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Modes of Action of Antimicrobial Agents

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I. Introduction

Actions of chemotherapeutic drugs have been investigated for more than three decades. The bibliography of source articles in this research field comprises more than 10,000 original papers; a number of textbooks and monograph collections have been published[1-7]. The purpose of this present article can not be an attempt at reviewing even a small part of the large literature on modes and mechanisms of action of chemotherapeutic drugs. What is intended, instead, is to present some of the conceptual outlines and fundamental considerations which have shaped and advanced this field of science.

When the author of this essay was confronted in 1949 with the problem of elucidating the mode of action of chloramphenicol, there existed virtually no methodological or conceptual guidelines which could be applied to this task. A review on the mode of action of sulfonamides in 1943[8] had occupied 87 pages, cited 291 references and did not come to any clearcut explanation of what is considered today one of the better understood modes of chemotherapeutic drug action.

The reason for these initial difficulties were clearly that scientists did not know conceptually what to look for, nor methodologically how to look. A review of the mechanism of action of the tetracyclines, as late as 1967, offered 6 pages of tabulation of "activities" which had been found to be inhibited by these antibiotics [9]. While it is not unusual to read that the advances in the last 15 years are methodological in nature, it is not always appreciated that answers began to come forth only after investigators had learned to ask pertinent questions.

II. What Constitutes the Mode of Action?

It is appropriate, therefore, to reprint here the postulates of this author which were offered in a symposium lecture on Modes of Action of Antibiotics at the Fourth International Congress of Biochemistry in 1958 [10].

"The problem of elucidating the mode of growth inhibition of an antibiotic is, ... not simply one of discovering some action upon some physiological process or biochemical reaction. Many such findings come up during extended investigations and are not infrequently misinterpreted as modes of action. The real problem is conceptual, viz., elimination of unimportant or secondary effects and identification of the primary process or reaction whose inhibition is originally responsible for growth inhibition."

"We have adopted a set of criteria which a physiological process or biochemical reaction must fulfill in order to be considered the key process whose inhibition leads to the overall result of growth inhibition.

1. *The inhibited reaction must be of vital necessity for the economy of the microbial cell.*
2. *The inhibition must be produced specifically in organisms whose growth is susceptible to the action of the drug.*

3. *The inhibition must be produced by an antibiotic concentration that is of the same order as the growth-inhibitory concentration range.*
4. *The degree of inhibition must approach an all-or-none effect.*
5. *The inhibition must depend upon the specific chemical structure of the antibiotic molecule in precisely the same manner as does the growth-inhibitory effect."*

Here are some selected examples of research studies which did not consider the above postulates. In 1949 a paper was published which reported inhibitions of bacterial esterases by chloramphenicol [11]. No thought was given to the requirement that the inhibited reaction must be vitally important to growth of susceptible cells.

One year later appeared a paper with the title, on the mechanism of action of aureomycin [12]. Here the effect of chlortetracycline was studied in a mammalian liver mitochondrial system in which the drug interfered with oxidative phosphorylation. It was completely missed that liver cells are not target organisms for the antibiotic action of tetracyclines. This violates the second postulate, *viz.*, the inhibition must be produced specifically in organisms whose growth is susceptible to the drug under study. Within a short time, it was shown [13, 14] that chlortetracycline binds Mg^{2+} by chelation and that the "inhibition" of oxidative phosphorylation was the simple result of magnesium deficiency. Even recently, a paper was published which reported effects of the antimalarials, chloroquine and primaquine, on polypeptide synthesis in a cell-free system from *rat liver* [15].

One of the most frequently neglected rules in the investigation of modes of action pertains to the use of excessive drug concentrations. In a study of the effects of chlortetracycline on a (mammalian) D-amino acid oxidase [16], the antibiotic concentration was 1.2×10^{-3}M which is 575 μg/ml, *i.e.* more than two orders of magnitude higher than the growth-inhibitory concentrations for susceptible bacteria.

The literature is replete with reports of minor inhibitions of some biochemical or physiological process by chemotherapeutic drugs. In my own laboratory it was found that quinacrine caused a slight inhibition of protein biosynthesis in susceptible bacteria [17] which subsequently was verified in a cell-free poly U system, polymerizing phenylalanine [18]. Since it was also established, however, that the drug is a strong inhibitor of DNA biosynthesis [17] and that this effect accounts for the bactericidal action of quinacrine, the slight effect on protein biosynthesis was not mistaken as the mode of action.

An interesting body of investigations was published on the effects of chlortetracycline on bacterial nitro-reductases (reviewed in [9]). Since a reductase enzyme isolated from chlortetracycline-resistant bacteria was less sensitive to the antibiotic, one could have hoped that these studies were in some manner directed towards the mode of action of chlortetracycline. However, antibiotically inactive degradation products of chlortetracycline also inhibited nitro-reductases from bacteria [19]. This was in disagreement with the last postulate that the observed inhibition must depend upon the specific chemical structure of the antibiotic molecule in precisely the same manner as does the growth-inhibitory effect. In our extensive studies on the mode of action of chloramphenicol, we carried out routinely control experiments with the antibiotically non-active enantiomer of the drug in order to disallow non-specific effects which conceivably might have been followed up.

An interesting exception to the absolute validity of the tifth postulate is the considerable activity of chloramphenicol derivatives in cell-free model systems of protein synthesis when these derivatives are substituted with amino acyl residues instead of with dichloroacetyl as is the antibiotic itself (rev. in [20]). This has been traced to the necessity of the dichloroacetyl grouping in aiding in the permeation of the antibiotic through the bacterial envelope [21]. The amino acyl derivatives have very low antibacterial activity [20]. Permeation failures of actinomycin D, macrolides and distamycin A with respect to certain families of bacteria occlude the action of these antibiotics on their intracellular drug receptors and target reactions but can be overcome experimentally by measures which render test organisms permeable.

III. Test Organisms and Drug Effects Upon Them

Since quantitative drug actions on the viability and/or growth of microorganisms are the basic reference parameters of all mode of action studies, the selection of a test organism and the determination of the effects of a drug upon it are the essential first steps in all investigations on modes and mechanisms of chemotherapeutic drugs.

If the drug under study is a reasonably broad spectrum antibacterial agent, it is easy to select a non-pathogenic test bacterium which lends itself to extensive laboratory investigations. Many such studies have therefore been carried out with prototrophic strains of *Escherichia coli* which grow in mineral medium supplied with a source of carbon. With a view to anticipated testing for the incorporation of building blocks into the macromolecules of a test organism, it can be advantageous to select auxotrophic mutants which may require a certain amino acid (such as phenylalanine) or thymine for growth. A frequently used test strain is *E. coli* TAU^- which requires thymine, uracil and arginine.

Chemotherapeutic drugs with an extremely narrow spectrum of activity such as isoniazid or p-aminosalicylic acid which act exclusively on *Mycobacteria* are difficult to study, because no convenient test organism is available.

A special problem arose with respect to the antiplasmodial drugs chloroquine (Resochin) and quinacrine (Atebrin). Plasmodia can not be propagated in cell-free lifeless media and the limited availability of erythrocytic plasmodial cultures severely limits biochemical or molecular pharmacological studies on the action of antimalarials. The actions of chloroquine and quinacrine were, therefore, studied in *Bacillus megaterium* or *E. coli*, respectively [22].

The selection of bacteria as test organisms for the investigation of antimalarial drugs did, at first, not go unchallenged and required a scientific justification which was given as follows [22]:

"Owing to the near universality of the molecular processes, fundamental to microbial growth and replication, the choice of test organisms or cell-free test systems is dictated not so much by the pathogenic properties of specific microorganisms but rather by considerations of methodological feasibility and of the opportunity of physical and conceptual isolation of a given phenomenon to be studied. In no instance in which a problem of the mechanism [of action] of a chemotherapeutic drug has been solved, was such a solution accomplished through

work on a major pathogenic target organism or has required that the study be extended to such pathogens. In this respect the investigative perspective in molecular pharmacology differs from that in pathology, immunology or medical microbiology which must be concerned with the pathogenic, immunogenic or diagnostic properties of specific etiological agents of communicable diseases."

"It would be erroneous to consider an investigation of the inhibition of major biosynthetic pathways or of the mechanistic details of such inhibitions merely a "model" until such a time when a confirmatory analogy study was carried out with intact of fractionated cells of a pathogenic microorganism for no deeper reason than that the given chemotherapeutic molecule can be used to cure clinical illness produced by the growth and replication of such an organism. Essentially, any microorganism that is subject to growth inhibition by critical concentrations of a given chemotherapeutic drug is, for that very reason, a suitable test organism in mode of action studies."

This reasonig has been fully justified in the sense that subsequent studies on the action of chloroquine on plasmodia have confirmed the results of preceding work[22] which used bacteria as test organisms (rev.[23]). There are instances, however, in which no suitable test organism can be found despite a systematic search for it. In the laboratory of this writer, no test bacterium was discovered which was susceptible to reasonably low concentrations of quinine and certain new investigational antimalarial drugs could not be studied in bacterial cultures because of their limited solubility in aqueous media.

Among the many microbiological methods of demonstrating growth inhibitions stands out the determination of decreases in *growth rates* by graded drug concentrations. Growth rates of cultures which have entered the exponential phase of multiplication (measured either turbidimetrically, by electronic counting such as in a Coulter Counter, or by plating and colony counts) are systematically reduced, yielding families of growth curves with decreasing slopes. The regression coefficients of such exponential growth curves can be expressed in per cents of inhibition by (logarithmically) graded drug concentrations, and these percentages, converted to their probits, are linear functions of the logarithm of drug concentration and permit interpolation to the 50 per cent inhibitory concentration, ID_{50}, which is the most precise measure of antibacterial potency [24]. Furthermore, the ID_{50} in molar concentration represents the dissociation constant of a drug-receptor complex which is formed and its reciprocal value is the apparent association constant. This does not specify if the receptor is on the cell surface where its occupancy precedes entry of the drug into the cell or is intracellular and causally responsible for the molecular mechanism of action of the drug.

This type of kinetic analysis of growth inhibition works with predominantly bacteriostatic drugs such as chloramphenicol or the tetracyclines[25] but has been extended to the study of the bactericidal antibiotic, streptomycin[26].

Brought to logical conclusion, it was shown that the probit transformations of bacterial growth inhibition and inhibition of DNA biosynthesis by Nitroakridin 3582 were superposable while the same functions for inhibition of RNA and protein biosyntheses were superposable upon each other but indicated a lesser susceptibility of the test organism. This led to the conclusion that the mode of antibacterial action of the nitroacridine was its inhibition of DNA biosynthesis[27].

Kinetic analysis of growth inhibitions by graded concentrations of a chemotherapeutic drug failed with penicillin[25]. Up to a certain threshold drug concentration and for a fraction of one doubling time, the antibiotic had little influence (turbidimetrically) on growth rates; beyond these critical time and concentration limits, the test culture underwent rapid lysis, *i.e.* morphological destruction of the bacterial cells. The same problem can logically be predicted for all drugs which interfere with the integrity of the cell wall, resulting in lysis and physical disassembly of the test cells.

The concentration of choice for mode of action studies is the lowest drug concentration which inhibits growth entirely. This can either be estimated by extrapolation of the probit-transformed log dosage response correlation or by determination of the MIC, *i.e.* minimal inhibitory concentration, by the method of serial twofold dilution of drug-containing medium in test tubes, inoculation of these media and visual observation of growth after incubation overnight.

IV. The Strategy of Mode of Action Studies

At the early beginning of mode of action studies, the field was without a systematic approach and, hence, relegated to empirical inquiry in which scores of enzyme reactions and physiological processes had to be tested for susceptibility to a chemotherapeutic drug under investigation in the hope that some major effect would be uncovered which could be considered significant. An early review on sulfonamides [8] and a long tabulation of actions of tetracyclines[9] are indicative of the results which were empirically obtained.

A review of the mode of action field today leads to the conclusion that there exists a very linited number of interferences with physiological processes of microbial cells which result in cell death or in inhibitions of cellular replication. Regardless of the underlying mechanistic reasons, there are mainly five categories of action which lead to chemotherapeutic potency.

1. Inhibition of DNA synthesis,
2. inhibition of RNA synthesis,
3. inhibition of protein synthesis,
4. inhibition of cell wall synthesis and
5. interference with the structural and functional integrity of cell membranes.

Substances such as the antibiotics of the antimycin family which interfere with electron transport in the respiratory chain are generally toxic and, for this reason, unsuited as chemotherapeutic drugs. The only practical use of antimycin is that as a fish poison.

After the selection of a suitable test organism, the strategy of mode of action studies involves basically the testing of the compound under study for its effects on each of the five processes listed above. This requires invariably the study of actively growing cultures or, better, of organisms which are incubated in a medium which supports growth and multiplication. Bactericidal drugs, for example, penicillin

and streptomycin have no lethal effects on non-growing bacterial suspensions and actions on macromolecular syntheses can, of course, not be investigated in "resting" organisms, *i.e.* in suspensions of bacteria in buffered salt solutions devoid of nutrients. The resting cell technique was mostly used in manometric experiments on gas exchanges in cell suspensions, oxidizing a variety of substrates[28].

1. Inhibition of DNA Synthesis is usually signalled by bactericidal actions of the drug under investigation: organisms which can not replicate their chromosomes cannot produce a viable progeny. In addition, inhibitions of DNA synthesis frequently give rise to long filamentous forms of bacteria. It is believed that the completion of one round of chromosomal DNA biosynthesis produces a "signal" which actuates cross septation and cytokinesis. In its absence, the cells grow longer but do not divide. Figure 1 shows such filaments of *E. coli* after exposure to 2×10^{-4} M quinacrine for 24 hours [17] and Fig. 2 shows another microscopic field photographed in the fluorescence microscope and demonstrating the fluorescent staining of the bacterial filaments by the drug.

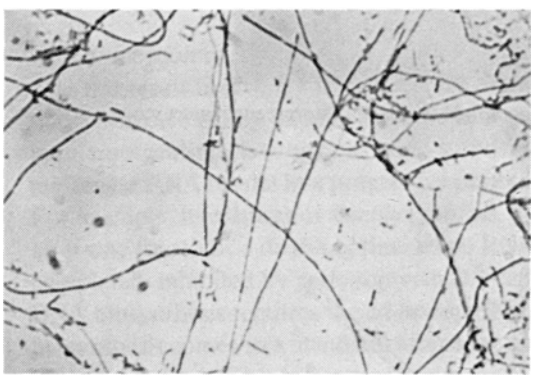

Fig. 1. Filaments of *Escherichia coli*, formed during 24 hours of growth in the presence of 2×10^{-4} M quinacrine[17]

One classical and early example of selective inhibition of DNA biosynthesis is shown in Fig. 3 for the anitbiotic, mitomycin C[29]. A concentration of 0.1 μg/ml (3×10^{-7} M) completely inhibited DNA synthesis in *E. coli* B, while RNA synthesis, protein synthesis and "growth" meaning turbidity, *i.e.* cell mass increase, continued. However, after the experimental period of only 90 min, the number of viable bacteria had decreased by 85 per cent. By that time, bacterial filaments were visible under the microscope.

With the introduction of radiochemical methods, DNA biosynthesis and its inhibition is usually followed either by measuring the incorporation of radioactive thymine into thymine auxotrophs of bacteria or the incorporation of radioactive thymidine into prototrophic organisms. In the latter instance, it is practical to include in the experimental medium a large excess of non-radioactive deoxyadenosine in order

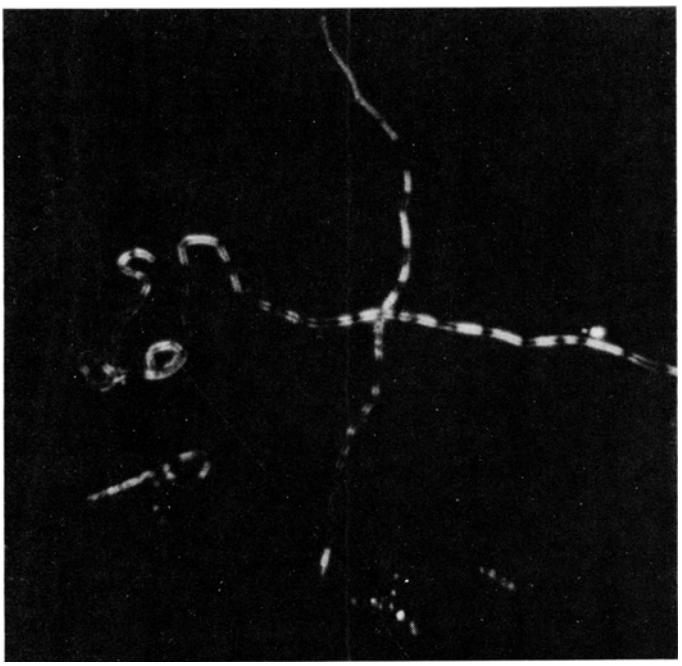

Fig. 2. Same object as in Fig. 1 but photographed under the fluorescence microscope

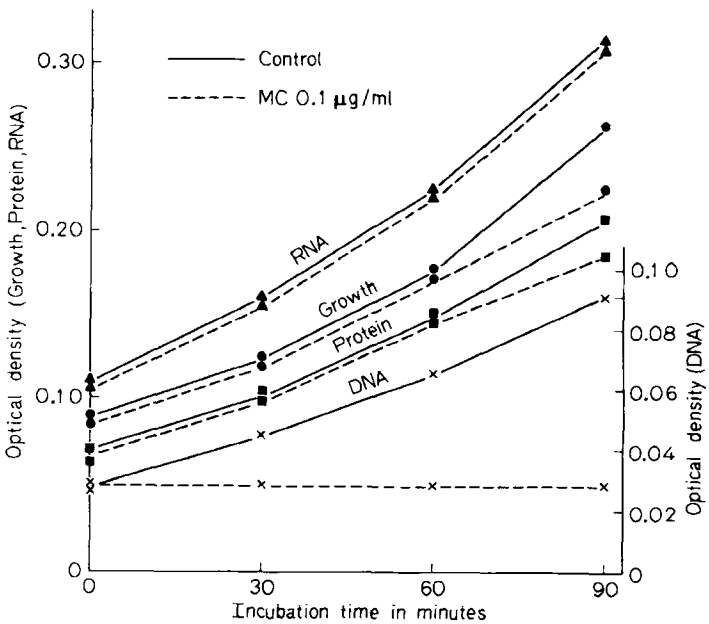

Fig. 3. Selective inhibition of DNA biosynthesis in *E. coli* by 0,1 µg/ml of mitomycin C[29]

to decrease the hydrolysis of the labelled precursor by a deoxynucleosidase which bacteria excrete into their growth medium. Without this precaution the labelled compound in the medium is quickly dissipated and incorporation levels off owing to an exhaustion of thymidine.

A large number of antibiotic and synthetic inhibitors of DNA biosynthesis form complexes with DNA and act as template poisons for the replication of chromosomal DNA[6,7]. These substances are preferential inhibitors of eucaryotic cells such as protozoa or neoplastic cells. The only antibacterial chemotherapeutic drug which is a selective inhibitor of DNA biosynthesis is nalidixic acid. This synthetic compound does not form complexes with DNA but acts in an unknown manner which appears to involve a perturbation of the interaction of DNA with a bacterial membrane system which is instrumental in DNA replication[30].

In bacteria, DNA complexing drugs or experimental DNA complexers have been found selectively to inhibit the replication of plasmidic, especially of R-factor, DNAs and, hence, to eliminate resistance determinants and restore antibiotic sensitivity to such organisms[31]. This has opened the prospect of developing a special category of drugs for the combination (with antibiotics) treatment of Gram-negative bacterial infections[32].

2. Inhibition of RNA Synthesis usually has bacteriostatic consequences since it does not result in severely unbalanced biosynthesis and growth. When RNA biosynthesis is turned off by an inhibitor, the cell cannot replenish messenger RNA which, in microorganisms, is subject to rapid turnover. The progressive deficiency in messenger RNA results in a progressive decay in the rate of protein biosynthesis. For example, in cultures of *Bacillus subtilis,* protein synthesis had ceased entirely after one fourth of a doubling time when RNA synthesis was specifically and completely inhibited by actionomycin D[33]. Since the initiation of a new round of DNA biosynthesis requires the *ad hoc* synthesis of "initiator protein(s)," DNA biosynthesis comes to a standstill after a lag as the result of the failure of initiator protein synthesis. This interesting sequence of events was first described by Kirk[34] for the action of actinomycin D in *Staphylococcus aureus.*

Actinomycin D is a selective inhibitor of RNA synthesis but its use in cancer chemotherapy is severely limited owing to its toxicity. It is thought that the two cyclic peptide moieties of the antibiotic whose three-membered aromatic ring system is intercalated into doublestranded DNA occlude the minor groove of duplex DNA and impede the progression of the enzyme, RNA polymerase.

An important family of inhibitors of procaryotic RNA synthesis are the antibiotics of the ansamycin series whose prototype is rifampicin. It inhibits bacterial RNA synthesis at the extremely low concentration of 0.01 μg/ml (1.4×10^{-8} M) by binding to bacterial RNA polymerase[35].

With the exceptions of mitomycin C and actinomycin D which are, at growth-inhibitory concentrations, specific inhibitors of DNA or RNA biosyntheses, other DNA-complexing chemotherapeutic drugs inhibit both categories of biosyntheses although usually to different extents which depend not only on the drug concentrations but also on the test organism. For example, quinacrine is a preferential inhibitor of DNA biosynthesis in *E. coli*[17], while in *Bacillus cereus* the drug acted

predominantly on RNA biosynthesis[36]. While such species-dependent specificities in the actions of DNA-complexing drugs are fairly generally recognized, the underlying reasons are not clear. In *in vitro* studies on inhibitions of DNA and RNA polymerase reactions by quinacrine, the two dosage response correlations were not significantly different from each other[22].

3. Inhibition of Protein Synthesis is a most predominant mode of action among antibiotics. There are more than 20 single antibiotics or congeneric families of antibiotics which act in this manner. With the exception of the aminoglycosides which are bactericidal drugs, inhibitions of protein synthesis produce bacteriostasis. Interestingly, there is no synthetic compound among clinical chemotherapeutic drugs which inhibit protein biosynthesis, although a number of experimental amino acid antimetabolites affect protein synthesis by inhibiting amino acyl transfer RNA synthetases.

Chloramphenicol and chlortetracycline were the first antibiotics recognized to be inhibitors of protein synthesis on the basis of findings that they inhibited induced enzyme syntheses in bacteria *after* induction had occurred and syntheses had been under way for considerable periods of time[37].

Inhibitions of protein synthesis were subsequently confirmed by chemical analyses for protein in growing bacterial cultures[38, 39]. Figure 4[39] shows the specific inhibition of protein biosynthesis by chloramphenicol in *E. coli* and the continuation of RNA ("ribose") and DNA biosynthesis for an experimental period of 50 minutes. Similar results have been typically obtained with other inhibitors of protein biosynthesis.

While the synthesis of DNA levels off for reasons discussed in the preceding section, RNA synthesis in chloramphenicol-exposed bacteria can continue for extended periods of time. Some of this excess RNA is transfer RNA or is found in incomplete (with respect to protein) ribosomelike particles (rev. in[40]), but much of the bacterial RNA, accumulating during inhibition of protein synthesis is messenger RNA[41]. When bacteria are released from inhibition of protein synthesis, growth does not resume immediately in minmal medium. Instead, there is considerable messenger RNA breakdown and excretion of its products into the medium[41, 42] before balanced growth begins again. However, if the antibiotic-free organisms are resuspended in amino-acids containing media[43, 44], no breakdown of RNA is observed and the organisms resume protein synthesis without delay, soon accompanied by RNA synthesis in the manner characteristic of balanced growth[44]. Evidently, the unbalanced synthesis of excess RNA during inhibition of protein synthesis is not bactericidal.

The "bacteriostatic" effect of chloramphenicol has been investigated quantitatively[45]. Addition of the antibiotic to cultures of *E. coli* B/r growing exponentially in brain-heart infusion broth with a generation time of 21.90 min was followed by considerable further increase in total cell number (as measured in a Coulter counter) and an initial increase in the number of colony-forming bacteria which then progressively decreased by $\sim$75 per cent during continued incubation for a total of 13.7 generation times (Fig. 5). Division of cells in chloramphenicol-containing cultures was observed under the microscope[45]. It is possible that "bacteriostasis"

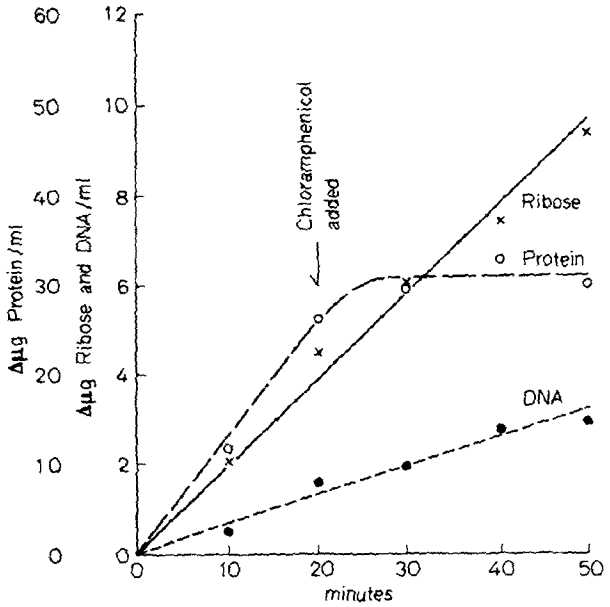

Fig. 4. Selective inhibition of protein biosynthesis in *E. coli* by 1.9×10^{-4} M chloramphenicol[39]

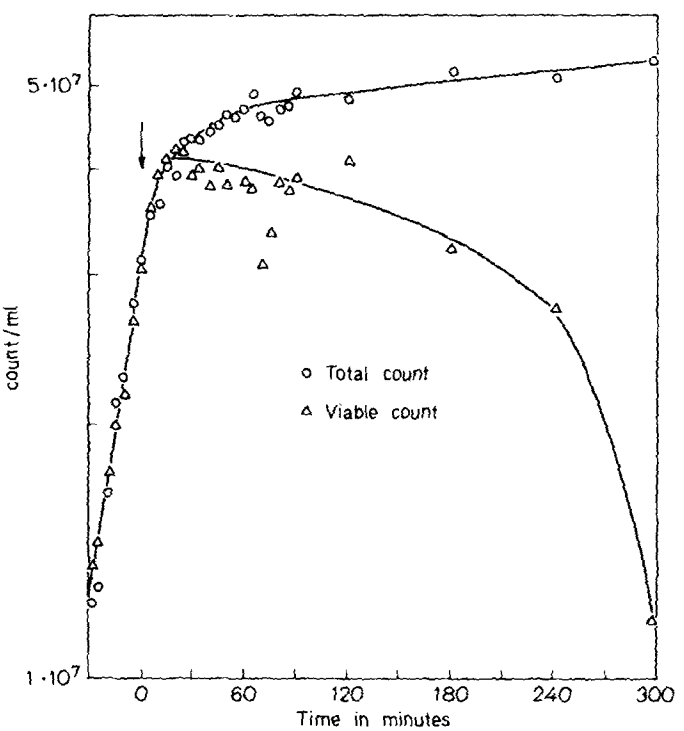

Fig. 5. Changes in total and in viable count of *E. coli* B/r before and after addition (arrow) of chloramphenicol to 1.5×10^{-4} M[45]

is an oversimplified concept supported by not more than conventional serial dilution of cultures, followed by plating and colony counting.

