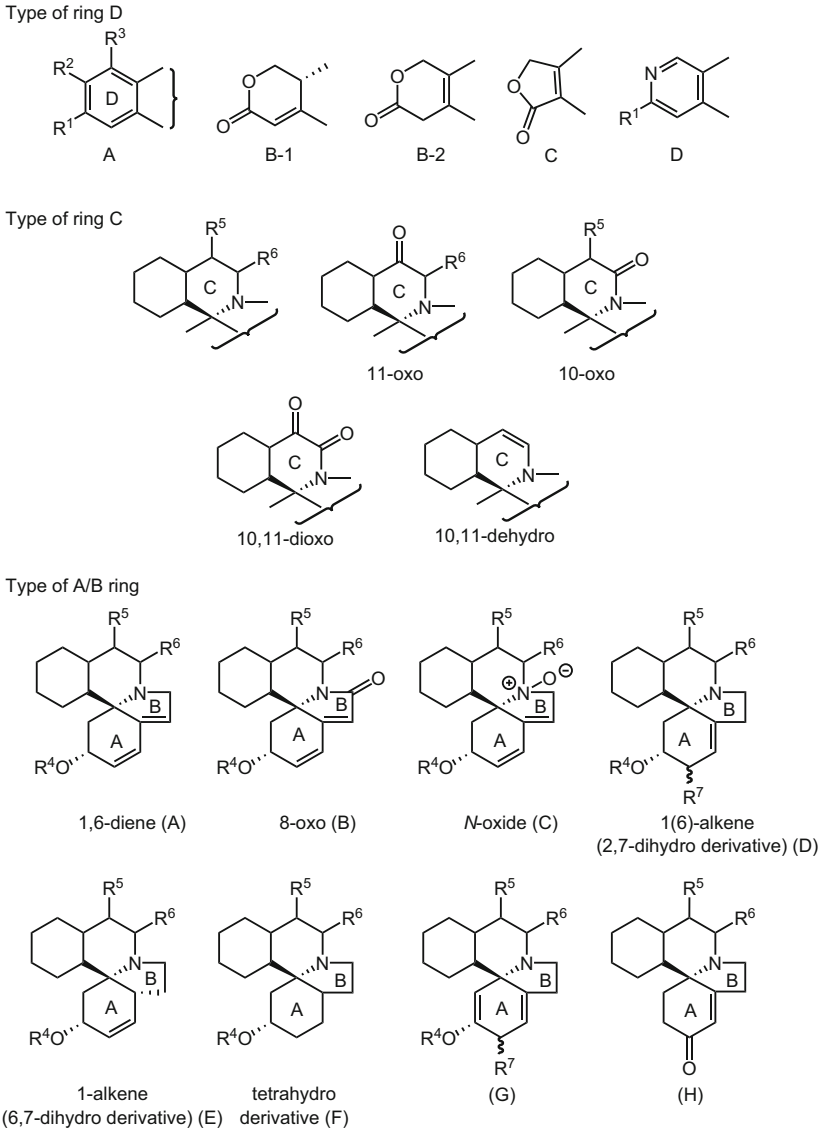


Fig. 2 Structural variants of erythrinan alkaloids

emphasis on synthesis strategies for *Erythrina* and related alkaloids. In the review, 7 more erythrinan alkaloids were reported, bringing the total of known alkaloids to 112. The current chapter continues where previous reviews have left off, and has uncovered 40 further erythrinan alkaloids giving a total of 154 known structures. The new alkaloids display a wide array of structural diversity including conjugate (hydrolyzable) dimeric, true dimeric, and trimeric structures with novel skeletons as

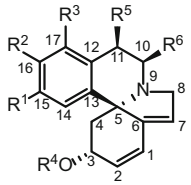
well as other adducts, for which the structures have not been described previously. It has proven necessary to reclassify what Amer et al. [21] termed dimeric dienoid alkaloids as conjugate dienoids. The current contribution has examined the previous reviews and where structural corrections and revisions were required these have been made. Included also are any structures that were omitted from other reviews.

The stereochemical aspects of erythrinan alkaloids derived from X-ray diffraction analysis and chemical correlation so far suggests that all natural erythrinan alkaloids reported to date have a (5*S*) absolute configuration and those with 3-oxygenation have a (3*R*) absolute configuration. In turn, all 11-methoxy or hydroxy- or glycosyloxy-analogs have a (11*S*)-configuration, or a β-orientation [14]. Some alkaloids in addition to C-3 oxygenation have 2-hydroxylation, such as erythratine (**100**) (2*R*,3*R*,5*S*) [20, 22], erythratidine (**95**) (2*S*,3*R*,5*S*) [20, 26], and 2-epierythratidine (**96**) [11]. The last-mentioned alkaloid initially was assigned (2*R*,3*R*,5*S*) based only on its simple ^{1}H and ^{13}C NMR spectra and by comparison with the literature data. It had to be revised to (2*S*,3*R*,5*S*), because the experimental data from detailed 2D NMR experiments, substantiated by a molecular dynamics search followed by quantum–mechanical calculations using density function theory/gauge invariant atomic orbitals, thereby supported the new assignment made. A cautionary word is that any future isolation of these compounds should be accompanied by a thorough study of their NMR spectra and where possible theoretical calculations should be performed to support the experimental data obtained.

The current contribution compiles all of the known erythrinan alkaloids to date as shown in Tables 1–10 and Figs. 3–7, totaling 154 compounds [9, 26–58]. The structures were organized into easily recognizable and user-friendly groups that hopefully should enable the reader to track and identify structures readily. Therefore, instead of putting the new alkaloids at the end of the contribution, which has been the format adopted in most past reviews, these have been placed in the relevant groups, and highlighted by means of the superscripts [a] and [b]. Several structures in previous reviews that were wrong were corrected in this current contribution, while those omitted in previous reviews were also included in this compilation, for the sake of completeness.

It is the author's opinion that compound **26** (erythascine; 11-acetoxyerysotrine), first reported by Ghosal et al. in 1972 [59] from the seeds of *Erythrina arborescens* (Plate 1), and compound **41** (Table 2), isolated by Hussain in 2002 [36] from the flowers of *E. stricta* (Plate 2) and named 11-acetylerysotrine, may be the same compound. Hussain provided only ^{1}H NMR data (no ^{13}C NMR data nor those from other 2D NMR experiments) and low-resolution MS data as the basis of the structural assignment made. The IR spectrum showed an absorption at 1760 cm^{-1}, pointing to an ester rather than a ketone carbonyl functional group.

Table 1 Erythrinan alkaloids of the dienoid type—unsubstituted C ring ($R^5 = R^6 = H$)

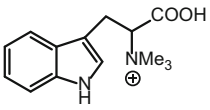


No	R^1	R^2	R^3	R^4	Name	Comment/remarks	Ref.
1	OMe	OMe	H	Me	Erysotrine		[14, 20, 29]
2	OMe	OH	H	Me	Erysodine		[14, 20]
3	OH	OMe	H	Me	Erysovine		[14, 20, 29]
4	OH	OH	H	Me	Erysopine		[14, 20, 29]
5	OH	OMe	H	H	Erysoline		[14, 20, 29]
6	OMe	OH	H	H	Erysonine		[14, 20]
7	OMe	OMe	H	H	Erythravine		[14, 20, 29]
8	OMe	OGlc	H	Me	Glucoerysodine		[14, 20]
9[a]	OMe	ORha	H	Me	Rhamnoerysodine?	Quinovoerysodine	[14, 30]
10	–OCH_2O–		H	Me	Erythraline		[14, 20, 29]
11	–OCH_2O–		H	H	Erythrocarine		[14, 31]
12	S_1	OMe	H	Me	Erysothiovine		[14, 20, 29]
13	OH	S_1	H	Me	Erysothiopine		[14, 20, 29]
14	OMe	H	H	Me	Coccuvinine		[14, 20]
15	OH	H	H	Me	Coccuvine	[20] gives incorrect structure	[14, 20]
16	OGlc	OH	H	Me	(+)-15-*O*-*β*-D-glucoerysopine		[12, 32]
17	OH	OGlc	H	Me	(+)-16-*O*-*β*-D-glucoerysopine		[12, 32]
18	OMe	Hyp	H	Me	Erysodinophorine hypaphorine conjugate ester		[14, 21]
19	OH	Hyp	H	Me	Erysopinophorine hypaphorine conjugate ester		[14, 21]
20	Hyp	OH	H	Me	Isoerysopinophorine	Hypaphorine conjugate ester	[14, 21]
21	Hyp	OMe	H	Me	Erysophorine	(should be erysovinophorine)	[14, 21]
22[b]	S_2	OMe	H	Me	Erysovine-15-*O*-sulfate	(sodium salt)	[33]
23[b]	S_3	OH	H	Me	Erysopine-15-*O*-sulfate		[33]

S_1: –OSO_2CH_2COOH; Glc: –*O*-*β*-D-glucosyl; S_2: –OSO_3Na; S_3: –OSO_3H; Rha: –*O*-*α*-L-rhamnosyl; Hyp: hypaphorine ester

[a]Close examination of the data shows that the sugar is quinovose and not rhamnose

[b]New alkaloid



hypaphorine

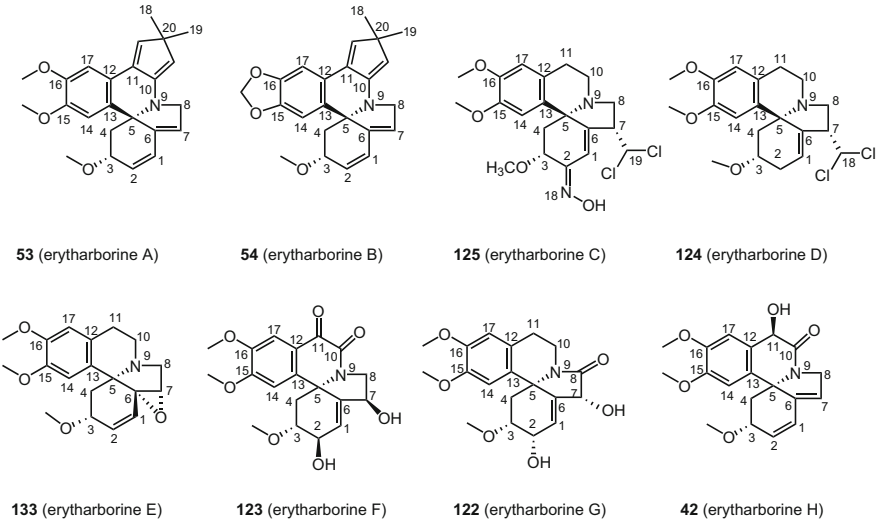


Fig. 3 Erytharborines A–H from *E. arborescens*

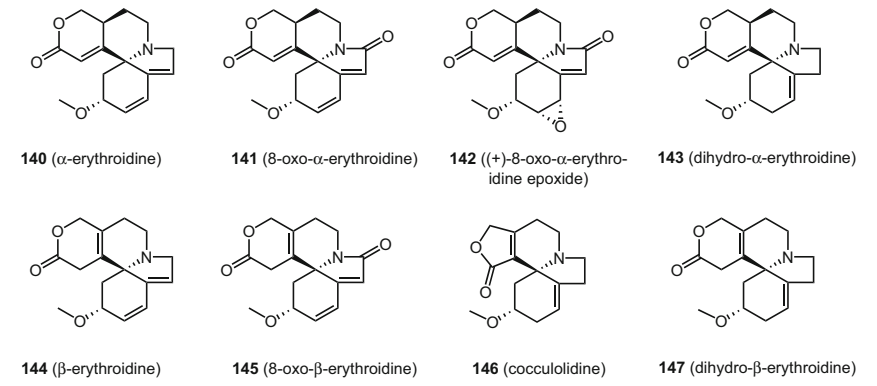
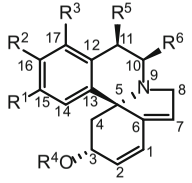


Fig. 4 Erythrinan alkaloids of the lactone type

Table 2 Erythrinan alkaloids of the dienoid type—substituted C ring ($R^5 \neq H$, $R^6 = H$, -H or not H)



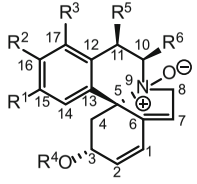
No	R^1	R^2	R^3	R^4	R^5	R^6	Name	Comments/remarks	Ref.
24	OMe	OMe	H	Me	OH	H	Erythrartine	11-Hydroxyerysotrine	[14, 20]
25	OMe	OMe	H	Me	OMe	H	Erythristemine	11-Methoxyerysotrine	[14, 20, 26]
26[a]	OMe	OMe	H	Me	OAc	H	Erythrascine	11-Acetoxyerysotrine (cf structure **41**)	[14, 20, 29]
27	–OCH_2O–		H	Me	OH	H	Erythrinine	11-Hydroxyerythraline	[14, 20, 29]
28	–OCH_2O–		H	Me	OMe	H	11-Methoxyerythraline		[14, 20, 29]
29	OH	OMe	H	Me	OH	H	11-Hydroxyerysovine		[14, 20, 29]
30	OH	OMe	H	Me	OMe	H	11-Methoxyerysovine		[14, 20, 29]
31	OMe	OH	H	Me	OH	H	11-Hydroxyerysodine		[14, 20, 29]
32	OMe	OH	H	Me	OMe	H	11-Methoxyerysodine		[14, 20, 29]
33	OH	OH	H	Me	OMe	H	11-Methoxyerysopine		[14, 20, 29]
34	OMe	OGlc	H	Me	OMe	H	11-Methoxyglucoerysodine		[14, 30]
35[b]	OMe	OGlc	H	Me	OH	H	11β-Hydroxyerysodine-glucose	Minimal structural proof	[60]
36	OGlc	OMe	H	Me	OMe	H	11-Methoxyglucoerysovine		[14, 30]
37[b]	–OCH_2O–		H	Me	OGlc	H	Erythraline-11β-*O*-glucopyranoside		[9]
38[b]	OMe	OMe	H	H	α-OH	H	(+)-11α-Hydroxyerythravine?	(**39** and **40** missing from review [12])	[34]
39[b]	OMe	OMe	H	H	α-OH	H	11β-Hydroxyerysotrine?	Should be (+)-11α-hydroxyerysotrine	[34]
40[b]	–OCH_2O–		H	Me	α-OH	H	(+)-Epierythrinine?	(as inseparable mixture with erythrinine)	[12, 35]

41[a]	OMe	OMe	H	Me	Ac	H	11-Acetylerysotrine?	(min. structural proof; no ^{13}C and 2D NMR)	[12, 36]
42[b]	OMe	OMe	H	Me	OH	=O	Erytharborine H		[37]
43[b]	OMe	OMe	H	Me	=O	OH	10-Hydroxy-11-oxoerysotrine	(missed from previous review [25])	[38]
44	OMe	OMe	H	Me	=O	=O	(+)-10,11-Dioxoerysotrine		[12, 39]
45	$-OCH_2O-$		H	Me	=O	=O	(+)-10,11-Dioxoerythraline		[12, 40]
46	$-OCH_2O-$		H	Me	=O	OH	(+)-Erythbidin B	First C-10 oxygenated alkaloid reported	[12, 41]
47	$-OCH_2O-$		H	Me	=O	H	11-Oxoerythraline		[14, 20, 29]
48	OH	OMe	H	Me	=O	H	11-Oxoerysovine		[14, 20, 29]
49	OMe	OH	H	Me	=O	H	11-Oxoerysodine		[14, 20, 29]
50	OH	OH	H	Me	=O	H	11-Oxoerysopine		[14, 20, 29]
51	OH	OMe	H	Me	*	*	10,11-Dehydroerysovine	10,11-Double bond (artifact?)	[14, 20, 29]
52	OMe	OH	H	Me	*	*	10,11-Dehydroerysodine	10,11-Double bond (artifact?)	[14, 20, 29]
53[b]	OMe	OMe	H	Me	S^o		Erytharborine A	R^5+R^6 form a 2*H*-imidazole ring	[37]
54[b]	$-OCH_2O-$		H	Me	S^o		Erythrarborine B	R^5+R^6 form a 2*H*-imidazole ring	[37]

S^o = R^5+ R^6 = 

[a]Compounds **26** and **41** may be the same
[b]New alkaloid
*10,11-double bond

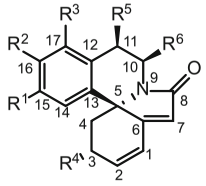
Table 3 Erythrinan alkaloids of the dienoid type—*N*-oxides



No	R^1	R^2	R^3	R^4	R^5	R^6	Name	Comments/remarks	Ref.
55	OMe	OMe	H	Me	H	H	Erysotrine *N*-oxide	(Juma and Majinda [42] not first to report)	[14, 42, 43]
56	OMe	OMe	H	Me	OMe	H	Erythristemine *N*-oxide		[14]
57	OMe	OMe	H	Me	OH	H	Erythrartine *N*-oxide		[14, 43]
58	–OCH_2O–		H	Me	H	H	(Erythraline *N*-oxide)	(same as cristanine A)	[14, 37, 44]
59	–OCH_2O–		H	Me	OMe	H	11-Methoxyerythraline *N*-oxide		[14]
60	OMe	OMe	H	Me	OH	H	(+)-11β-Hydroxyerysotrine *N*-oxide	(structure in review [25] wrong)	[25, 42]
61[a]	OMe	OMe	H	Me	α-OH	H	(+)-11α-Hydroxyerysotrine *N*-oxide		[42]
62[a]	OMe	OMe	H	Me	OMe	H	(+)-11β-Methoxyerysotrine *N*-oxide		[42]
63[a]	S_1	OMe	H	Me	H	H	Erysovine-*N*-oxy-15-*O*-sulfate	(sodium salt)	[33]
64[a]	OMe	OH	H	Me	H	H	Erysodine *N*-oxide	(missed from previous reviews)	[61]
65[a]	OH	OMe	H	Me	H	H	Erysovine-*N*-oxide	(missed in previous review [25])	[45]