Inhibition of protein synthesis by aminoglycoside antibiotics, especially by streptomycin, is bactericidal (rev.[46]). The antibiotic binds to the smaller ribosomal subunit and leads to the formation of abortive initiation complexes of ribosomes, streptomycin and amino acyl tRNA which progressively trap ribosomes in the form of such biologically irreversible complexes. When protein synthesis is prematurely terminated by puromycin and ribosomes are thus made available for reinitiation of *de novo* protein biosynthesis, the bactericidal action of streptomycin is accelerated[47]. Destruction of ribosomes under the influence of primaquine operationally also results in non-occurrence of protein synthesis and in a marked bactericidal effect[48, 49].

4. Inhibitions of Cell Wall Biosynthesis. All bactericidal modes of action involve the alteration or destruction of some component (s) of the cell whose physiological function is of vital importance and cannot be compensated for or repaired by other cell constituents. This has been described above for the bacterial chromosome (DNA) and for the total population of bacterial ribosomes. The most prominent bactericidal effect, however, is caused by interferences with the biosynthesis of the cell wall polymer. Such a mode of action was proposed for penicillin on the basis of morphological observations in 1946[50] when the underlying biochemistry was still unknown.

"The morphological changes described . . . . . in particular the failure of proper cell division and the ready occurrence of swelling and protoplasmic protrusion suggest that penicillin interferes specifically with the formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis[50]*."*

Such experiments with *E. coli* were subsequently refined through the introduction into the medium of sucrose for osmotic protection of the spheroplasts formed, and the sequence of events during the action of penicillin was photographed under the phase contrast microscope (Fig. 6)[51].

After Park isolated and identified uridine-diphosphate-peptidoglycan products that accumulate to high concentrations in penicillin-exposed *Staphylococcus aureus*[52], it took the results of several years of independent investigations on the chemical composition of bacterial cell walls to recognize that the products were biosynthetic precursors of the cell wall polymer[53]. The biochemistry of bacterial cell wall synthesis was subsequently studied in great detail by Strominger and his associates with the conclusion that penicillins interfere with a final cross- linking step in the assembly of the cell wall polymer (rev.[54]). The morphological manifestation of bacterial destruction in Fig. 6 are, hence, the results of a defective growth of the cell wall which weakens this structure to an extent that it can no longer withstand the internal osmotic pressure. Disruption and lysis of Gram-positive bacteria under the influence of penicillin has also been demonstrated[55] although the morphology of these events is not so dramatic as that in Gram-negative bacteria (Fig. 6).

Additional antibiotics which interfere with cell wall synthesis are the cephalosporins whose mode of actionis similar to that of penicillins, cycloserine which

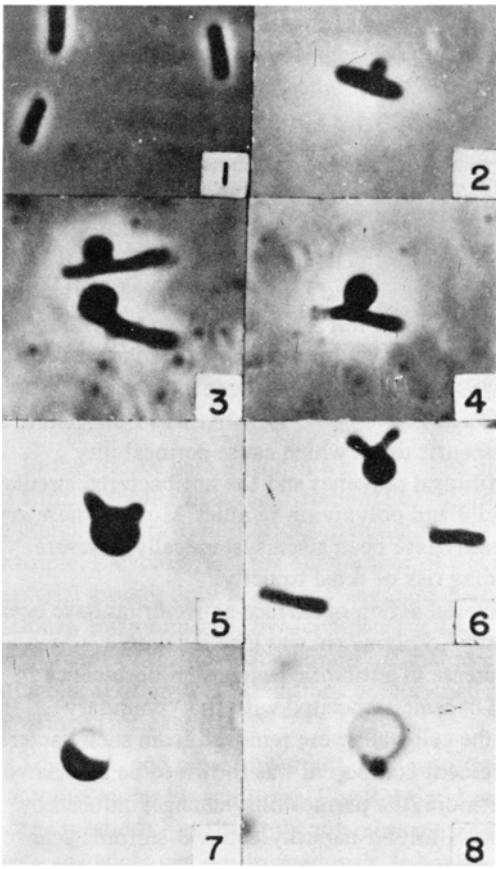

Fig. 6. Sequential phases of penicillin-induced fromation of spheroplasts of *E. coli* B as photographed under the phase contrast microscope[51]

inhibits the formation of the D-alanyl-D-alanine moiety of the peptidoglycan (rev.[56]) and phosphonomycin which acts on an earlier step of peptidoglycan synthesis as an antagonist of phosphoenolpyruvate[57].

A synthetic inhibitor of cell wall synthesis is 3-fluoro-D-alanine which inhibits bacterial growth in competition with D-alanine and exhibits the "chemotherapeutic paradox" of being more active *in vivo* than *in vitro*[58].

5. Interference with the Structural and Functional Integrity of Membranes. The membrane is another structure whose alteration of partial destruction has a bactericidal effect. Its vital importance to the economy of the bacterial cell is that it controls and mediates permeation, *i.e.* it determines which chemicals enter or leave the cell and which do not. Sterilizing agents such as phenols or detergents and also non-polar solvents such as toluene affect the lipid constituents of the membranes and cause chaotic and uncontrolled permeability through which the cell loses much of its essential biochemicals such as intermediates of synthetic pathways (the metabolic "pool") and coenzymes into the surrounding medium. This leakage produces irreversible metabolic starvation as indicated by the simultaneous failure of all macromolecular biosyntheses and, hence, is bactericidal.

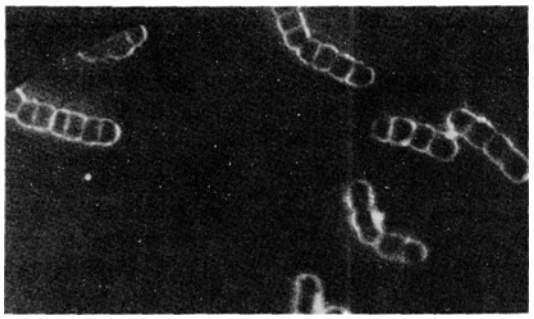

Fig. 7. Fluorescence photomicrograph of *Bacillus megaterium* after treatment with fluorescent polymyxin[67]

There are relatively few chemotherapeutic drugs which cause permeability increases in membranes such as the antifungal polyenes and the antibacterial circular oligopeptides of the tyrocidin, gramicidin and polymyxin families. Most of these are too toxic for systemic use but polymyxins have been given systemically in severe *Pseudomonas* infections with an attending risk of renal toxicity.

Studies on the nature of the antibacterial action of surface-active drugs have been carried out and/or reviewed by Newton[59]. Polymyxin was labelled with a fluorescent dye without significant decrease in antibiotic potency. Fluorescence microscopy showed that the antibiotic became associated with the "boundary structures" of bacteria (Fig. 7). When the cell walls were removed from such bacteria by treatment with lysozyme, the fluorescent compound was shown to be associated with the protoplast membrane. Furthermore, the permeability change, induced by polymyxin was demonstrated by adding N-tolyl-α-naphthylamine-8-sulfonic acid to polymyxin-treated bacteria. Aqueous solutions of this dye do not fluoresce but when it reacts with proteins, the conjugates are strongly fluorescent. Suspensions of *Pseudomonas aeruginosa* did not take up the dye and, hence, did not become fluorescent. However, the addition of polymyxin produced immediate bacterial fluorescence, thereby indicating that the bacteria had been rendered permeable to the compound and their proteins had reacted with it[59].

A second category of membrane-active antibiotics are the depsipeptides with valinomycin as the prototype. These substances act as iononophores and increase permeation of K^+ ions. The antibacterial effect of 10^{-6} M valinomycin can be reversed by supplying an excess of K^+ [60].

Finally, one 2-hydroxy-3-alkyl-1,4-naphthoquinone has been shown to be bactericidal and to produce a shut-down of all major biosynthetic processes. This is the result of an inhibition of the transport of nutrients/precursors through the membrane into the bacterial cell. Detailed experiments on the permeation of uracil demonstrated that ingress *and* egress were inhibited by the substituted naphthoquinone[61].

Following this capsule review of the five major categories of modes of action, it is now possible to outline the strategy of mode of action studies which leads to the classification of drugs as belonging into one of these categories.

After the selection of a suitable test organism, the first step is to measure the response of the growth rate to graded concentrations of the drug under study, as

outlined in Section III. When this is carried out turbidimetrically, observations of decreases in absorbance will indicate lysis of the test cells such as can result from inhibitions of cell wall biosynthesis or from the effects of membrane-active substances.

A second step is to determine if the drug under study is predominantly bacteriostatic or bactericidal. While there will always occur some decrease in the number of colony-forming cells under the influence of a growth-inhibiting drug, a bactericidal effect is considered one in which an exponential decrease in the number of viable cells is two decadic logarithms or greater within one doubling time. When such studies of viability are carried out, it is not an infrequent occurrence to find a narrow range of lowest drug concentrations which are merely bacteriostatic. A marked bactericidal action can signal effects on DNA synthesis, cell wall synthesis or membrane integrity as underlying causes.

In the special case in which a growth inhibitor has a structure, reminiscent of an antimetabolite of some metabolite of intermediary metabolism, it can be useful to compare growth inhibitions in minimal medium with those in broth media. For example, L-cycloserine, the enantiomer of the antibiotic D-cycloserine, inhibits bacterial growth only in minimal medium devoid of alanine (unpublished observations). Furthermore, DNA-complexing drugs can be sequestered when DNA is a constituent of the growth medium[34], and in certain reported instances, the antagonism of growth inhibitions by enriched media has not been satisfactorily explained (for example,[62, 63]).

The next, and most important, step is to test the effects of the drug on macromolecular biosyntheses. For RNA and protein there exist sensitive colorimetric methods but the diphenylamine method for determination of DNA is in many instances not sensitive enough. It has become customary to follow macromolecular biosyntheses by measuring incorporations of radioactive precursors into the cellular polymers. Reference has already been made to the use of thymidine (or thymine) in the study of DNA biosynthesis. The label of choice for following RNA biosynthesis is uracil. Protein synthesis can be followed by using any of a number of radioactive amino acids. Care should be taken to avoid glycine which is an intermediate in a number of biosynthetic pathways or those amino acids (alanine, glutamic acid, aspartic acid, valine or lysine) whose biosyntheses are not regulated by the rate of protein synthesis or which also become constituents of the cell wall polymer. Cell wall biosynthesis can be studied by measuring the incorporation of α-ϵ-diaminopimelic acid if the wall of the test organism contains this compound instead of lysine[48].

Specific inhibitions of DNA or protein biosyntheses have been illustrated in Figs. 3 and 4. Inhibition of protein synthesis results, after some delay, in the gradual decay of the rate of DNA synthesis as reviewed earlier in this section. The more complicated biosynthetic interrelations and consequences of inhibitions of RNA synthesis have also been reviewed with special reference to the action of actinomycin D[34]. In such instances it is important to concentrate on the early changes in rates of macromolecular syntheses: after some delay, inhibitions of RNA synthesis will have produced shutdowns in protein and DNA syntheses.

Inhibitions of cell wall biosynthesis can be determined independently from DNA, RNA and protein biosyntheses. They will usually have been signaled by bactericidal effects and lysis of the test culture. In many instances, inhibition of cell wall synthesis results in the progressive accumulation of intermediates which can be assayed colorimetrically[64, 65] for N-acylaminohexose in acid-soluble fractions of the test bacteria.

In those instances in which all categories of macromolecular biosyntheses fail entirely[61], the explanation can be sought either in effects on the bacterial membrane or in a failure of energy generation or transduction.

It is apparent that the definition of a mode of action in physiological/biochemical terms can be accomplished by a conventional set of tests for which the strategy has been outlined in this section. This also applies to substances which interfere with intermediary metabolism such as the inhibitors of the synthesis or transformations of folic acid. The task is relatively simple and unambiguous for predominantly bacteriostatic drugs. However, in the case of a rapid bactericidal effect, late biosynthetic failures after most cells have lost viability are in the nature of *post mortem* findings which must be interpreted with caution. When rapid killing of the test organism has been found in the preliminary stages of the investigation, attention must become focused on the *earliest* effects which can be determined and considered responsible for the ensuing cell death.

V. Research on Mechanisms of Action

After the *mode* of action of a chemotherapeutic drug has been defined and categorized through the application of the strategy outlined in the preceding section, the scientific task arises to investigate the underlying *mechanism* in molecular terms. Although a very considerable scientific effort has been made and continues in research on mechanisms of action of antimicrobial and antitumor agents, there exist relatively few instances in which definitive explanations of drug actions have been achieved in molecular pharmacological terms.

Even for antimicrobial drugs wich have the same *mode* of action, the *mechanisms* of actions in molecular terms can be quite different. An instructive example of this is the body of knowledge on actions of inhibitors of protein synthesis at the ribosomal level[66]. Since the mechanistic details of protein biosynthesis as well as of DNA replication are still incompletely resolved, studies on mechanisms of action of inhibitors of these biosynthetic processes frequently remain inconclusive when the target reaction is not known. Each problem of the *mechanism* of action of a chemotherapeutic drug becomes an individual research problem in its own right, after the *mode* of action has been elucidated in the manner described in this article. For this reason, a clearcut general strategy of this type of research can not be formulated. This is especially the case for all antibiotics and synthetic drugs which have been discovered empirically.

In contrast, substances which have been premeditatively developed as antimetabolites and possess growth-inhibitory activity for microorganisms, lend themselves to

investigation of the antimetabolic relationship for which they have been deliberately designet. While it may be tempting to review the few instances of successful elucidation of mechanisms of action, no systematic strategic approaches to such investigations would emerge, and such a review of existing knowledge which has accumlated over the years would have no place in a volume is dedicated to Topics in Current Chemistry.

VI. Conclusions

This article has traced the development of a strategy for mode of action studies of chemotherapeutic drugs from its blindfolded empirical beginnings to the current state in which it is possible to assign a category of mode of action to a given substance within a limited period of investigative time, provided a suitable test organism can be found. This current research strategy has been described.

Successful solutions to the problems of mechanisms of action which always emerge after a mode of action has been pinpointed can not yet be organized systematically, and critical consideration of instances in which such studies have been definitively successful do not offer much guidance to an investigator who is faced with a new problem. It will take considerable time until the biochemical process patterns of microorganisms are known to such an extent that a systematic research strategy can be developed on the basis of this knowledge.

VII. References

1) Newton, B. A., Reynolds, P.E. (eds.): Biochemical studies of antimicrobial drugs. Cambridge: University Press 1966
2) Gottlieb, D., Shaw P.D., (eds.): Antibiotics I. Mechanism of action. Berlin-Heidelberg-New York: Springer 1967
3) Franklin, T.J., Snow, G.A.: Biochemistry of antimicrobial action. New York: Academic Press 1971
4) Schönfeld, H., DeWeck, A., (eds.): Antibiotics and chemotherapy 17. Mode of action. Basel: S. Karger 1971
5) Gale, E.F., Cundliffe, E. Reynolds, P.E., Richmond, M.H., Waring, M.J.: The molecular basis of antibiotic action. London: Wiley 1972
6) Kersten, H., Kersten, W.: Inhibitors of nucleic acid synthesis. New-York-Heidelberg-Berlin: Springer 1974
7) Corcoran, J.W., Hahn, F.E, (eds.): Antibiotics III. Mechanism of action of antimicrobial and antitumor agents. Berlin-Heidelberg-New York: Springer 1975
8) Henry, R.J.: Bacteriol. Rev. *7*, 175 (1943)
9) Laskin, A.I., in: Antibiotics I. Mechanism of action. Berlin-Heidelberg-New York: Springer 1967, p. 331
10) Hahn, F.E.: Proc. Fourth Internat. Congr. Biochem. *5*, 104 (1958)
11) Smith, F.N., Worrel, C.S., Swanson, A.I.: J. Bacteriol. *58*, 803 (1949)
12) Loomis, W.F.: Science *111*, 474 (1950)
13) Van Meter, J.C., Oleson, J.J.: Science *113*, 273 (1951)
14) Van Meter, J.C., Spector, A., Oleson, J.J., Williams, J.H.: Proc. Soc. Exptl. Biol. Med. *81*, 215 (1952)
15) Roskoski, Jr., R., Jaskunas, S.R.: Biochem. Pharmacol. *21*, 391 (1972)
16) Yagi, K., Okuda, J. Ozawa, T., Okada, K.: Biochim. Biophys. Acta *34*, 372 (1959)
17) Ciak, J., Hahn, F.E.: Science *156*, 655 (1967)
18) Wolfe, A.D., Hahn, F.E.: Naturwissensch. *59*, 277 (1972)
19) Saz, A.K., Weiss Brownell, L., Slide, R.B.: J. Bacteriol. *71*, 421 (1956)
20) Hahn, F.E., Gund, P., in: Topics in infectious diseases 1. Drug receptor interactions in antimicrobial chemotherapy. Wien: Springer 1974, p. 245
21) Telesnina, G.N., Novikova, M.A., Zhdanov, G.L., Kolosov, M.N., Shemiakin, M.M.: Experientia *23*, 427 (1964)
22) Hahn, F.E., O'Brien, R.L., Ciak, J., Allison, J.L., Olenick, J.G.: Military Med. *131*, 1971 (1966)
23) Hahn, F.E., in: Antibiotics III. Mechanism of action of antimicrobial and antitumor agents. Berlin-Heidelberg-New York: Springer 1975, p. 58
24) Hahn, F.E., in: Topics in infectious diseases. Vol. 1. Wien: Springer 1974, p. 3
25) Ciak, J., Hahn, F.E.: J. Bacteriol. *75*, 125 (1958)
26) Treffers, H.P.: J. Bacteriol. *72*, 108 (1956)
27) Wolfe, A.D., Cook, T.M., Hahn, F.E.: J. Bacteriol. *108*, 320 (1971)
28) Umbreit, W.W., Burris, R.H., Stauffer, J.F.: Monometric techniques and related methods for the study of tissue metabolism. Minneapolis: Burgess 1945
29) Shiba, S., Terawaki, A., Taguchi, T., Kawamata, J.: Biken's Journal *1*, 179 (1958)
30) Goss, W.A., Cook, T.M., in: Antibiotics III. Mechanism of action of antimicrobial and antitumor agents. Berlin-Heidelberg-New York: Springer 1975, p. 174
31) Hahn, F.E., Ciak, J.: Antimicrobial agents and chemotherapy. *9*, 77 (1976)
32) Hahn, F.E., in: Antibiotics and chemotherapy. Vol. *20*, Basel: S. Karger 1976, p. 196
33) Levinthal, C., Keynan, A., Higa, A.: Proc. Nat. Acad. Sci. U.S. *48*, 1631 (1962)
34) Kirk, J.: Biochim. Biophys. Acta *42*, 167 (1960)
35) Wehrli, W., Staehelin, M., in: Antibiotics III. Mechanism of action of antimicrobial and antitumor agents. Heidelberg-New York: Springer 1975, p. 252
36) Seligman, M.L., Mandel, H.G. J. Gen. Microbiol. *68*, 135 (1971)
37) Hahn, F.E., Wisseman, C.L.: Proc. Soc. Exp. Biol. Med. *76*, 533 (1951)

38) Gale, E.F., Folkes, J.P.: Biochem. J. *53*, 493 (1953)
39) Wisseman, C.L., Smadel, J.E. Hahn, F.E., Hopps, H.E.: J. Bacteriol. *67*, 662 (1954)
40) Hahn, F.E., in: Antibiotics I. Mechanism of action. Berlin-Heidelberg-New York: Springer 1967, p. 308
41) Hahn, F.E., Wolfe, A.D.: Biochem. Biophys. Res. Comm. *6*, 464 (1961)
42) Hahn, F.E., Schaechter, M., Ceglowski, W.S., Hopps, H.E., Ciak, J.: Biochim. Biophys. Acta *26*, 469 (1957)
43) Horiuchi, T., Sunakawa, S., Mizuno, D.: J. Biochem. (Japan) *45*, 875 (1958)
44) Aronson, A.I., Spiegelman, S.: Biochim. Biophys. Acta *53*, 84 (1961)
45) Allison, J.L., Hartman, R.E., Hartman, R.S., Wolfe, A.D., Ciak, J., Hahn, F.E.: J. Bacteriol. *83*, 609 (1962)
46) Hahn, F.E., in: Antibiotics and chemotherapy. Vol. *17*, Basel: S. Karger 1971, p. 29
47) White, J.R., White, H.L.: Science *146*, 772 (1964)
48) Olenick, J.G., Hahn, F.E.: Antimicrobial agents and chemotherapy. *1*, 259 (1972)
49) Olenick, H.G.: Antimicrobial agents and chemotherapy. *8*, 754 (1975)
50) Duguid, J.P.: Edinburgh Med. J. *53*, 401 (1946)
51) Hahn, F.E., Ciak, J.: Science *125*, 119 (1957)
52) Park, J.T.: J. Biol. Chem. *194*, 877 (1952)
53) Park, J.T., Strominger, J. L.: Science *125*, 99 (1957)
54) Strominger, J.L., Willoughby, E., Kamiryo, T. Blumber, P.B., Yocum, R.R.: Ann. New York Acad. Sci. *235*, 210 (1974)
55) Ciak, J., Hahn, F.E.: Science *137*, 982 (1962)
56) Neuhaus, F.C., in: Antibiotics I. Mechanism of action. Berlin-Heidelberg-New York: Springer 1967, p. 40
57) Kahan, F.M., Kahan, J.S., Cassidy, P.J., Kropp, H.: Ann. New York Acad. Sci. *235*, 364 (1974)
58) Kollonitsch, J., Barash, L., Kahan, F.M., Kropp, H.: Nature *243*, 346 (1973)
59) Newton, B.A., in: The strategy of chemotherapy. Cambridge: University Press 1958, p. 62
60) Harold, F.M., Baarda, J.R.: J. Bacteriol. *94*, 53 (1967)
61) Olenick, J. G., Hahn, F.E.: Ann. New York Acad. Sci. *235*, 542 (1974)
62) Wacker, A.: Zschr. Naturforsch. *6B*, 173 (1951)
63) Zygmunt, W.A.: Biochem. Biophys. Res. Comm. *6*, 324 (1961)
64) Reissig, J.L., Strominger, J.L., Leloir, L.F.: J. Biol. Chem. *217*, 959 (1955)
65) Ciak, J., Hahn, F.E.: Antibiotics and chemotherapy. *9*, 47 (1959)
66) Weisblum, B., Davies, J.: Bacteriol. Rev. *32*, 493 (1968)
67) Gale, E.F.: Synthesis an organization in the bacterial cell. New York: Wiley, 1959, p. 19

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Ansamycins
Chemistry, Biosynthesis and Biological Activity

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Table of Contents

Introduction

The ansamycin antibiotics derive their name from the characteristic configuration of their molecule, which consists of a flat aromatic nucleus and a long aliphatic bridge, shaped like a handle (L. *ansa*), joining two non-adjacent positions of the nucleus (Fig. 1)[1, 2]. The molecules thus formed are very rigid and compact (Fig. 2), a fact which leads to unique chemical properties and a variety of specific biological actions. The first compounds isolated some 20 years ago belonged to the group of rifamycins[3]. Since then several other groups have been identified. In the following article the chemistry, the biosynthesis and the biological activities of the various ansamycins will be discussed.

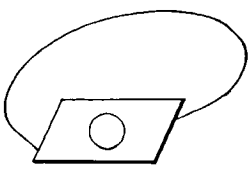


Fig. 1. Schematic structure of the ansamycins

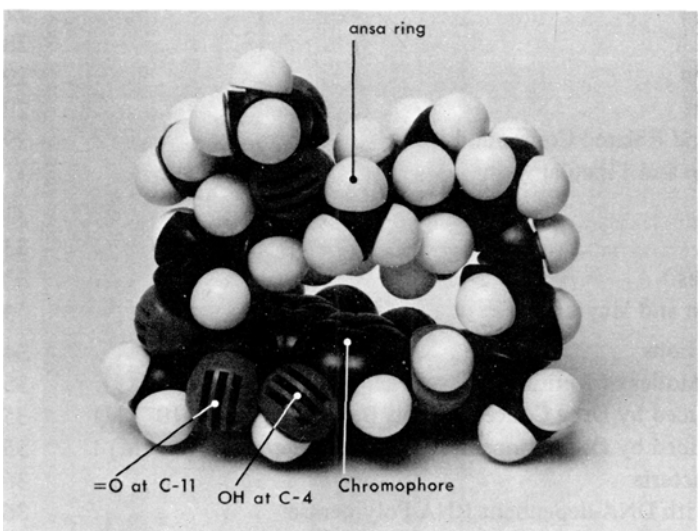


Fig. 2a. Stereomodel of rifamycin SV. The hydrophobic face

1. Chemistry

Figure 3 shows the chemical structures of the various groups of ansamycins. Two types of aromatic nucleus can be distinguished: a naphthalenic ring system, as in the rifamycins, streptovaricins, tolypomycins and naphthomycin and a benzenic ring system as in geldanamycin and the maytansine group. Except in this last group, the nuclei are substituted in positions 1 and 4, in many cases forming quinone-hydro-

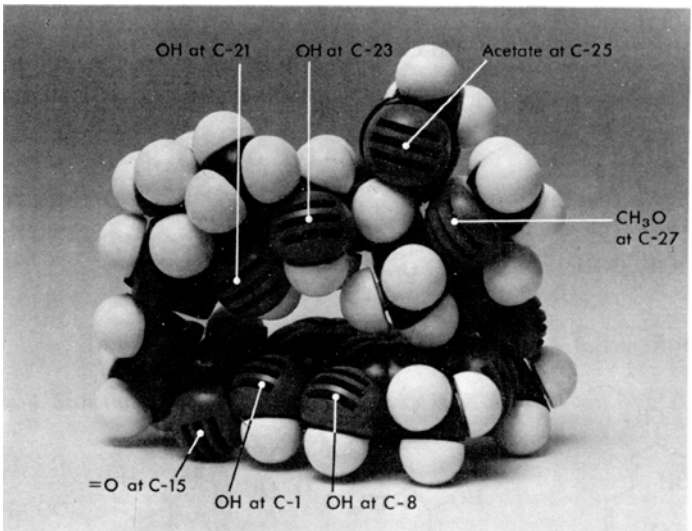


Fig. 2b. Stereomodel of rifamycin SV. The hydrophilic face

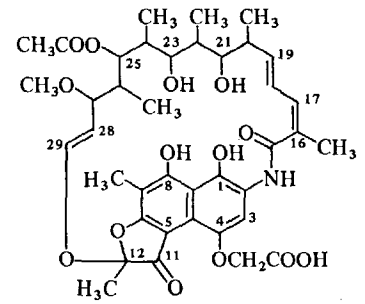
quinone systems or being easily converted into such systems. The *ansa* bridge is always an aliphatic chain containing no lactone bonds, and is linked as an amide to an amino group of the nucleus. This feature is in contrast to the structure of the macrolide antibiotics, which characteristically contain a large lactone ring.

The chemistry of the various groups of ansamycins is only discussed briefly, as several excellent reviews have already been published in recent years[1, 4, 5]. These should be consulted for detailed chemical descriptions and extensive literature references.

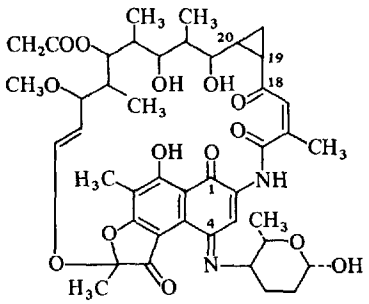
1.1. Rifamycins

The rifamycins were first isolated by Sensi *et al.*[3] from *Nocardia mediterranei* as a complex mixture (Rifamycins A–E). Addition of diethylbarbiturate to the fermentation medium led to the sole production of rifamycin B[6] which was obtained in crystalline form. Its structure has been determined by chemical[7, 8] and X-ray analysis[9]. The rifamycins might easily have excaped detection altogether, since rifamycin B has no antibacterial activity. However, it is spontaneously oxidized to rifamycin O and hydrolyzed to rifamycin S, a naphthoquinone derivative; reduction yields the naphthohydroquinone derivative rifamycin SV (Fig. 4). These compounds inhibit the growth of Gram-positive bacteria at concentration as low as 0.0025 μg/ml.

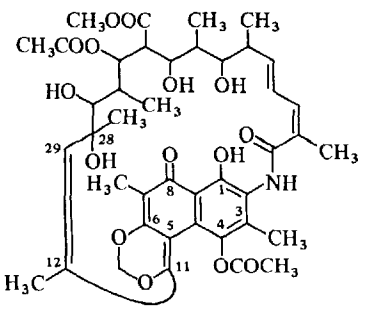
The rifamycins have some remarkable chemical properties. Rifamycin SV can be obtained from the quinone rifamycin S by treating the latter with weak reducing agents such as ascorbic acid. It is fairly stable against oxidation by air and can form a stable sodium salt. This behaviour can be ascribed to the acylamino group in position 2. Unsubstituted naphthoquinone can only be reduced under much more drastic conditions, and once in the hydroquinone form it is very sensitive to oxidation.



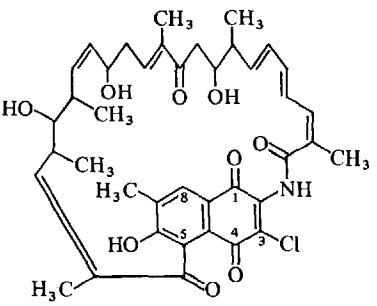
Rifamycin B
(756)



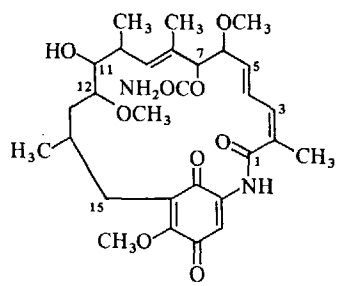
Tolypomycin Y
(823)



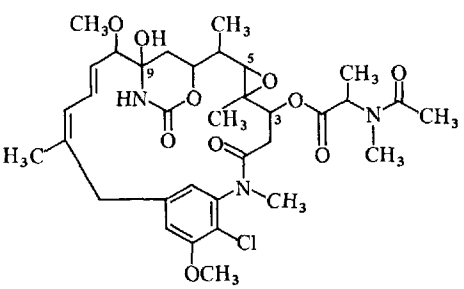
Streptovaricin B
(812)



Naphthomycin
(720)



Geldanamycin
(560)



Maytansine
(692)

Fig. 3. Structure of various ansamycins. (Numbers in brackets indicate molecular weights. For all naphthalenic ansamycins the numbering system as proposed by Prelog[7, 8] has been used.)

Another peculiar property of the rifamycins is their high lipophilicity. Even the sodium salt of rifamycin SV is easily soluble in organic solvents such as chloroform. One striking feature that emerges upon analysis of the tertiary structure is that all the oxygen functions of the *ansa* ring are situated on the same side as the C–1 and C–8 hydroxyls of the chromophore (Fig. 2). The rifamycin molecule thus has two faces differing in lipophilicity. The sodium salts of many rifamycin derivatives have a tendency to form gels (Dr. W. Kump, personal communication). Furthermore, derivatives with a lipophilic side chain in position 3 at concentrations as low as

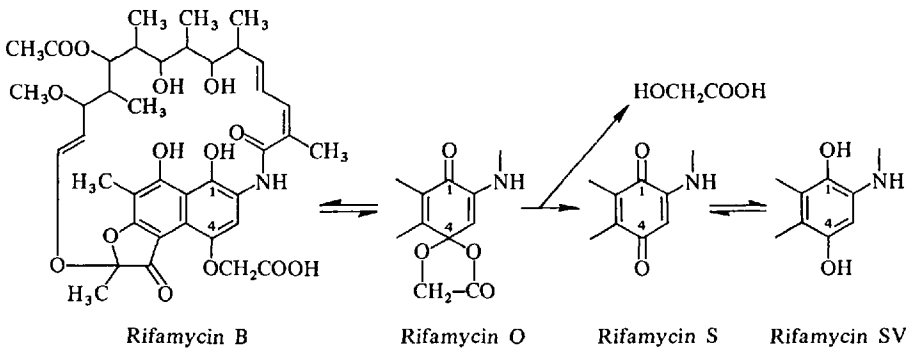


Fig. 4. Structural relations between rifamycins B, O, S and SV

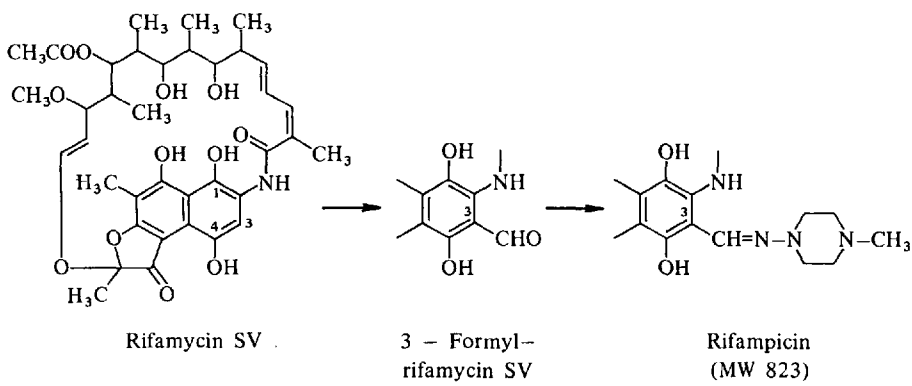
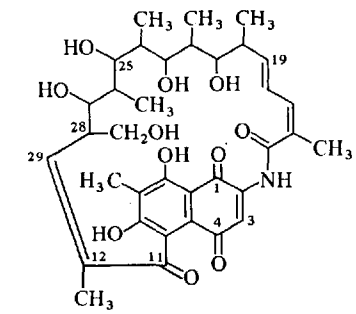


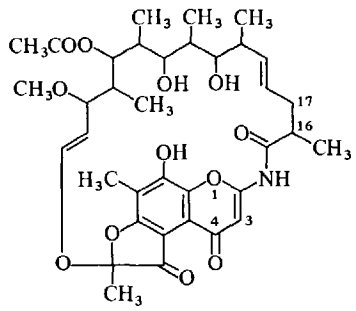
Fig. 5. Structure of rifampicin and its synthesis from rifamycin SV

50 μg/ml have been found to form viscous solutions and to sediment when centrifuged at high speed (unpublished results). These observations point to the existance of intermolecular interactions leading to the formation of aggregates and micelles, a behaviour comparable to that found with detergents. These chemical properties should be kept in mind, when rifamycins and other ansamycins are used at high concentrations as inhibitors of biological systems.

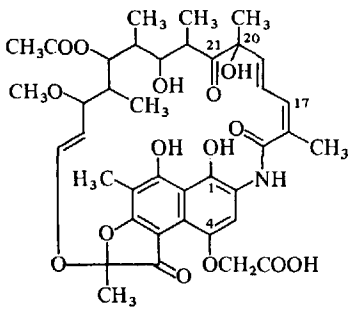
Several hundred semisynthetic derivatives have been prepared in an effort to obtain substances with better biological activities (for references see Ref.[5]). Particularly positions 3 and 4 of the naphthoquinone ring system (numbering system as proposed by Prelog[7, 8]) have been extensively substituted, since it has been shown that structural changes in these two positions do not critically affect the action of the substance on the target enzyme, the bacterial RNA polymerase (cf. Chapter 3.). They can, however, influence other parameters such as its ability to penetrate into cells, its pharmacokinetic properties and resorption, which are all important for clinical use as an antibiotic. Rifampicin (U.S.: rifampin), which is a widely used orally active tuberculostatic agent, is a 3-(4-methyl piperazinyl)-iminomethyl derivative of rifamycin SV, synthesized *via* the 3-formyl derivative (Fig. 5)[10].



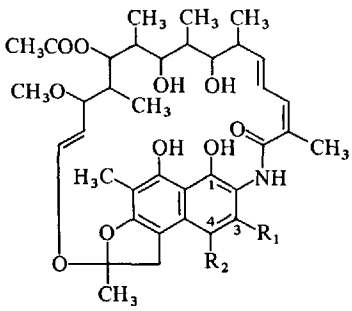
Rifamycin W



Rifamycin G



Rifamycin Y



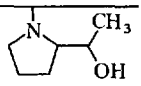
	R_1	R_2
3–Methylthiorifamycin SV	SCH_3	OH
Halomicin B	H	

Fig. 6. Structure of some naturally occurring rifamycins and halomicin B

Many chemically different rifamycins have been isolated from the fermentation broth of naturally occurring strains of Actinomycetes, or from selected mutants[5, 11]. The most interesting ones are rifamycin W[12, 13], G[14] and Y[15, 16] Fig. 6). The structure of rifamycin W bears a remarkable resemblance to the streptovaricins, because it lacks the ketal linkage between the *ansa* chain and the chromophore and has an extra carbon on C–28. The isolation of rifamycin W contributed greatly towards the understanding of the biosynthesis of the ansamycins (cf. Chapter 2.). Rifamycin G differs from rifamycin S in having no double bond C-16 – C-17 and no C-1. Rifamycin Y is similar to rifamycin B, but has an additional hydroxyl group at C-20 and a keto instead of a hydroxyl group at C-21. These three compounds have proved very valuable in studies of the relations between the chemical structure and the biological activity: they are totally inactive in inhibiting bacterial growth as well as the enzymatic activity of bacterial RNA polymerase (cf. Chap. 3.). Recently, structural studies of the group of halomicins isolated from Micromonospora halophytica[17] have shown that halomicin B[18] is closely related to rifamycin B, having a pyrrolidine

group on C-4 (Fig. 6). Furthermore, 3-methylthiorifamycin SV has been isolated from a species of Micromonospora[19]. Thus, *Nocardia* is not the only producer of rifamycins.

1.2. Streptovaricins

The streptovaricins, produced by *Streptomyces spectabilis*, are a complex mixture of closely related substances. Figure 7 shows the formulas of streptovaricins A–G and J. A summary account of the structural studies undertaken so far has been published by Rinehart[4]. The streptovaricins are chemically related to the rifamycins, but there are a number of important structural differences between the two groups, *e.g.*:

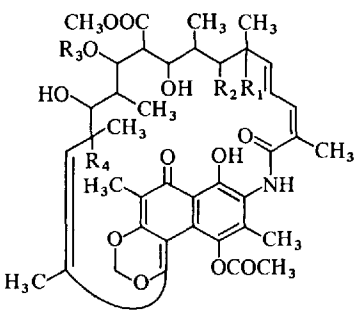
a) the *ansa* chain is linked to the chromophore by a C–C double bond and not *via* oxygen;

b) the configuration of the conjugated double bonds in the *ansa* ring is different;

c) the B-ring of the naphthalene chromophore is not a benzene ring, but part of a quinone methide system;

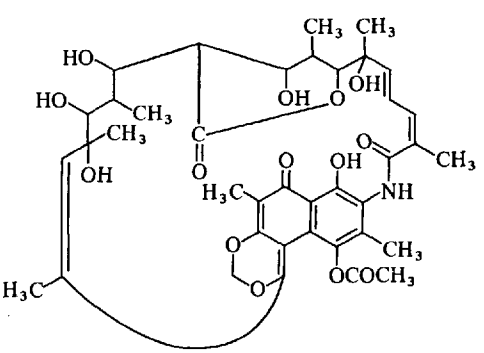
d) the hydroxyl group at C-4 is acetylated;

e) C-6 and C-11 are linked *via* a methylenedioxi-bridge.



	R_1	R_2	R_3	R_4
A	OH	OH	$COCH_3$	OH
B	H	OH	$COCH_3$	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	=O	H	OH
G	OH	OH	H	OH
J	H	$OCOCH_3$	H	OH

Streptovaricins A–E, G and J.

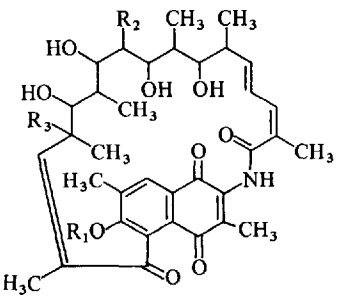


Streptovaricin F

Fig. 7. Structure of various streptovaricins

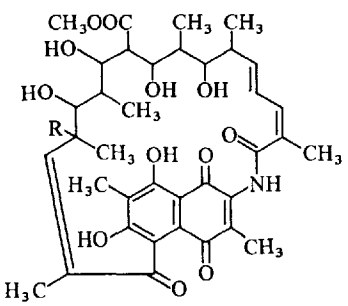
On the other hand, the configuration of the 8 chiral centres C-20 to C-27 and of the *ansa* system is identical to that found in the rifamycins and in tolypomycin Y.

The streptovaricins differ from each other mainly in the different extent of oxidation of the *ansa* bridge[20]. Very recently, protostreptovaricins I–V[21] and damavaricin C and D[22] have been isolated and structurally analyzed (Fig. 8). It has been postulated that these compounds are biosynthetic precursors of the streptovaricins[21] (cf. Chap. 2.). Damavaricin C can also be generated from streptovaricin C by oxidative hydrolysis[23].



	R_1	R_2	R_3
I	H	CH_3	H
II	CH_3	CH_3	H
III	H	CH_3	OH
IV	CH_3	CH_3	OH
V	H	H	H

Protostreptovaricins I–V



Damavaricin

Fig. 8. Structure of the protostreptovaricins and damavaricins

1.3. Tolypomycin Y

Tolypomycin Y has been isolated from *Streptomyces tolypophorus*[24–26] together with rifamycin B and O. Its structure has been elucidated chemically and by X-ray analysis[27] and closely resembles that of the rifamycins, especially rifamycin S (Fig. 3). In position 4 the chromophore contains a tolyposamin residue which upon mild acid hydrolysis can be split off yielding the 1,4-naphthoquinone tolypomycinone[24]. In the *ansa* ring, C-19, C-20 and C-31 form a cyclopropane ring and at C-18 a keto group is found. It should be noted that tolypomycin Y bears no particular resemblance to rifamycin Y; the latter contains a keto group at C-21 and is biologically inactive, whereas tolypomycin Y has the normal C-21 hydroxyl function and is biologically active.

1.4. Naphthomycin

Naphthomycin has been isolated from a *Streptomyces* species[28]. Its structure has been elucidated and shown to belong to the class of naphthalenic ansamycins (Fig. 3)[29]. In contrast to these compounds, however, the skeleton of the *ansa* chain contains six additional C-atoms. Very recent structural studies of the chromophore have shown that naphthomycin possesses a hydroxyl group in position 6 and is unsubstituted in position 8 (W. Keller-Schierlein, personal communication). It thus resembles the streptovaricin precursor protostreptovaricin I (Fig. 8). A further characteristic of naphthomycin is that it contains a halogen in the chromophore. As a result of these structural properties, its biological effects differ from those of the other naphthalenic ansamycins (cf. Chap. 3.).

1.5. Geldanamycin

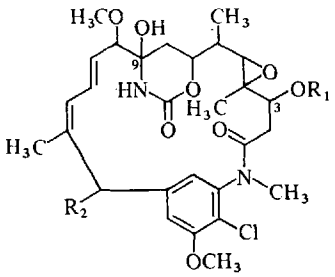
In contrast to the foregoing ansamycins which all contain a naphthalenic ring system, the chromophore of geldanamycin, an antibiotic isolated from *Streptomyces hygroscopicus*[30], is a benzoquinone derivative (Fig. 3)[31]. The *ansa* ring between C-1 and C-11 resembles the part of the other ansamycins between C-15 and C-25. C-12 to C-15 seem to correspond to C-5 to C-8 of the naphthalene skeleton. At C-21 a carbamoyl residue is found. Considering all these marked structural disparities, it is not surprising that the biological activity of geldanamycin differs from that of the naphthalenic ansamycins (cf. Chap. 3.) However, an analogous biosynthesis (Chap. 2.) clearly assigns it to the class of ansamycins.

1.6. Maytansine and Related Compounds

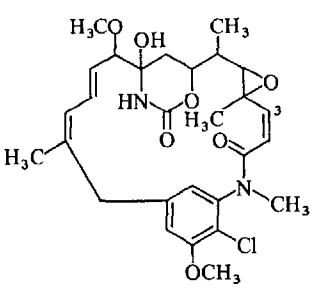
Maytansine and other maytansinoids[32–37] are the only ansamycins so far found to occur in plants (*Celastraceae, Rhamnaceae,* see Table 1). Since wood and bark of the plants in question only contain about 0.7 ppm, 10 tons had to be processed to isolated some 6 g of maytansine. The chemical structure (Fig. 9) resembles that of geldanamycin, having a benzenic chromophore and the same number of C-atoms in the skeleton of the *ansa* ring. However, C-7 and C-9 are linked forming a carbinolamide, and maytansine and other members of the group contain a fairly large substituent at C-3. In addition, an epoxy group is found in a position adjacent to that in tolypomycins and a chlorine in the same position as in naphthomycin. X-ray analysis of (3-bromopropyl)-maytansine revealed that the 19-membered *ansa* ring has two roughly parallel sides, C-1 to C-6 and C-10 to C-15, which are linked on one side by the chromophore and on the other side by the six-membered carbinolamide ring[32, 37]. Thus a rather flat molecule is formed with two faces of different character: that with the C-3 ester and C-9 hydroxyl group is hydrophilic, the other predominantly hydrophobic, a situation analogous to that found in the rifamycins.

A large number of maytansinoids have been isolated and chemically characterized, and interesting structure-activity relationships have been deduced, suggesting

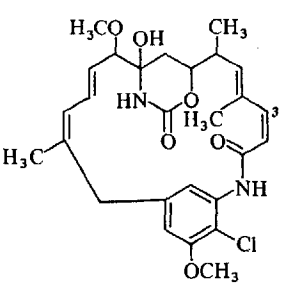
that the C-3 ester and the C-9 hydroxyl group are essential for biological activity (Chap. 3.).



	R_1	R_2
Maytansine	$-C(=O)-CH(CH_3)-N(CH_3)-C(=O)-CH_3$	H
Maytanbutine	$-C(=O)-CH(CH_3)-N(CH_3)-C(=O)-CH(CH_3)-CH_3$	H
Maytanvaline	$-C(=O)-CH(CH_3)-N(CH_3)-C(=O)-CH_2-CH(CH_3)-CH_3$	H
Maytanacine	$-C(=O)-CH_3$	H
Maytansinol	$-H$	H
Colubrinol	$-C(=O)-CH(CH_3)-N(CH_3)-C(=O)-CH(CH_3)-CH_3$	OH



Maysine

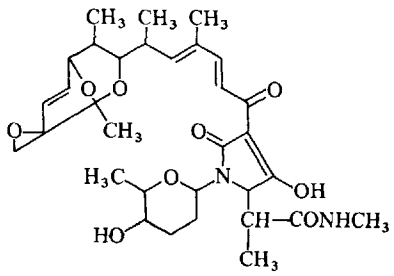


Maysenine

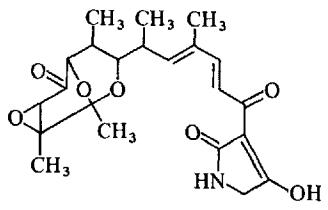
Fig. 9 Structure of maytansine and related compounds

1.7. Streptolydigin and Tirandamycin

Neither streptolydigin[38] nor tirandamycin[39] belong to the class of ansamycins, but they have some biological activities resembling those of the ansamycins (cf. Chap. 3.), and also certain notable structural analogies (Fig. 10): both streptolydigin and tirandamycin contain a great part of the *ansa* ring system found in the ansamycins. Thus it could be postulated that the similarity of the *ansa* rings is the reason for the analogies found in their biological action.



Streptolydigin



Tirandamycin

Fig. 10. Structure of streptolydigin and tirandamycin

2. Biosynthesis

Although all ansamycins consist of an aromatic nucleus spanned by an aliphatic bridge, the chemical structures of these two parts vary considerably. Moreover, ansamycins are isolated from a variety of actinomycetes and even from plants (Table 1). On the other hand, the similarities between the various members of the group are striking. An analogous configuration occurs, for instance, at all 8 asymmetrical C-atoms (C-20–C-27) of the *ansa* ring in rifamycins, streptovaricins and tolypomycins[1]. It could therefore be postulated that the ansamycins have a common route of biosynthesis. Certain similarities to macrolide antibiotics such as erythromycin suggested that the ansamycins might be synthesized in a similar manner. Woodward[40] proposed that macrolides are formed from acetic acid and propionic acid residues in an analogous way as fatty acids are synthesized from acetic acid. Birch[41] put forward the hypothesis that the methyl groups might be introduced into an intermediate polyketide chain by transmethylation through compounds such as methionine or choline.

Table 1. Origin and biological activities of ansamycins

Compound	Origin	Inhibition of bacterial RNA polymerase	Biological activities
Rifamycins	Nocardia mediterranea Micromonospora halophytica etc. Streptomyces tolypophorus	+	Antibacterial (antifungal, antiviral, antitumour)
Streptovaricins	Streptomyces spectabilis	+	
Tolypomycin	Streptomyces tolypophorus	+	Antibacterial
Naphthomycin	Streptomyces collinus	–	Antibacterial, antifungal
Geldanamycin	Streptomyces hygroscopicus	–	Antibacterial, antiprotozoal
Maytansines	Maytenus serrata Maytenus buchananii (Celastraceae) Putterlickia verrucosa Colubrina texensis (Rhamnaceae)	–	Antimitotic, antileukaemic, antitumour

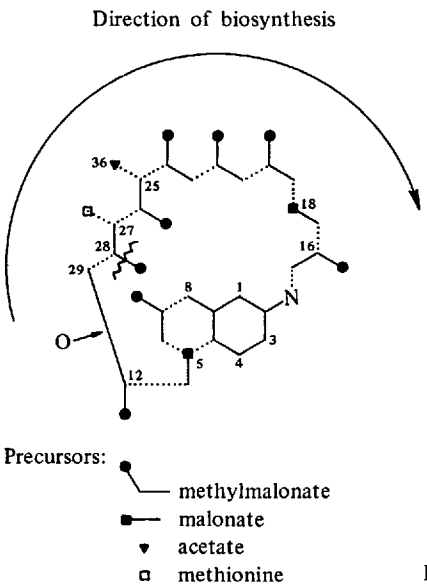


Fig. 11. Scheme of the biosynthesis of rifamycin S

Investigations of the biosynthesis of the rifamycins, streptovaricins and geldanamycin proved that the *ansa* chain of the ansamycins is synthesized as proposed by Woodward. The isolation of the precursors rifamycin W, damavaricin C and D and the protostreptovaricins, together with the fact that rifamycin B and tolypomycin Y are cosynthesized by *Streptomyces tolypophorus*[26] make it probable that the rifamycins, streptovaricins and tolypomycin Y have a common progenitor.

2.1. Rifamycins

The biosynthesis of the rifamycins was studied by using either ^{14}C- or ^{3}H-labelled precursors and subjecting the labelled product to extensive chemical degradation and analysis, or using ^{13}C-enriched precursors and analyzing the product by carbon-13 magnetic resonance spectroscopy[42–44]. These experiments have shown (Fig. 11) that to a C_7N piece of as yet uncertain origin[45] eight propionate and two acetate units are added to form the chromophore and the *ansa* ring of rifamycin S, whereby the incorporation of the propionate occurs *via* methylmalonate and the acetate incorporation *via* malonate. Only the methyl of the $-OCH_3$ group at C-27 stems from methionine, and it is introduced after completion of the *ansa* ring. The *ansa* chain grows clockwise and ends at the C-15 carbon atom (Fig. 11). The first precursor product is thought to be modified by cleavage of the methyl group on C-28 and by oxidation of the *ansa* chain between C-12 and C-29 to yield a ketal linkage. That this does in fact occur has been neatly demonstrated by the isolation of rifamycin W[12] (Fig. 6), in which C-12 and C-29 are directly linked by a C–C double bond, and a hydroxymethyl group is still found at C-28. Rifamycin W can be transformed by the parent Nocardia strain to rifamycin B, and it is thus thought to be a normal intermediate in the formation of the other rifamycins.