S_1: –OSO_3Na
[a]New alkaloid

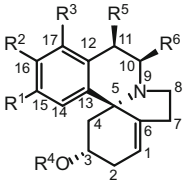
Table 4 Erythrinan alkaloids of the dienoid type—8-oxo derivatives



No	R^1	R^2	R^3	R^4	R^5	R^6	Name	Comments/remarks	Ref.
66	OMe	OMe	H	OMe	H	H	Erysotramidine	8-Oxoerysotrine	[14, 20]
67	OMe	OMe	H	OMe	OH	H	(+)-11*β*-Hydroxyerysotramidine		[42]
68	OMe	OMe	H	OMe	OMe	H	(+)-11*β*-Methoxyerysotramidine		[42]
69	–OCH_2O–		H	OMe	H	H	8-Oxoerythraline		[14]
70	–OCH_2O–		H	OMe	OH	H	8-Oxoerythrinine		[14]
71	H	OMe	H	OMe	H	H	Isococcolinine		[14, 46]
72	H	OH	H	OMe	H	H	(Isococcoline)		[14, 46]
73	OMe	H	H	OMe	H	H	Coccolinine	8-Oxococcuvinine	[14, 20]
74	OH	H	H	OMe	H	H	Coccoline	8-Oxococcuvine	[14, 20]
75	OMe	OMe	H	OMe	*	*	Erytharbine	10,11-Dehydroerysotramidine	[14, 20]
76	–OCH_2O–		H	OMe	*	*	Crystamidine	10,11-Dehydro-8-oxoerythraline	[14, 20]
77[a]	OMe	OMe	H	OMe	H	OH	Cristanine(s) C	10*β*-Hydroxyerysotramidine	[47]
78[a]	OMe	OGlc	H	OMe	H	H	16-*O*-*β*-D-Glucopyranosylcoccoline	16-*β*-D-Gluco-8-oxoerysodine	[33]
79	OH	OMe	H	H	H	H	Erythromotidienone	3-Deoxy derivative (rare)	[12, 48]
80	–OCH_2O–		H	H	H	H	Erythrosotidienone	3-Deoxy derivative (rare)	[12, 48]
81[a]	OMe	OMe	H	OMe	=O	=O	(+)-10,11-Dioxoerysotramidine	(missing in the last review)	[62]
82[a]	OMe	OMe	H	OMe	OH	=O	(+)-11*β*-Methoxy-10-oxoerysotramidine	(missing in the last review)	[62]

[a]New alkaloid
*10,11-double bond

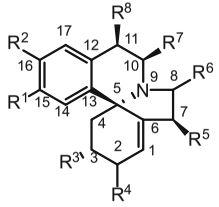
Table 5 Erythrinan alkaloids of the 1(6)-alkenoid type—2,7-dihydro derivatives—unsubstituted ring C



No	R^1	R^2	R^3	R^4	R^5	R^6	Name	Comments/remarks	Ref.
83	OMe	OMe	H	Me	H	H	(Dihydroerysotrine)	2,7-Dihydro	[14, 20]
84	OH	OMe	H	Me	H	H	Dihydroerysodine		[14, 20]
85	OH	OMe	H	Me	H	H	Dihydoerysovine		[14, 20]
86	S_4	OMe	H	Me	H	H	Erythroculine		[14, 20]
87	S_5	OMe	H	Me	H	H	Erythramide		[14, 49]
88	S_4	OMe	OH	Me	H	H	Erythlaurine		[14, 49]
89	–OCH_2O–		H	Me	H	H	Erythramine		[14]
90	OMe	H	H	Me	H	H	Cocculidine	*O*-Methylcocculine	[14, 20]
91	OH	H	H	Me	H	H	Cocculine		[14]
92[a]	OH	H	H	Me	H	H	Pachygonine	Cocculine metho-salt	[14, 50]
93	OH	H	H	H	H	H	Cocclafine		[14]
94	OH	H	OMe	Me	H	H	Coccutrine		[14, 20]

S_4: -$COOCH_3$; S_5: -$CONH_2$
[a]N-9 methylated to form a metho-salt

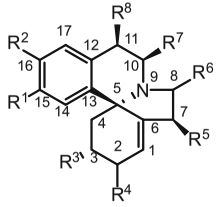
Table 6 Erythrinan alkaloids of the 1(6)-alkenoid type—2,7-dihydro derivatives



No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
95	OMe	OMe	OMe	α-OH*	H	H	H	H	Erythratidine	(*may need revision to β-OH)	[14, 20]
96	OMe	OMe	OMe	β-OH*	H	H	H	H	2-Epierythratidine	(*recently revised to α-OH Ref. [11])	[11, 14]
97	OH	OMe	OMe	OH	H	H	H	H	Erysalvine		[14, 20]
98	OMe	OH	OMe	α-OH	H	H	H	H	Erysotine		[14, 20]
99	OH	OH	OMe	OH	H	H	H	H	Erysopitine		[14, 20]
100	–OCH_2O–		OMe	β-OH	H	H	H	H	Erythratine		[14, 20]
101	OMe	H	OMe	β-OH	H	H	H	H	Cocculitine	*O*-Methylcocculitinine	[14]
102	OH	H	OMe	β-OHe	H	H	H	H	Cocculitinine		[14]
103	OMe	OMe	OMe	=O	H	H	H	H	Erythratidinone		[14, 20]
104	–OCH_2O–		OMe	=O	H	H	H	H	Erythratinone		[14, 20]
105	OMe	OH	OMe	=O	H	H	H	H	Erysotinone		[14, 20]
106[a]	OMe	Rha	OMe	=O	H	H	H	OH	11-Hydroxyerysotinone-rhamnoside	(minimal structural proof)	[60]
107	OH	OH	OMe	=O	H	H	H	H	Erysoflorinone	Erysopitinone	[14, 20]
108	OH	OMe	OMe	=O	H	H	H	H	Erysalvinone		[14, 20]
109	OMe	H	OMe	=O	H	H	H	H	Coccudinone		[14]
110	OMe	OMe	OMe	β-OH	H	H	H	OH	11-Hydroxyepierythratidine		[14, 51]

(continued)

Table 6 (continued)



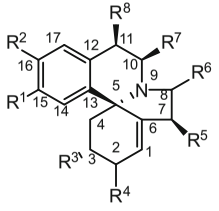
No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
111	OMe	OMe	OMe	*α*-OH	H	H	H	OMe	11-Methoxyerythratidine		[14, 31]
112	–OCH_2O–		OMe	*β*-OH	H	H	H	OMe	11-Methoxyerythratine		[14, 52]
113[b]	OMe	H	OMe	H	H	H	H	H	Cocculidine *N*-oxide	*N*-oxide at N-9	[12, 53]
114	–OCH_2O–		OMe	*β*-OH	H	H	=O	=O	(+)-10,11-Dioxoerythratine		[25, 54]
115	OMe	OMe	OMe	*β*-OH	H	H	=O	=O	(+)-10,11-Dioxoepierythratidine		[25, 54]
116	OMe	OMe	OMe	=O	H	H	=O	=O	(+)-10,11-Dioxoerythratidinone		[25, 54]

Rha: *α*-L-rhamnopyranoside

[a]New alkaloid

[b]Note the presence of *N*-oxide at N-9, though not shown in the parent base structure

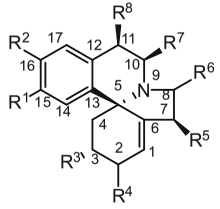
Table 7 Erythrinan alkaloids of the 1(6)-alkenoid type—2,7-dihydro derivatives



No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
117	S_4	OMe	-CH_2O-		H	H	H	H	3-Demethoxy-2α,3α-methylenedioxyerythroculine		[14, 55]
118[a]	S_5	OMe	-CH_2O-		H	H	H	H	15-Amido-3-demethoxy-2α,3α-methylenedioxyerythroculine		[56]
119[a]	OMe	OMe	OMe	β-OH*	H	H	H	H	Cristanine E	*N*-oxide (*may need revision to α-OH	[47]
120[a]	–OCH_2O–		OMe	β-OH*	H	H	β-OH	H	Cristanine D	(*may need to revision to α-OH [11])	[47]
121[a]	–OCH_2O–		OMe	β-OH*	α-OH	H	H	H	Cristanine B	(*may need revision to α-OH [11])	[44]
122[a]	OMe	OMe	OMe	α-OH	α-OH	=O	H	H	Erytharborine G		[37]

(continued)

Table 7 (continued)

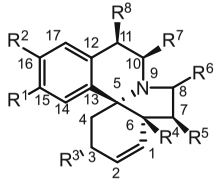


No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
123[a]	OMe	OMe	OMe	β-OH	β-OH	H	=O	=O	Erytharborine F		[37]
124[a]	OMe	OMe	OMe	H	S_6	H	H	H	Erytharborine D		[37]
125[a]	OMe	OMe	OMe	S_7	S_6	H	H	H	Erytharborine C		[37]
126	OMe	OMe	H	=O	H	H	H	H	3-Demethoxyerythratidinone		[14, 20]
127	OMe	H	OMe	=O	H	H	H	H	Coccudinone		[57]

S_4: -$COOCH_3$; S_5: -$CONH_2$; S_6: α-$CHCl_2$; S_7: =N-OH

[a]New alkaloid

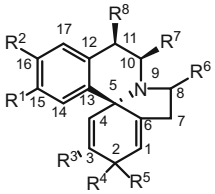
Table 8 Erythrinan alkaloids of the 1(2)-alkenoid type—6,7-dihydro derivatives



No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
128	OMe	H	OMe	H	H	H	H	H	Isococculidine	Dihydrococcuvinine	[14, 20, 57]
129	OH	H	OMe	H	H	H	H	H	Isococculine	Dihydrococcuvine	[14, 20, 57]
130	OH	H	OMe	H	H	H	H	OMe	Cocculimine	Dihydrococcuvinine	[14, 20, 57]
131[a]	OMe	OMe	OMe	H	H	H	H	OMe	6,7-Dihydro-11-methoxyerysotrine	(minimal structural proof)	[62]
132[a,b]	OMe	OMe	OMe	H	H	H	H	H	6,7-Dihydro-17-hydroxyerysotrine	(minimal structural proof)	[62]
133[a]	OMe	OMe	OMe	—O—		H	H	H	Erytharborine E	6,7-Epoxy	[37]
134[a,c]	-OCH_2O-		OMe	[c]	[c]	=O	H	H	8-Oxoerythraline epoxide	(1,2-α-epoxy/6,7-double bond)	[37, 40]

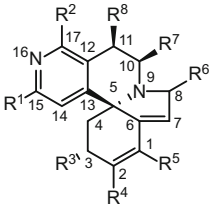
[a]New alkaloid
[b]Note additional 17-hydroxy group, although not shown in the parent structure
[c]Note presence of 1,2-epoxy group and 6,7-double bond

Table 9 Erythrinan alkaloids of the 1(6), 3(4)-dienoid type



No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/ remarks	Ref.
135	OMe	OMe	OMe	H	OH	H	H	H	Erythritol		[14, 46, 58]
136	OH	H	OMe	=O		H	H	H	Erysodienone	R^4+ R^5 is =O	[14, 20, 29]
137	OMe	H	OMe	=O		H	H	H	Coccudienone	R^4+ R^5 is =O	[57]

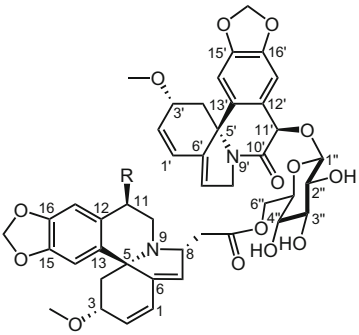
Table 10 Erythrinan alkaloids of the 16-aza-type



No	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Name	Comments/remarks	Ref.
138	S_4	H	OMe	H	H	H	H	H	(+)-Erymelanthine		[14, 21, 27]
139	S_4	H	OMe	H	H	=O	H	H	(+)-Melanacanthine	8-Oxoerymelanthine (minimal structural proof)	[21, 28]

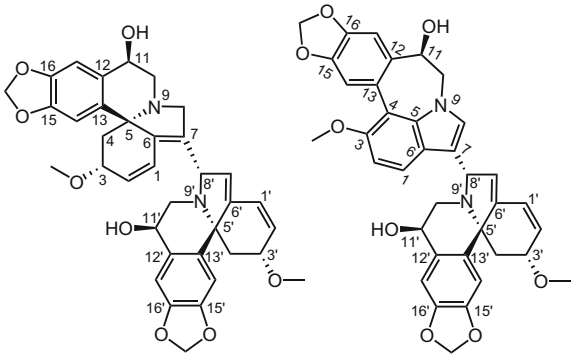
S_4: -$COOCH_3$

Fig. 5 Conjugate (hydrolyzable) dimeric erythrinan alkaloids



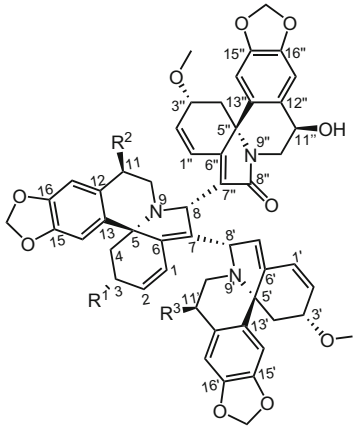
148 R = OH (erythrivarine C)
149 R = OCH_3 (erythrivarine D)

Fig. 6 Structures of erythrivarine A and B, true dimeric erythrinan alkaloids



150 (erythrivarine A) **151** (erythrivarine B)

Fig. 7 Trimeric erythrinan alkaloids



152 R^1 = OCH_3 R^2 = OH R^3 = OH (erythrivarine E)
153 R^1 = OCH_3 R^2 = OCH_3 R^3 = OH (erythrivarine F)
154 R^1 = =O R^2 = OH R^3 = OCH_3 (erythrivarine H)

Plate 1 *Erythrina arborescens*; photograph: Woudloper, Wikimedia Creative Commons

Plate 2 *Erythrina stricta*; photograph: Sailesh, Wikimedia Creative Commons

2 An Update of the Erythrinan Alkaloids

Ozawa, Kishida, and Ohsaki [33] reported the four new erythrinan alkaloids erysovine-15-*O*-sulfate (**22**), erysopine-15-*O*-sulfate (**23**), erysovine *N*-oxy-15-*O*-sulfate (**63**), and 16-*O*-*β*-D-glucopyranosylcoccoline (**78**), isolated from the methanolic and aqueous methanolic extracts of the seeds of *Erythrina velutina* (Plate 3). Their structures were elucidated using spectroscopic techniques including HR-FAB (+)-MS and 1D- and 2D-NMR. The appropriate name for compound **78**, in the opinion of the author, would be 16*β*-D-gluco-8-oxoerysodine, since hydrolyzing the sulfate group would yield 8-oxoerysodine and not coccoline (**74**). Compounds **12** (erysothiovine) and **13** (erysothiopine), along with **22**, **23** (Table 1), and **63** (Table 3) are, so far, the only five reported sulfated erythrinan alkaloids.

Plate 3 *Erythrina velutina*; photograph: D. Keats, Wikimedia Creative Commons

Plate 4 *Erythrina crista-galli*; photograph: P. D. Flores, Wikimedia Creative Commons

Tan et al. in 2017 reported the new alkaloid, erythraline-11-*β*-*O*-glucopyranoside (**37**) (Table 2), along with the known alkaloids erythraline (**10**), erythrartine (**24**), erysodine (**2**), erysotrine (**1**), and (+)-16*β*-D-glucoerysodine (**17**) from the methanolic extract of the seeds of *Erythrina crista-galli* (Plate 4). The structures of the new and known compounds were elucidated using techniques including HR-ESI-MS and 1D and 2D-NMR spectroscopy.

The new erythrinan alkaloid (+)-11*α*-hydroxyerythravine (**38**), and the known *α*-hydroxyerysotrine (actually 11*α*-hydroxyerysotrine) (**39**), and erythravine (**7**) were isolated from the alkaloidal fraction of the flowers of *Erythrina mulungu* (Plate 5) [34].

The structures of alkaloids **7**, **38**, and **39** were again elucidated using techniques including HR-ESI-MS-MS and 1D and 2D-NMR spectroscopy. Although

Plate 5 *Erythrina mulungu* (= *E. verna*); photograph: J. Medeiros, Wikimedia Creative Commons

compound **39** was not reported as new, there is no report of this compound from Nature except its C-11 epimer. What is interesting is that all C-11 oxygenated erythrinan alkaloids show a β-orientation, with only three compounds (**38**–**40**) to date known to show α-orientation. It is even more interesting that the work by García-Beltrán et al., 2012 [11] suggests that the future characterization of 2- and/or 11-oxygenated erythrinan alkaloids need a very thorough NMR analysis, preferably accompanied by theoretical calculations to confirm the NMR assignments made. These researchers also revised the structure of 2-epierythratidine (**96**) from 2α-OH to 2β-OH, sounding a caution that many related structures may have to be revised in the light of these findings.

The compound 10-hydroxy-11-oxoerysotrine (**43**) was reported for the first time by Tanaka et al., 2008 [38] from the flowers of *Erythrina herbacea* along with the known compounds erytharbine (**75**), 10,11-dioxoerysotrine (**44**), erythrartine (**24**), erysotramidine (**66**), and erysotrine *N*-oxide (**55**). The structure of **43** and other known isolates were determined using conventional spectroscopic methods including HR-EI-MS and 1D and 2D-NMR spectroscopy. This was the second report of an erythrinan alkaloid with a hydroxy group at C-10, with the first one being (+)-erythbidin B (**46**) [41]. The third member, cristanine(s) C (**77**) has been reported very recently by Wang et al. in 2018, from the seeds of *Erythrina crista-galli* (Plate 4) [47]. The absolute stereochemistry of each of **43**, **77**, and **46** at C-3, C-5, and C-10 was determined as (3*R*,5*S*,10*R*). The compound erysovine *N*-oxide (**65**) was isolated from the seeds of *Erythrina addisoniae* (Plate 6) along with the known alkaloids erysosalvinone (**108**), erysodine (**2**), glucoerysodine (**8**), erysotrine (**1**), erysovine (**3**), erythraline (**10**), erysopine (**4**), and an indole derivative [45].

The structures of **65** and some known alkaloids were determined using spectroscopic techniques including HR-ESI-MS and 1D and 2D-NMR spectroscopy. The erythrinan alkaloid 15-amido-3-demethoxy-2α,3α-methylenedioxyerythroculine (**118**)

Plate 6 *Erythrina addisoniae*; photograph: D. Avery, Wikimedia Creative Commons

was isolated from the leaves of *Hyperbaena valida* (Menispermaceae) [56] along with the known 3-demethoxy-2α,3α-methylenedioxyerythroculine (**117**). Compound **118**, containing a carboxamido group at the C-15 position, is related structurally only to erythramide (**87**), first isolated from *Cocculus laurifolius* (Menispermaceae) [49]. Compound **87** is also related structurally to erythroculine (**86**) and erythlaurine (**88**). These compounds with S_4 [-$CONH_2$, carboxamido] (**118**, **87**) and S_5 [-$COOCH_3$, methyl carboxy ester] (**86**, **88**, **138**, **139**) functionalities at C-15 pose some very interesting questions as to their biosynthesis and, as yet, no sound proposal has been made as to how these may be produced in menispermaceous plants. The compound, 8-oxoerythraline epoxide (**134**), was first isolated by Tanaka et al. in 1999 from the flowers of *Erythrina* x *bidwilli* [40] and then re-isolated by Wu et al. in 2017 from the flowers of *Erythrina arborescens* (Plate 1) [37]. Although not new, this natural product seems to have been missed from previous reviews. This is one of only three erythrinan alkaloid epoxides reported to date, with the other two being erytharborine E (**133**) and (+)-8-oxo-α-erythroidine epoxide (**142**). Ozawa et al. in 2010 [44] reported the two new compounds cristanine A (**58**) and cristanine B (**121**) from the bark of *Erythrina crista-galli* (Plate 4) along with the known erythrinan alkaloids, erythratine (**100**), crystamidine (**76**), erysovine (**3**), erysotrine (**1**), erythraline (**10**), 8-oxoerythraline (**69**), erythrinine (**27**), 8-oxoerythrinine (**70**), erythratidine (**95**), and 2-epierythratidine (**96**). Cristanine A turned out to be the already known compound erythraline-

N-oxide (**58**). Previously, erythrinan alkaloids with C-7 substitution were unknown and cristanine B was the first example having C-7 hydroxylation. Recently, two more C-7 hydroxylated erythrinan alkaloids, erytharborine G (**122**) and erytharborine F (**123**), were reported [37], as well as the 6,7-epoxy derivative, erytharborine E (**122**) [37]. Wang et al. in 2018 [47] isolated three new erythrinan alkaloids from the seeds of *Erythrina crista-galli* (Plate 4), (3*R*,5*S*,10*R*)-10-hydroxyerysotramidine (named cristanine(s) C) (**77**), (3*R*,5*S*,10*R*)-10-hydroxyerythratine (named cristanine(s) D) (**120**) and erythratidine *N*-oxide (named cristanine(s) E) (**119**), along with the 11 known alkaloids erysotramidine (**66**), 8-oxoerythraline (**69**), erythratidine (**95**), erythratine (**100**), erysotrine (**1**), erysodine (**2**), erysovine (**3**), erythraline (**10**), cristamidine (**76**), erythrarbine (**75**), and erysotrine *N*-oxide (**55**), and their structures were elucidated using spectroscopic techniques including HR-ESI-MS and 1D and 2D-NMR. Recent compound isolation work by Wu et al. [37] has afforded structural diversity in types of erythrinan alkaloids that was hitherto rare or uncommon. As mentioned above, substitution at C-7 is very infrequent, with cristanine B (**121**), in having a C-7-hydroxylation, being the only known example, as reported in 2010 by Ozawa et al. [44]. Wu et al. in 2017 [37] isolated the eight new erythrinan alkaloids erytharborines A (**53**), B (**54**), C (**125**), D (**124**), E (**133**), F (**123**), G (**122**), and H (**42**) from the flowers of *E. arborescens* (Plate 1), along with 17 known alkaloids. These eight new alkaloids exhibited some structural features (Fig. 3) not found before among the erythrinan alkaloids, such an additional fifth ring with a 2*H*-imidazole moiety (erytharborines A and B (**53**) and (**54**)), a dichloromethyl substituent at C-7 (erytharborines C and D (**125**) and (**124**)), an (*E*)-oxime group at C-2 (erytharborine C (**125**), a 6,7-epoxy group (erytharborine E (**133**)), and the rare 7α-hydroxy (erytharborine G (**122**)) and 7β-hydroxy substitution (erytharborine F (**123**)).

The structures of these erytharborines were elucidated using, among other techniques, HR-ESI-MS and 1D and 2D-NMR experiments, such as HMQC, HMBC, NOESY, and ROESY. The known alkaloids were erytharbine (**75**), 8-oxoerythraline epoxide (**134**), erythratidinone (**103**), erythratine (**100**), erysotramidine (**66**), 10,11-dioxoerysotrine (**44**), 11β-hydroxyerysotramidine (**67**), erythrartine (**24**), erythrartine *N*-oxide (**57**), erysotrine (**1**), 8-oxoerythrinine (**70**), 8-oxoerythraline (**69**), erythraline (**10**), erythraline *N*-oxide (**58**), erythrinine (**27**), erysodine (**2**), and erysovine (**3**).

The structures of erytharborines A–D are quite interesting from a biosynthesis point of view, in that it is difficult to envision how a 2*H*-imidazole ring is formed in erytharborine A (**53**) and B (**54**), and also the biosynthesis origins of a dichloromethyl group in erytharborine C (**125**) and D (**124**) in addition to an (*E*)-oxime functionality in **125** are obscure. These compounds (erytharborines A–G) were isolated in very small quantities (only 1–2 mg from 6.5 kg powdered sample!) and it is possible that these are products formed when already pre-formed secondary metabolites undergo secondary reactions with some exogenous reagents. Erytharborines A and B may have arisen from 10,11-dioxo derivatives, and, among the known isolated alkaloids, was 10,11-dioxoerysotrine (**44**). Erytharborine A (**53**) could have been generated from a reaction between **44** and a diamine, probably ethane- or propane-2,2-diamine. In turn, erytharborine B (**54**) may result from an erysodine or erysovine precursor of **44** where either 15-hydroxy/16-methoxy or 16-hydroxy/15-methoxy groups could have formed

a methylenedioxy unit, leading to the formation of 10,11-dioxoerythraline (**45**), which would further react with a diamine to form erytharborine B (**54**). A pertinent question is where did the diamine originate? It is equally difficult to explain how an oxime functionality could be formed at C-2 in erytharborine C (**125**), except to speculate that it may have arisen from a reaction of erythratidinone (**103**) (co-isolated with **125**) with hydroxylamine. The dichloromethyl group attached at C-7 is also difficult to explain for both erytharborines C and D. Erytharborine E (**133**) is the sole example of an erythrinan alkaloid with a 6,7-epoxy moiety and only one of three epoxides reported among the erythrinan alkaloids. Some 6,7-epoxides, however, been reported in the related homoerythrinan alkaloids, where at least seven members occur [14].

2.1 D-oxa or Lactone Erythrinan Alkaloids

These are erythrinan alkaloids in which the D-ring has been modified by oxidation and then lactonized to give either a six-membered ring (Fig. 4 structures **140**–**147**) or a five-membered ring (**146**). The compounds α- and β-erythroidines (**140** and **144**) were documented initially as a mixture by Folkers and Majors in 1937 [63] from *Erythrina americana* (Plate 7), and the structure of β-erythroidine was later confirmed by Boekelheide et al. [64] in 1953. The two compounds were later described as constituents of the flowers of *Erythrina americana* [14, 65] while their 8-oxo-derivatives, 8-oxo-α-erythroidine (**141**) and 8-oxo-β-erythroidine (**145**), were isolated subsequently from the seeds and leaves of *E. berteroana*, together with α- and β-erythroidines [14, 66]. Tanaka et al. in 2001 [67] purified a 2,3-epoxide named (+)-8-oxo-α-erythroidine epoxide (**142**) from the wood of *Erythrina poeppigiana*

Plate 7 *Erythrina americana* (with hummingbird); photograph: F. E. Guerrero, Wikimedia Creative Commons

Plate 8 *Erythrina poeppigiana*; photograph: Veronidae, Wikimedia Creative Commons

(Plate 8) [12, 67] together with 8-oxo-α-erythroidine (**141**) and another 2,3-epoxide named 8-oxoerythraline epoxide (**134**). The only five-membered lactone derivative described to date is cocculolidine (**146**), which was first reported by Wada et al. in 1966 [68] from the fresh leaves of *Cocculus trilobus* (Menispermaceae) (Plate 9) and later re-isolated by Elsohly et al. in 1976 [69] from an ethanolic extract of the fruits of *Cocculus carolinus*, together with cocculine (**91**).

Plate 9 *Cocculus trilobus*; photograph: Dalgial, Wikimedia Creative Commons

2.2 Conjugate (Hydrolyzable) Dimeric Erythrinan Alkaloids

Alkaloids of the conjugated type, where an erythrinan alkaloid is esterified with the quaternary base, hypaphorine, were first reported from *Erythrina arborescens* (Plate 1). Ghosal and Srivastava [70] isolated the first conjugated alkaloid erysophorine (**21**), from the seeds of this species. The pod walls gave erysodinophorine (**18**) [71] and erysopinophorine (**19**) hypaphorine conjugate esters [72] and the ethanolic extract of the seeds provided isoerysopinophorine hypaphorine conjugate esters (**20**) [73]. These compounds strictly speaking are not dimeric compounds but conjugates wherein a tryptophan moiety (hypaphorine) has been incorporated into an erythrinan alkaloid via an ester linkage. In terms of true dimeric structures, polymerization, a specialized form of natural product biogenesis, serves not only to increase structural diversity but also affects the physicochemical and pharmacological properties. Natural polymeric compounds are usually derived from direct polymerization or indirect polymerization. In direct polymerization, units are linked directly while indirect polymerization utilizes "spacers" in which unit combination occurs via a bridge such as a methylene group, an acetate group, or atoms such as oxygen. It was only recently that true dimeric alkaloids have been reported among the erythrinan alkaloids. These structures, probably due to their high molecular masses, were shown to display particular chromatographic characteristics, such as high retention times, both on normal- and reversed-phase silica gel procedures, making it easier to search for them in a complex plant extract matrix. Using HPLC, Zhang et al. [8] isolated from the flowers of *Erythrina variegata* five new polymeric erythrinan alkaloids, of which two proved to be the conjugate dimeric erythrivarines C (**148**) and D (**149**) (Fig. 5), and three were the trimeric erythrivarines E (**152**), F (**153**), and G (**154**) (Fig. 7) (Plate 10).

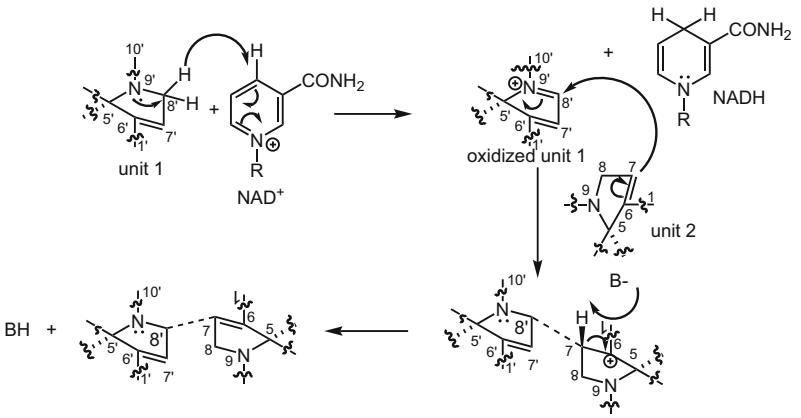
Plate 10 *Erythrina variegata*; photograph: Vinayaraj, Wikimedia Creative Commons

In the structures of erythrivarines C (**148**) and D (**149**) the upper erythrinan moiety is joined to a glucose moiety via an ether linkage at C-11′ of the alkaloid to the C-1″ of glucose, while the lower erythrinan alkaloid moiety is linked to the glucose C-6″ via an acetyl spacer at C-8. The two units can be separated by hydrolysis at the C-8 acetyl group, thus making these two alkaloids conjugates. The structures of these alkaloids were determined using spectroscopic techniques including HR-ESI-MS and 1D and 2D-NMR experiments. The two erythrinine analogues (erythrinine and 10-oxoerythrinine), the β-D-glucose moiety, and the acetyl group were deduced from the NMR data. The linkage between the 10-oxoerythrinine and the glucose moiety was through the alkaloid C-1 position and glucose C-1 (C-11′-O-C-1″), and this was confirmed by HMBC correlations. On the other hand, the erythrinine moiety with an acetyl group at C-8 is linked to glucose C-6 (C-8-CH_2CO-OC-6″-). The absolute configuration was determined as (3(3′)*R*,5(5′)*S*,11(11′)*R*) by hydrolysis and then comparing with erythrinine (**27**), which is a component of the units of the two alkaloids, and since its absolute configuration has been determined previously using X-ray diffraction [74]. What is interesting is that unlike the large positive optical rotations given by monomeric erythrinan alkaloids, erythrivarines C (**148**) and D (**149**) exhibited very small negative specific rotations ($[\alpha]_D^{23} -7^\circ$ cm^2 g^{-1} and $[\alpha]_D^{23} -13^\circ$ cm^2 g^{-1}, $c = 0.10$, CH_3OH). The values of the specific rotations in being close to zero strongly suggest that some step in the biosynthesis pathway may have led to epimerization/racemization. Otherwise, the low specific rotation could be due to a intramolecular "canceling" effect of the optical activities of the two structural subunits, as a combination (cancellation of mirror image parts) of the two units could dramatically reduce this value without any racemization.

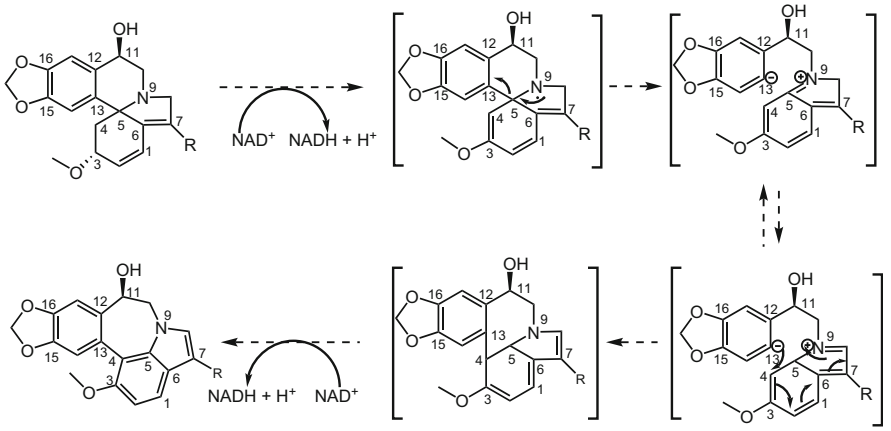
2.3 True Dimeric Erythrinan Alkaloids

True dimeric erythrinan alkaloids where the units are directly linked were unknown until 2014 when Zhang and co-workers [74] isolated and reported the two dimeric alkaloids, erythrivarine A (**150**) and erythrivarine B (**151**) (Fig. 6) from the flowers of cultivated *Erythrina variegata* plants (Plate 10), along with a large amount (31.2 g from 10.0 kg powdered biomass) of erythrinine (**27**).

These structures were elucidated using, among other techniques, 1D- and 2D-NMR spectroscopy, FT-IR, UV, HR-ESI-MS, and X-ray crystallographic diffraction. Erythrivarine A (**150**) is composed of two erythrinine units directly linked at C-7 of one and at C-8 (i.e. C-7/C-8′) of the other moiety. The connectivity of the two units was established by observed HMBC correlations between H-8′ and C-6, C-7, C-8, C-5′, C-6′, C-7′, and C-10′. The relative orientations of the H-8′ and other important groups were determined by ROESY and NOE experiments. The absolute stereochemistry was determined using the known absolute stereochemistry of erythrinine (**27**), which was determined by X-ray crystallography to be (3*R*,5*S*,11*R*). Thus, the absolute configuration of erythrivarine A (**150**) was assigned as (3(3′)*R*,5*R*,5′*S*,8′*S*,11(11′)*R*), rather than the expected (3(3′)*R*,5(5′)*S*,8′*S*,11(11′)*R*), which is probably due to the polymerization (dimerization) process. In erythrivarine B (**151**), the second erythrinine unit has undergone a structural modification due to a rearrangement reaction. Erythrivarine B thus represents the first example of an erythrinan alkaloid where a normal 6,5,6,6-membered indoloisoquinoline spirocyclic core [10, 11] has rearranged to a spiro-fused 6,5,7,6-skeleton. The difference in molecular formula between **150** and **151** is that the latter is only four hydrogens less, suggesting two additional degrees of unsaturation compared to the former. This was supported by the ^{13}C NMR data of erythrivarine B (**151**), which, when compared to erythrivarine (**150**), showed the following changes: an oxymethine carbon resonance at δ_C 77.0 ppm (CH, C-3) was replaced by an olefinic quaternary carbon at δ_C 153.5 ppm (C), a methylene carbon resonance δ_C 41.0 ppm (CH_2, C-4) was replaced by an olefinic quaternary carbon resonance at δ_C 112.5 ppm (C), a quaternary aliphatic (sp^3) carbon at δ_C 69.6 ppm (C, C-5) was replaced by an olefinic quaternary carbon resonance at δ_C 135.9 ppm (C), and the oxymethylene carbon at δ_C 60.2 ppm (CH_2, C-8) was substituted by an olefinic methine carbon at 130.0 ppm (CH). The absence of the aliphatic quaternary carbon and its replacement by an olefinic quaternary carbon strongly suggested that the erythrinine core underwent a rearrangement involving C-5. These inferences were all confirmed by HMBC NMR spectroscopic correlations. The absolute configurations of the remaining chiral centers probably correspond to those of **150** based on their ^{1}H NMR chemical shifts, ROESY correlations, and on biosynthesis grounds. The two erythrinine units in erythrivarine A (**150**) and B (**151**) are linked via C-7 of one unit and C-8 of the other, forming a C-7/C-8′ connecting bond. The probable mechanism for the formation of this linkage is shown in Scheme 1, while the possible rearrangement of **150** to **151** is illustrated in Scheme 2.