Rifamycin S has been shown to be further processed to rifamycin B[46, 47] or rifamycin G[14]. Rifamycin B and rifamycin S can be oxidized to yield rifamycin Y and rifamycin YS respectively[48]. Figure 12 summarizes the route of biosynthesis of the rifamycins based on these findings.

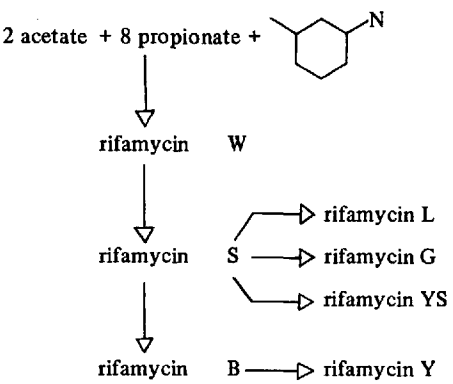


Fig. 12. Proposed route of the biosynthesis of the rifamycins[44]

2.2. Streptovaricins

Using carbon-13 magnetic resonance spectroscopy, Rinehart and his collaborators have shown[49] that the biosynthesis of the streptovaricins is very similar to that of the rifamycins. Streptovaricin D is synthesized from a C_7N unit of unknown origin to which 8 propionic acid residues and two acetic acid residues are attached, whereby the direction of growth is the same as that of the rifamycins. In contrast to the

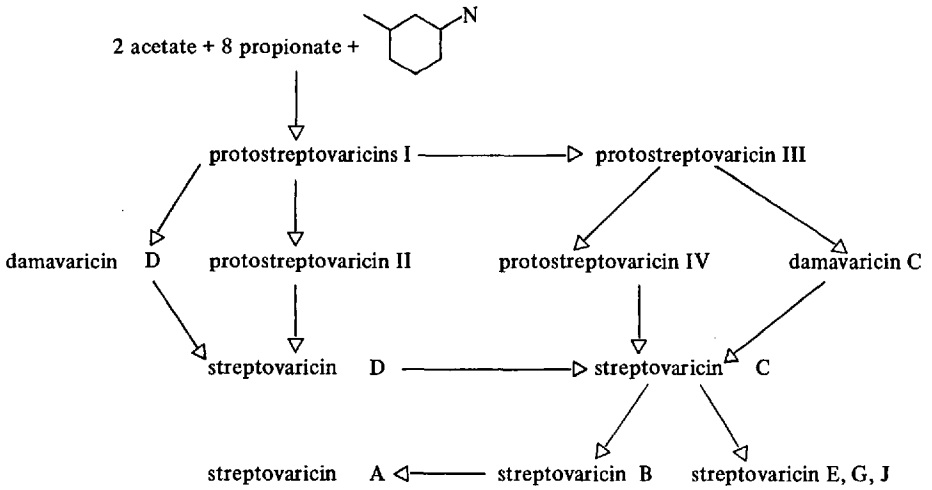


Fig. 13. Proposed routes of biosynthesis of the streptovaricins[21)]

rifamycins, the streptovaricins retain an intact double bond between the C-12 and C-29 and the methyl group on C-28 is not split off. On the other hand, the hydroxyl group in position 6 on the chromophore is methylated, methionine being the methyl donor, then processed in such a way that C-6 and C-11 are linked by a methylene-dioxi-bridge. Besides, the methyl group at C-3 also stems from methionine[22)].

As possible biogenetic precursors of the streptovaricins, damavaricin C and D and the protostreptovaricins I–V have been isolated and characterized (Fig. 8)[21, 22)]. Streptovaricin C and D are the precursors of the other streptovaricins[22)]. Figure 13 summarizes the various supposed routes of biosynthesis of the streptovaricins.

2.3. Geldanamycin and Maytansine

Studies using ^{14}C-labelled precursors and C-13 carbon magnetic resonance have suggested that the biosynthesis of the benzenic ansamycin geldanamycin follows essentially the same pathway as that of the rifamycins and streptovaricins[50)]. Geldanamycin is composed of 3 acetate and four propionate units which are attached to a C_7N unit in the same direction of growth as is found in rifamycins and streptovaricins. The incorporation of three acetate units into the *ansa* chain, as opposed to two in the naphthalenic ansamycins, excludes the existance of a common precursor.

Nothing is known about the biosynthesis of the maytansine group, the only ansamycins so far identified as being of plant origin. Their similarity to the bacterial metabolite geldanamycin has led to the hypothesis that despite their occurrence in plants, microorganisms might be involved in their production.

3. Biological Actions

The ansamycins have a very broad spectrum of biological effects which are of great significance for both scientific and practical reasons. One member of the rifamycins,

rifampicin, has proved to be an excellent, orally active antibiotic and is now in widespread clinical use, especially as an antituberculous agent but also in the treatment of various other infective diseases.

From the biochemical point of view, the unique mode of action of some of the ansamycins, in particular of rifampicin, has aroused much interest. Rifampicin and several other ansamycins have been shown to inhibit bacterial transcription very specifically and at extremely low concentrations by interacting exclusively with DNA-dependent RNA polymerase. This unique action has spurred many investigations on the effects of ansamycins in a variety of viral and eukaryotic systems. More recently maytansine and related compounds have been found to be very potent antimitotic agents and to have interesting antitumour activities.

The published literature on the biological and medical actions of ansamycins is too vast to be dealt with at length in this article. Several reviews have appeared, however, dealing with the rifamycins[51–53].

3.1. The Various Modes of Action

Table 1 indicates the main biological activities of ansamycins. In analysing their great variety it seems useful to group them according to the drug concentration needed to elicit an effect *in vitro* on isolated enzyme systems or on intact bacterial or eukaryotic cells. The reason for this is that effects evoked by low drug concentrations point to a specific interaction with a defined receptor molecule, whereas at high drug levels "effects" may be artifacts or due to unspecific actions, as has unfortunately been the case with many experiments done with ansamycins.

3.1.1. Effects Produced by Drug Concentrations Below 1 μg/ml ($<10^{-6}$ M)

I. Effect on DNA transcription in bacteria:

The rifamycins, streptovaricins and tolypomycins are very effective antibacterial agents. They all inhibit the synthesis of RNA by inactivating the DNA-dependent RNA polymerase. This effect occurs at low concentrations (0.01 μg/ml, 10^{-8} M) and is highly specific. It is the most thoroughly investigated and clearly defined biological action of the ansamycins.

II. Effect on mitosis:

Maytansine and related compounds inhibit cell division in sea urchin eggs at concentrations of 0.04 μg/ml (6×10^{-8} M), possibly by interfering with the polymerization of tubulin. This effect bears some resemblance to the action of the vinca alkaloids such as vincristine. Maytansine inhibits the growth of KB cells at levels of 10^{-5} μg/ml.

3.1.2. Effects Produced by Drug Concentrations Over 1 μg/ml ($>10^{-6}$ M)

I. Actions on eukaryotes:

Clear evidence exists to prove that ansamycins such as rifampicin have no effect on eukaryotic RNA polymerases, be they of nuclear, mitochondrial or chloroplastic

origin. Certain reports claiming inhibition of mitochondrial or chloroplastic RNA polymerase are doubtful because of the experimental conditions used or the high drug concentration needed as compared to that required for inhibition of the bacterial enzyme. Rifamycins with lipophilic side chains and some derivatives of streptovaricin and geldanamycin have been found to inhibit indiscriminately a large number of both DNA and RNA polymerases of bacterial, eukaryotic and viral origin. The 100–10'000-fold higher drug concentration needed for inhibition, as well as the lack of enzyme specificity, definitely distinguishes this effect from the inhibition of bacterial RNA polymerase.

II. Effects on RNA tumour viruses:

As has already been mentioned, some lipophilic rifamycins and some streptovaricins and geldanamycins affect the growth of cells transformed by RNA tumour viruses or the RNA-dependent DNA polymerase (reverse transcriptase) characteristic of these viruses. Again, high drug concentrations are needed to produce an effect and only partial, but never absolute, selectivity of enzyme inhibition has been found.

III. Effects on DNA viruses and larger infectious agents belonging to the genus of Chlamydiae:

Certain ansamycin derivatives, such as rifampicin, inhibit the growth of these organisms at high drug concentration. The underlying mechanism of action is poorly unterstood at present, but it does not seem to be related to RNA or DNA synthesis.

3.2. Effects on Bacteria

3.2.1. Interaction with DNA-dependent RNA Polymerase

The potent antibacterial activity of the rifamycins, streptovaricins and tolypomycins is a consequence of the specific inhibition of DNA-dependent RNA polymerase, the enzyme responsible for most of the transcription of DNA to RNA. The interaction between these ansamycins and the enzyme has accordingly been studied in great detail. Because of its easy availability, rifampicin has been used most often as the model compound in such studies, but it is reasonable to assume that the data obtained with rifampicin also hold true, at least qualitatively, for the other rifamycins, the streptovaricins and tolypomycins.

Rifampicin was first shown by Hartmann *et al.* [54] to have a specific inhibitory effect on RNA polymerase from *E. coli*. Later, other active ansamycins were found and RNA polymerases from a large variety of bacteria other than *E. coli* proved to be sensitive to the drug. More recently, an RNA polymerase from *E. coli* containing only one subunit and probably involved in the initiation of DNA replication (dna G gene product) has been shown to be resistant to rifampicin[55]. This holds true also for the various mammalian RNA polymerases. In contrast to non-specific inhibitors of transcription such as actinomycin and mitomycin, rifampicin interacts specifically with the bacterial enzyme itself. With the aid of ^{14}C-labelled rifampicin it could be shown that the drug forms a very stable complex with the enzyme in a molar ratio of 1 : 1[56, 57]. The dissociation constant of this complex is 10^{-9} M at 37 °C and

2.7×10^{-10} M at 0 °C[58, 59]. These relatively low constants explain why ansamycins, and in particular rifampicin, inhibit both the activity of RNA polymerase and the growth of bacteria at very low drug concentrations. Experiments with rifampicin-resistant mutants have shown[60, 61] that the β subunit of RNA polymerase contains the binding site for the drug. It could be demonstrated, however, that binding to the enzyme precursor $\alpha_2\beta$ is very weak[62]. To form the specific drug-binding site, the four subunits $\alpha_2\beta\beta'$, as they occur in the core enzyme, are required. σ, the subunit necessary for specific promoter recognition, affects only weakly the ansamycin binding site[58].

3.2.2. Mode of Action

Inhibition of RNA polymerase is a direct consequence of the binding of the drug to the enzyme: on the one hand, enzyme inhibition only occurs, when the ansamycin is bound, and on the other hand, drug binding never seems to occur without causing inhibition.

Two aspects of the mode of action must therefore be distinguished:

a) what determines and influences the binding of ansamycins to RNA polymerase

b) which function of RNA polymerase is impaired when ansamycins are bound to the enzyme.

Recent investigations of the rate of binding of rifampicin to RNA polymerase at various stages of transcription have shown that nucleic acids bound to the enzyme strongly decrease the on-rate of the antibiotic to the enzyme[58]. Rifampicin binds 500 times slower to a specific RNA polymerase -T7 DNA complex than to the free enzyme. The rate of drug binding to a ternary enzyme-DNA-RNA complex, as it occurs during RNA chain elongation is at least 5 orders of magnitude slower. These direct kinetic measurements are in line with earlier observations that DNA can protect RNA polymerase against inactivation by rifampicin[63–65] and that ansamycins inhibit RNA chain initiation, but not elongation[66]. The rate of enzyme inhibition by ansamycins therefore parallels the on-rate of drug binding and can vary greatly depending on the stage of transcription.

For a long time there was no clear picture as to which function of the RNA polymerase was impaired by ansamycins. Very recently, McClure and collaborators clarified the problem in a series of very elegant experiments[67], personal communication. Upon analyzing the initiation of RNA synthesis on the P_R promoter of phage λ, McClure found that rifampicin leads to abortive initiation, *i.e.* to the formation of the dinucleotide pppApU, and inhibits the formation of the second phosphodiester bond. A model is proposed in which rifampicin binding to RNA polymerase sterically blocks the translocation of pppApU. The drug does not affect the recognition of the specific promoter by the enzyme, since the only dinucleotide formed is pppApU, which corresponds to the first two bases of the gene starting at P_R.

3.2.3. Structure-Activity Relations

The antibacterial activity of the ansamycins varies to a large extent. In most cases, antibacterial activity parallels the inhibition of the bacterial RNA polymerase and

can therefore be predicted to some extent on the basis of structure-activity studies with the enzyme. The availability of many natural and semisynthetic derivatives, especially in the rifamycin and streptovaricin groups, has made it possible to get an idea of the structural parameters necessary to inhibit RNA polymerase. For the rifamycins, some of the main features are the following[68, 69]:

1. Free hydroxyl or keto groups at C_1 and C_8;
2. unbroken *ansa*-bridge;
3. free hydroxyl groups at C_{21} and C_{23}.

Analysis by X-ray[69] and NMR spectroscopy[70] shows that the rifamycin molecule is very rigid (Fig. 2). The four oxygen functions at C_1, C_8, C_{21} and C_{23} all lie on the same side of the molecule. Being required for enzyme inhibition it is reasonable to assume that they are involved in the binding of the drug to the enzyme. However, kinetic studies of the interaction of rifampicin with RNA polymerase in various solvents indicate that the bonds forming the drug-enzyme complex are mostly of a lipophilic nature[58]. Large parts of the *ansa* ring thus seem to participate in the binding to the enzyme as well as the chromophore which possibly interacts with an aromatic amino acid, since rifampicin shows a characteristic bathochromic shift when bound to the enzyme. The nature of the interactions between the drug and the enzyme could therefore conceivably be represented by the model shown in Fig. 14, in which the enzyme closes on the drug molecule from three sides. In such a model, positions 3 and 4 on the chromophore do not take part in the binding. This would be consistent with the finding that chemical substitutions at C-3 and C-4 of the molecule in many cases have little effect on its interaction with the enzyme[68, 71]. Effects on eukaryotic and viral enzymes observed after the introduction of substituents in these positions are most probably not due to specific binding such as is found with the bacterial enzyme.

Although many facts are known about the structure-activity relations of ansamycins, some problems remain unsolved. Rifamycin W, for instance, (Fig. 6) does not

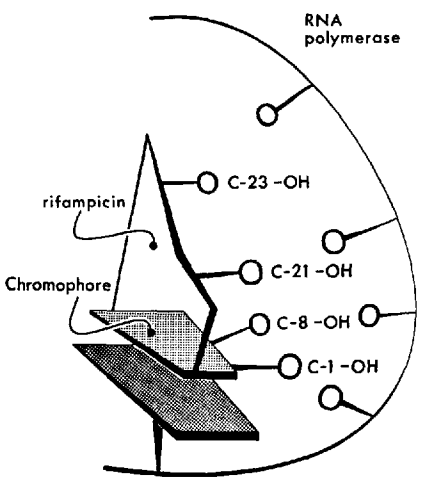


Fig. 14. Model of the interaction between rifampicin and RNA polymerase

seem to inhibit RNA polymerase[11], although the structural requirements mentioned above are fulfilled. On the other hand, damavaricin C, which is assumed to be a precursor of streptovaricin C and is chemically very similar to rifamycin W, is a powerful inhibitor of the enzyme[23]. Moreover, among the streptovaricins, streptovaricin J (acetate at C-21) and streptovaricin E (keto group at C-21) still show some activity, whereas the corresponding rifamycin-C-21-acetate and rifamycin Y are inactive[20]. The structure-activity relations of the rifamycins and the streptovaricins therefore do not seem to be identical.

Another puzzle is posed by naphthomycin. The fact that it does not inhibit RNA polymerase can be explained by the absence of a hydroxyl group at C-8. However, it shows antibacterial activity against gram-positive strains that is counteracted by SH-containing substances such as cystein[28]. Naphthomycin is therefore an example of an ansamycin with antibacterial activity not due to inhibition of RNA polymerase.

The structure of the ansamycins determines not only their activity on RNA polymerase, but also other important characteristics such as their ability to penetrate into bacteria and their pharmacokinetics and absorption in the host. To cite just a few examples: rifamycin B, containing a free carboxylic acid group, has no antibacterial activity, although it inhibits RNA polymerase as strongly as rifampicin. Damavaricin C behaves similarly to rifamycin B, whereas its 6-methyl ether inhibits RNA polymerase to a lesser extent, but has good antibacterial activity[23]. Rifampicin owes its widespread clinical use to the fact that, in contrast to most other rifamycin derivatives, it is well absorbed when given orally.

3.2.4. Nature of Ansamycin Resistance in Mutants

Mutants resistant to rifamycins have been isolated from a variety of micro-organisms. The mutation rate of *E. coli* is in the region of 10^{-8}, and the conversion is apparently due to a one-step mutation. The mutants analyzed so far map at a single position (79 minutes) on the *E. coli* chromosome [72]. RNA polymerase has been prepared from rifamycin-resistant mutants of *E. coli* and *S. aureus*. In both cases it was found that the enzyme differed from the corresponding enzyme of the sensitive strain in that it is no longer inhibited by rifampicin and, in addition, could no longer bind the drug[56, 73, 74]. Resistance to rifampicin was therefore caused by an alteration in the structure of the enzyme, probably by the replacement of one single amino acid. In a number of resistant mutants, the site of the mutation was located in the β subunit[60, 61].

Resistance to rifampicin is not an all-or-nothing phenomenon. Mutants can be selected that are resistant to various concentrations of the drug. As the bacteria become more resistant to rifampicin, the sensitivity of the corresponding RNA polymerase decreases, and the enzyme-antibiotic complex becomes less stable[75]. Hence, the degree to which the enzyme and the antibiotic fit each other varies widely, indicating that there are many possibilities for the location and the nature of the amino acid substitution in the enzyme molecule. These substitutions would be expected to occur mainly in the β subunit, but mutations in other subunits such as β' or α cannot be excluded, since, as discussed above, the core enzyme $\alpha_2\beta\beta'$ has to be present for the formation of the specific drug-binding site.

The possible occurrence of ansamycin resistant mutants in which at the same time the correct functioning of the RNA polymerase has been affected poses an interesting problem. Recently such mutants have been found in *Lactobacillus casei* which were resistant to rifampicin and in the same mutational event had developed auxotrophy for glutamine[76]. A rifampicin resistant mutant of *E. coli* W has been found to contain an alteration in some parameters regulating the arginine biosynthesis[77]. Such pleiotropic effects could be of great help in elucidating the different modes of the regulation of gene expression.

As would be expected from their similar mechanisms of action, resistance to streptovaricins and tolypomycins develops in a way analogous to that found with the rifamycins, and cross-resistance is observed between all three groups of antibiotics[78, 79].

So far no enzyme has been found that can inactivate ansamycins either by cleavage or by chemical modification.

3.2.5. Other Effects on Bacteria

As has already been mentioned, naphthomycin inhibits grampositive bacteria, although it does not inhibit RNA polymerase. An antagonism between naphthomycin and vitamin K has been observed[28], but the underlying mode of action is not known.

The two structurally related antibiotics streptolydigin and tirandamycin should be mentioned at this point. They do not belong to the ansamycins, but show some striking similarities in their chemical structure (Fig. 10). Surprisingly enough, these two compounds also inhibit RNA polymerase by binding to the β subunit, although higher drug concentrations are required and their mode of action differs from that of the ansamycins[60, 80–82]. Genetic studies of *E. coli* have shown that the loci for rifampicin and streptolydigin resistance map very closely together[83]. It could be postulated that the binding sites for streptolydigin and rifampicin partially overlap.

3.3. Effects on Eukaryotes

The rifamycins, streptovaricins and tolypomycins which inhibit bacterial RNA polymerase, do not in general affect eukaryotic RNA polymerases, whether they are of nuclear, mitochondrial or chloroplastic origin[52]. The antibacterial action of these ansamycins resides in the recognition of a specific binding site on the bacterial enzyme that is absent in eukaryotic enzymes. Certain ansamycins such as lipophilic rifamycin derivatives at high concentrations show some activity on a large variety of mammalian DNA and RNA polymerases as well as on rifampicin-resistant bacterial RNA polymerase[52, 84–87]. Even enzymes other than polymerases, such as glutamate-oxaloacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT) and alkaline-phosphatases, and polyphenylalanine synthesis in a cell-free system are inhibited to some extent[87, 88]. This inhibition is due to non-specific binding of the drug to

these enzymes, whereby the number of drug molecules bound depends on their concentration and can amount to several hundreds per molecule of the enzyme[88]. Studies of sedimentation and viscosity show that lipophilic rifamycins form large aggregates at the same concentrations as those leading to enzyme inhibition (unpublished observation). These data clearly demonstrate that the inhibitory action on these enzymes cannot be related to the very specific interaction of some ansamycins with bacterial RNA polymerase, which occurs at 1000 to 10'000 times lower concentrations and in a very well defined stoichiometric ratio of 1:1.

Nevertheless, under certain conditions some ansamycins seem to affect the growth of eukaryotic cells. Medoff and collaborators[89, 90], for instance, have reported that rifampicin together with amphotericin B inhibited the growth of certain yeasts such as *S. cerevisiae* and *C. albicans,* whereas neither antibiotic alone had any effect at the concentrations tested. This observation has been confirmed by other authors with a variety of different fungi[91–94] and even with mouse L-cells[95]. The underlying mechanism of action is rather puzzling. On one hand, there seems to be some indication that RNA synthesis is inhibited, which has led to the conclusion that amphotericin B facilitated the permeation of rifampicin through the cell membrane, allowing rifampicin to act on RNA synthesis much as it does in bacteria[89]. On the other hand, careful studies have shown that neither the various nuclear[96] nor mitochondrial[97] RNA polymerases of yeast are inhibited, even by very high concentrations of rifampicin.

The results obtained in experiments with a fragile *Saccharomyces cerevisiae* mutant are even more puzzling; in contrast to the wild type it was found to be inhibited by rifampicin alone and even seems to contain an RNA polymerase sensitive to the drug, whereas the analogous enzyme from the wild type is rifampicin-resistant[98]. Similar results have been obtained with vesicular stomatitis virus, an RNA virus containing an endogenous RNA transcriptase. Here again, both the growth of the mutant strain and its enzyme are drug sensitive, but the wild type is drug resistant[99]. Further studies are needed to clarify the situation, and in this respect determinations of the rate of mutation to drug resistance might be of great value.

Very interesting effects on eukaryotic cells result after treatment with ansamycins containing a benzene chromophore, especially with compounds of the maytansine group which are the only ansamycins found so far to occur in plants.

Maytansine and some of its congeners affect the growth of KB cells at concentrations as low as 10^{-4} to 10^{-5} μg/ml[32]. They irreversibly inhibit cell division in the eggs of sea urchins and clams, causing the disappearance of the mitotic apparatus. They inhibit the polymerization of tubulin *in vitro*[100] and thus act similarly to the vinca alkaloids such as vincristine. Bacterial RNA polymerase is not affected up to a concentration of 60 μg/ml[101]. Despite its highly toxic effects on cells *in vitro,* maytansine and other maytansinoids are highly potent antitumour drugs with a relatively low toxicity *in vivo.* The reason for their specific action remains unclear, but differences in the cell surface affecting drug attachment and permeation might be of importance. Structure-activity studies have revealed the crucial role of a free hydroxy group in position 9 and an ester group in position 3[35, 36] (see Fig. 9). Maytansine and other maytansinoids esterified at C-3 have a potent antileukaemic effect in the μg/kg dose range and are very cytotoxic, whereas compounds without an ester such

as maytansinol, maysine, maysenine and also geldanamycin lack an antitumour effect and have a cytotoxicity 3–5 orders of magnitude lower. Conversion of the free C-9 hydroxyl group to the C-9 ethyl ether yields an inactive compound. As to the mechanism of action, it has been postulated that after specific binding to the target protein(s), maytansine acts as an alkylating agent of free thio- or amino-groups, preferably through C-9.

3.4. Effects on RNA Tumour Viruses

Oncogenic RNA viruses contain an RNA-dependent DNA polymerase (reverse transcriptase) that is characteristic of this group of viruses and seems to be of importance for viral proliferation and the transformation of its host cell[102, 103]. Since RNA tumour viruses might be implicated in some human cancers, inhibitors of reverse transcriptase could be very valuable research tools in defining the exact function of this enzyme in viral carcinogenesis and they might even be of clinical value. The early finding that certain lipophilic rifamycin derivatives inhibit reverse transcriptase and have some effects on transformed cells[104, 105] led to great efforts both to synthesize new ansamycin derivatives and to analyse them in a variety of biological systems[5, 52, 106]. A large number of rifamycin, streptovaricin and geldanamycin derivatives have been synthesized, in most cases through substitution of the chromophore. Many of them inhibit reverse transcriptase to some extent, but none of the substances so far tested acts in an absolutely selective way and at concentrations comparable to those that inhibit bacterial RNA polymerase. In fact, as has already been discussed in the preceeding section, many other nucleic acid polymerases of bacterial, eukaryotic and viral origin, as well as other enzymes such as phosphatases, are also inhibited to a similar extent by these derivatives. For all these enzymes, including reverse transcriptase, the drug concentrations required to bring about this effect are relatively high (> 1 μg/ml). As in the case of eukaryotic polymerases there is no clearly defined stoichiometry of binding to reverse transcriptase independent of the drug concentration.

It can therefore be concluded that inhibition of reverse transcriptase caused by ansamycins with a modified chromophore is not due to a strong and defined interaction between the drug and enzyme, but rather results from the weak binding of a varying number of aggregated drug molecules to a relatively undefined protein region occurring in many enzymes. There is little chance that further chemical modification of the ansamycin chromophore will lead to compounds with a selective activity on reverse transcriptase at low concentrations.

3.5. Effects on DNA Viruses and Larger Infectious Agents such as Chlamydiae

Most studies about the effects of ansamycins on DNA viruses have been made with vaccinia virus[51]. It has been found that some derivatives, such as rifampicin, inhibit the growth of this virus. There is no doubt, however, that this inhibition is not due to a block in RNA synthesis, as was found in bacteria, but apparently the assembly

of immature virus particles is affected[107–109]. As in the case of the RNA viruses, very large concentrations of antibiotic are needed to inhibit the virus growth. Thus, clinical application is not possible.

The infectious elementary bodies of trachoma agent belong to the *Chlamydiae* which are parasites of mammalian cells and are considered unusually small bacterial cells. As in the case of vaccinia virus, only certain ansamycins, *e.g.* rifampicin at very high concentrations, affect the growth of trachoma agent. The mechanism of action is not known, but again RNA synthesis is not involved.

4. Conclusions and Summary

The ansamycins are a remarkable group of natural compounds varying widely in both their chemical structure and their biological activities. They have mostly been isolated from prokaryotic microorganisms, but one group, the maytansines, occurs in plants.

Their chemical structure consists of two parts, a chromophore and a long aliphatic bridge spanning it. The molecules thus formed are very compact and rigid. As a consequence, intramolecular and intermolecular interactions lead to unexpected chemical properties. In particular, those derivatives with lipophilic side chains tend to aggregate and behave like detergents even in dilute solutions. This property should be taken into account, when ansamycins are used at high concentrations in biological systems. The ansamysins do not contain lactone bonds in their *ansa* ring, which sets them clearly apart from the macrolide antibiotics.

Studies of the biosynthesis of the substances have shown that starting from a nucleus of unknown origin a varying number of acetate and propionate units can be linked to yield ansamycins differing in both the structure of the chromophore and the *ansa* chain. Continued research will very probably disclose new types of ansamycins with novel biological actions. One interesting problem in this context is to determine what modes of regulation exist to direct the biosynthesis of common building blocks into specific compounds, and whether it might even be possible to influence and modify this regulation in such a way that new biosynthetic pathways yield novel compounds.

Ansamycins have been shown to cause a large variety of biological effects on bacteria, eukaryotes and viruses. Two of these are very powerful and selective. One is the specific inhibition of bacterial RNA synthesis by rifamycins, streptovaricins and tolypomycins. Detailed investigations have proved that DNA-dependent RNA polymerase, the enzyme responsible for DNA transcription, forms a very stable 1 : 1 complex with these ansamycins and as a consequence, is inactivated. Eukaryotic and viral enzymes do not interact with the drug in this selective manner. The binding of ansamycins to bacterial RNA polymerase is a good example of a specific drug-receptor interaction; a chemically complex molecule with a rigid shape is linked by physical bonds to a complementary site of a macromolecule. This results in a tight complex and, as a consequence, in a dramatic change of the functional properties of the macromolecule.

More recently, the maytansines have been found to exert a very potent antimitotic action on eukaryotic cells and to show interesting antitumour activity. The

drug concentrations necessary to cause these effects are very low which suggests that the mode of action although not yet known in all its details is selective.

Besides these two specific actions, many other biological effects caused by ansamycins have been reported. Most of them are unspecific and are only observed at high drug concentrations, at which the derivatives used have the detergent-like properties mentioned above. To this category belongs the inhibition of the various mammalian and viral nucleic acid polymerases, including reverse transcriptase. It must be stressed very strongly that an effect on a mammalian enzyme at drug concentrations of 5–200 μg/ml cannot be interpreted in the same way as the inhibition of bacterial RNA polymerase at 10^{-2} μg/ml.

Finally, there remain some effects of ansamycins of which the significance and the biochemical targets are as yet unknown; these include the combined action of rifamycin and amphotericin B on fungal cells, and the antibacterial and antifungal activity of naphthomycin and geldanamycin.

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5. References

1) Prelog, V., Oppolzer, W.: Ansamycine, eine neuartige Klasse von mikrobiellen Stoffwechselprodukten. Helv. Chim. Acta *56*, 2279 (1973)
2) Lüttringhaus, A., Gralheer, H.: Über eine neue Art atropisomerer Verbindungen. Liebigs Ann. Chem. *550*, 67 (1942)
3) Sensi, P., Margalith, P., Timbal, M.T.: Rifomycin, a new antibiotic-preliminary report. Il Farmaco, Ed. Sci. *14*, 146 (1959)
4) Rinehart, K.L.: Antibiotics with *ansa* rings. Accounts Chem. Res. *5*, 57 (1972)
5) Sensi, P.: Recent progress in the chemistry and biochemistry of rifamycins. Pure Appl. Chem. *41*, 15 (1975)
6) Margalith, P., Pagani, H.: Rifomycins. XIV. Production of Rifomycin B. Appl. Microbiol. *9*, 325 (1961)
7) Oppolzer, W., Prelog, V., Sensi, P.: Konstitution des Rifamycins B und verwandter Rifamycine. Experientia *20*, 336 (1964)
8) Oppolzer, W., Prelog, V.: Über die Konstitution und Konfiguration der Rifamycine B, O, S und SV. Helv. Chim. Acta *56*, 2287 (1973)
9) Brufani, M., Cerrini, S., Fedeli, W., Vaciago, A.: Rifamycins, an insight into biological activity based on structural investigations. J. Mol. Biol. *87*, 409 (1974)
10) Maggi, N., Pasqualucci, C.R. Ballotta, R., Sensi, P.: Rifampicin, a new orally active rifamycin. Chemotherapia *11*, 285 (1966)
11) White, R.J., Lancini, G., Sensi, P.: New natural rifamycins. In: Proc. Ist intersectional congress of Intern. assoc. microbiol. Soc. Hasegawa, T. (ed.) Vol. 3. Tokyo 1975, p. 483
12) White, R.J., Martinelli, E., Lancini, G.: Ansamycin biogenesis: studies on a novel rifamycin isolated from a mutant strain of *Nocardia mediterranei*. Proc. Nat. Acad. Sci. USA *71*, 3260 (1974)
13) Martinelli, E., Gallo, G.G., Antonini, P. White, R.J.: Structure of rifamycin W. A novel ansamycin from a mutant of *Nocardia mediterranei*. Tetrahedron *30*, 3087 (1974)
14) Lancini, G., Sartori, G.: Rifamycin G, a further product of Nocardia mediterranei metabolism. J. Antibiotics *29*, 466 (1976)
15) Leitich, J., Prelog, V., Sensi, P.: Rifamycin Y und seine Verwandlungsprodukte. Experientia *23*, 505 (1967)
16) Brufani, M., Fedeli, W., Giacomello, G., Vaciago, A.: The X-ray analysis of the structure of rifamycin Y. Experientia *23*, 508 (1967)
17) Weinstein, M.J., Luedemann, G.M., Oden, E.M., Wagmann, G.H.: Halomicin, a new Micromonospora-produced antibiotic. Antimicrobial Agents and Chemotherapy *1967*, 435
18) Ganguly, A.K., Szmulewicz, S., Sarre, O. Z., Greeves, D., Morton, J., McGlotten, J.: Structure of Halomicin B. J. Chem. Soc. Chem. Comm. *1974*, 395
19) Celmer, W.D., Cullen, W.P. English, A.R., Jefferson, M.T., Oscarson, J.R., Routien, J.B., Sciavolino, F.C.: 3-Methylthiorifamycin SV and CP-32'656, novel sulfur containing ansamycins from a new species of Micromonospora. In: 15th Intersci. Conf. Antimicrob. Ag. Chemotherapy, abstract *1975*, 260
20) Rinehart, K.L., Antosz, F.J., Sasaki, K., Martin, P.K., Masheshwari, M.L., Reusser, F., Li, L.H., Moran, D., Wiley, P.R.: Relative biological activities of individual streptovaricins and streptovaricin acetates. Biochem. *13*, 861 (1974)
21) Deshmukh, P.V., Kakinuma, K., Ameel, J.J., Rinehart, K.L., Wiley, P.F., Li, L.H.: Protostreptovaricins I–V. J. Amer. Chem. Soc. *98*, 870 (1976)
22) Rinehart, K.L., Antosz, F.J., Deshmukh, P.V., Kakinuma, K., Martin, P.K., Milavetz, B.J., Sasaki, K., Witty, T.R., Li, L.H., Reusser, F.: Identification and preparation of damavaricins, biologically active precursors of streptovaricins. J. Antibiotics *29*, 201 (1976)
23) Sasaki, K., Naito, T., Satomi, T.: Chemical modification of streptovaricin C. I. 19–0–substituted damavaricin C. J. Antibiotics *29*, 147 (1976)
24) Kishi, T., Asai, M., Muroi, M., Harada, S., Mizuta, E., Terao, S., Miki, T., Mizuno, K.: Tolypomycin. I. Structure of Tolypomycinone. Tetrahedr. Letters *1969*, 91

25) Shibata, M., Hasegawa, T., Higashide, E.: Tolypomycin, a new antibiotic I. *Streptomyces tolypophorus* nov. sp., a new antibiotic, tolypomycin producer. J. Antibiotics *24*, 810 (1971)
26) Kishi, T., Yamana, H., Muroi, M., Harada, S., Asai, M., Hasegawa, T., Mizuno, K.: Tolypomycin, a new antibiotic. III. Isolation and characterization of tolypomycin Y. J. Antibiotics *25*, 11 (1972)
27) Kamiga, K., Sugino, T., Wada, Y., Nishihawa, M., Kishi, T.: The X-ray analysis of tolypomycinone tri-m-bromobenzoate. Experientia *25*, 901 (1969)
28) Balerna, M., Keller-Schierlein, W., Martins, C., Wolf, H., Zähner, H.: Naphtohomycin, an antimetabolite of vitamine K_1. Arch. Mikrobiol. *65*, 303 (1969)
29) Williams, T.H.: Naphthomycin, a novel *ansa* macrocyclic antimetabolite. Proton NMR spectra and structure elucidation using lanthanide shift reagent. J. Antibiotics *28*, 85 (1975)
30) Sasaki, K., Rinehart, K.L., Slomp, G., Grostic, M.F. Olson, E.C.: Geldanamycin. I. Structure assignment. J. Amer. Chem. Soc. *92*, 7591 (1970)
31) De Boer, C., Meulman, P.A., Wuuk, R.J., Peterson, D.H.: Geldanamycin, a new antibiotic. J. Antibiotics *23*, 442 (1970)
32) Kupchan, S.M., Komoda, Y., Court, W.A., Thomas, G.J., Smith, R.M., Karim, A., Gilmore, C.J., Haltiwanger, R.C., Bryon, R.F.: Maytansine, a novel antileukaemic *ansa* macrolide from *Maytenus ovatus*. J. Amer. Chem. Soc. *94*, 1354 (1972)
33) Kupchan, S.M., Komoda, Y., Thomas, G.J., Hintz, H.P.J.: Maytanprine and maytanbutine, new antileukaemic *ansa* macrolides from *Maytenus buchananii*. J. Chem. Soc. Chem. Comm. *1972*, 1065
34) Wani, M.C., Taylor, H.L., Wall, M.E.: Plant Antitumor agents: colubrinol acetate and colubrinol, antileukaemic *ansa* macrolides from *Colubrina texensis*. J. Chem. Soc. Chem. Comm. *1973*, 390
35) Kupchan, S.M., Komoda, Y., Branfman, A.R., Dailey, R.G., Zimmerly, V.A.: Novel maytansioids. Structural interrelations and requirements for antileukaemic activity. J. Amer. Chem. Soc. *96*, 3706 (1974)
36) Kupchan, S.M., Branfman, A.R., Sueden, A.R., Verma, A.K., Dailey, R.G., Komoda, Y., Nagao, Y.: Novel Maytansinoids. Naturally occurring and synthetic antileukaemic esters of maytansinol. J. Amer. Chem. Soc. *97*, 5294 (1975)
37) Kupchan, S.M.: Novel natural products with antitumor activity. Fed. Proc. *33*, 2288 (1974)
38) Rinehart, K.L., Beck, J.R., Borders, D.B., Kinstle, T.H., Krauss, D.: Streptolidigin. III. Chromophore and structure. J. Amer. Chem. Soc. *85*, 4038 (1963)
39) Mac Kellar, F.A., Grostic, M.F., Olson, E.C., Wuuk, R.J., Branfman, A.R., Rinehart, K.L.: Tirandamycin. I. Structure assignment. J. Amer. Chem. Soc. *93*, 4943 (1971)
40) Woodward, R.B.: Struktur und Biogenese der Macrolide. Angew. Chem. *69*, 50 (1957)
41) Birch, A.J.: Biosynthetic relations of some natural phenolic and enolic compounds. Fortschr. Chem. org. Naturstoffe *14*, 186 (1957)
42) Brufani, M., Kluepfel, D., Lancini, G., Leitich, J., Mesentser, A.S., Prelog, V., Schook, F.P., Sensi, P.: Über die Biogenese des Rifamycins S. Helv. Chim. Acta *56*, 2315 (1973)
43) White, R.J., Martinelli, E., Gallo, G.G., Lancini, G.: Rifamycin biosynthesis studied with ^{13}C-enriched precursors and carbon magnetic resonance. Nature *243*, 273 (1973)
44) Karlsson, A., Sartori, G., White, R.J.: Rifamycin Biosynthesis: further studies on origin of the *ansa* chain and chromophore, Europ. J. Biochem. *47*, 251 (1974)
45) White, R.J., Martinelli, E.: Ansamycin biogenesis: incorporation of (1–^{13}C) glucose and (1–^{13}C) glycerate into the chromophore of rifamycin S. FEBS Letters *49*, 233 (1974)
46) Liersch, M., Auden, J., Gruner, J., Nüesch, J.: Biosynthesis of rifamycine. Path. Microbiol. *34*, 243 (1969)
47) Lancini, G.C., Gallo, G.G., Sartori, G., Sensi, P.: Isolation and structure of rifamycin L its biogenetic relationship with other rifamycines. J. Antibiotics *22*, 369 (1969)
48) Lancini, G.C., Thiemann, J.E. , Sartori, G. Sensi, P.: Biogenesis of rifamycins. The conversion of rifamycin B into rifamycin Y. Experientia *23*, 899 (1967)
49) Milavetz, B., Kakinuma, K., Rinehart, K.L., Rolls, J.P., Haak, W.J.: Carbon-13 magnetic

resonance spectroscopy and the biosynthesis of streptovaricin. J. Amer. Chem. Soc. *95*, 5793 (1973)
50) Johnson, R.D., Haber, A., Rinehart, K.L.: Geldanamycin biosynthesis and carbon magnetic resonance. J. Amer. Chem. Soc. *96*, 3316 (1974)
51) Wehrli, W., Staehelin, M.: Actions of the rifamycins. Bacteriol. Rev. *35*, 290 (1971)
52) Wehrli, W., Staehelin, M.: Rifamycins and other ansamycins. In: Antibiotics Vol. 3. Mechanism of action of antimicrobial and antitumor agents. Corcoran, J. W. and Hahn, F. E., (ed). Berlin-Heidelberg-New York: Springer 1975, p. 252
53) Binda, G., Domenichini, E., Gottardi, A., Orlandi, B., Ortelli, E., Pacini, B., Forwst, G.: Rifampicin, a general review. Arzneimittelforsch. *21*, 1907 (1971)
54) Hartmann, G., Honikel, K.O., Knüsel, F., Nüesch, J.; The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim. Biophys. Acta *145*, 843 (1967)
55) Bouché, J.-P., Zechel, K., Kornberg, A.: DNA G gene product, a rifampicin resistant RNA polymerase, initiates the conversion of a single-stranded coliphage DNA to its duplex replicative form. J. Biol. Chem. *250*, 5995 (1975)
56) Wehrli, W., Knüsel, F., Schmid, K., Staehelin, M.: Interaction of rifamycin with bacterial RNA polymerase. Proc. Natl. Acad. Sci. USA *61*, 667 (1968)
57) Wehrli, W., Staehelin, M.: Interaction of rifamycin with RNA polymerase. In: Proc. Ist Int. Lepetit Colloq. RNA Polymerase. Transcript. Amsterdam-London: North-Holland Publ. Co. 1970, p. 65
58) Wehrli, W., Handschin, J., Wunderli, W.: Interaction between rifampicin and DNA-dependent RNA polymerase of *E. coli*. In: RNA Polymerase. Cold Spring Harbor Laboratory 1976, p. 397
59) Bähr, W., Stender, W., Scheit, H.-M., Jovin, T. M.: Binding of rifampicin to *E. coli* RNA polymerase. Thermodynamic and kinetic studies. In: RNA Polymerase. Cold Spring Harbor Laboratory 1976, p. 369
60) Heil, A., Zillig, W.: Reconstitution of bacterial DNA-dependent RNA polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. FEBS Letters *11*, 165 (1970)
61) Boyd, D.H., Zillig, W., Scaife, J.: Reference mutations for the β subunit of RNA polymerase. Molec. Gen. Genet. *130*, 315 (1974)
62) Handschin, J.: Kinetische Untersuchungen über den Komplex zwischen Rifampicin und RNS Polymerase aus *E. coli*. Dissertation, Basel 1976
63) Sippel, A., Hartmann, G.: Rifampicin resistance of RNA polymerase in the binary complex with DNA. Europ. J. Biochem. *16*, 152 (1970)
64) Bautz, E.K.F., Bautz, F.A.: Initiation of RNA synthesis: the function of sigma in the binding of RNA polymerase to promoter sites. Nature *226*, 1219 (1970)
65) Hinkle, D.C., Mangel, W.F. Chamberlin, M.J.: Studies of the binding of *E. coli* RNA polymerase to DNA. IV. The effect of rifampicin on binding and on RNA chain initiation. J. Mol. Biol. *70*, 209 (1972)
66) Sippel, A., Hartmann, G.: Mode of action of rifamycin on the RNA polymerase reaction. Biochim. Biophys. Acta *157*, 218 (1968)
67) Johnston, D.E., Mc Clure, W.R.: Abortive initiation of *in vitro* RNA synthesis on bacteriophage λ DNA. In: RNA Polymerase. Cold Spring Harbor Laboratory 1976, p. 413
68) Wehrli, W., Staehelin, M.: The rifamycins – relation of chemical structure and action on RNA polymerase. Biochim. Biophys. Acta *182*, 24 (1969)
69) Brufani, M., Cerrini, S., Fedeli, W., Vaciago, S.: Rifamycins: an insight into biological activity based on structural investigations. J. Mol. Biol. *87*, 409 (1974)
70) Gallo, G.G., Martinelli, E., Pagani, V., Sensi, P.: The confirmation of rifamycin S in solution by ^{1}H NMR spectroscopy. Tetrahedron *30*, 3093 (1974)
71) Dampier, M.F., Whitlock, H.W.: Electronegative groups at C-3 of rifamycin S enhance its activity toward DNA-dependent RNA polymerase. J. Amer. Chem. Soc. *97*, 6254 (1975)
72) Taylor, A.L., Trotter, C.D.: Linkage map of *E. coli* strain K-12 Bact. Rev. *36*, 504 (1972)
73) Wehrli, W., Knüsel, F. Staehelin, M.: Action of rifamycin on RNA polymerase from sensitive and resistant bacteria. Biochim. Biophys. Res. Commun. *32*, 284 (1968)

74) White, R.J., Lancini, G.C.: Uptake and binding of ^{3}H-rifampicin by *E. coli* and *Staph. aureus*. Biochim. Biophys. Acta *240*, 429 (1971)
75) Zimmermann, W., Wehrli, W.: Rifampicin resistance in *E. coli:* comparison of microbiological and enzymatic properties. Experientia *33*, 132 (1976)
76) Morishita, T., Yura, T.: Altered nutritional requirements associated with mutations affecting the structure of RNA polymerase in *Lactobacillus casei*. J. Bact. *125*, 416 (1976)
77) Wozny, M.E., Carnevale, H.N., Jones, E.E.: Alteration of regulation of arginine biosynthesis in *E. coli* W by mutation to rifampicin resistance. Biochim. Biophys. Acta *383*, 106 (1975)
78) Nitta, K., Mizuno, S. Yamazaki, H., Umezawa, H.: Streptovaricin and rifampicin resistance of RNA polymerase in a resistant clone of *E. coli* B.J. Antibiotics *21*, 521 (1968)
79) Kondo, M., Oishi, T., Tsuchiya, K.: Tolypomycin, a new antibiotic. V. *In vitro* and *in vivo* antimicrobial activity. J. antibiotics *25*, 16 (1972)
80) Cassani, G., Burgess, R.R., Goodman, H.M., Gold, L.: Inhibition of RNA polymerase by streptolydigin. Nature, New Biol. *230*, 197 (1971)
81) v. d. Helm, K., Krakow, J. S.: Inhibition of RNA polymerase by streptolydigin. Nature, New Biol. *235*, 82 (1972)
82) Reusser, F.: Tirandamycin, inhibition of RNA polymerase. Infect. Immun. *2*, 77, 82 (1970)
83) Sokolova, E.V., Ovadis, M.I., Gorlenko, Zh. M., Khesin, R.B.: Localization of streptolydigin resistant mutation in *E. coli* chromosome. Biochim. Biophys. Res. Commun. *41*, 870 (1970)
84) Wu, G., Dawid, I.B.: Purification and properties of mitochondrial DNA-dependent RNA polymerase from ovaries of Xenopus laevis. Biochem. *11*, 3589 (1972)
85) Follett, E.A.C., Pennington,T.H.: A direct effect of some rifamycin derivatives on the morphology of mammalian mitochondria. Exp. Cell. Res. *77*, 47 (1973)
86) Meilhac, M., Typser, Z., Chambon, P.: Animal DNA-dependent RNA polymerases. 4. Studies on inhibition by rifamycin derivatives. Europ. J. Biochem. *28*, 291 (1972)
87) Busiello, E., di Girolamo, A., di Girolamo, M., Fischer-Fantuzzi, L., Vesco, C.: Multiple effects of rifamycin derivatives on animal-cell metabolism of macromolecules. J. Europ. Biochem. *35*, 251 (1973)
88) Riva, S., Fietta, A. Silvestri, L.G.: Mechanism of action of a rifamycin dervative (AF/013) which is active on the nucleic acid polymerases insensitive to rifampicin. Biochim. Biophys. Acta *49*, 1263 (1972)
89) Medoff, G., Kobayashi, G.S., Kwan, C.N., Schlessinger, D., Venkov, P.: Potentiation of rifampicin and 5-Fluorocytosine as antifungal antibiotics by Amphotericin B. Proc. Natl. Acad. Sci. *69*, 196 (1972)
90) Kobayashi, G.S., Cheung, S.C., Schlessinger, D., Medoff, G.: Effect of rifamycin derivatives, alone and in combination with amphotericin B, against Histoplasma capsulation. Antimicrob. Ag. Chemother. *1974*, 16
91) Battaner, E., Kumar, B.V.: Rifampin: inhibition of RNA synthesis after potentiation by amphotericin B in Saccharomyces cerevisiae. Antimicrob. Ag. Chemother. *1974*, 371
92) Beggs, W.H., Sarosi, G.A., Andrews, F.A.: Synergistic action of amphotericin B and rifampin an Candida albicans. Amer. Rev. Resp. Dis. *110*, 671 (1974)
93) Rifkind, D., Crowder, E.D., Hyland, R.N: *In vitro* inhibition of *Coccidioides immitis* strains with Amphotericin B plus rifampicin. Antimicrob. Ag. Chemother. *1974*, 783
94) Huppert, M., Pappagianis, D., Sun, S.H., Gleason-Jordan, I., Collins, M.S., Vukovich, K.R.: Effect of Amphotericin B and rifampin against *Coccidioides immitis in vitro* and *in vivo*. Antimicrob. Ag. Chemother. *1976*, 406
95) Medoff, G., Kwan, C.N., Schlessinger, D., Kobayashi, G.S.: Permeability control in animal cells by polyenes: a possibility. Antimicrob. Ag. Chemother. *1973*, 441
96) Adman, R., Schultz, L.D., Hall, B.: Transcription in yeast: separation and properties of multiple RNA polymerases. Proc. Natl. Acad. Sci. *69*, 1702 (1972)
97) Wintersberger, E.: Isolation of a distinct rifampicin-resistant RNA polymerase from mitochondria of yeast, neurospora and liver. Biochem. Biophys. Res. Comm. *48*, 1287 (1972)
98) Venkov, P.V., Milchev, G.I., Hadjiolov, A.A.: Rifampin susceptibility of RNA synthesis in a fragile Saccharomyces cerevisiae mutant. Antimicrob. Ag. Chemother. *1975*, 627

99) Moreau, M.-C.: Inhibition of a vesicular stomatitis virus mutant by rifampin. J. Virol. *14*, 517 (74)
100) Remillard, S., Rebhun, L.I.: Antimitotic activity of the potent tumor inhibitor Maytansine. Science *189*, 1002 (1975)
101) Wolpert-Dufilippes, R.K., Adamson, R.H., Cysyk, R.L., Johns, D.G.: Initial studies on the cytotoxic action of maytansine, a novel *ansa* macrolide. Biochem. Pharmacol. *24*, 751 (1975)
102) Green, M., Gerard, G.F.: RNA-directed DNA polymerase properties and functions in oncogenic RNA viruses and cells. In: Progress in nucleic acid res. and molec. biol. Vol. 14, p. 188. New York and London: Academic Press 1974
103) Wu, A.M., Gallo, R.C.: Reverse transcriptase. CRC Critical Rev. Biochem. *1975*, 289
104) Gallo, R.C., Young, S.S., Ting, R.C.: RNA-dependent DNA polymerase of human acute leukaemic cells. Nature *228*, 927 (1970)
105) Gurgo, C., Ray, R.K., Thiry, L., Green, M.: Inhibitors of the RNA and DNA-dependent polymerase activities of RNA tumor viruses. Nature New Biol. *229*, 111 (1971)
106) Szabo, C., Bissell, M.J., Calvin, M.: Inhibition of infectious rous sarcoma virus production by a rifamycin derivative. J. Virol. *18*, 445 (1976)
107) Pennington, T.H., Follett, E.A.C.: Inhibition of pox virus maturation by rifamycin derivatives and related compounds. J. Virol. *7*, 821 (1971)
108) Szilagyi, J.F., Pennington, T.H.: Effect of rifamycins and related antibiotics on the DNA-dependent RNA polymerase of vaccinia virus particles. J. Virol. *8*, 133 (1971)
109) Grimley, P.M., Moss, B.: Similar effect of rifampin and other rifamycin derivatives on vaccinia virus morphogenesis. J. Virol. *8*, 225 (1971)
110) Becker, Y.: Antitrachoma activity of rifamycin B and 8-O-acetylrifamycin S. Nature *231*, 115 (1971)

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Syntheses and Activity of Heteroprostanoids

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Dedicated to Professor Theodor Wieland on the occasion of his 65th birthday (June 1978)

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1. Introduction

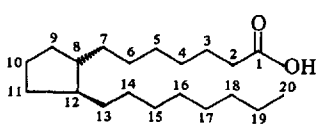
Several reasons are responsible for the fact that prostaglandins – a group of natural occurring substances – have been studied in biochemical, medical and chemical experiments more intensively than any other group of substances in the last years[1–6].

There are few if any classes of compounds that have so many different actions in cells, tissues and organs. Although they may have a wide range of effects, they nevertheless exhibit strict structure-activity specifity, thereby raising the question how those relatively simple molecules can exert so many disparate effects.

The standard nomenclature for this family of readily released fatty acids from tissues and organs and the synthetic prostaglandin-like compounds is derived from the C-20 cyclopentane acid skeleton of the hypothetical prostanoic acid *1*.