Scheme 1 Possible biosynthesis pathway for the formation of the C-7/C-8′ linkage in erythrivarines A (**150**) and B (**151**)



Scheme 2 Probable skeletal rearrangement between erythrivarine A (**150**) and erythrivarine B (**151**)

The new fused ring system found in erythrivarine B (**151**) is possibly derived from an erythrinine unit in erythriarine A (**150**) undergoing initial oxidation and subsequent rearrangement. The initial oxidation probably utilizes either NAD^+ or FAD as a co-enzyme to give a triene system, namely, 3,4-dehydroerythrivarine A. This was followed by generation of an intermediate with a C_5/N_9 iminium ion and an anion in the benzene ring (ring D). The C_5/N_9 iminium intermediate may have tautomerized spontaneously to the better and more effective C_8/N_9 iminium ion. Subsequent attack by the anion on the benzene ring on the A ring and successive migration of double bonds would give the new seven-membered ring C. Further oxidation of ring

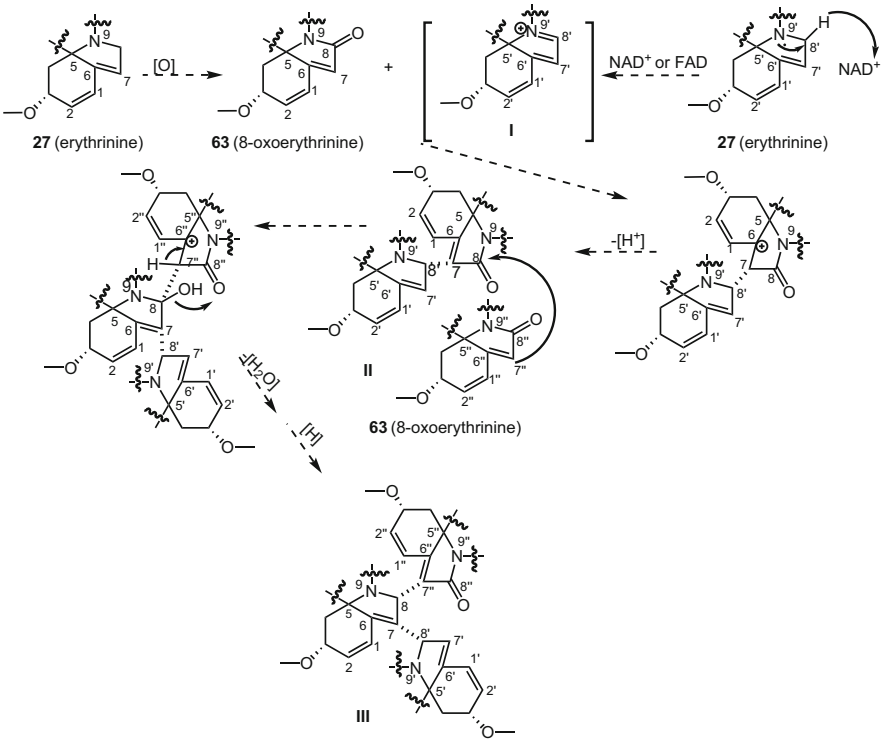
A by either NAD^+ or FAD could lead to erythrivarine B (**151**), in which both rings A and B are aromatic. The aromatization of both rings A and B may have been the driving force for the reaction. It is important to note that erythrivarines A and B, in contrast to monomeric erythrinan alkaloids that have large and positive specific rotations, have large but negative specific rotations ($[\alpha]_D^{23}$ −165 and −114° $cm^2\ g^{-1}$, $c = 0.10$, CH_3OH).

2.4 Trimeric Erythrinan Alkaloids

Trimeric erythrinan alkaloids were unknown until 2016 when Zhang et al. [8] reported three of these, namely, erythrivarine E (**152**), erythrivarine F (**153**), and erythrivarine G (**154**) (Fig. 7), from the flowers of *Erythrina variegata* (Plate 10). Their structures were elucidated by intensive use of spectroscopic techniques including HR-ESI-MS and 1D and 2D-NMR experiments. The ^1H and ^{13}C NMR pattern of erythrivarine E (**152**) displayed three pairs of signals reminiscent of erythrinine or erythrinine analogue signals, thereby suggesting that it was trimeric. A comparison of the NMR data with those of erythrivarine A (**150**) revealed that **152** in addition to the two erythrinine units found in **150**, also had an oxoerythrinine unit, a presumption supported by the molecular formula. The additional unit connection via C-8/C-7″ was confirmed by an observed HMBC correlation between H-8 and C-6″ and C-8″. The NMR data of erythrivarine F (**153**) were very similar to those of erythrivarine E (**152**), with the former showing an *O*-methyl group instead of an OH group. The observed HMBC correlation between the methoxy group and C-11 and that from H-8 to C-10 as well as a ^1H-^1H COSY correlation between H-10 and H-11 helped place the methoxy group at C-11. Erythrivarine G (**154**) is similar in constitution to erythrivarine F (**153**), with the only difference being that the C-3 methoxy group in the latter is replaced by a C-3 oxo group in the former. The position of the C-3 oxo substituent was confirmed by a HMBC NMR correlations between the coupled protons H-1 and H-2 with C-6 and C-3. The linkage between this unit and the other was deduced from a HMBC correlation between H-8 and C-6 and C-8″. The methoxy group was located at C-11′ based on the HMBC correlation between the methoxy protons and C-11′. With both having a similarity to erythrivarine A (**139**), the configurations for erythrivarine E (**152**) and F (**153**) were determined as (3(3′/3″)*R*,5(5′)*R*,5″*S*,8′(8″)*S*,11(11′/11″)*R*), and that for erythrivarine G (**154**) as (3(3″)*R*,5(5′)*R*,5″*S*,8′(8″)*S*,11(11′,11″)*R*). Erythrivarines E–G (**152**–**154**) have large but negative specific rotations ($[\alpha]_D^{23}$ −24, −205, and −180° $cm^2\ g^{-1}$, $c = 0.10$, CH_3OH).

To prove whether erythrivarines E–G (**152**–**154**) were obtained as artifacts or are true natural products, UPLC-MS-MS analysis was performed, and these alkaloids were detected as trace compounds in the total alkaloid fraction, proving them to be true natural products. Biosynthetically, the trimeric alkaloids erythrivarines E–G

may be derived from the base unit erythrinine by a combination of oxidation and addition (reduction) reactions. The erythrinine (**27**) unit is first oxidized either by coenzyme NAD^+ or FAD to give 8-oxoerythrinine (**70**) and a triene cation system (I) (Scheme 3). These two units are then coupled with subsequent dehydration to give a dimeric intermediate (II), which further reacts with another 8-oxoerythrinine unit. This reaction leads to further dehydration followed by reduction to give the trimeric skeleton (III), which is the basic skeleton for erythrivarines E–G (**152**–**154**) (Scheme 3). Erythrivarine G (**154**) is the only erythrinan alkaloid to date with a 3-oxo functionality. What may result are the 3-oxy (hydroxy or methoxy) and the 3-deoxy analogs, with the former group representing the majority of the erythrinan alkaloids, and the latter group having only three representative members, namely, erythromotidienone (**79**), erythrosotidienone (**80**), and 3-demethoxyerythratidinone (**126**). Biosynthetically speaking, erythrivarine G (**154**) may arise from III by a C-3 demethylation followed by oxidation of the resulting C-3 hydroxy to a keto carbonyl group.



Scheme 3 Possible biosynthesis pathway for the formation of trimeric erythrivarines E–G (**152**–**154**)

3 Pharmacological Properties of Erythrinan Alkaloids

The biological activities of extracts of *Erythrina* species have been known for many years. Concentrated *Erythrina* extracts were used by indigenous South Americans as arrow poisons, as an antidote against strychnine, and as a hypnotic and antiepileptic [75]. It was further observed that an alcoholic extract of the seeds of *Erythrina americana* when administered to dogs, provoked a response similar to that of tubocurarine, which was later confirmed by Lozoya and Lozoya in 1982 [76]. The first crystalline erythrinan alkaloid, called erythroidine, which also proved to be pharmacologically active, was isolated by Folkers and Major in 1937 [63]. Further analysis showed erythroidine to be actually a mixture of the two isomers α-erythroidine (**140**) and β-erythroidine (**144**).

As early as the 1930s the curare-like action, in addition to the diuretic, laxative, hypotensive, sedative and CNS-depressant properties of extracts of seeds of various *Erythrina* species, were reported [25, 77, 78] and it was considered that erythrinan alkaloids may be responsible for these observations. Further activities such as anticonvulsant, hypnotic, analgesic [79], nicotinic [80] and anxiolytic effects [34, 81] were also reported. A systematic review of more than 50 *Erythrina* species between 1940 and 1950 showed that all the isolated alkaloids produced similar effects to those of the curare alkaloids [82], with selected representatives of the latter substances once used as adjuvants in surgical anesthesia. This observation stimulated a systematic investigation of *Erythrina* species with a view to finding the constituent alkaloids responsible for the observed biological properties of these *Erythrina* extracts.

3.1 Central Nervous System and Related Activities

Acetylcholine receptors are divided into nicotinic receptors that activate ion channels (pentameric ion channels) and muscarinic receptors that couple to phospholipase C or to adenylyl cyclase through G-proteins. The results of binding assay studies led to the identification of three major subtypes of nicotinic acetylcholine receptors (nAChRs), namely, the α4β2 subtype found predominantly in the central nervous system (CNS), the α3β4 subtype that occurs primarily in the ganglionic nervous system, and the α7 subtype, also found in the CNS. There are, however, other subtypes present such as α3β2 in the CNS and α1β1γδ at neuromuscular junctions [83], and the α4β4 and α6β4 subtypes occurring in the chick retina [84]. The nAChR α4β2 subtype is composed of α4 and β2 subunits and is the most prevalent subtype expressed in the mammalian brain, where it constitutes a high-affinity binding site for nicotine [85, 86]. It belongs to the Cys loop ligand-gated ion channel family that also includes the muscle nAChR, the γ-butyric acid (GABA) receptors types A and C, the glycine receptors, and the serotonin type 3 receptors [86, 87]. In vivo, nicotinic agents have a variety of effects on both

peripheral and central targets, affecting locomotion, cognition, anxiety, and pain perception [83]. There is increasing clinical and preclinical data showing that α4β2 antagonists may offer a potential treatment approach for depression and anxiety [88]. Even though dihydro-α-erythroidine (**143**) (DHαE) (Fig. 4) and dihydro-β-erythroidine (**147**) (DHβE) (Fig. 4) are mentioned frequently as having been isolated from *Erythrina* species, it has been difficult to find the primary references concerned, although it has been reported that these are from *Erythrina americana* (Plate 7) [89]. The compound **147** is a neuromuscular nAChR competitive antagonist. Thus, as a nAChR antagonist, **147** was assessed for its in vivo modulation of behavioral effects (antinociception, hypomotility, motor impairment, and hypothermia) of nicotine. It was found to indeed block some of the central actions of nicotine, except for antinociception, with the same potency as mecamylamine, a classical ganglionic antagonist. The mechanism of blockage proved to be different from that of mecamylamine and may thus involve a direct action of **147** on the nicotinic receptor [90]. Furthermore, it was found that neuronal nicotinic acetylcholine receptors were differentially sensitive to blockade by **147** and that both the α and β subunits are involved. The sensitivity to **147** when comparing the α4β4 and α3β4 subunits showed a 120-fold decrease. The IC_{50} values for blocking α4β4 and α3β4, responding to EC_{20} concentrations of acetylcholine, were 0.19 ± 0.06 and 23.1 ± 10.2 μ*M* [91].

Erysodine (**2**), an erythrinan alkaloid related to DHβE (**147**), was found to be a more potent inhibitor (K_i = 5.0 ± 1.3 n*M*) of [^{3}H]-cytisine binding at neuronal nicotinic receptors (α4β2) than **147** ($Ki = 35 \pm 3$ n*M*). In contrast to its activity at the α4β2 nAChR subtype labeled by [^{3}H]-cytisine, erysodine was approximately three orders of magnitude less potent (K_i = 4000 ± 900 n*M*) in displacing [^{125}I]-α-bungarotoxin binding from the α-bungarotoxin-sensitive nAChR subtype present in the rat brain. Dihydro-β-erythroidine (**147**) was also less potent (K_i = 9000 ± 1200 n*M*) in this assay than erysodine (**2**) but was at least tenfold more potent (K_i = 11,000 n*M*) than erysodine (K_i > 100,000 n*M*) in displacing the binding of [^{125}I]-α-bungarotoxin to the muscle-type α1β1δγ nAChR subtype found in *Torpedo electroplax* membranes [80]. Erysodine (**2**) was also found to be a competitive, reversible antagonist of (−)-nicotine-induced dopamine release from striatal slices and was found to be equipotent with **147** (**2**: $IC_{50} = 58 \pm 3$ n*M*; **147**: $IC_{50} = 58 \pm 5$ n*M*). Erysodine (**2**) furthermore inhibited (–)-nicotine-induced $^{86}Rb^+$ efflux from the human neoblastoma cell line, IMR-32, in a concentration-dependent manner, and was found to be approximately ten-fold more potent than **147** (**2**: $IC_{50} = 7 \pm 2$ μ*M*, **147**: $IC_{50} = 84 \pm 17$ μ*M*) in this assay [80]. While it is clear that the erythrinan alkaloids erysodine (**2**) and DHβE (**147**) are potent and selective competitive inhibitors of α4β2 nAChRs, not much was known about the molecular determinants of the sensitivity of this receptor subtype to inhibition by this class of antagonist. To answer this question, Iturriaga-Vásquez and co-workers examined the effects of **147**, **2**, and several more erythrinan alkaloids on [^{3}H]-cytisine binding and receptor function in conjunction with homology models of α4β2 nAChR, mutagenesis, and functional assays [86]. They tested six alkaloids, namely, DHβE (**147**), erysodine (**2**), *O*-acetylerysonine (semi-synthetic acetylation derivative of

Table 11 Effects of erythrinan alkaloids on [^{3}H]-cytisine binding to α4β2 nAChR and [^{3}H]-epibatidine binding to α7 nAChR

Compound	K_i/n*M*	
	[^{3}H]-cystine binding to α4β2 nAChR	[^{3}H]-epibatidine binding to α7 nAChR
DHβE (**147**)	98 ± 6	10,500 ± 400
Erysodine (**2**)	50 ± 3	7500 ± 150
O-Acetylerysodine[a]	79 ± 8	7340 ± 230
Erysopine (**4**)	154 ± 6	14,600 ± 1000
Erysotrine (**1**)	604 ± 5	NE
2-Epierytratidine (**96**)	710 ± 9	NE

NE: no effects at the highest concentration (1 n*M*) of inhibitor tested
[a]Semi-synthetic

Table 12 Functional effects of some erythrinan alkaloids on the function of recombinant and native nAChRs

Compound	IC_{50}/n*M*		
	nAChR		Nicotine-mediated [^{3}H]-dopamine release
	α4β2	α7	
DHβE (**147**)	110 ± 11	10,101 ± 1870	ND
Erysodine (**2**)	96 ± 25	9532 ± 2000	108 ± 11
O-Acetylerysodine[a]	105 ± 43	14,543 ± 5400	120 ± 16
Erysopine (**4**)	201 ± 20	16,676 ± 6000	250 ± 20
Erysotrine (**1**)	367 ± 16	16,987 ± 3000	402 ± 33
2-Epierytratidine (**96**)	4923 ± 150	NE	5400 ± 40

ND: not determined; NE: no functional effects at the highest concentration (1 n*M*) of inhibitor tested
[a]Semi-synthetic

erysodine), erysopine (**4**), erysotine (**1**), and 2-epierythratidine (**96**), and the results are shown in Tables 11 and 12.

The rank order of potency for the displacement of [^{3}H]-cytisine from α4β2 nAChRs by these alkaloids was erysodine > *O*-acetylerysodine ≈ DHβE > erysopine > erysotrine > 2-epierythratidine. Data for ACh-evoked currents (Table 12) were concentration-dependent with IC_{50} values ranging from 96 n*M* (erysodine) to 4923 n*M* (2-epierythratidine). The rank order of potency for functional inhibition of [^{3}H]-cytisine of α4β2 AChR mirrored that found for the inhibition of [^{3}H]-cytisine binding (Table 11), except that the potencies of **147**, **2**, and *O*-acetylerysodine statistically were not different from each other [86]. The similarity between the rank order of potency of inhibition of α4β2 nAChR function and the binding of [^{3}H]-cytisine to α4β2 suggests that the structural determinants that affect the binding of the alkaloids to α4β2 AChRs also influence their functional effects, as expected for competitive antagonists. To make a determination of whether these alkaloids inhibit native α4β2 nAChRs with the same rank order of potency that they inhibit recombinant α4β2 nAChRs, their ability to inhibit nicotine-induced [^{3}H]-dopamine release

from rat striatal slices was assessed. The results showed that all six alkaloids inhibited [^{3}H]-dopamine release fully and with potencies closely matching those obtained from electrophysiological measurements on recombinant α4β2 nAChRs and with a comparable rank order of potency of inhibition (Table 12). The lactone group of **147** and a C-16 hydroxy group were identified as major determinants of potency for the aromatic erythrinan alkaloids, which was decreased when the conserved residue Tyr126 in loop A of the α4 subunit is substituted with alanine. The conserved residue α4Tyr126 seems to act as a hydrogen-bonding acceptor or donor depending on whether the bonding partner is erysodine, erysopine, DHβE, or *O*-acetylerysodine. Site-directed mutagenesis studies also suggested that β2Asp196 within the α4β2 ligand binding domain (LBD) may be a major contributor to sensitivity of α4β2 nAChRs to inhibition by erythrinan alkaloids. When mutating β2Asp196 to alanine (i.e. β2D196A) the ability of both DHβE and erysodine to inhibit the function of α4β2 nAChRs was totally lost. Homology modeling predicted a strong ionic interaction between the ammonium center of the erythrinan alkaloid and β2Asp196 leading to the uncapping of loop C [86].