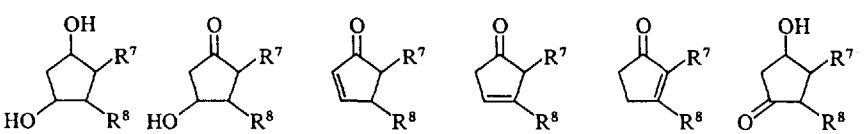
The numbering of the carbon atoms is, according to the nomenclature rules used by the Chemical Abstracts[7, 8], consistent with the numbering for fatty acids. The compounds are typed depending upon the substitution pattern of the cyclopentane ring and classified by the degree of unsaturation in the side chains; see Scheme 1.

E. J. Corey[9] proposed the introduction of the term *Prostanoids* to designate the family of natural prostaglandins and synthetic prostaglandin-like compounds. Following this proposal the authors of this review want to designate the prostaglandin-like compounds which contain one or more heteroatoms in the prostanoic acid skeleton at the place of one or more carbon atoms as *Heteroprostanoids.* The



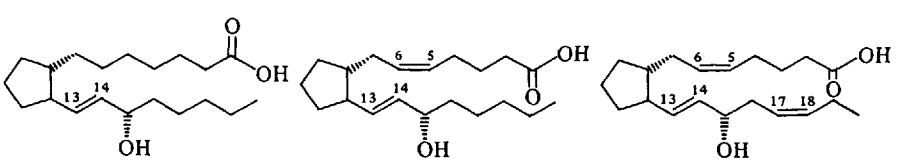
1

Types (ring dependent)



F *E* *A* *C* *B* *D*

Unsaturated classes (number of carbon–carbon double bonds)



1 *2* *3*

(mono) *(bis)* *(tris)*

Scheme 1. Classification of prostanoids

following review is concerned with syntheses leading to these not naturally occurring substances and comparison of their biological activities, as far as they have been reported.

Before starting to present this fascinating chapter of chemistry, we have to mention, however briefly, the biological potencies of the natural occurring prostanoids[10]. The wide range of biological response they produce and the potency of action they have was and is still yet the main driving force behind the enormous synthetic effort all over the world.

After their discovery and after von Euler[11] had coined the term prostaglandins which was in fact a misnomer, the prostanoids remained unnoticed for a long period of time. The situation changed however completely, when S. Bergström and his co-workers[12] succeeded in isolating the active compounds and elucidating their chemical structures. Before satisfactory studies of their pharmacologic effects could be concluded, methods for the preparation of adequate quantities of relatively pure prostanoids had to be evaluated.

Extraction and purification of prostanoids from animal tissues was insufficient because the tissue levels are very low.

The development of bioconversion processes from particular polyunsaturated fatty acids[13, 14] yielded however sufficient amounts of various prostanoids to study their chemistry[15] and biology and allowed preliminary clinical evaluation. With the rapidly increasing knowledge of their pharmacologic actions the chances of a practical medical use for induction of labor at term[16], for inducing therapeutic abortion[17] for fertility control in man[18] and farm animals[19] for treatment of thrombosis and for certain types of stomach ulcers[20] rose.

The recent introduction of PGF_2 as *Prostin* $F_{2\alpha}$® is an example for the clinical application of a natural occurring prostanoid.

Serious drawbacks of the natural occurring compounds however do not allow their general use in therapy. The rapid metabolic deactivation[21–25] and the too wide range of activity are the main problems which have to be overcome. The development of other clinical applications awaits further progress in prostanoid research and this meant a challenge to the synthetic chemists to develop chemical total syntheses which could effectively compete with biosynthesis and which could be easily modified in order to prepare analogs with improved selectivity and stability to metabolic deactivation.

For planning and conducting these total syntheses the exact knowledge of the chemical properties of the prostanoids[15] which had been studied with biosynthetic prepared prostanoids proved to be very helpful. Relatively soon it was discovered how prostanoids can be metabolised and which parts of the molecules are the targets of metabolic inactivation. Metabolism is mainly confined to the side chains. β-Oxidation of the acid side chain leads to the essentially inactive bis- and tetra-nor acids and the oxidation of the 15-hydroxygroups results in the formation of inactive 15-ketones[26–29].

The plans for effective synthesis therefore aimed at common intermediates suitable for the synthesis of the natural prostanoids in high yield and for the synthesis of desirable analogues with variations in the molecule around those areas where metabolic variations occur.

Many groups of synthetic chemists have succeeded in the meantime in solving the problem of total synthesis of the natural prostanoids by developing more or less elegant routes[30a, 30b, 31–33]. The total synthesis developed by Corey[34] however which has been modified in many ways[35] is without doubt the most successful and most commonly used synthetic method in the prostanoid field.

There is no other synthesis which allows both the preparation of all natural prostanoids even in the optically active form[36, 37a, b] as well as the variation of the structure of the prostanoids in a very wide range.

At the same time as the attempt to slowdown or prevent the fast metabolism and the inactivation of prostanoids through chemical variations of the side chains, was the hope also to find substances which act more specific and longer with lower dosis. Many thousands of analogues have already been synthesized all over the world by variation of Corey's method. The first successes to prevent the metabolic oxydation of 15-OH as much as possible came with the introduction of the methyl group in position C_{15}[38] and/or C_{16}[39] and the partial substitution from the pentyl chain (C_{17}-C_{20}) by aromatic ring systems[40, 41]. For example, it was proved that these compounds were no substrates for the 15-hydroxydehydrogenase (isolated from human placenta)[42, 43]. The β-oxydation of the acid side chain can be suppressed by substitution of the C_3-methylengroup through an oxygen atom or through alkyl-[44] or halogensubstituents[45] in the C_3-position. In all of these cases an extension of activity and a partial specifity of activity could be observed.

Parallel to these concepts with biochemical background other research groups proceeded purely phenomenological. In this way the cyclopentane ring was substituted either by a cyclohexane ring[46, 47] or cyclobutane ring[48] or by 5 or 6 ring aromates[49–52]. These analogues show no or only very minor biological activity. Apparently by these analogues the special arrangement of the side chains responsible for the activity is changed to much a degree that no or only insufficient receptor bonding is possible.

N. H. Andersen *et al.*[53–55] succeeded in their attempt to qualify the structure-activity relationship. Through their research results they proved the concept, that the decisive prerequisite for the specific biological activity of each prostanoid is to achieve easily a "hairpin" conformation in which both side chains are arranged in a certain way to each other.

It can be seen from model observations that this hairpin conformation is only then optimally attained, if both side chains are bonded to a cyclopentane, cyclopentene, cyclopentanone or cyclopentenone. Therefore the chances to obtain a final molecule showing good biological activity seemed to be optimal, when the prostanoid ring system is kept intact, while the ring substitution is varied.

This was experimented, for example, by introducing methyl groups into the positions C_8[56] C_9[57], C_{10}[58, 59], C_{11}[57] and C_{12}[60–62]. The Syntex research group under Crabbé tried to improve activity by shifting the 5-ringhydroxyl group(s)[124] or by introducing an additional hydroxylgroup[125, 126]. Finally concepts of synthesis were developed to vary the side chains.

Even though at the present time no conclusive judgement can be made about the most favorable or acceptable changes with improvement of activity, nevertheless the variation of the side chains seems to be the most promising.

In accordance with published and our own results the prostanoid analogues should show biological activity, when the entire molecule conformation does not differ greatly from that of natural prototype.

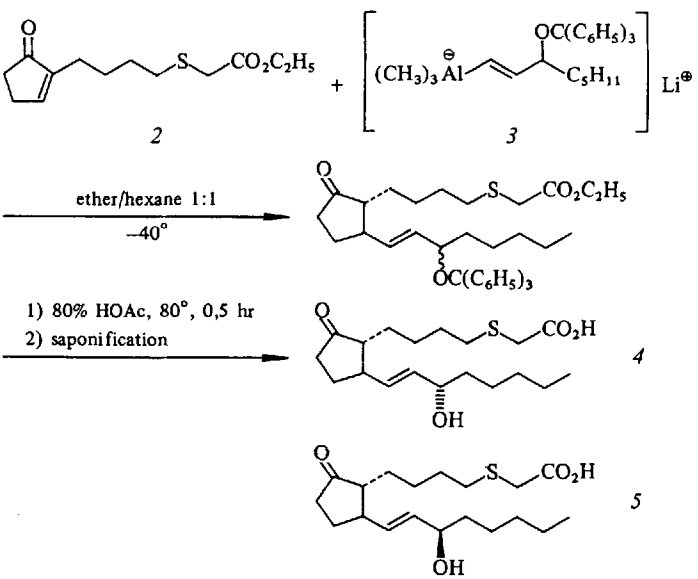
The substitution of one or more C-atoms of the prostanoids acid structure by hetero atoms is one of the conceivable possibilities to fulfill this assumption. In the following these ideas of synthesis will be presented and the attempt made as much as possible, to find out by using published biological data, where the introduction of hetero atoms caused qualitative and/or quantitative variations in activity.

2. Synthesis of Heteroprostanoids

2.1. 3-Thioprostanoids

K. F. Bernady and co-workers[63] from the Lederle Laboratories have described a useful procedure for the synthesis of *dl*-11-deoxy-3-thiaprostanoids based upon the conjugate addition of E-l-alkenyl ligands from lithium E-l-alkenyltrialkylalanate reagent *3* to the sulfur containing cyclopentenone (2)[64].

It was found that the "ate"-complex formed by treatment of alkenyl-lithium with trialkylaluminum conjugatively transfers the alkenyl ligand to cyclopentenone in relatively good yield. It is noteworthy that the total yield of the expected product depends on the solvents used. The results – obtained in an analogous reaction – indicate that the addition in hydrocarbons gives as by-products more 1,4-reduction product than cyclopentenone polymer. On the other hand the cyclopentenone derived polymer becomes significant in the more basic THF. The michael addition

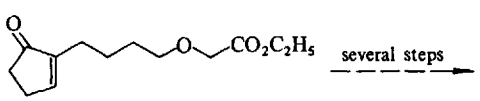


obviously afforded only products in which the side chains are exclusively *trans* orientated. The *trans*-configuration of the double bond in the lithium alanate reagent can be taken for granted. That means, that this process is accompanied by retention of configuration. Protolytic work up of the reaction mixture, followed by detritylation, dry column chromatography upon silica gel and saponification gave in 27% yield the C_{15}-epimers, *dl*-11-deoxy-3-thia-PGE_1, *4* and *dl*-15-epi-11-deoxy-3-thia-PGE_1, *5* in a ratio of approximately 45:55.

The biological activities of these new analogs are not reported until now.

2.2. 3-Oxaprostanoids

The stereospecific synthesis of 3-oxa-prostanoids is described in the same publication as for 3-thia-prostanoids by K. F. Bernady *et al.*[63]. The principle of this synthesis is also analogous. In that case, the starting material is the corresponding 3-oxa-cyclopentenone, *6*. Work up, followed by deprotection of the ether, chromatography and saponification gave in 38% yield the free acids of *dl*-11-deoxy-3-oxa-PGE_1 *7* and its C_{15}-epimer *8*, *dl*-15-epi-11-deoxy-3-oxa-PGE_1, in a ratio of approximately 45:55.



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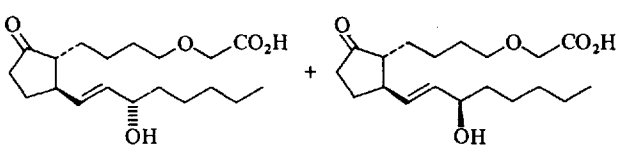
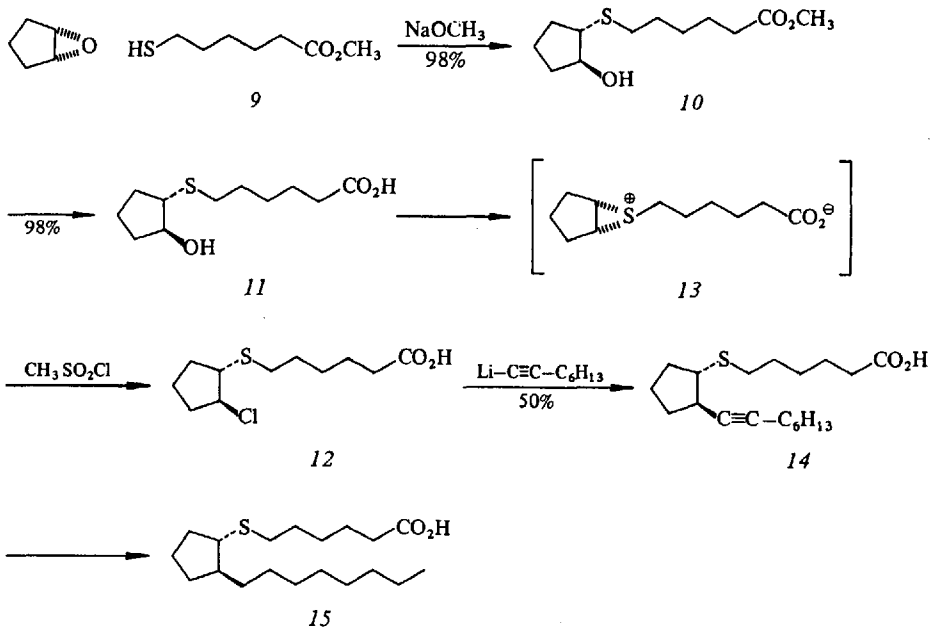


7 *8*

2.3. 7-Thiaprostanoids

J. Fried *et al.*[65] reported a stereospecific synthesis of nat.-7-thia-$PGF_{1\alpha}$, *24*, ent-15-epi-7-thia-$PGF_{1\alpha}$, *25*, and racemic 7-thia-13-prostynoic acid *14*. The elaboration of the basic skeletal structure is exemplified by the synthesis of *14*, which is compatible with the additional functionality required for *24* and *25*.

Reaction of cyclopentene oxide with mercaptohexanoate *9* in the presence of sodium methoxide in methanol at room temperature produced the *trans*-hydroxyester *10*. This ester was hydrolyzed to the acid *11*, which was treated with methanesulfonyl chloride in pyridine and afforded the *trans*-chloro acid *12* in 82% yield. The fact, that no *cis*-chloro acid was obtained is an evidence for the formation of a symmetrical episulfonium intermediate *13*.

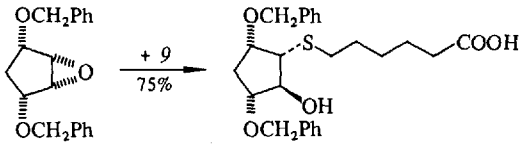


The sodium salt of the chloro acid *12* was converted to racemic 7-thia-13-prostynoic acid *14* by reaction with 5 equivalents of 1-octynyllithium in DMF. Catalytic reduction of *14* with excess palladium in ethyl acetate afforded the cristalline 7-thia-prostanoic acid *15*, m.p. 40–41 °C. This synthetic procedure was successful also in the synthesis of 7-thia-$PGF_{1\alpha}$, *25*. Reaction of the protected dihydroxyepoxide *16* with the anion of methyl-6-mercaptohexanoate *9*, followed by hydrolysis furnished the hydroxy acid *17*. This acid was converted in two steps into the bromo acid *18*. The sodium salt of *18* was treated with (S)-3-*tert.*butyloxy-1-octynyllithium in DMF/hexane at room temperature to form, after chromatography, in 33% yield the mixture of diastereomeric acids. After conversion into the methyl esters, debutylation and reduction with lithium alanate in boiling THF the chromatographic separation gave the corresponding diastereomeric alcohols *20* and *21*.

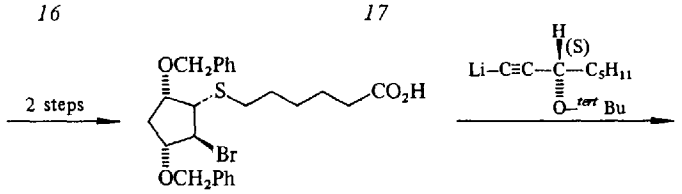
Debenzylation was achieved in 63% yield by converting the alcoholic groups to their anions with sodium hydride in THF and followed by reduction with lithium in ammonia-THF at −78 °C.

A crucial step in this synthesis is the selective oxidation of the tetrols *22* and *23* but the application of a very interesting method, published by J. Fried and J. C. Sih[66] with platinium in aqueous acetone in the presence of sodium bicarbonate gave *24* and *25*, respectively, in 50% yield. The absolute configuration of the products *24* and *25* were carefully determined by chemical and physical methods.

All of these three 7-thia-prostanoids show interesting biological activities. 7-Thia-13-prostynoic acid *14* is an inhibitor of the contraction of the gerbil colon, and of the stimulation of adenylate cyclase in the mouse ovary caused by prostaglandin E_1. This inhibition is as effective as for the 7-oxa-analog. Compound *14*



16 17



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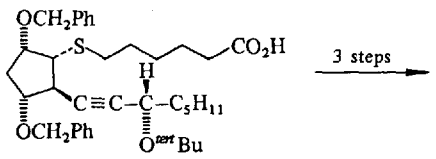

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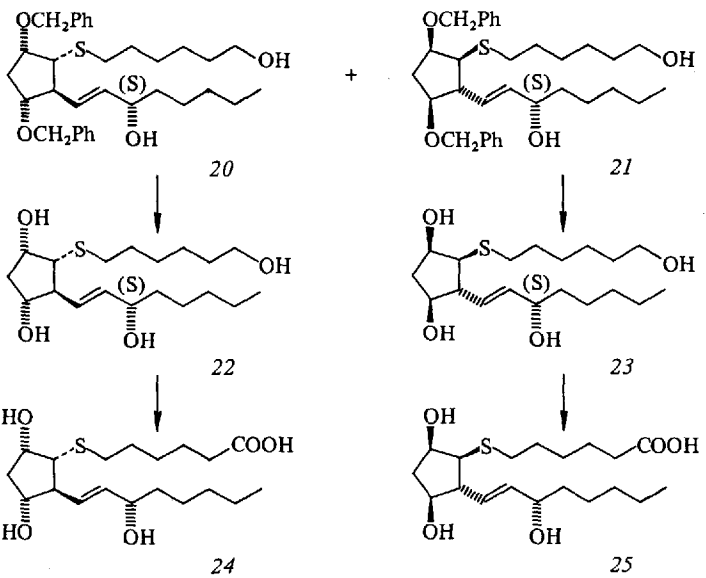


m.p. 94–96°C
$[\alpha]_D$ + 5.50° (c 0,43; CH_3OH)

oel
$[\alpha]_D$ – 4,4° (c 0,27; CH_3OH)

inhibits also the placental prostaglandin-dehydrogenase 5–10 times more than the 7-oxa-analog[67].

The 7-Thia-prostanoid with the natural configuration of all chiral centers, *24*, stimulates the c-AMP synthesis in the mouse ovary, whereas *25*, which possesses

unnatural configuration at four centers shows no remarkable activity in this model. The heteroprostanoid *24* shows binding to a bovine corpus luteum receptor with 1/10th of the affinity of $PGF_{1\alpha}$, while *25* exhibits 1/100th the binding of *24*. Both, *24* and *25* are inhibitors of the placental prostaglandin-15-dehydrogenase at $(I)_{50}$ = 5,2 μM and $(I)_{50}$ = 8,8 μM, respectively.

2.4. 7-Oxaprostanoids

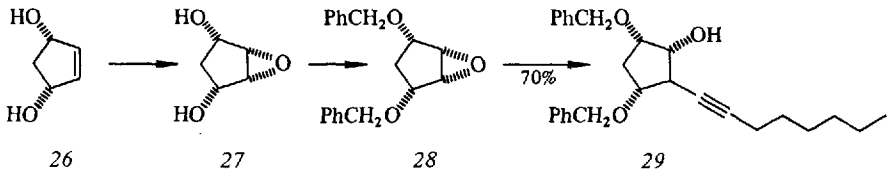
The structural similarity of $PGF_{1\alpha}$ and the substance in which the 7-methylene group has been replaced by ether oxygen, stimulated the research group of J. Fried (University of Chicago) to look for a stereo-specific approach for the synthesis of 7-oxaprostanoids[68]. Fried was fascinated by the fact, that the stereochemistry of the 7-oxaprostanoid and that of $PGF_{1\alpha}$ is fully analogous and that its geometry differs only slightly because the C–O–C bond angle is somewhat larger (111,5 °C) than that described by the C–C–C bond (109,5 °C). The proximity of the 7-ether group and the 9-hydroxylgroup enables the formation of a stable hydrogen bond in the 7-oxaprostanoid which could be responsible for conformational changes. The known differences in activity between natural prostanoids of the E and F series which likewise differ in the same region of the molecule which was planned to modify in Fried's work made it very interesting to study the biological properties of these molecules.

The synthesis[69] started with *cis*-cyclopentene-3,5-diol[70] *26* which had been obtained in a stereospecific reaction by addition of singlet oxygen to cyclopentadiene and reduction of the intermediate cycloperoxide.

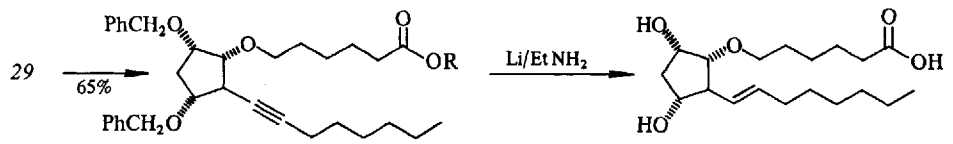
The oxydation of the *cis*-diol gave exclusively the all-*cis*-oxido-diol[71] *27* which was converted to the cristalline dibenzylether *28*. For the introduction of the eight carbon chain with and without a 15-hydroxyfunction Fried and his co-workers developed a very elegant aluminium-organic method. They succeeded in conversion of the 3,5-dibenzyloxyepoxide by a very efficient alkynylation reaction into the acetylenic alcohol *29*. They could show that the opening of the epoxide ring had occurred exclusively with the formation of the *trans* alcohol, as expected.

This intermediate was alkylated with *tert.*-butyl-ω-iodohexanoate to the ester *30*. Conversion to the acid *31* was achieved by cleavage of the *tert.*-butylester with trifluoroacetic acid at low temperature. The triple bond was reduced to a *trans*-double bond and simultanously the benzylether groups had been removed with lithium in ethylamine, under formation of the desired 15-deoxy-7-oxaprostaglandin $F_{1\alpha}$ *32* in crystalline form. The *cis*-isomer was prepared by first reducing the triple bond of compound *30* with palladium on barium sulfate to *33*, removal of the *tert.*-butylgroup with formic acid to *34* and debenzylation of the acid with lithium in ethylamine to *35*.

The fully saturated analogue was synthesized by catalytic reduction of *30* with palladium on charcoal in ethyl acetate to *36* and hydrogenolysis of the benzylether groups with palladium on charcoal in acetic acid to *37*. The *tert.*-butylester function was then cleaved with trifluoroacetic acid in hexane to *38*.

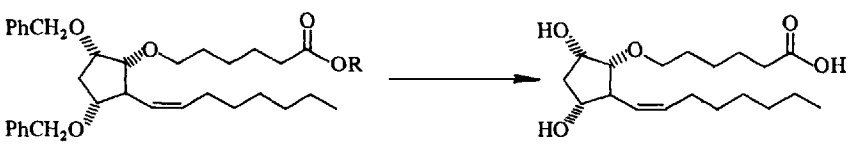


26 *27* *28* *29*



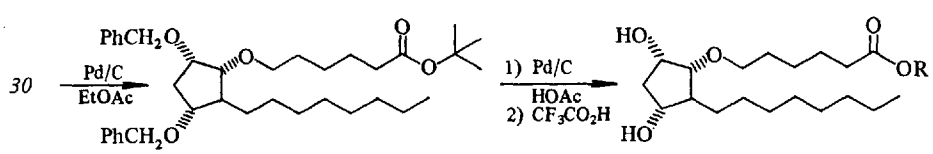
30 R = *tert.*-butyl
31 R = H
$Pd/BaSO_4$

32



33 R = *tert.*-butyl
34 R = H

35



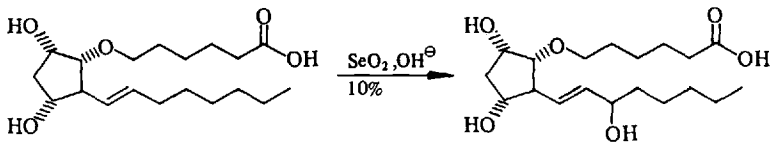
36

37 R = *tert.*-butyl
38 R = H

The introduction of the 15-hydroxygroup was possible by hydroxylation of *32* with SeO_2 [69] to yield the (±)-7-oxa-$PGF_{1\alpha}$ or its 15-epimer as a crystalline substance *39*.

Although the 7-oxa-$PGF_{1\alpha}$ obtained by the sequence mentioned above has the absolute configuration of the natural prostanoids the substances synthesized were racemic because the sequence of reactions was carried out on racemic material.

The synthesis of (+) -7-oxa-PGF_1 and (+) -7-oxa-15-epi-PGF_1 and their enantiomers was then started by Fried and his co-workers[72] by using the opening of the meso-cyclopentene oxide *28* with a dialkylalkynylalane *45* which contained the completely functionalized eight-carbon side chain in optically active form.



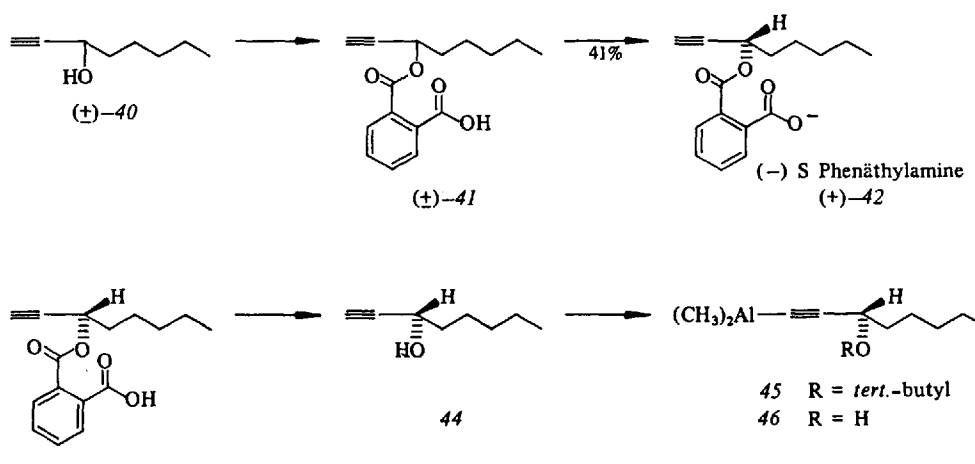
32 *39*

This led to the formation of two diastereomeric alcohols *47a* and *47b* which were readily separated by chromatography.