Two erythrinan alkaloids isolated from the leaves of *Hyperbeana valida* (Menispermaceae), 15-amido-3-demethoxy-2α,3α-methylenedioxyerythroculine (**118**, Table 7) and 3-demethoxy-2α,3α-methylenedioxyerythroculine (**117**, Table 7), showed no agonist activity, but were found to be antagonists at nicotinic receptors [56]. Compounds **118** and **117** showed antagonism of a 100 μ*M* nicotine response, with IC_{50} values of 94 ± 8 μ*M* and 77 ± 19 μ*M*. These compounds seem to be equipotent inhibitors of α3β4 nicotinic receptors. Their potencies are similar to that of DHβE (**147**) a blocker of nicotinic channels. DHβE is known to be a weak competitive blocker of these cells, with an IC_{50} value, based on an ^{86}Rb efflux of ~100 μ*M* [56, 92]. While weak for the α3β4 subtype, **147** has proved to be much more potent for the α4β2 subtype (IC_{50} 0.19 μ*M*), the predominant subtype in the brain [91].

The Pakistani medicinal plant, *Erythrina suberosa*, is employed among other uses, as a calming agent. Two alkaloids, erysotrine (**1**) and erysodine (**2**), with anxiolytic properties, were isolated from the flowers of this taxon [93]. The results demonstrated that acute p.o. treatment with **1** and **2** produced anxiolytic-like effects in mice when evaluated in two widely used anxiety tests, namely, the elevated plus-maze (EPM) assay and the light-dark transition model (LDTM) [93]. The results strongly suggested that **1** and **2** significantly contribute to the antianxiety activities of *E. suberosa*.

A tincture prepared from the leaf or bark decoction of *Erythrina mulungu* (Plate 5) is used to calm agitation and other disorders of the central nervous system, such as insomnia and depression [34]. Further work showed that both acute and chronic treatment with a semi-polar extract from *E. mulungu* produced anxiolytic-like effects on a specific subset of defensive behavior in rats exposed to the elevated T-maze (ETM) apparatus and to the LDTM, exhibiting antianxiety effects mimicking those provoked by diazepam (Valium®), a well-known anxiolytic drug [34, 94, 95]. Purification of a hydroalcoholic extract of the flowers of *E. mulungu* led to the identification of three anxiolytic erythrinan alkaloids, namely,

(+)-11α-hydroxyerythravine (**38**) (Table 2), (+)-erythravine (**7**) (Table 1), and α-hydroxyerysotrine or 11α-hydroxyerysotrine (**39**) (Table 2). The anxiolytic effects of the hydroalcoholic crude extract and of alkaloids **7**, **38**, and **39** were evaluated using the ETM test [34, 81]. In the LDTM assay, the alkaloid erythravine (**7**), at doses of 3 and 10 mg kg^{-1}, and the alkaloid 11α-hydroxyerythravine (**39**), at a dose of 10 mg kg^{-1}, were able to induce enhancements of the time spent by mice in the illuminated compartment, while 11α-hydroxyerythravine, also at the dose of 3 mg kg^{-1} increased the number of transitions of animals between the light-dark compartments [34, 81]. Erythravine (**7**) and 11α-hydroxyerythravine (**39**) were further shown to induce potent anticonvulsant effects against seizures induced by glutamate agonists (*N*-methyl-D-aspartate and kainic acid) as well as GABA antagonists (bicuculline and pentylenetetrazole (PTZ)) [96].

The erythrinan alkaloid erysotrine ((**1**) Table 1), also isolated from a hydroalcoholic extract of the flowers of *E. mulungu* (Plate 5), was analyzed for central nervous system effects such as potential anticonvulsant and anxiolytic activities and modulation of GABA and glutamate uptake and their binding systems, using different experimental approaches [97]. The results indicated that erysotrine (**1**) inhibited seizures evoked by bicuculline, PTZ, NMDA, and most remarkably, kainic acid. It also induced an increase in the number of entries but not in the time spent in the open arms using the elevated plus-maze (EPM) test apparatus. The results further showed that erysotrine (0.001–10 μg cm^{-3}) did not alter the GABA or glutamate synaptosomal uptake and binding, leading to the conclusion that erysotrine (**1**) has anticonvulsant and mild anxiolytic activities [97]. Investigation into whether the alkaloids **7**, **38**, and **39**, could affect nicotinic receptors and if they are selective for different central nervous system (CNS) subtypes was carried out by Setti-Perdigão et al. [98]. These alkaloids were screened using a single concentration of the alkaloid co-applied with acetylcholine in whole cell patch-clamp recordings in three different cell models expressing α3* (PC12 cells), α7* (hippocampal neurons), and α4β2 (HEK 293 cells) nicotinic acetylcholine receptors (nAChRs). The percentage inhibition of acetylcholine-activated currents by (+)-11α-hydroxyerysotine (**39**) was the lowest, whereas those by (+)-erythravine (**7**) and (+)-11α-hydroxyerythravine (**38**) were significantly higher. Concentration response curves with a pre-application protocol for the α7* and α4β2 nAChRs for (+)-erythravine (**7**) and (+)-11α-hydroxyerythravine (**38**) were obtained from which IC_{50} values were extracted. Erythravine and 11α-hydroxyerythravine gave IC_{50} values of 6 and 5 μ*M* for the α7* receptors, and 13 and 4 n*M* for the α4β2 receptors, respectively. The data obtained suggested that these erythrinan alkaloids may exert their behavioral effects through inhibition of the CNS nicotinic acetylcholine receptors, particularly the α4β2 subtype [98].

The polymeric *Erythrina* alkaloids, erythrivarine C (**148**), erythrivarine D (**149**), erythrivarine E (**152**), erythrivarine F (**153**), and erythrivarine G (**154**), isolated from the flowers of *Erythrina variegata* [8], were assayed for acetylcholinesterase (AChE) inhibition activity using the Ellmann method [99], and only erythrivarine F (**153**) showed potential acetylcholinesterase inhibitory activity, with an IC_{50} value of 12.5 μ*M*, as compared to 0.03 μ*M* for a standard, huperzine [8].

The hypotensive potential of *Erythrina falcata* extracts obtained by maceration and infusion has been studied and a potent dose-dependent hypotensive effect was observed [60], which may be correlated to an interaction with β-adrenergic receptors. This activity could be associated with alkaloids in the extract, which were determined to be erythristemine (**25**), 11β-methoxyglucoerysodine (**34**), 11β-hydroxyerysodine-glucose (**35**), erysothiopine (**13**), and 11-hydroxyerysotinone-rhamnoside (**106**) [60].

3.2 Antioxidant Activities

An alkaloidal fraction of the seeds of *Erythrina americana* (Plate 7) and the alkaloid, erysodine (**2**) were evaluated using the stable radical–2,2-diphenyl-1-picrylhydrazyl (DPPH) method and measured using a UV-visible spectrophotometric method. The alkaloidal fraction gave an IC_{50} value of 0.1593 ± 0.0305 mg cm^{-3} and pure erysodine isolated from the fraction showed a strong DPPH inhibition (IC_{50} 0.0212 ± 0.0080 mg cm^{-3}) comparable to that of ascorbic acid (IC_{50} 0.0068 ± 0.0007 mg cm^{-3}). Erysodine (**2**) also caused 94% inhibition at 0.5 mg cm^{-3} [100]. These data were comparable to those obtained from the crude extract from the flowers and pods of *Erythrina lysistemon*, which gave an IC_{50} value of 86 μg cm^{-3} while erysodine (**2**) and 11β-hydroxyerysodine (**31**, Table 2) gave IC_{50} values of 90 and 160 μg cm^{-3} (ascorbic acid IC_{50} 16 μg cm^{-3}) [42]. Six erythrinan alkaloids (all erysotrine derivatives) isolated from the flowers of *Erythrina herbacea*, were evaluated for their ability to scavenge free radicals of peroxynitrite in vitro [38] and the results are shown in Table 13. Erythrartine (**24**) exhibited the most potent antioxidant activity followed by erysotramidine or 8-oxoerysotrine (**66**), while the rest of the alkaloids, 10-hydroxy-11-oxoerysotrine (**43**), erysotrine *N*-oxide (**55**), erythrarbine (**75**), and 10,11-dioxoerysotrine (**44**) all showed weak antioxidant activity. It appears 16-hydroxy and 11-hydroxy substituents, and, to a lesser extent, an 8-oxo moiety are structural requirements for the mediation of antioxidant activity by the erythrinan alkaloids.

Table 13 Antioxidant activities of erythrinan alkaloids from the flowers of *Erythrina herbacea*

Compound	Antioxidant activity/mg cm^{-3} (500 μ*M*)*
Erythrartine (11β-hydroxyerysotrine) (**24**)	37.8 ± 0.3
10-Hydroxy-11-oxoerysotrine (**43**)	8.0 ± 1.0
Erysotrine *N*-oxide (**55**)	9.3 ± 1.9
Erysotramidine (8-oxoerysotrine) (**66**)	21.9 ± 0.3
Erythrarbine (10,11-dehydroerysotrine) (**75**)	6.7 ± 2.1
10,11-Dioxoerysotrine (**44**)	3.5 ± 0.7
Butylated hydroxytoluene (BHT)	61.2 ± 1.6 (400 μ*M*)*

Note: Higher values mean higher activity in this assay
*Final concentration of each sample

3.3 Anti-inflammatory and Related Activities

The bark of *Erythrina crista-galli* (Plate 4) yielded the erythrinan alkaloids erysotrine (**1**), erysovine (**3**), erythraline (**10**), erythrinine (**27**), cristanine A (or erythraline *N*-oxide) (**58**), 8-oxoerythraline (**69**), 8-oxoerthrinine (**70**), cristamidine (**76**), erythratidine (**95**), 2-epierytratidine (**96**), erythratine (**100**), and cristanine B (**121**) [44]. These compounds were examined for inhibitory activity on lipopolysaccharide (LPS)-induced nitric oxide (NO) production and cell viability in RAW 264.7 macrophages. Two of these erythrinan alkaloids, erythraline (**10**) and erythrinine (**27**) showed inhibitory activity on LPS-induced NO production with IC_{50} values of 8.8 and 3.4 μg cm^{-3}, respectively, and did not exhibit any cytotoxicity for the host murine macrophages RAW 246.7 used in this assay. The other tested compounds, **1**, **3**, **58**, **69**, **70**, **76**, **95**, **96**, **100**, and **121** were inactive ($IC_{50} > 25$ μg cm^{-3}) at the tested concentrations. It has been observed that overproduction of nitric oxide (NO) is associated with oxidative stress and with the pathophysiology of various diseases such as rheumatism, diabetes, and cardiovascular diseases with chronic inflammation [44]. The structure-activity requirements for LPS-induced NO inhibitory activity for the erythrinan alkaloids seem to require the presence of the following structural moieties: (i) a 1,6-conjugated ($\Delta^{1,2}$,$\Delta^{6,7}$) diene system, (ii) a C-15−C-16–methylenedioxy functionality, (iii) a 11-hydroxy group, (iv) a C-8 sp^3 methylene unit, and (v) a N-9 tertiary nitrogen, i.e. a non-oxygenated nitrogen.

3.4 Antimicrobial Activities

Erythrinan alkaloids appear to be devoid of antibacterial activity. So far, there is no record of antibacterial activity reported for these substances. Three alkaloids, (+)-10,11-dioxoerythratine (**114**), (+)-10,11-dioxoepierythratidine (**115**), and (+)-10,11-dioxoerythratidinone (**116**), isolated from *Erythrina subumbrans*, showed no discernible activity against a H37Ra strain of *Mycobacterium tuberculosis* [54].

The antimycotic (antifungal) activity of erysovine (**3**) isolated from the seeds of *Erythrina americana* (Plate 7) was evaluated against the phytopathogenic fungi *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Monilinia fructicola*, *Penicillium* sp., and *Trichoderma harzianum*, using a paper disk diffusion method [101]. *B. cinerea*, *F. oxysporum*, and *M. fructicola* displayed greater susceptibility when exposed to a dose of 8 mg cm^{-3} of erysodine, which inhibited the growth of the mycelia by 88%, 57%, and 43%, respectively. The other phytopathogens *A. solani*, *Penicillium* sp., and *T. harzianum* were only inhibited in mycelial growth by 27%, when compared to a control [101].

The erythrinan alkaloids erythraline-11*β*-*O*-glucopyranoside (**37**), erythraline (**10**), erythrartine (**24**), erysodine (**2**), erysotrine (**1**), and (+)-16-*O*-*β*-D-glucoerysopine (**17**), isolated from *Erythrina crista-galli*, were evaluated for their inhibitory activity

against the tobacco mosaic virus (TMV), using a leaf-disk method [9]. The compounds showed inhibition against the replication of TMV, at a concentration of 500 μg cm^{-3}, with inhibitory rates varying from 52.5 to 79.7%, with ningnanmycin, a commercial antiviral agent, exhibiting a 91.7% inhibition under the same conditions. The IC_{50} values of the erythrinan alkaloids **37**, **10**, **24**, **2**, **1**, and **17** were determined to be 0.59, 1.52, 1.04, 1.48, 1.28, and 0.74 m*M*, using this leaf-disk method, while ningnanmycin, under the same conditions, gave an IC_{50} value of 0.18 m*M* [9]. It appears that the presence of a 1,6-conjugated ($\Delta^{1,2}$,$\Delta^{6,7}$) diene system and a glucose moiety are essential structural features for TMV inhibition.

A crude alkaloidal fraction (CAF) of the seeds of *Erythrina abyssinica* was tested for anti-HIV-1 activity using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) method [102]. A reduction in the viability of mock-infected MT-4 cells with a $CC_{50} = 53$ μ*M* and a 50% protection of MT-4 cells from HIV-1-induced cytopathogeneticity with an EC_{50} of ≥53 μ*M* (control efavirenz, EFV: CC_{50} 45 μ*M*; $EC_{50} = 0.003$ μ*M*) were observed. The resulting cytotoxicity of the mock-infected MT-4 cells ($IC_{50} = 53$ μ*M*) was attributed to isoquinoline-type alkaloids, which are biosynthetic precursors to the *Erythrina* alkaloids [103]. These alkaloids have been shown to inhibit the replication cycle of the human immunodeficiency virus HIV-1 by virus adsorption or the reverse transcription process [104]. The CAF yielded the erythrinan alkaloids, erythraline (**10**), erysodine (**2**), erysotrine (**1**), 8-oxoerythraline (**69**), and 11-methoxyerysodine (**32**) and it is likely that all or some of these have some anti-HIV-1 activity.

3.5 Antiprotozoal Activities

Three erythrinan alkaloids, (+)-10,11-dioxoerythratine (**114**), (+)-10,11-dioxoepierythratidine (**115**), and 10,11-dioxoerythratidinone (**116**), isolated from *Erythrina subumbrans* [54], were tested for antiplasmodial activity against *Plasmodium falciparum* (K1, a multidrug-resistant strain). However, the compounds were not active at the highest concentration tested [54].

Seven erythrinan alkaloids, namely, erythraline (**10**), 8-oxoerythraline (**66**), 8-oxoerythraline epoxide (**134**), crystamidine (**76**), erythrinine (**27**), erysovine (**3**), and erythratidinone (**103**), isolated from the bark of *Erythrina verna* (Plate 5), were evaluated for leishmanicidal activity against *Leishmania amazonensis* [105]. These compounds were tested against the promastigote form of the parasite and their cytotoxicity was assessed against the J774 macrophage cell line in vitro, with the results shown in Table 14. These alkaloids showed a very low selectivity index suggesting possible cytotoxicity to the mammalian cells at the concentration active against the promastigote form of *L. amazonensis*. The first five of the above-mentioned alkaloids all have in common a 1,6-conjugated diene system and a 15,16-methylenedioxy unit. A hydroxy group at C-11, a 1,2-epoxide ring, and a 10,11-double bond seem to increase activity, while the presence of an 8-oxo moiety

Table 14 Leishmanicidal activity of erythrinan alkaloids from *E. verna* against *Leishmania amazonensis*

Compound	*L. amazonensis* IC_{50}/μg cm^{-3}	Macrophage cells (J774) CC_{50}/μg cm^{-3}	SI
Erythraline (**10**)	65.27	69.33	1.06
8-Oxoerythraline (**69**)	>1000	>1000	ND
8-Oxoerythraline epoxide (**134**)	48.17	47.66	0.98
Crystamidine (**76**)	71.53	58.97	0.82
Erythrinine (**27**)	39.53	59.97	1.52
Erysovine (**3**)	>1000	>1000	ND
Erythratidinone (**103**)	>1000	>1000	ND
Amphotericin B	0.20	NT	NT

SI: selectivity index = CC_{50} test compound/IC_{50} test compound; ND: not determined; NT: not tested

appears to abrogate activity, i.e. a C-8 sp^3 methylene unit is required. The most active compound in this assay was erythrinine (**27**), followed, in turn, by 8-oxoerythraline epoxide (**134**) and erythraline (**10**). It appears that the presence of a 1,2-epoxide more than compensates for the negative effects of an 8-oxo group.

3.6 Antifeedant and Insecticidal Activities

The antifeedant activities of erythrinan alkaloids from the seeds, seed pods and flowers of *Erythrina latissima* (Plate 11) were investigated in laboratory dual-choice bioassays using third-instar *Spodoptera littoralis* (Boisduval) larvae [62]. The isolated alkaloids were (+)-10,11-dioxoerysotrine (**44**), erysotrine (**1**), (+)-11β-methoxyerysotramidine (**68**), (+)-11β-methoxy-10-oxoerysotramidine (**82**), (+)-10,11-dioxoerysotramidine (**81**), erysotramidine (**66**), 8-oxoerythraline (**69**), glucoerysodine (**8**), erysovine (**3**), erythraline (**10**), and 11β-hydroxyerysotramidine (**67**). The compounds erysotrine (**1**), erysotramidine (**66**), erythraline (**10**), and (+)-11β-hydroxyerysotramidine (**67**) exhibited dose-dependent antifeedant activity at concentrations of $\geq$100 ppm, while (+)-10,11-dioxoerysotramidine (**44**) and (+)-11β-methoxy-10-oxoerysotramidine (**82**) showed dose-dependent activity at concentrations of $\geq$300 and $\geq$500 ppm, respectively. The compounds (+)-11β-methoxyerysotramidine (**68**), 8-oxoerythraline (**69**), and glucoerysodine (**8**) showed no appreciable change in antifeedant activity with an increase in concentration [62].

A bioassay-guided method to isolate insecticidal erythrinan alkaloids from the seeds of *Erythrina crista-galli* (Plate 4), and verification of their aphicidal potential against the cotton aphid (*Aphis gossypii* Glover), an extraordinarily crucial pest mainly feeding on industrial cotton, was adopted by Wang and co-workers [47]. This led to the isolation of three new erythrinan alkaloids, cristanines C (**77**),

Plate 11 *Erythrina latissima*; photograph: JMK, Wikimedia Creative Common

Table 15 Contact aphicidal activities of crude extract, alkaloidal fraction, non-alkaloidal fractions, fractions, subfractions, and pure isolates against *Aphis gossypii* 24 h after treatment and the Potter spray tower assay of the most active isolates

Sample/compound	Contact aphicidal assay LD_{50}/ng aphid^{-1}	Potter spray tower assay LC_{50}/μg cm^{-3}
Crude extract (CE)	372.50 ± 56.51	
Alkaloidal fraction (AF)	162.96 ± 37.92	
Non-alkaloidal fraction (NAF)	>1000	
Fraction 2	77.60 ± 10.75	
Fraction 4	50.65 ± 8.03	
Subfraction 2b	34.39 ± 5.02	
Subfraction 2c	50. 02 ± 6.03	
Subfraction 4c	42.36 ± 5.77	
Subfraction 4d	29.57 ± 3.56	
55, **66**, **69**, **76**, **77**, **95**, **100**, **119**, and **120**	>530	
Erysotrine (**1**)	5.13 ± 1.10	163.74 ± 22.67
Erysodine (**2**)	7.48 ± 1.55	186.81 ± 25.49
Erysovine (**3**)	6.68 ± 1.16	165.35 ± 14.12
Erythraline (**10**)	4.67 ± 0.73	112.78 ± 11.95
Imidacloprid[a]	1.84 ± 0.40	45.40 ± 10.18

[a]Commercial aphicide imidacloprid used as a positive control

D (**120**), and E (**119**), along with 11 known alkaloids erysotramidine (**66**), 8-oxoerythraline (**69**), erythratidine (**95**), erythratine (**100**), erysotrine (**1**), erysodine (**2**), erysovine (**3**), erythraline (**10**), crystamidine (**76**), and erysotrine *N*-oxide (**55**). During the course of the bioassay-guided isolation procedure, the contact aphicidal activity of all of the crude extract, the alkaloidal and non-alkaloidal portions, fractions 1–6, and subfractions 2a–2f and 4a–4f were evaluated against *A. gossypii*

after 24 h, and the results are shown in Table 15. The new compounds cristanines C (**77**), D (**120**) and E (**119**) did not exhibit any aphicidal activity at the highest test dose used (530 ng aphid^{-1}), while the known compound erythraline (**10**) displayed the most potent aphicidal activity with a LD_{50} value of 4.67 ng aphid^{-1}, which was of the same order of magnitude but slightly less active than the commercial systemic aphicide imadacloprid (1.84 ng aphid^{-1}). The other active compounds, erysotrine (**1**), erysodine (**2**) and erysovine (**3**) showed moderate aphicidal activities, with LD_{50} values of 5.13, 7.48, and 6.68 ng aphid^{-1}. The Potter spray tower assay, under controlled experimental conditions, is used routinely to simulate the spray effects of insecticides in the field. The results of the Potter spray tower assay (Table 15) for the active compounds erythraline, erysotrine, erysodine, and erysovine mirrored those in the contact aphicidal assay, with erythraline being the most potent compared to the other three aphicidal compounds evaluated, although its potency (LC_{50}: 112.78 μg cm^{-3}) was of an order of magnitude lower than imidacloprid (LC_{50}: 45.40 μg cm^{-3}). The most highly active compound, erythraline (**10**), produced significant elevation in the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) in *A. gossypii* at its LC_{50} and LC_{75} doses. The activity of the detoxification enzyme glutathione-*S*-transferase (GST) in *A. gossypii* showed a significant increase that was found to correlate with erythraline concentration [47]. Structure-activity analysis using the contact aphicidal and Potter spray assays suggested that an erythrinan alkaloid substructure possessing a conjugated diene system ($\Delta^{1,2}$,$\Delta^{6,7}$; compounds **1**, **2**, **3**, **10**), a sp^3 methylene at C-8 (compounds **1**, **2**, **3**, **10** vs. **119** and **120**), a non-oxygenated site at N-9 (compound **1** vs. **55**), and modulated by substitution in ring D (activity trends of **1**, **2**, **3**, **10**) all play a crucial role in modulating the resultant aphicidal activity.

The new conjugate dimeric erythrinan alkaloids erythrivarines C (**148**) and D (**149**), and the trimeric alkaloids erythrivarines E (**152**), F (**153**) and G (**154**), isolated from the flowers of *Erythrina variegata* by Zhang and co-workers [8], were assessed for their insecticidal activity against the aphid *Rhodobium porosum*. While these three trimeric alkaloids were inactive at the highest concentration tested (400 μg cm^{-3}), erythrivarines C (**148**) and D (**149**) exhibited moderate insecticidal activity against the aphid *R. porosum*, with IC_{50} values of 121.8 and 77.1 μ*M*, respectively, when compared with imidacloprid (IC_{50}: 49.1 μ*M*), a positive control and a commercial aphicide [8].

3.7 Cytotoxicity and Other Activities Related to Cancer

Three erythrinan alkaloids, (+)-10,11-dioxoerythratine (**114**), 10,11-dioxoepierythratidine (**115**), and 10,11-dioxoerythratidinone (**116**), isolated from the stem bark of *Erythrina subumbrans*, were assessed for cytotoxicity against oral human epidermal carcinoma (KB), human breast cancer (BC), and human small cell lung cancer (NCI-H187) cell lines, using a standard method. While the standard drug, ellipticine, exhibited IC_{50} values against these cell lines of 1.33, 1.46, and 0.39 μg cm^{-3}, respectively, the alkaloids **114**–**116** were found to be inactive

[54]. Similarly, the new dimeric erythrinan alkaloids erythrivarines A (**150**) and B (**151**), isolated from the flowers of *Erythrina variegata* by Zhang and co-workers, were also evaluated against three human cancer cell lines, namely, HeLa, gastric cancer (SGC-7901), and lung cancer (A-549) using a MTT method, and were also found to be inactive [74]. Further work by Zhang and co-workers on the same extracts yielded two new conjugate dimeric erythrinan alkaloids, erythrivarines C (**148**) and D (**149**), and three new trimeric erythrinan alkaloids, erythrivarines E (**152**), F (**153**), and G (**154**), and all these compounds were inactive against the three immediately above-mentioned cell lines [8]. Erysotrine (**1**) and 11-methoxy-glucoerysovine (**36**), isolated from the fruits of *Erythrina vespertilio*, were evaluated against a metastatic prostate cancer cell line (PC-3) and neonatal foreskin fibroblasts, using a new xCELLigence system, a real-time label-free analyzer, and they were also found to be inactive in both cases [106].

The seeds of *Erythrina abyssinica* (Plate 12) growing in Sudan were investigated for their alkaloidal constituents, anti-HIV-1 activity and cytotoxicity against six cancer cell lines [102]. The in vivo cytotoxicity of the crude alkaloidal fraction (CAF) against the cell lines HeLa, Hep-G2, HEP-2, HCT116, MCF-7, and HFB4 showed activity. A bioassay-guided fractionation of the CAF led to the isolation of the erythrinan alkaloids erythraline (**10**), erysodine (**2**), erysotrine (**1**), 8-oxoerythraline (**69**), and 11-methoxyerysodine (**32**), for which the cytotoxic activities were also assessed (Table 16).

The results of the in vitro cytotoxicity assessment gave the following IC_{50} values (in μg cm^{-3}), in comparison to a positive control, doxorubicin (Dox): HeLa (13.8; Dox 3.64), Hep-G2 (10.1; Dox 4.57), HEP-2 (8.16; Dox 4.89), HC116 (13.9; Dox

Plate 12 *Erythrina abyssinica*; photograph: P. Weigell, Wikimedia Creative Common

Table 16 Cytotoxic activities of the crude alkaloidal fraction and pure alkaloids from *E. abyssinica* against some cancer cell lines

	IC_{50}/μg cm^{-3}					
	Cell line					
Sample/compound	HeLa	Hep-G2	HEP-2	HCT116	MCF-7	HFB4
CAF	13.8	10.1	8.16	13.9	11.4	12.2
Doxorubicin (+ve control)	3.64	4.57	4.89	3.74	2.97	3.96
Erythraline (**10**)	–	17.60	15.90	–	–	–
Erysodine (**2**)	–	11.80	19.90	–	–	–
Erysotrine (**1**)	–	15.80	21.60	–	–	–
8-Oxoerysotrine (**69**)	–	3.89	18.50	–	–	–
11-Methoxyerysodine (**32**)	–	11.40	11.50	–	–	–

3.74), MCF-7 (11.4; Dox 2.97), and HFB4 (12.2; Dox 3.96). These results (Table 16) also revealed that the CAF was more potent against the Hep-G2 (IC_{50}: 10.1; Dox 4.57) and HEP-2 (IC_{50}: 8.16; Dox 4.89) cell lines, and therefore the isolated compounds subsequently were assessed for activity using these particular cell lines. The isolated compounds gave the following IC_{50} values (in μg cm^{-3}) against the two cell lines: erythraline (**10**) (Hep-G2: 17.60; HEP-2: 15.90), erysodine (**2**) (Hep-G2: 11.80; HEP-2: 19.90), erysotrine (**1**) (Hep-G2: 15.80; HEP-2: 21.60), 8-oxoerysotrine (**69**) (Hep-G2: 3.89; HEP-2: 18.50), and 11-methoxyerysodine (**32**) (Hep-G2: 11.40; HEP-2: 11.50). A structure-activity relationship determination showed that all these alkaloids have a 1,6 conjugated diene system as a basic substructure and for the Hep-G2 activity, this seems to be greatly enhanced by an 8-oxo group. 8-Oxoerythraline (**69**) was more potent (IC_{50}: 3.89 μg cm^{-3}) than the doxorubicin standard (IC_{50}: 4.57 μg cm^{-3}). It seems also that the presence of an 11-methoxy group enhances the activity of these compounds against the Hep-G2 cell line, while a 15,16-methylendioxy moiety decreases the resultant cytotoxicity.

The term "apoptosis" was first coined in 1972 by John Kerr, an Australian pathologist, who observed that certain dying cells share a number of common morphological features [107]. The criteria for describing apoptosis are all morphological, and they include condensation and margination of chromatin, cytoplasmic vacuolization, cellular shrinkage, increases in cellular density, nuclear fragmentation, and apoptotic body formation [107, 108].

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or Apo 2 ligand (Apo2L) is an important member of the TNF family of ligands, and is capable of initiating apoptosis through engagement of its death receptors [109]. TRAIL selectively induces apoptosis of a variety of tumor cells and transformed cells, but not most normal cells, and therefore has garnered much interest as a promising approach to cancer therapy [109]. Accordingly, compounds that have synergistic activity with TRAIL but have minimal cytotoxicity may be promising tools in this regard.

The eight erythrinan alkaloids, erysodine *N*-oxide (**64**), erythraline (**10**), 8-oxoerythraline (**69**), erysotrine (**1**), erysodine (**2**) erysovine (**3**), glucoerysodine

Table 17 Values of $IC_{50}/g_{ligand}\ cm^{-3}$ of erythrinan alkaloids isolated from *E. velutina* with TRAIL for Jurkat cells

Compound	No TRAIL	With TRAIL	Selectivity index (SI)
Erysodine *N*-oxide (**64**)	>50	>50	No toxicity, no activity
Erythraline (**10**)	23.0	5.0	4.6
8-Oxoerythraline (**69**)	>50	22.0	>2.3
Erysotrine (**1**)	>50	7.2	>6.9
Erysodine (**2**)	10.8	4.9	2.2
Erysovine (**3**)	24.0	14.2	1.7
Glucoerysodine (**8**)	>50	44.0	>1.1
Erymelanthine (**138**)	>50	>50	No toxicity, no activity
Apigenin[a]	30.0	6.7	4.5
Vincristine[b]	3.1	ND	

ND: not determined
[a]Positive control as TRAIL enhancement
[b]Positive control for Jurkat cells

(**8**), and erymelanthine (**138**), isolated from the seeds of *Erythrina velutina* (Plate 3), were evaluated for their TRAIL-enhancing activity using Jurkat (an immortalized line of human T-lymphocyte) cells [61]. The results, shown in Table 17, indicated that most of the alkaloids exhibited an enhanced cytotoxicity against Jurkat cells when combined with TRAIL. Erythraline (**10**), erysodine (**2**), and erysovine (**3**) showed IC_{50} values of 23.0, 10.8, and 24.0 $g_{ligand}\ cm^{-3}$, respectively, and their cytotoxic effects were further enhanced by addition of TRAIL, down to IC_{50} values of 5.0, 4.9, and 14.2 $g_{ligand}\ cm^{-3}$. These alkaloids, however, showed some cytotoxicity, albeit moderate, in the absence of TRAIL. The other alkaloids, 8-oxoerythraline (**69**), erysotrine (**1**), and glucoerysodine (**8**) presented some promise in that they exhibited no cytotoxicity by themselves but acted synergistically when combined with TRAIL. Erysotrine (**1**) demonstrated a marked enhancement when combined with TRAIL (0.125 $g_{ligand}\ cm^{-3}$), while on its own it exhibited no discernible cytotoxicity (>50 $g_{ligand}\ cm^{-3}$) [61].

The alkaloids tested all have a 1,6-conjugated diene ($\Delta^{1,2}$, $\Delta^{6,7}$) system as a basic substructure. Cytotoxicity to Jurkat cells was of the order: 16-hydroxy > 15,16-methylenedioxy > 8-oxo > 17-hydroxy, while TRAIL enhancement followed the order: 16-hydroxy > 15,16-methylenedioxy > 16-methoxy > 15-hydroxy > 15-glucosyl substitutions. From the selectivity index, although erythraline (**10**) is moderately cytotoxic to Jurkat cells, its safety margin demonstrated supports the further investigation of this compound as a potential anticancer agent.

The three erythrinan alkaloids, (+)-11α-hydroxyerysotrine *N*-oxide (**61**), (+)-11β-hydroxyerysotrine *N*-oxide (**60**), and (+)-11β-methoxyerysotrine *N*-oxide (**62**), isolated from the flowers and pods of *Erythrina lysistemon* [42], were found to activate human recombinant caspase-3 in a dose-dependent manner [107]. Compounds **61** and **60** caused a five-fold induction, whilst **62** induced a ten-fold increase in activity [107]. The ability of these alkaloids to activate caspase-3 makes them of interest for

Table 18 Ligand binding data for eythroidine alkaloids from *E. poeppigiana*

Compound	Relative binding affinity (RBA[a]/%)		IC_{50}/μ*M*	
	ERα	ERβ	ERα	ERβ
α-Erythroidine (**140**)	0.015 ± 0.010	n.m.	57.3	n.m.
β-Erythroidine (**144**)	0.005 ± 0.010	0.006 ± 0.010	111	96.1
8-Oxo-α-erythroidine (**141**)	n.m.	n.m.	n.m.	n.m.
8-Oxo-β-erythroidine (**145**)	n.m.	n.m.	n.m.	n.m.
17*β*-Estradiol[b]	100	100	0.0083	0.0055

n.m.: not measurable
[a]RBA = (IC_{50} 17*β*-estradiol/IC_{50} compound) × 100
[b]Positive control

more detailed evaluation of their ability to induce apoptosis in cancer cells. Whereas the presence of N-9 oxidation in erythrinan alkaloids tends to lead to a decrease in activity, for this particular biological activity, this seems to be an exception.

Djiogue et al. reported the isolation of four erythroidine erythrinan alkaloids, namely, α-erythroidine (**140**), β-erythroidine (**144**), and their 8-oxo derivatives, 8-oxo-α-erythroidine (**141**) and 8-oxo-β-erythroidine (**145**), from the methanolic extract of the stem bark of *Erythrina poeppigiana* [110]. The estrogenic properties of the isolated erythroidines were assayed using various estrogen receptor (ER)-dependent test systems, which included receptor-binding affinity, cell culture-based ER-dependent reporter gene assays, and gene expression studies in cultured cells using the reverse transcription polymerase chain reaction (PCR). The results (Table 18) showed the relative binding affinities (RBA) to Erα and ERβ and the α- and β-estrogen receptors. α-Erythroidine (**140**) and β-erythroidine (**144**) displayed RBAs for the ERα of 0.015 ± 0.010 and 0.005 ± 0.010. The 8-oxo-derivatives **141** and **145** did not show any RBA, implying either a very low or no affinity for ERα. Only β-erythroidine (**144**) showed an RBA value of 0.006 ± 0.010 for the ERβ receptor. The IC_{50} values for 17*β*-estradiol, **140**, and **144** were 8.3×10^{-9}, 5.73×10^{-5}, and 1.11×10^{-4} *M*, respectively for ERα, while for ERβ the values were 5.5×10^{-9} and 9.61×10^{-5} *M* for 17*β*-estradiol and **144**, respectively (Table 18).