This procedure required but one resolution of the acetylenic alcohol *40* which then served to resolve the remaining chiral portion of the molecule. The resolution of octyn-3-ol *40* therefore was the start of the synthesis of the optically active 7-oxaprostanoids. Reaction of the racemic octyn-3-ol *40* with phthalic anhydride gave the phthalyl acid *41* which formed the crystalline salt *42* by reaction with (-)-α-phenethylamine with the absolute configuration shown.

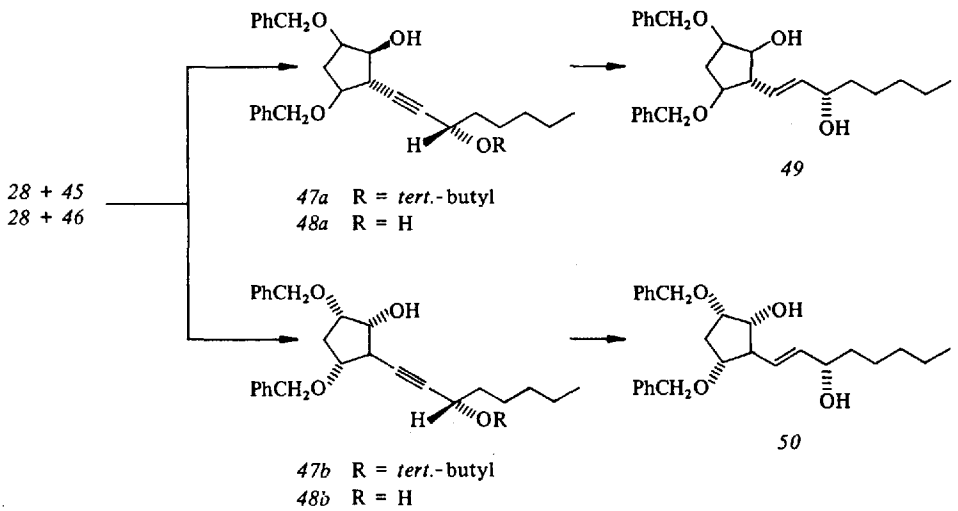
Optically active octyn-3-ol was obtained by first converting the salt *42* to the ester acids *43* and then hydrolysis of the ester to compound *44*. Optical purity and assignment of the absolute configuration as *S* was established by methods known from analogous compounds from the literature. Fried and his co-worker proved that preparation of the *tert.*-butyl-ether derivatives and deprotection with trifluoroacetic acid is possible without a trace of racemisation. This guarantees that the *S* (normal) or the *R* (epi) 15-hydroxyprostanoids can be synthesized by using the resolved *tert.*-butylethers in the alkylation reaction.

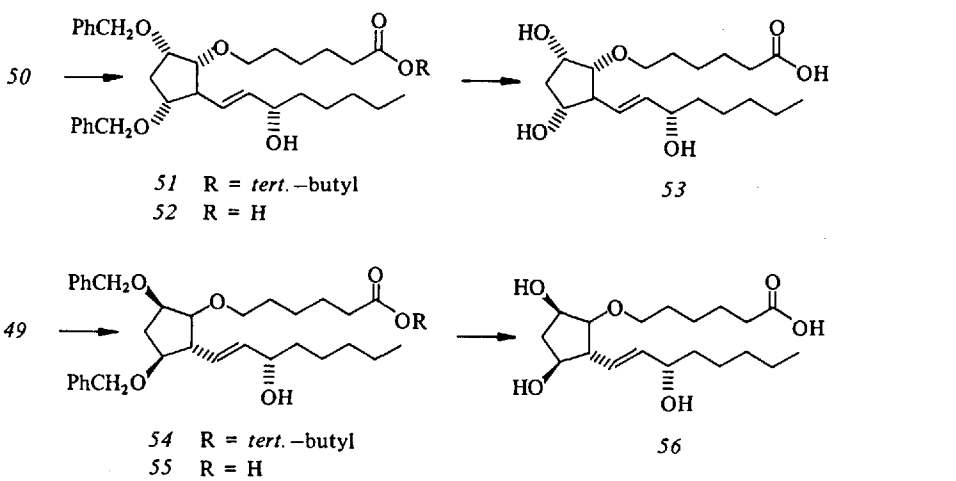
Dimethyl (*S*)-(-)-3-*tert.*-butyloxy-1-octynylalane *45* was therefore prepared from (*S*)-(-)-3-hydroxy-1-ocyne *44* with isobutylene in methylene chloride, followed by lithiation and reaction with dimethylchloroalane. Condensation of all-*cis*-cyclopentane-3,5-dibenzylepoxide with this reagent in toluene formed the mixture of diastereomeric butylethers *47a* and *47b*.



Debutylation gave the acetylenic alcohols *48a* and *48b* which could not be separated by chromatography. The acetylenic alcohols could be prepared also directly by reaction of the epoxide with the free alcohol *46* instead of the *tert.*-butylether reagent *45*.

Reduction with $LiAlH_4$ in THF produced a mixture of olefins which was readily separated by chromatography into the diastereomeric olefinic alcohols *49* and *50*. The olefinic diols were alkylated with remarkable specificity for the ring alkylation to *51* and *54* with *tert.*-butyl-ω-iodohexanoate using dimsyl anion in DMSO. Cleavage of the ester function to *52* and/or *55* and respectively debenzylation furnished after column chromatography on silica gel crystalline (+)-7-oxa-PGF_1 (mp. 65–67 °C and *53* (+)-15-epi-7-oxa-PGF_1 *56*. Repeating the sequence of





reactions with (R)-(+)-3-*tert*-butyloxy-1-octynyldimethylalane instead of its antipode furnished (−)-7-oxa-$PGF_{1\alpha}$ and (−)-15-epi-7-oxa-$PGF_{1\alpha}$.

On the way to 7-oxa-PGE_1 [123] starting with the same all-*cis*-1,2-epoxycyclopentane-3,5-diol *27* as used for the synthesis of 7-oxa-PGF-derivatives, Fried and co-workers utilized some unusual selective reactivity of polyhydroxylated cyclopentanes.

The disilyl derivative of all-*cis*-1,2-epoxycyclopentane-3,5-diol *57* was treated with diethyloctynylalane to afford after cleavage of the silylether function the triol *58*. This triol was convered *via* the acetonide to the benzyl ether *59*. Hydrolysis with aqueous trifluoroacetic acid yielded the diol benzylether *60* which could be prepared by an alternative route as well. This route proceeds *via* the monotrityl epoxide *61* and benzylation to the trityl benzylether *62* and then reaction with diethyl octynyl alane to the diolbenzylether *60* and the isomeric 1,3-diol.

Alkylation of the diol as mentioned above with *tert*-butyl-ω-iodo-hexanoate gave the desired ether *63* and the isomeric ether, readily separated by chromatography.

The corresponding acid *64* was prepared with anhydrous trifluoroacetic acid. This same acid was also obtained by selective debenzylation of the above mentioned dibenzylether *30*, which links this synthesis with that of 7-oxa-$PGF_{1\alpha}$. The keto acid *65* was prepared with Jones reagent and the ketogroup then protected as the ethylene ketal by converting the acid simultanously to the ester *66*.

Alkaline hydrolysis led to the acid *67* which was debenzylated and reduced with lithium in methylamine to the olefinic acid *68*. Introduction of the 15-hydroxy group *69* into the 7-oxaprostanoid was possible as in the PGF_1 series with SeO_2. (±)-7-oxa-PGE_1 *70* and its 15-epimer were then obtained by removal of the ketal group with trifluoroacetic acid and chromatography on silica gel columns.

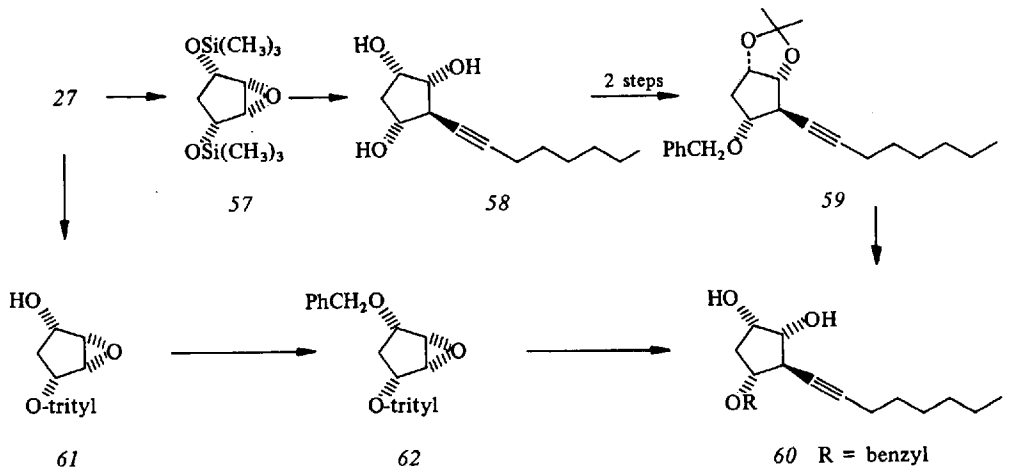
The final product obtained ca. 10% of 7-oxa-PGA_1 the product of the β-elimination of the 11-hydroxy group. This β-elimination took place also in neutral solvents that means that this reaction is more easily happening than in the series of natural E-prostanoids. Besides the above mentioned syntheses of 7-oxaprostanoids of the E and F series Fried and its co-workers synthesized quite a lot of 7-oxaprostanoids with different structures.

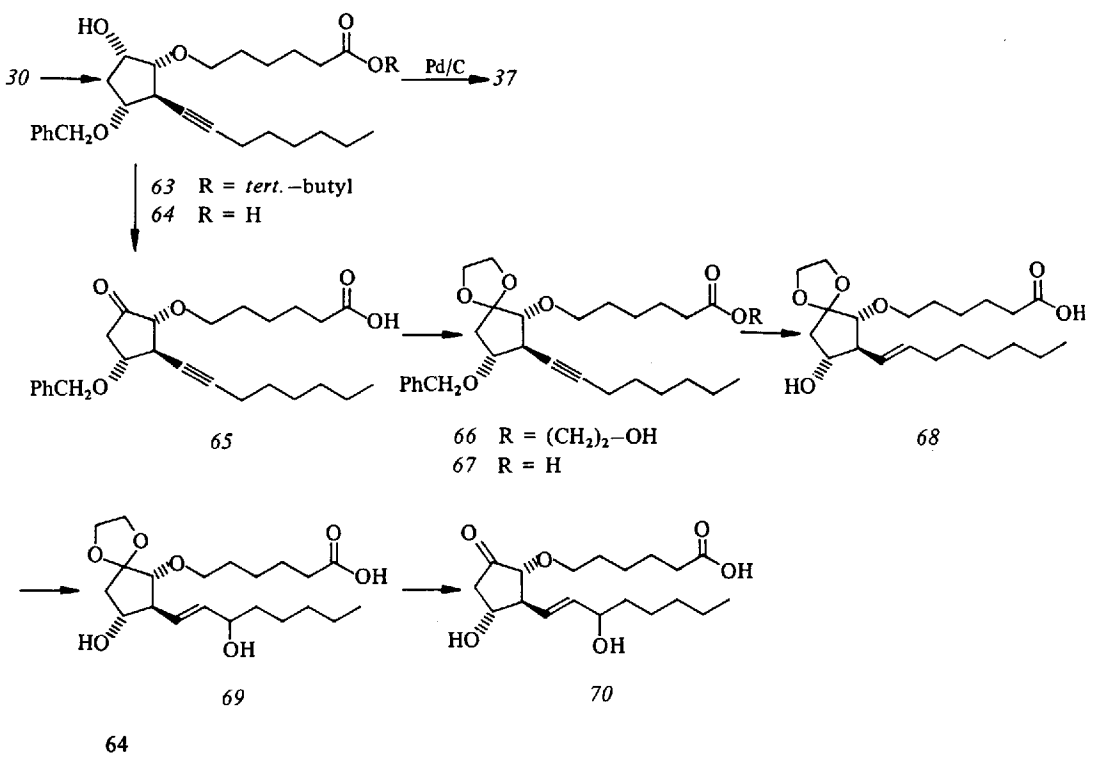
These syntheses will not be reported here and those who want to know more details about the preparation of these derivatives can find these in the original papers published by Fried and co-workers.

2.5. 8-Azaprostanoids

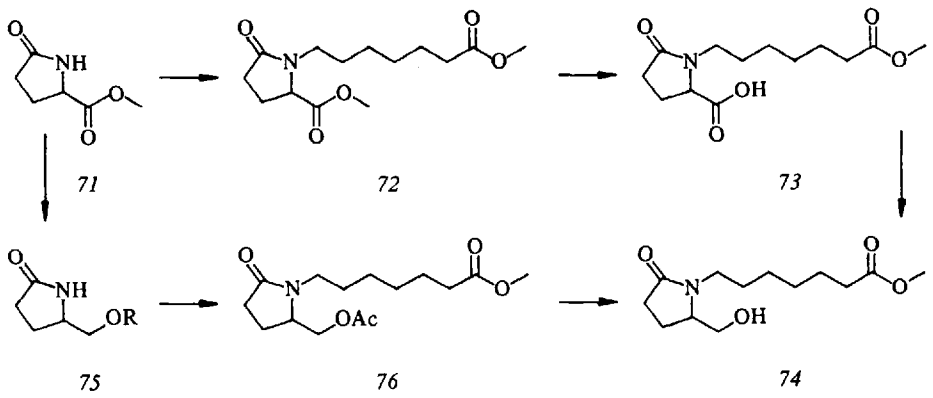
Two very similar independent approaches to 11-Deoxy-8-azaprostanoids have been published in 1975 [73, 74].

Both synthetic routes started with methyl pyroglutamate *71* that means from a starting material already containing the heterocyclopentanone system. Introduction of the side chains was achieved in different ways. G. Bollinger and Joseph M. Muchowski [73] prepared the half acid *73* by first N-alkylating the sodium salt of the pyroglutamate with methyl-7-bromoheptanoate and then selective hydrolysis of


27
OSi(CH3)3
OSi(CH3)3
57
HO
OH
HO
58
2 steps
O
O
PhCH2O
59
HO
O
O-trityl
61
PhCH2O
O
O-trityl
62
HO
OH
OR
60 R = benzyl


30
HO
O
OR
Pd/C
37
PhCH2O
63 R = tert.–butyl
64 R = H
O
O
OH
PhCH2O
65
O
O
O
OR
PhCH2O
66 R = (CH2)2–OH
67 R = H
O
O
O
OH
HO
68
O
O
O
OH
HO
OH
69
O
O
O
OH
HO
OH
70

64



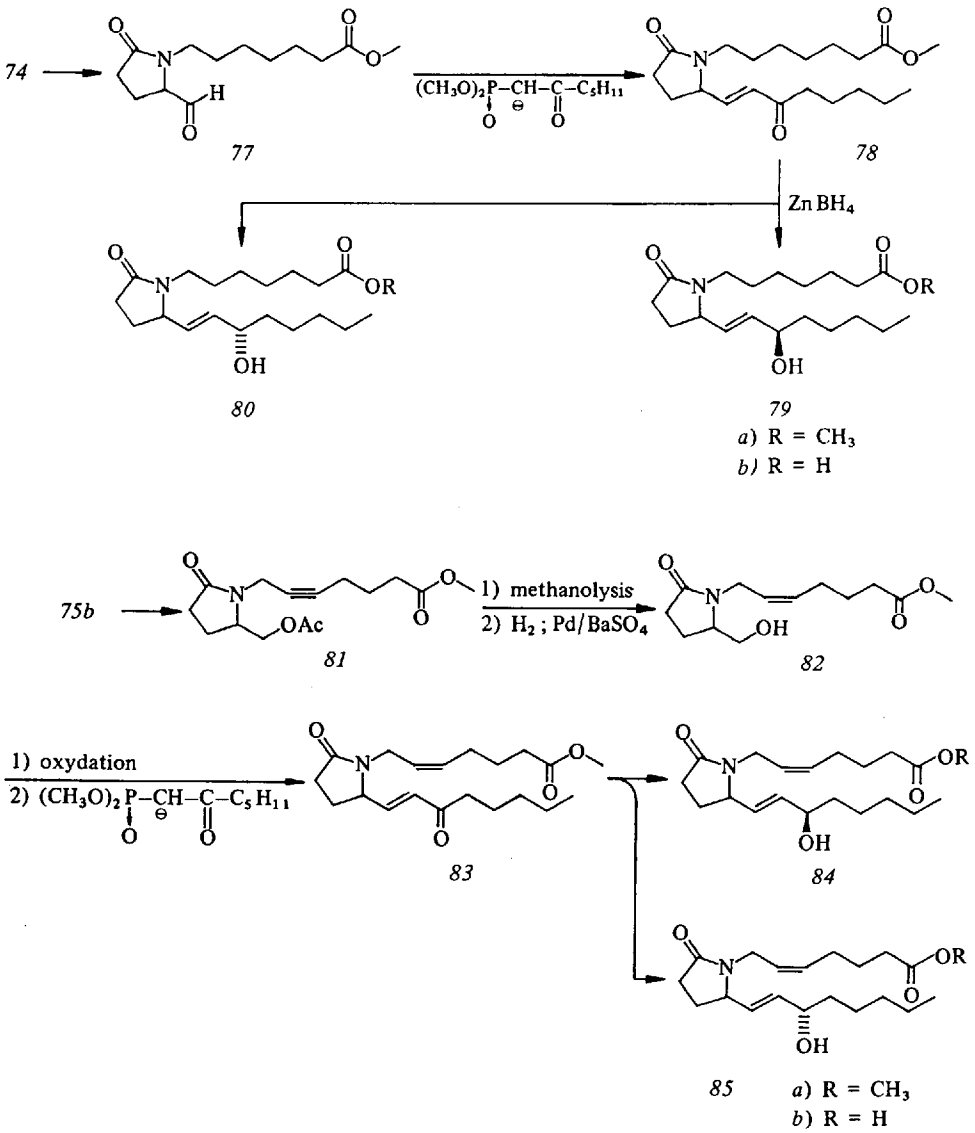
a) R = H
b) R = OAc

the diester *72*. Reduction of the acid *73 via* the mixed anhydride led to the primary alcohol *74* which was used for preparation of the enone *78* by Wittig-Horner reaction similar to the way reported by Corey and others for introduction of the "lower" side chain during the syntheses leading to prostanoids without hetero atoms.
The same intermediate *74* was synthesized by J. W. Bruin *et al.*[74] by an alternative route. They reduced the ester function of the pyroglutamate with $LiBH_4$ to the primary alcohol *75a* which was protected as the acetate *75b* to prevent O-alkylation. Reaction of the sodium salt of *75b* formed with NaH in dimethylformamide, with methyl-7-bromoheptanoate followed by methanolysis of the acetate function gave *74*. Oxydation of the alcohol *74* by Pfitzner-Moffat-oxydation or oxydation with Collins reagent led to the unstable aldehyde *77*.

Both teams reduced the enone to the mixture of the C_{15}-epimeric alcohols and separated the mixture by preparative TLC on silica gel or by column chromatography into a more polar *80* and a less polar *79* isomer with very similar spectral data.
The relative configuration at C_{15} was tentatively assigned to be as in the natural prostanoids to the more polar isomer by analogy with the chromatographic behavior of similar derivatives of prostanoids reported in the literature.
In addition G. Bollinger and Joseph M. Muchowski took the chemical shift in the ^{13}C NMR in which the α-isomer had resonance of carbon-13 at a lower field than the β-isomer as another strong argument for the correlation of the more polar isomer to the α-series.

J. W. Bruin *et al.* were able to prepare the 11-deoxy-8-aza-PGF_2, too by alkylating *75b* with methyl-bromo-5-heptynoate to *81*, methanolysis of *81* and partial catalytic hydrogenation of the triple bond to compound *82*. The next steps *83* → *84* and *85* were analogous to the procedure used for the synthesis of the 11-desoxy-PGE_1-series.

The fact that the more polar ester or acid was more active in several biological assays and that only the analogs *80* and *85* but not *79* or *84* had been shown to



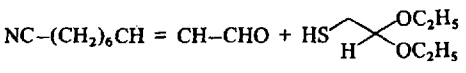
be substrates for 15-hydroxy-prostaglandin dehydrogenase supports further that the assignments mentioned above were correct.

2.6. 9-Thiaprostanoids

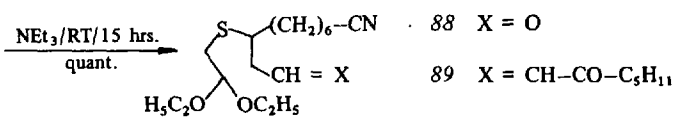
J. Vlattas *et al.*[75] from the Ciba-Geigy-Corporation, USA, have considered the replacement of the C_9-carbinol or C_9-ketone functionalities by a sulfur atom in the prostanoic skeleton.

The preparation of these compounds is an acceptance and extension of the scheme previously used in the syntheses of natural according prostanoids by E. J. Corey and co-workers[76].

The michael addition of mercaptoacetaldehyde diethyl acetal *87* and 9-cyanononenal *86* in the presence of triethylamine gave the adduct *88* quantitatively. 1-Tributylphosphoranylidene-2-heptanone was reacted with the aldehyde and produced the conjugated enone *89*, which ketalizated with four equivalents of ethylene glycol and catalytic amounts of *p*-toluenesulfonic acid in refluxing benzene to the bisdioxolane *90*. Surprisingly, the isomeric bisdioxolane with the double bond at positions $C_{13,\ 14}$ was not detected.

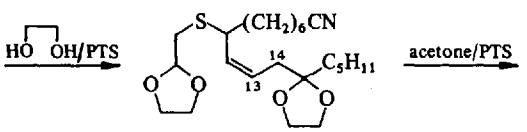


86 *87*

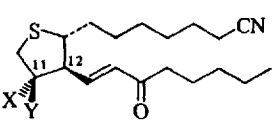


88 X = O

89 X = CH–CO–C_5H_{11}

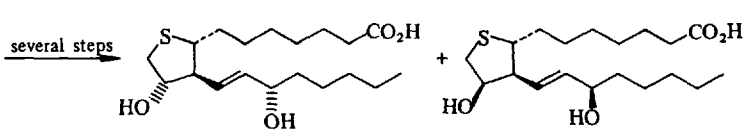


90

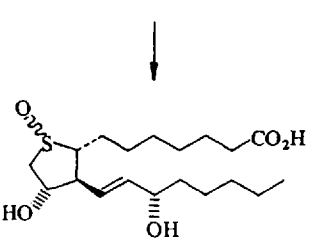


91 X = OH; Y = OH

92 X = H; Y = OH

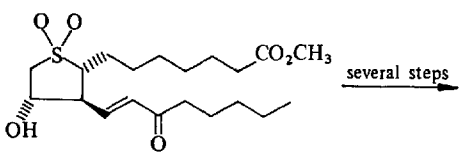


93 *94*

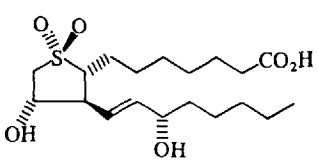


95

The intermediate *90* was treated with a small amount of PTS in acetone at room temperature followed by chromatographic separation of the reaction mixture. The enone *91* is the less polar epimer and its isolated yield, obtained by Vlattas *et al.* is two times more than *92*. The stereochemical assignments in *91* and *92* were made by virtue of the difference between the resonances of their low field olefinic protons. In the isomer with 11β, 12β-configuration, *92*, the low field olefinic proton appears 0,31 ppm downfield of the 11α, 12β-configurated isomer *91*. The upfield olefinic protons were in approximately the same position.
Reduction of *91*, respectively *92*, with zinc borohydride, separation of the C_{15}-isomers, followed by hydrolysis gave (*dl*)-9-deoxy-9-thiaprostanoid *93* and (*dl*)-11,15-epi-9-deoxy-9-thia-prostanoid *94*. Oxidation of *93* with sodium periodate produced a mixture of two epimeric sulfoxides *95*, which were partially separated by preparative thin layer chromatography.
In another paper J. Vlattas and co-workers[77] published an alternative synthetic route leading to the preparation of racemic *93*, as well as optically active forms of 9,9-dioxy-9-thia-analog, *96*.
The enantiomeric forms of *96* were obtained by resolution of the intermediate enone *97*. Esterification of this sulfone with R-(−)-α-methoxybenzeneacetyl chloride, produced a mixture of diastereomers which were separated by preparative TLC. Each diastereomer was converted to the enantiomeric alcohols by zinc borohydride reduction, followed by chromatography of the C_{15}-epimeric mixtures. Saponification of the diolester gave the enantiomeric 9,9-dioxy-9-thia-prostanoids *96*.



97

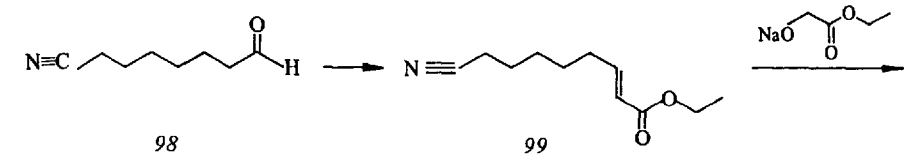


96

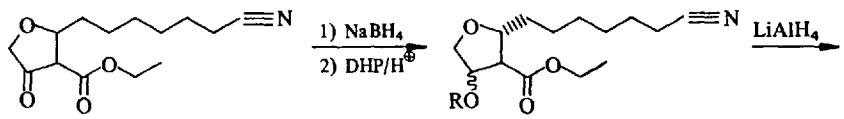
2.7. 9-Oxaprostanoids

The displacement of the C-9 by an oxygen atom has been planned by a research group of Ciba-Geigy[78].
Their synthetic approach for the construction of the tetrahydrofuranone ring started with 7-cyanoheptanal[79] *98* which was converted in the first step into 9-cyano-2-nonenoate *99* by reaction with sodium triethyl phosphonoacetate.