In a reporter gene assay, both α- and β-erythroidines (**140**, **144**) exhibited significant dose-dependent estrogenic stimulation ER-dependent receptor gene activity in osteosarcoma cells (U2OS) detectable at as low a concentration as 10 n*M*. The results were confirmed in MVLN cells, a bioluminescent variant of MCF-7 breast cancer cells. These two erythroidines, in addition, both induced the enhanced expression of the specific ERα-dependent genes trefoil factor-1 (TFF1, formerly called sP2 gene) and serum/glucocorticoid regulated kinase 3 (SGK3) in MCF-7 cells, thus confirming their estrogenicity. Furthermore, molecular docking calculations and simulations on the possible mode of binding on the ERα ligand binding domain (LBD), supported the binding of **140** and **144** on ERα [110].

From a methanolic extract of the leaves of *Erythrina poeppigiana* that showed cytotoxic activity against the MCF-7 breast cancer cell line, Herlina and co-workers

Table 19 Gibbs free energy of binding and inhibition constants for compounds **52**, **131**, and **132**

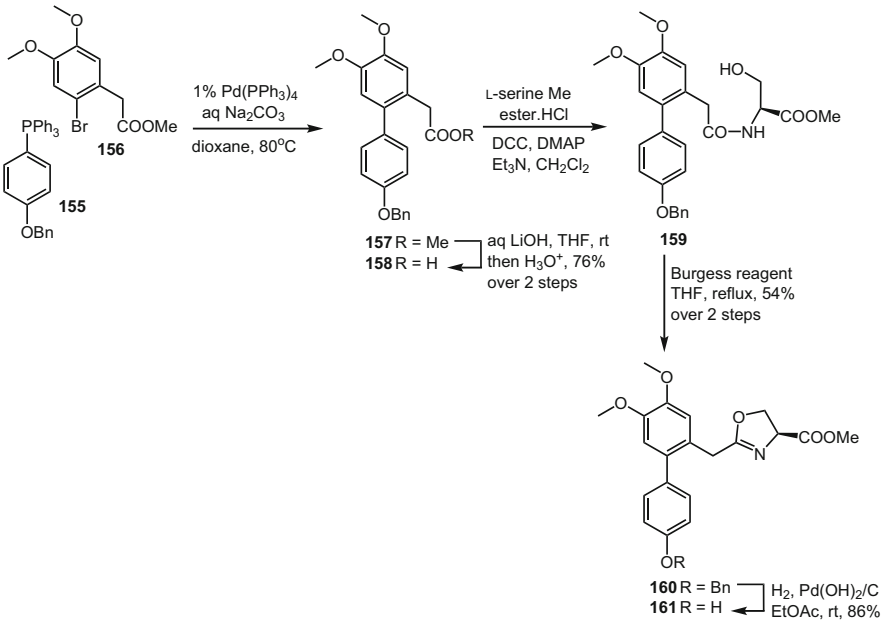
Compound	ΔG/kJ mol^{-1}	*Ki*/*M*
10,11-Dehydroerysodine (**52**)	–36.03	4.84×10^{-7}
6,7-Dihydro-11-methoxyerysotrine (**131**)	–30.71	4.14×10^{-6}
6,7-Dihydro-17-hydroxyerysotrine (**132**)	–33.95	1.12×10^{-6}
Canertinib[a]	–33.95	1.12×10^{-6}

[a]Positive standard

isolated three erythrinan alkaloids, 10,11-dehydroerysodine (**52**), 6,7-dihydro-17-hydroxyerysotrine (**132**), and 6,7-dihydro-11-methoxyerysotrine (**131**) [111]. Since one of the proposed mechanisms for cytotoxicity to breast cancer cell lines is based on the inhibition of the epidermal growth factor receptor 2 (EGFR 2), an in silico method using molecular docking was employed to monitor the potential effects on the EGFR2 receptor of the alkaloids and of a reference compound, canertinib. The latter substance is a known EGFR-2 inhibitor and therefore molecular docking in silico studies on the interaction of the EGFR 2 LBD was considered useful to predict potential cytotoxicity against the MCF-7 cell line. The three compounds **52**, **131**, and **132** (Table 19) were predicted to inhibit the MCF-7 cell line using this in silico method, with bond Gibbs free energy (ΔG) values of -36.03, -33.95, and -30.71 kJ mol^{-1} and inhibition constants, *Ki*/n*M*, of 4.84×10^{-7}, 1.12×10^{-6}, and 4.14×10^{-6}. Canertinib, under the same conditions, gave a ΔG of -33.95 kJ mol^{-1}, and an inhibition constant of 1.12×10^{-6} *M*.

4 Synthesis Aspects

Given the rather small amounts of erythrinan alkaloids available from plants, synthesis of these substances has proven to be a viable option for obtaining them in sizable amounts, especially where larger quantities are required for biological activity work. The non-stereoselective synthesis aspects, often always leading to diastereomeric mixtures of target erythrinan alkaloids, are very well-covered in the literature with two excellent reviews by Reimann [12] in 2007 and Parsons and Palframan [25] in 2010 elegantly summing up the findings. Stereoselective synthesis, however, poses a problem of stereocontrol, particularly at the C-5 spiro carbon of the erythrinan core (Fig. 1). Past and recent synthesis strategies have addressed this problem by, among other methods, deriving key portions of the targets from chiral educts such as, for example, L-DOPA [112–114], malic acid [115, 116], certain lactams [117, 118], tartaric acid [119], material resolution [120]), by desymmetrization of *meso*-imides with chiral bases [121], by relaying axial chirality to spiro centers [122], or by nucleophilic addition of organometallic reagents to Ellman sulphinylimines derived from quinone monoketals [123]. Using some of these stereoselective methods, total syntheses of (−)-3-demethoxyerythratidinone

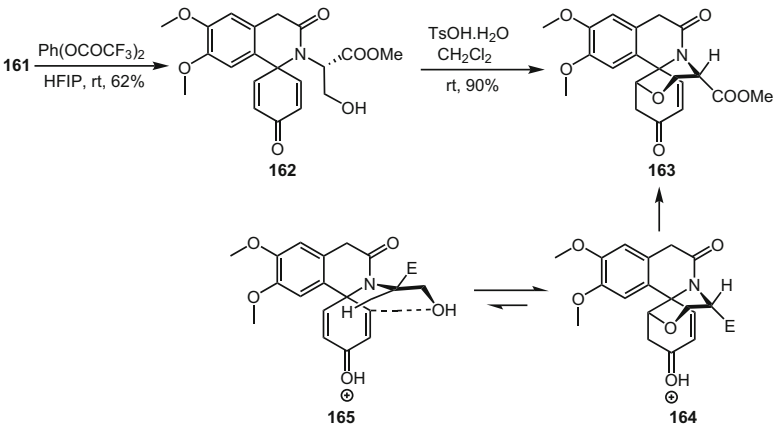


Scheme 4 Preparation of oxazoline **161**. OBn: benzyl ether; DCC: dicyclohexylcarbodiimide; DMAP: (4-dimethylamino)pyridine; Burgess reagent: methyl *N*-(triethylammoniumsulfonyl) carbamate

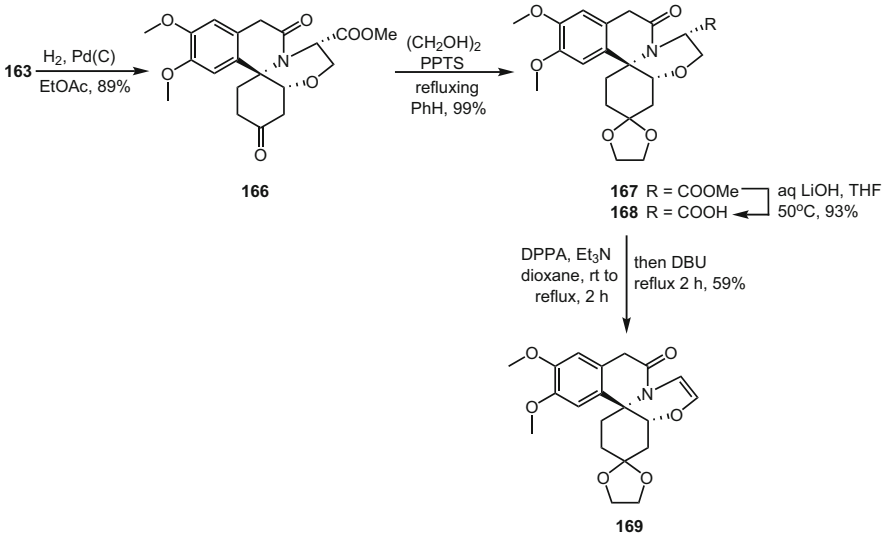
(**126**) [123], also called *enantio*-(−)-3-demethoxyerythratidinone, (+)-3-demethoxyerythratidinone (**126**) [115, 116] or natural 3-demethoxyerythratidinone, (+)-erythrartine (**24**) [124], and (+)-erysotrine (**1**) [113] were achieved successfully.

Paladino et al. [125] described a synthesis protocol where a common intermediate obtained by oxidative cyclization of a phenolic oxazoline en route to the synthesis of the two erythrinan alkaloids (+)-3-demethoxyerysotramidine (**126**) and (+)-erysotramidine (**66**). A highly diastereoselective Michael-type cyclization of the formed oxazoline adduct desymmetrizes a "local symmetrical" dienone [126], thus securing the correct configuration at the spirocenter. Oxazoline **161**, the first sub-target of the synthesis (Scheme 4), was obtained through Suzuki coupling of the commercial reagents **155** and **156** to give ester **157**, which, upon hydrolysis, gave acid **158**. Reaction of **158** with methylserinate. HCl yielded the adduct **159**, which upon cyclization and catalytic debenzylation gave the oxazoline **161**.

Oxidative cyclization of oxazoline **161** (Scheme 5) using [bis(trifluroacetoxy) iodo]benzene in 1,1,1,3,3,3-hexafluoroisopropanol gave the lactam intermediate **162** in 62% yield. Michael cyclization of lactam **162** using TsOH gave a spiro enone **163** as a single diastereomer thus establishing the correct (*R*)-configuration at the spiro carbon, which was confirmed by X-ray diffraction of the monohydrate of **163**. It thus appears that the stereorelay from the serine fragment (**165** = **164**) to the spirocenter occurred with perfect fidelity.

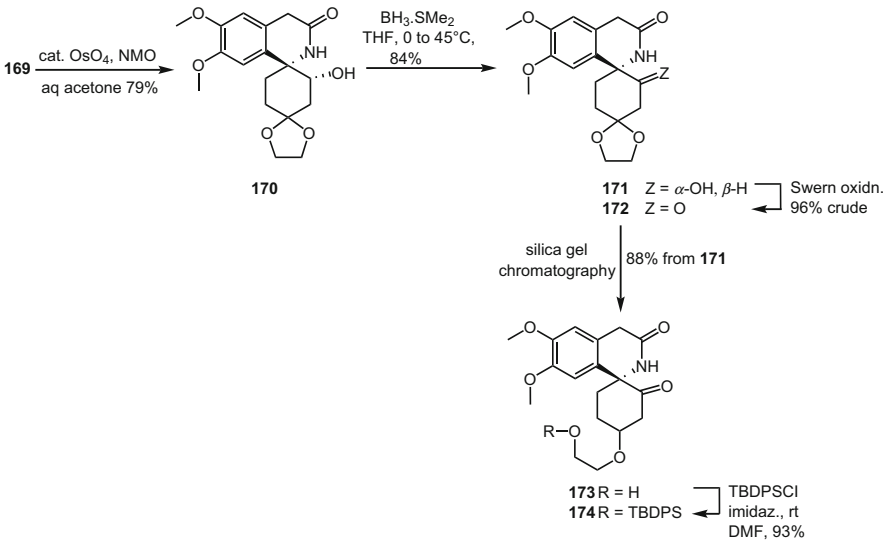


Scheme 5 Oxidative cyclization of **161** and stereoselective formation of **162**. HFIP: hexafluoroisopropanol; TsOH: *p*-toluenesulfonic acid



Scheme 6 Preparation of enamide **169**. PPTS: pyridinium *p*-toluenesulfonate; PhH: benzene; DPPA: diphenylphosphoryl azide; DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

Catalytic reduction of enone **163** gave **166** with full retention of configuration at the spiro center (Scheme 6). Acid **168** was obtained from **166** by protection of the keto group followed by base-catalyzed hydrolysis of the ester group. Reaction of **168** with diphenylphosphoryl azide and Et_3N in dioxane with a reflux time of 2 h,



Scheme 7 Preparation of ketones **172** and **174**. NMO: *N*-methylmorpholine *N*-oxide; $BH_3.SMe_2$: borane dimethyl sulfide; TBDPSCl: *tert*-butyldiphenylsilyl chloride

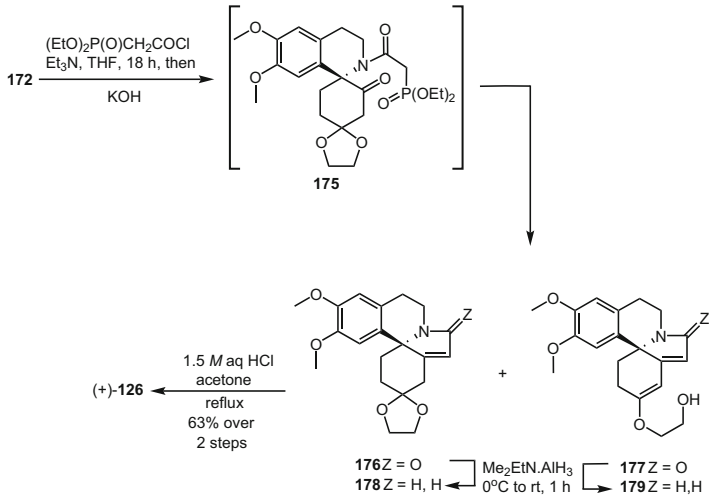
followed by addition of 1,8-diazbicyclo[5.4.0]undec-7-ene and a further 2-h reflux, gave **169** in 59% yield.

In work performed at The Upjohn Company, dihydroxylation [127] of **169** produced lactam **170** (Scheme 7), probably through release of the etheno bridge as a glyoxal. The carbonyl function in **170** opens a route to access the erythrinan alkaloids in which additional oxygen functionalities or double bonds (10,11-dehydro) are present in ring C of the erythrinan nucleus. Where this is not required, as in the present case, the carbonyl in **170** can be reduced to form **171**, which, on Swern oxidation [128, 129], would yield **172** (with an 8-oxo functionality).

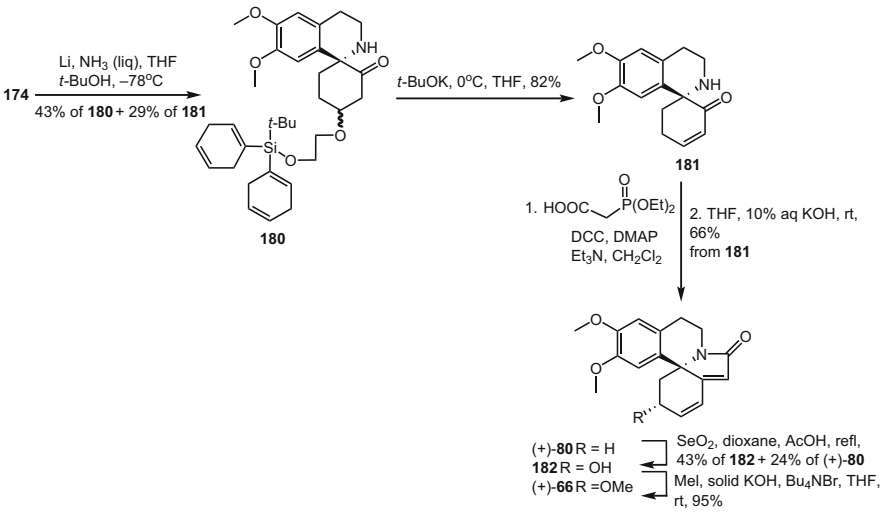
Reaction of crude **172** with $(EtO)_2P(O)CH_2COCl$ (Scheme 8) and subsequent treatment, in situ, of the resulting **175** with KOH yielded a mixture of **176** and **177** that was subsequently reduced (separation was not necessary) using $AlH_3.NEtMe_2$ and then acid hydrolyzed to give the target erythrinan alkaloid **126** ((+)-3-demethoxyerythratidinone) in 63% yield.

Compound **171** was found to be unstable on silica gel by which it was transformed to **173** in 88% yield. Compound **174** (the *tert*-butyldiphenylsilyl derivative of **173**) was obtained by reaction of **173** with *tert*-butyldiphenylsilyl chloride (TBDPSCl) in imidazole and then dimethyl formamide (DMF) in 93% yield (Scheme 7). Conjugate reduction of **174** with $Li/NH_3(l)$ followed by THF and then *tert*-BuOH gave a mixture of **180** (43%) and the expected product **181** (29%). However, treatment of **180** with potassium *tert*-butoxide transformed it into more **181** with an overall 64% yield.

Acylation of **181** (diethyl phosphonoacetic acid, DCC, DMAP, Et_3N, DCM) gave the rare erythrinan alkaloid (+)-erythrosotidienone (**80**) (Table 4). Allylic oxidation



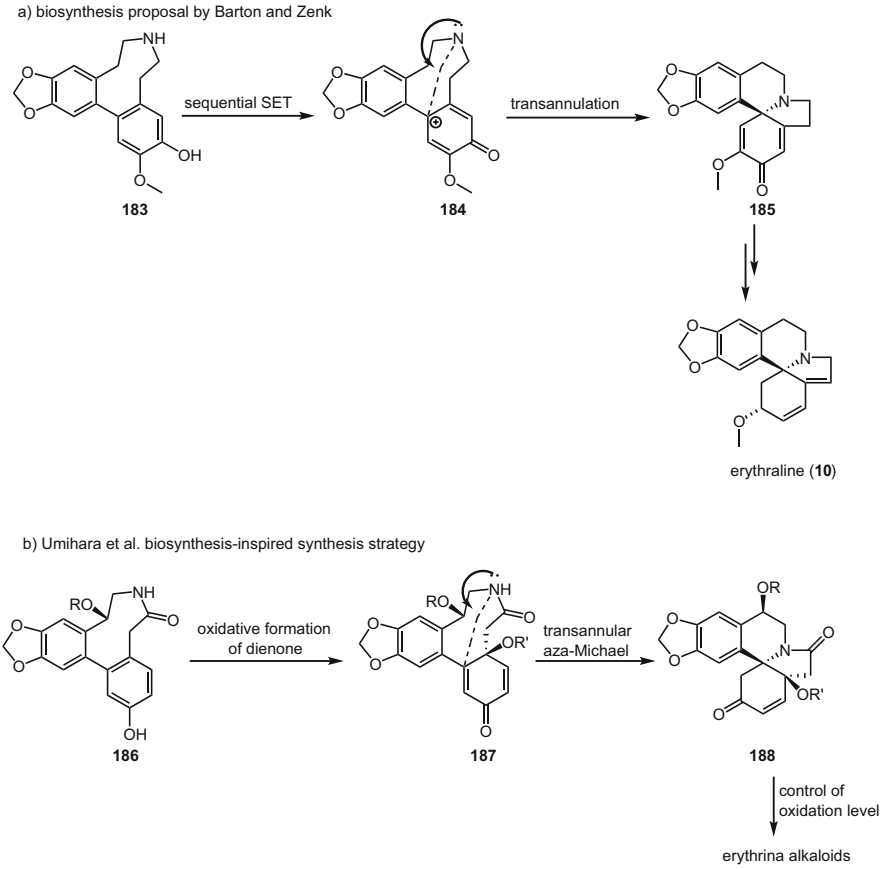
Scheme 8 Completion of the synthesis of **126** ((+)-3-demethoxyerythratidinone)



Scheme 9 Completion of the synthesis of **66** ((+)-erysotramidine). DCC: dicyclohexylcarbodiimide; DMAP: (4-dimethylamino)pyridine

of **80** with selenium dioxide gave a mixture of **80** (24%) and **182** (33%) and methylation of **182** (Scheme 9) gave (+)-erysotramidine (**66**).

The biosynthesis of the erythrinan core (Fig. 1) originally proposed by Barton [22–24] and later revised by Zenk [130] is known to involve a key nine-membered

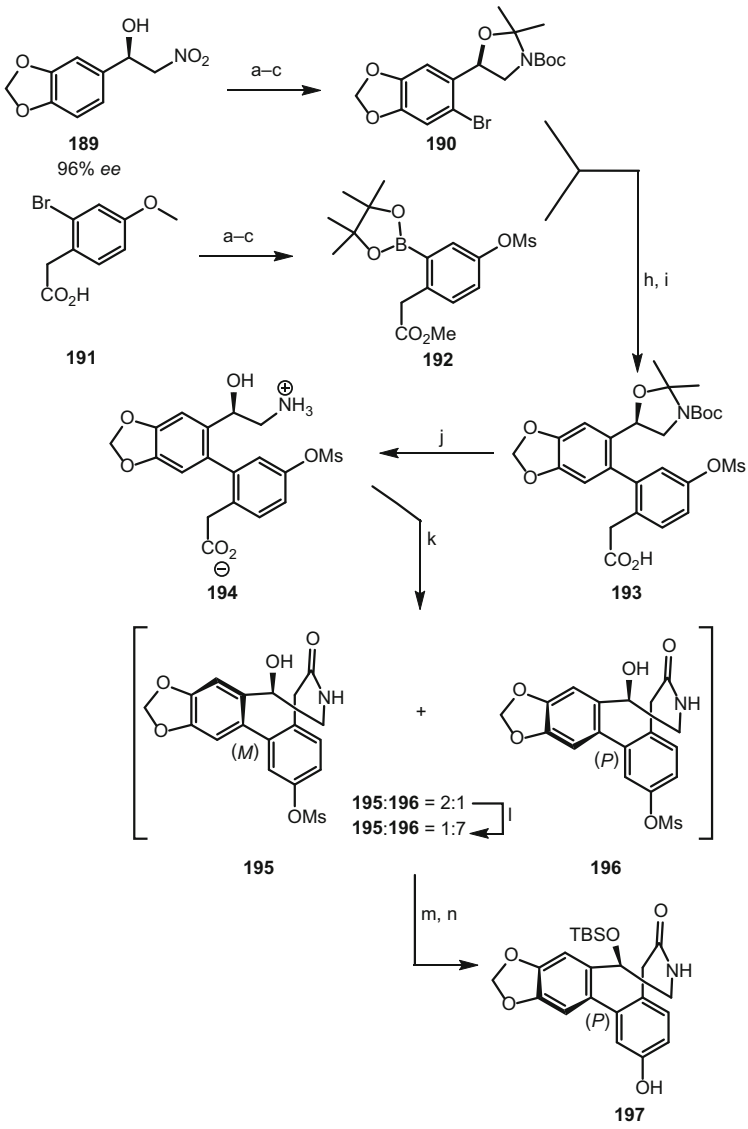


Scheme 10 Proposed biosynthesis and Umihara et al. synthesis strategy

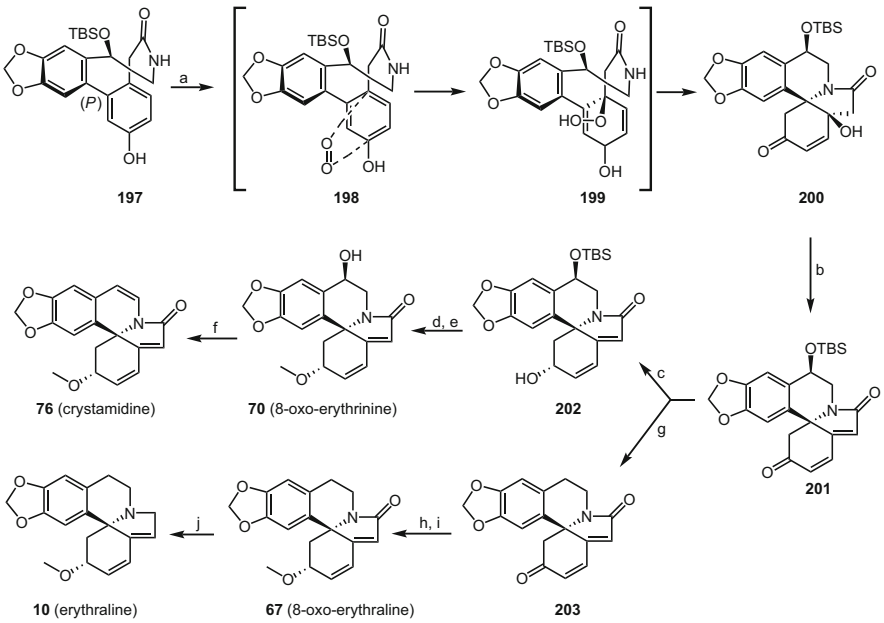
dibenz[*d*,f]azonine intermediate **183**, which undergoes a phenolic moiety oxidation by a two-step single electron transfer (SET) mechanism (Scheme 10a) to give a cationic intermediate **184**. This cationic intermediate **184** then undergoes a transannular aza-Michael-type reaction to give structure **185**, which has a tetrasubstituted C-5 spiro carbon center, thereby opening up the route to the biosynthesis of 15,16-methylenedioxy erythrinan alkaloids like erythraline (**10**) and its derivatives. This biosynthesis sequence inspired the Kitamura and Fukuyama group [131] to envision a novel asymmetric synthesis approach using also a nine-membered intermediate used previously by Teetz and Ito in *Erythrina* alkaloid syntheses [132–134]. Diastereoselective oxidative transformation of **186** into dienone **187** (Scheme 10b) provided access for the construction of the core structure of the tetracyclic moiety. Subsequent intramolecular reaction would form the C-5 tetrasubstituted chiral carbon center in the key intermediate **188**. The chirality at the C-11 benzylic oxygen functionality was introduced with the expectation of biasing the stereo induction during the oxidation process. Intermediate **188** thus provides a doorway for the synthesis of a variety *Erythrina* alkaloids having a methylenedioxy

moiety in ring D through a combination of elimination, reduction and oxidation of oxygen functionalities. A two-stage synthetic strategy initially involving construction of the common core, which is further functionalized at a later stage to provide key intermediates, would facilitate the divergent syntheses [135] of natural as well as non-natural derivatives of the *Erythrina* alkaloids.

The Kitamura-Fukuyama [131] synthesis strategy thus commenced with a chiral nitro alcohol **189** prepared using a method reported by Gong and co-workers [136]. This nitro alcohol was converted to the bromoaryl compound **190** in a three-pot sequence of reactions (Scheme 11). The aryl boronate **192** was similarly synthesized from a commercially available acid **191** in four steps. In a convergent synthesis, the bromoaryl **190** was coupled to the aryl boronate **192** using Suzuki-Mayaura cross-coupling [137] conditions to give the biaryl intermediate **193**, which upon removal of protecting groups (Boc and acetonide) gave the amino acid intermediate **194**. Using the reagent DMT-MM (Scheme 11) [138] in methanol as a condensation reagent ensured a nine-membered lactone was formed in preference to an eight-membered one. The formed expected product was found to be a mixture of two diastereomers of the stable biaryl atropisomers (*M*)-**195** and (*P*)-**196** in a 2:1 ratio, with the latter being thermodynamically more stable. The ratio was found to shift highly in favor of (*P*)-**196** when the mixture was heated continuously for 40 h where the composition was found to be 1:7 in favor of (*P*)-**196**. Selective silylation occurred only with (*P*)-**196** and subsequent demesylation gave **197** as a single isomer. The stereochemical configuration of the benzylic hydroxy group was thus successfully transferred to the atropisomeric stereochemistry of the biaryl moiety. The phenol moiety of **197** was oxidatively transformed (Scheme 12) by use of a singlet-oxygen oxidation [139–141] giving only a single diastereomer through the transition state **198** leading to dienone **199**, fortunately without forming the undesired orthoquinone derivatives. The C-5 position of the dienone **199** was attacked spontaneously by the amide nitrogen of the intermediate and in so doing creating the crucial tetrasubstituted center. The in situ reduction of the hydroperoxide by triphenyl phosphine (PPh_3) yielded the tertiary alcohol **200** bearing a C-11 oxygen functionality having the desired configuration. The stereochemistry of the biaryl atropisomer was thus successfully transferred to the tetrasubstituted point chirality at C-5. Elimination of the tertiary hydroxy group by thionyl chloride gave intermediate **201**. This intermediate provided a point of divergent synthesis leading on the one hand to 11-oxygenated erythrinan alkaloids and on the other hand to 11-deoxy analogues. Application of the diastereoselective Luche reduction [142, 143] of the enone moiety in **201** led to **202**, which was converted to the erythrinan alkaloid **70** (8-oxoerythrinine or 11-hydroxy-8-oxoerythraline) by methylation and treatment with TBAF. Subsequent dehydration under acidic conditions gave the erythrinan alkaloid **76** (crystamidine or 10,11-dehydro-8-oxoerythraline). Treating **201** with Et_3SiH under acidic conditions led to **203**, a precursor for alkaloids without C-11 oxygenation. A sequence involving a Luche reduction [142–144] and methylation on **203** led to the formation of the erythrinan alkaloid 8-oxoerythraline (**69**), and reduction of the amide moiety in **69** (8-oxoerythraline) using a combination of $LiAlH_4$ and $AlCl_3$ finally gave the erythrinan alkaloid **10** (erythraline).



Scheme 11 Construction of the nine-membered lactam intermediate. Reagents and conditions: a) H_2, Pd/C, EtOH, rt; b) Boc_2O, CH_3CN, 0°C to rt; evap.; 2,2-dimethoxypropane, $BF_3.OEt_2$, acetone, 0°C, 70% (2 steps); c) NBS, CH_3CN, 50°C, 88%; d) $SOCl_2$, CH_3OH, 0°C to rt, 95%; e) BBr_3, CH_2Cl_2, 0°C; f) MsCl, Et_3N, CH_2Cl_2, 0°C, 83% (2 steps); g) [$PdCl_2$(dppf)] (5mol%, $(Bpin)_2$, KOAc, 1,4-dioxane, 80°C, 59%; h) [$PdCl_2$(dppf)] (10mol%, Cs_2CO_3, DME, reflux; i) $LiOH.H_2O$, THF/H_2O (1:1), rt, 90% (two steps); j) H_2O/HCO_2H (1:2), 0°C to rt; k) DMT-MM, CH_3OH (2.0 m*M*), rt, 62% (two steps); l) 2-butanol, 90°C, 40 h; m) TBSCl, imidazole, $Cl(CH_2)_2Cl$, 40°C, 54% (two steps); n) KOH, CH_3OH, 50°C, 91%. Boc = *tert*-butoxycarbonyl, DME = dimethoxyethane, DMT-MM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, dppf = 1,1′-bis(diphenylphosphanyl)ferrocene, Ms = methanesulfonyl, NBS = *N*-bromosuccinimide, $(Bpin)_2$ = bis(pinacolato)boron, TBS = *tert*-butyldimethylsilyl, THF = tetrahydrofuran



Scheme 12 Synthesis of the common intermediate and total syntheses of four erythrina alkaloids. Reagents and conditions: a) O_2, *hv*, rose bengal, PPh_3, $NaHCO_3$, CH_3CN/H_2O (1:1), rt, 77%; b) $SOCl_2$, pyridine, 0°C, 84%; c) $NaBH_4$, $CeCl_3.7\ H_2O$, CH_3OH, rt, 75%; d) CH_3I, NaH, THF, rt; e) TBAF, THF, rt, 84% (two steps); f) $TsOH.H_2O$, (20mol%), toluene, reflux, 77%; g) Et_3SiH, $BF_3.OEt_2$, CH_2Cl_2, 0°C, 74%; h) $NaBH_4$, $CeCl_3.7H_2O$, CH_3OH, rt; i) CH_3I, KOH, Et_4NBr, THF, rt, 86% (two steps); j) $LiAlH_4$, $AlCl_3$, THF/Et_2O, (1:1), 0°C, 73%

5 Conclusions

Erythrinan alkaloids are found predominantly in the genus *Erythrina* of the Leguminosae family but are also reported as being present in the genera *Cocculus*, *Pachygon*e and *Hyperbaena* of the family Menispermaceae. Many reviews have been published on *Erythrina* and related alkaloids and by examining the literature it has become clear that a number of these structures have been missed from major reviews and that some structures needed correction, while a few others have been reported as new when in fact they have been known for some time. It has also become obvious that with the new and latest findings, there is a need to re-organize the existing data into a more user-friendly and easier-to-recognize and easier-to-identify format. Erythrinan alkaloids also have very similar and confusing names, which are easier appreciated when these structures are all included in one document. In this chapter, approximately 40 new structures have been added to those compiled earlier, bringing the total number of known erythrinan alkaloids to over 150. What is striking is the fact that in the past 5 years there has been a dramatic increase both in the number, structural variety, and complexity of erythrinan alkaloids reported. This

structural diversity seem to be more pronounced in the two *Erythrina* species *E. arborescens* (Plate 1) and *E. variegata* (Plate 10) which, between them, yielded new polymeric (dimeric and trimeric) erythrinan alkaloids, a first example in one case where a normal 6,5,6,6-membered indoloisoquinoline spiro-cyclic core has rearranged to a spiro-fused 6,5,7,6-skeleton. Furthermore, erythrinan alkaloids with a fifth ring containing a 2*H*-imidazole functionality were also reported for the first time, together with some new structures having unusual substitution and substituents at positions C-3 and C-7 of the erythrinan core.

The predominant pharmacological activity reported for the new and known erythrinan alkaloids is central nervous system (CNS) related, especially interactions with nicotinic acetylcholine receptors (nAChRs), where they act as reversible competitive nAChR antagonists. Other significant effects exhibited by these erythrinan alkaloids are antifeedant and insecticidal activity; cytotoxic and estrogenic activities; antiprotozoal activity; anti-inflammatory activity; antioxidant activity, and antifungal and antiviral activities. Although antibacterial activity has been reported frequently for other non-alkaloidal constituents of *Erythrina* species such as flavonoids, the erythrinan alkaloids surprisingly seem to be, by and large, devoid of this type of activity. The structural determinants for biological activity in general for the erythrinan alkaloids appear to be: a conjugated diene ($\Delta^{1,2}$, $\Delta^{6,7}$) system, a C-8 sp^3 methylene (as opposed to an 8-oxo group), a non-oxidized N-9 nitrogen (as opposed to a quaternary nitrogen), 11-oxidation (hydroxylation, methoxylation, glucosylation), and the nature of ring-D substituents (hydroxylation, or methoxylation, or methylenedioxy formation at C-15/C-16 or lactonization of ring D). The 1,2-epoxidation of the conjugated diene system also seems to enhance some biological activities even when some opposing structural features exist that seemingly mitigate against these effects. There are, however, one or two examples where an 8-oxo group has been shown to enhance compound cytotoxicity against a given cancer cell line and in another case where such activity was enhanced by the presence of an *N*-oxide group. It has been observed also that the recently described polymeric erythrinan alkaloids are generally devoid of activity (e.g. no perceived cytotoxicity against several human cancer cell lines), with two of these seven compounds showing moderate insecticidal activity, and another showing weak to moderate acetylcholinesterase inhibition.

Following two excellent previous reviews detailing the synthesis of erythrinan alkaloids that were published several years ago [12, 25], more recently useful methods have continued to be developed for the stereoselective chemical synthesis of these compounds. The availability of such methodogy will enhance the amounts of the erythrinan alkaloids available for future more detailed biological evaluation, particularly using experimental animals, since these compounds tend to be obtained only in low quantities from their plants of origin.

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