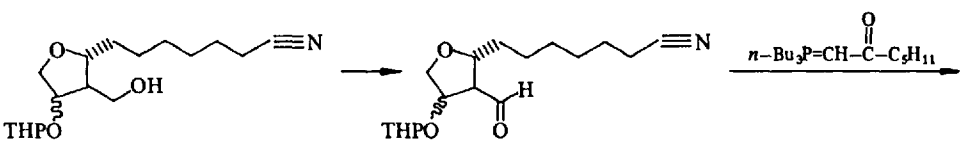
Addition of ethyl sodium glycolate to the β-unsaturated ester *99* led to the formation of the tetrahydrofuranone derivative *100*. The same reaction was used for the synthesis of 11-oxaprostanoids[80, 81]. This intermediate was reduced to a mixture of epimeric alcohols *101* which were protected as the tetrahydropyranylethers *102*. The ester group was then reduced to the primary alcohol function with $LiAlH_4$, the alcohol *103* oxydized with CrO_3 in pyridine to the aldehyde *104* and the aldehyde condensed with 1-tributylphosphoranylidene-2-heptanone to the epimeric prostanoid enones *105* and *107*, which were separated by preparative TLC on silica



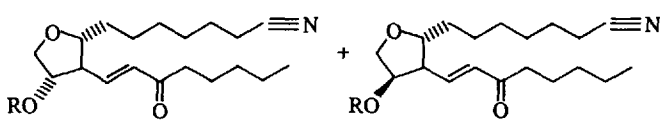
98 *99*



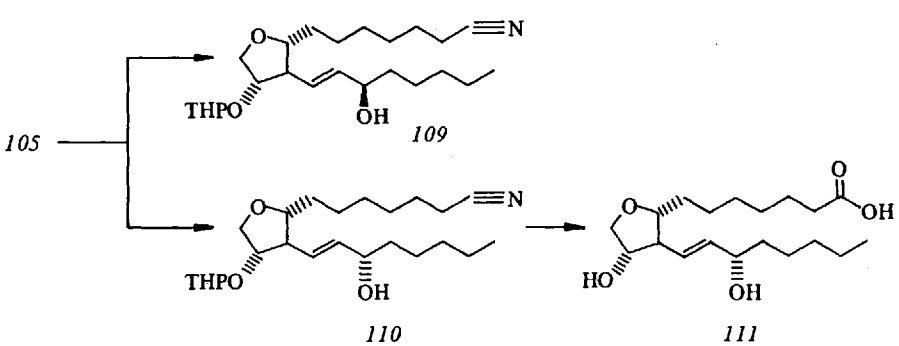
100 *101* R = H; *102* R = THP



103 *104*



105 R = THP; *106* R = H; *107* R = THP; *108* R = H



109 *110* *111*

gel. The stereochemical assignments in the formulas of *105* and *107* are based on the data of the NMR spectra of the corresponding alcohols *106* and *108* which had been obtained by hydrolysis of *105* and *107* respectively.

Compound *105* was used for the further synthesis and reduced to the mixture of the C-15 carbinols *109* and *110* which were again separated by TLC chromatography. The polar isomer *110* was converted by hydrolysis of the cyanogroup to the dl-9-desoxy-9-oxaprostaglandin E_1, *111*, as a cristalline derivative.

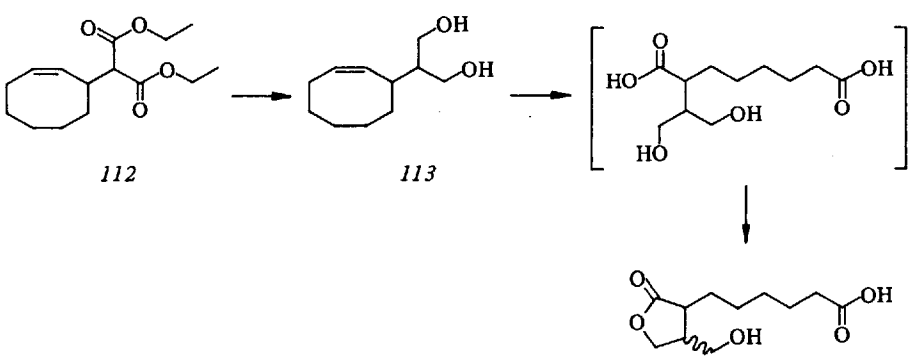
Recently, an Indian work group reported another synthesis of 9-oxa-13,14-dihydro-prostanoids[127].

2.8. 10-Oxaprostanoids

The synthesis of 10-oxa-11-deoxyprostanoids that is of prostanoids with a γ-lactone structure has been reported in the patent literature[82] and from a research group from the Research Triangle Institute[83]. The latter synthesis started with diethyl-2-(3-cyclooctenyl)-malonate *112* which can be prepared according to the literature [84, 85] by a treating the sodium salt of diethylmalonate with 3-bromocyclooctene or 1,2-dibromocyclooctane.

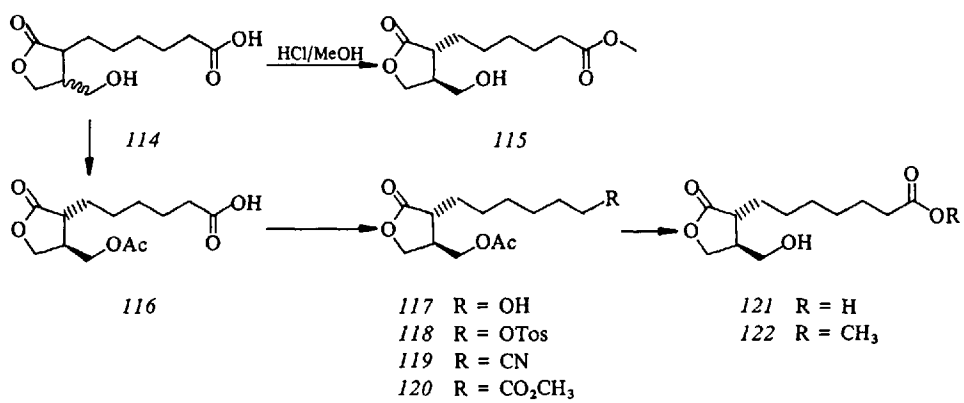
Reduction of the compound *112* with $LiAlH_4$ afforded 2-(3-cyclooctenyl)-1,3-propanediol *113* which was oxydized by reaction with ozone to the acid *114*. This acid is a mixture of *cis*- and *trans*-isomers. By esterifying the acid to the methyl ester with diazomethane a two compound mixture is formed, whereas ester formation under acidic conditions (MeOH/HCl) gives a single compound. This single compound *115* is thought to be the *trans*-isomer, because isomerization of the mixture of esters obtained by reaction with diazomethane to a single compound is apparently possible by an esterification relactonization mechanism by treating the mixture with acid in methanol. These results are consistent with the formulation of the ester *115* as the *trans*-isomer.

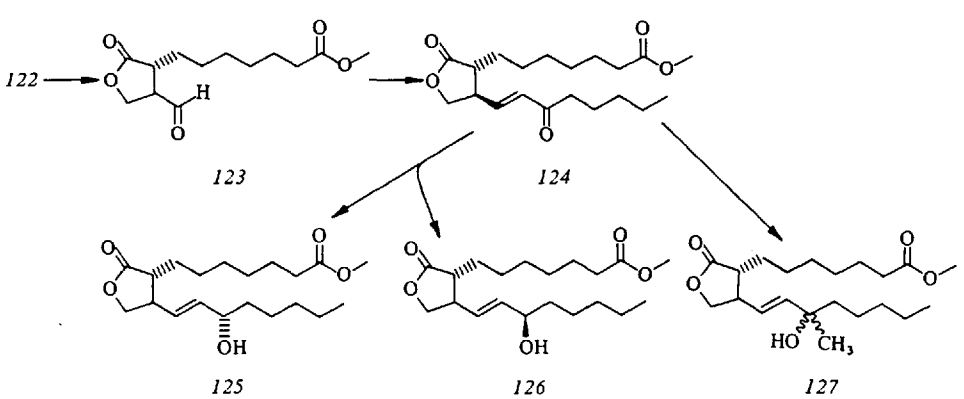
The intermediate carboxylic acid side chain in compound *114* is one methylene unit shorter than that of the natural prostanoids, therefore chain elongation



112 *113* *114*

starting with the carboxyl function had been necessary. As a first step on this way the primary alcohol function was protected by reaction with Ac_2O in pyridine. The resulting acetate *116* is formulated as the *trans*-isomer, too. The carboxylic group was then converted by selective reduction with diborane to the alcohol *117*. The alcohol function was converted to the nitrile *119 via* the tosylate *118* and displacement of the tosylate group with sodium cyanide. Methanolysis led to the methylester *120* because the acetate moiety was not cleaved under these conditions (MeOH/HCl). Hydrolysis with base yielded the deprotected lactone acid alcohol *121*, which was purified by converting it into the methylester *122*.
The primary alcohol function was then used for the introduction of the second side chain *via* the aldehyde *123*, the enone *124* and after reduction of the enone the mixture of the epimeric alcohols *125* and *126*, which could be separated by pressure column chromatography. Structural assignment is not given.
Reaction of the enone with methyl magnesium chloride gave rise to the tertiary alcohol *127* (which is a double racemate).

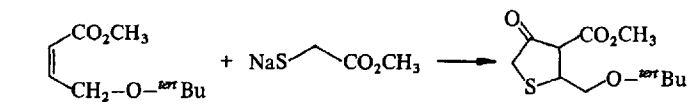




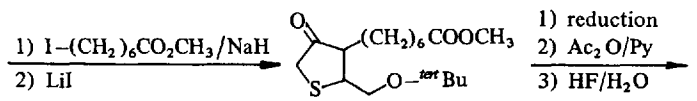
2.9. 11-Thiaprostanoids

The synthesis of some 11-thiaprostanoid analogs is described by a Syntex research group[86].

Tetrahydrothiophenone *130* was prepared in 52% yield by addition of sodium salt of methyl thioglycolate *129* and methyl-4-*tert*.-butoxybut-2-enoate *128*.



128 *129* *130*



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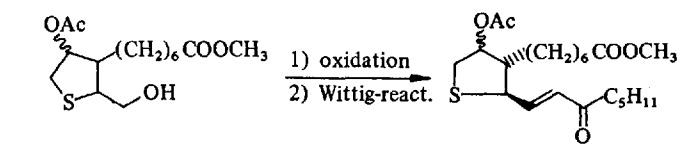
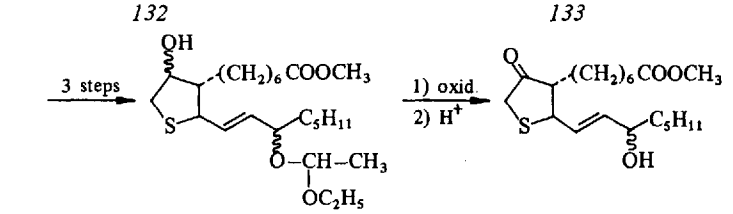
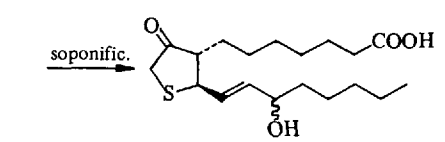


132 *133*



134 *135*



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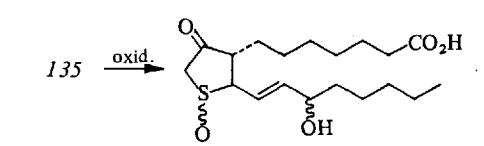

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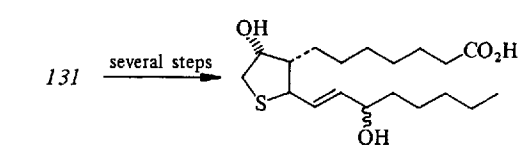


138

Alkylation of *130* with methyl-7-iodoheptanoate and subsequently decarboxylation with LiI in DMF under reflux gave the ketoester *131* in 24% yield. Reduction with sodium borohydride, acetylation, and cleavage of the *tert.*-butyl protecting group with 48% aqueous HF and THF afforded the alcohol *132*. The remaining steps in the synthesis were completed by well known methods: oxidation, followed by Wittig-Horner-reaction gave the enone *133* in which is assumed, that side chains at position 8 and 12 are orientated in the more stable *trans*-position. Zinc borohydride reduction, protection of the allylic alcohol, saponification of the acetate group, renewed oxidation and cleavage furnished *via 134* the methyl ester of 15-hydroxy-9-oxo-11-thia-prost-13-enonic acid *135*. Hydrolysis of the ester group gave 15-hydroxy-9-oxo-11-thia-prost-13-enoic acid *136*. The oxidation of *135* with one equivalent of *m*-chloroperbenzoic acid in methylene chloride produced the mixture of isomeric sulfoxides *137*.
For the synthesis of the 11-thia-prostanoid analog of the F-series *138*, the ketone *131* was reduced with potassium tri-sec.-butylborohydride (K-Selectride), followed by using the methods described above.

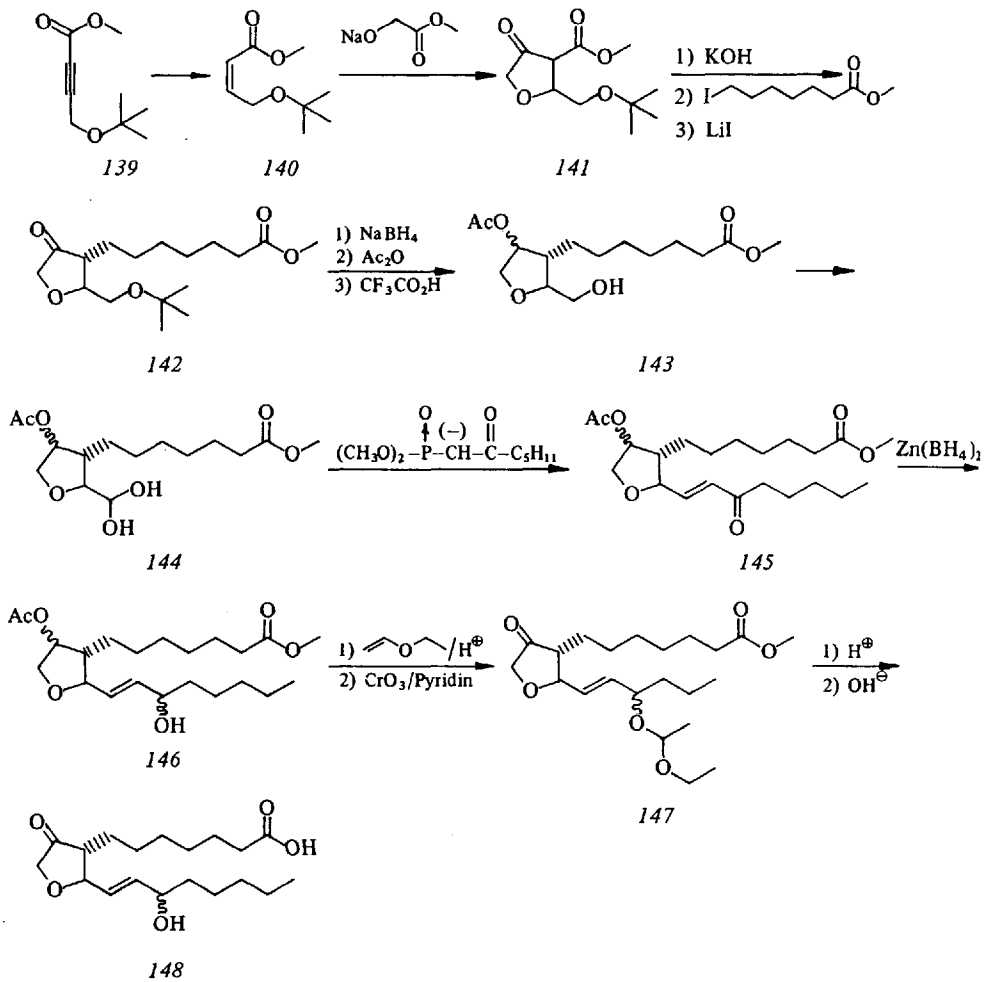
Both prostanoids *136* and *138* show a small activity (ca. 0,005 times the activity of PGE_2) in the gerbil colon assay. The activities of the corresponding sulfoxides or sulfones are not reported.

2.10. 11-Oxaprostanoids

The synthesis of 11-oxaprostanoids, that is the displacement of the carbonatom 11, which bears the 11-hydroxygroup of the E and F prostanoids by an oxygen has been studied in several research laboratories all over the world. At least four groups have published their results in 1974 and 1975.

The first synthesis reported was that of a Syntex-group[80]. They used the well known addition of the anion of methyl glycolate to α, β-unsaturated esters[87–90] for the construction of the oxacyclopentane-system.

The synthon they used for the reaction with the anion of methyl glycolate to the tetrahydrofuranone derivative *141* was methyl-4-*tert.*-butoxybut-2-enoate *140*. This compound had been prepared out of the corresponding acetylenic ester[91] *139* by partial hydrogenation. The β-ketoester *141* was then C-alkylated by treatment of its potassium salt with methyl-7-iodoheptanoate in DMSO and decarboxylated with lithium iodide in DMF to the ketoester *142* which was claimed to have the more stable conformation with *trans* side chains.
The intermediate primary alcohol *143* was obtained in three steps out of *142*. The further synthesis went *via* the aldehyde *144* (obtained as the hydrate), the enone *145* prepared by previously applied methods, to the mixture of the alcohols *146*. The corresponding 9-ketoprostanoid *148* was obtained in three additional steps *via* the protected intermediate *147* and in another two steps, as a mixture of the 15-epimeric alcohols *148*. Separation of the epimeric alcohols was possible by column chromatography on silica gel.
Another similar route using the same principle of addition reaction was studied by two independent groups[81, 92].



139 *140* *141* *142* *143* *144* *145* *146* *147* *148*

Both used methyl-4,4-diethoxycrotonate *149* as reagent for the synthesis of the tetrahydrofuranone derivative *150*. The carboxylic side chain however was introduced by different ways.

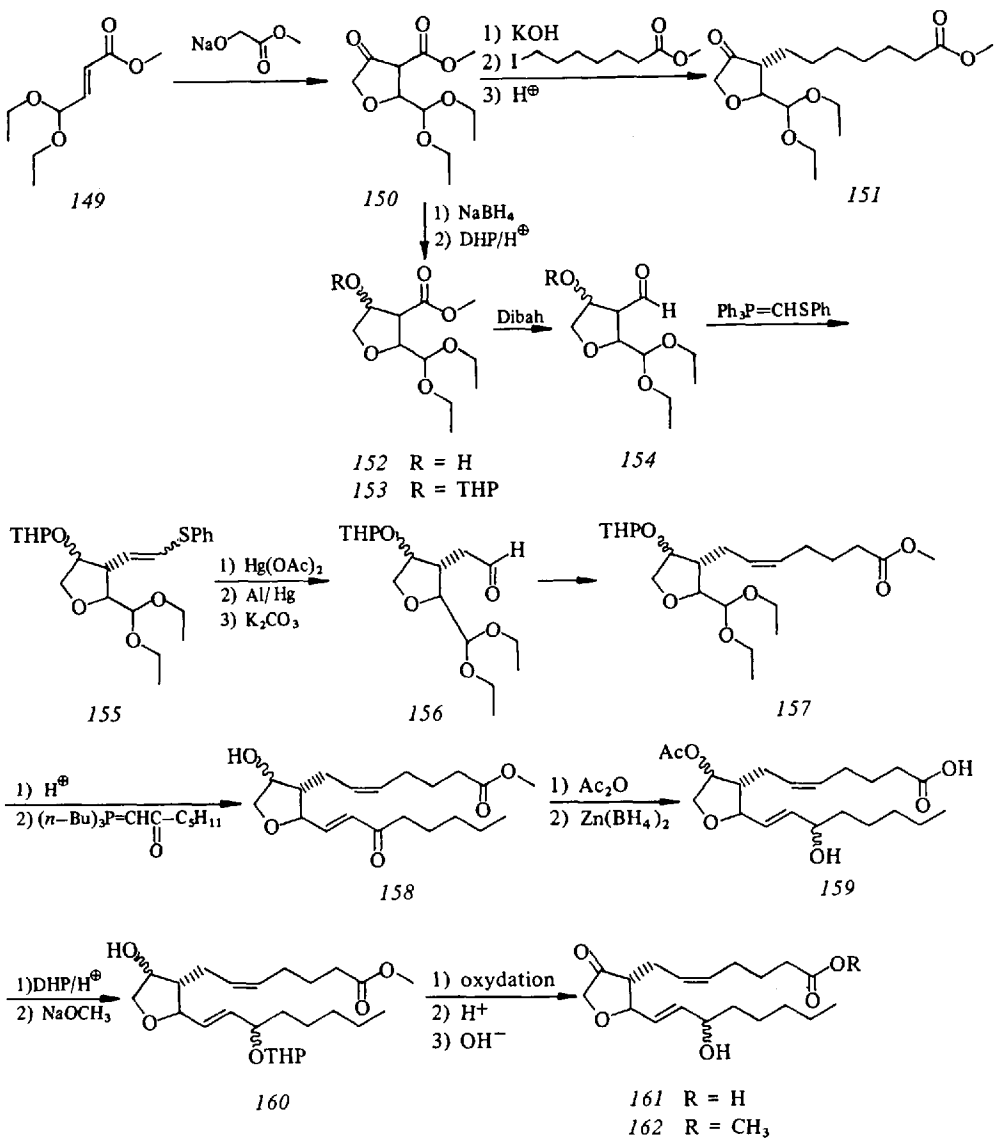
One group[92] chose the way analogous to the afore mentioned synthesis *i. e.* C-alkylation of the β-ketoester *150*, hydrolysis and decarboxylation to *151*. Interestingly enough the acetal function was not cleaved under these strongly acidic conditions.

As mentioned in the other publication[81], too, the cleavage of the acetal function is however readily achieved by reaction with mild acid yielding the desired aldehyde when the keto-function of the ring is reduced before.

Using the unprotected aldehyde function as mentioned above for construction of the remaining side chain, this approach leads in analogous manner as from *144* to 11-oxa-11-deoxyPGE$_1$. The other group[81] used the ester function in compound *150* for the construction of the carboxylic side chain by a novel method. This was

done *via* the compound *153* by reduction of the ester to the aldehyde *154* and conversion of *154* to the mixture of the *cis* and *trans* vinylthioethers *155*. Three additional steps were necessary to yield the elongated aldehyde *156*, which was converted to the intermediate *157* as usual.

The construction of the remaining side chain was possible in four steps *via* *158* to the mixture of the 15-epimeric alcohols *159* which were separated by preparative TLC on silica gel. The isomers were separately reacted with dihydropyrane, the acetoxygroups removed by methanolysis to *160* and the end products *161* and *162* prepared by two or three additional steps.



Structural similarity with furanose sugars was the common idea for two independent groups which published the synthesis of optically active 11-oxaprostanoids[93–95].

The first group[93] chose an approach by which the introduction of the "upper" side chain was achieved by reaction of the optically active epoxide *165* with the sodium derivative of diethylmalonate to the mixture of the isomers *166* and *167*. The desired isomer *166* was isolated in 20% yield by chromatography on silica gel. The stereocontrolled opening of the epoxide which had been prepared out of *163 via* the mesylate *164* was the prerequisite for the correct configuration of the prostanoid side chains in compound *171*.

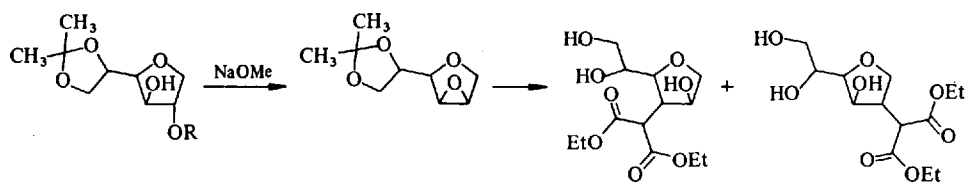
Compound *166* was converted to the diol *168* which then was oxydised with periodate to the aldehyde *169*.

The aldehyde function of *169* was used as usual for the construction of the "lower" side chain to compound *170* which seems to be a suitable intermediate for the synthesis of 11-oxaprostanoids.

The conversion of the intermediate *170* to optically active endproducts as *e. g.* *171* has not been published yet.

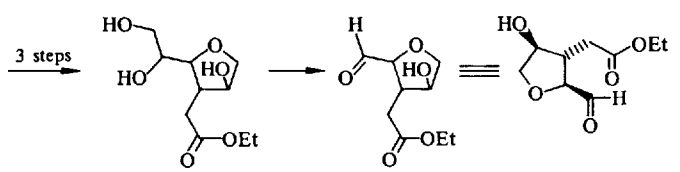
The other route[94] to 11-oxaprostanoids from branched ribofuranose sugars started with D-xylose which was converted by a method published by W. Sowa[96] to 1,2-isopropylidene-5-0-trityl-α-D-erythropentofuranos-3-ulose *172*.

Condensation of *172* with the potassium salt of trimethylphosphonoacetate followed by hydrogenation gave compound *173*, which had *trans* configurated side

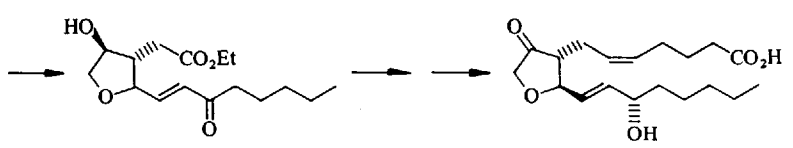


163 R = H
164 R = mesyl

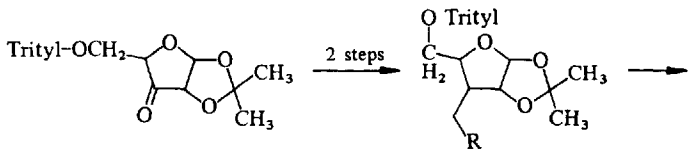
165 *166* *167*



168 *169*



170 *171*



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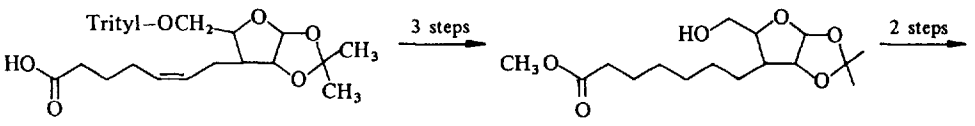
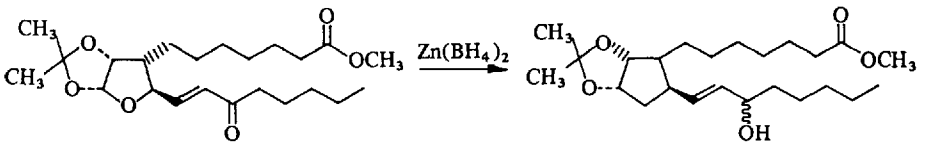
173 R = CO_2CH_3
174 R = CH_2–OH
175 R = CHO



176 *177*



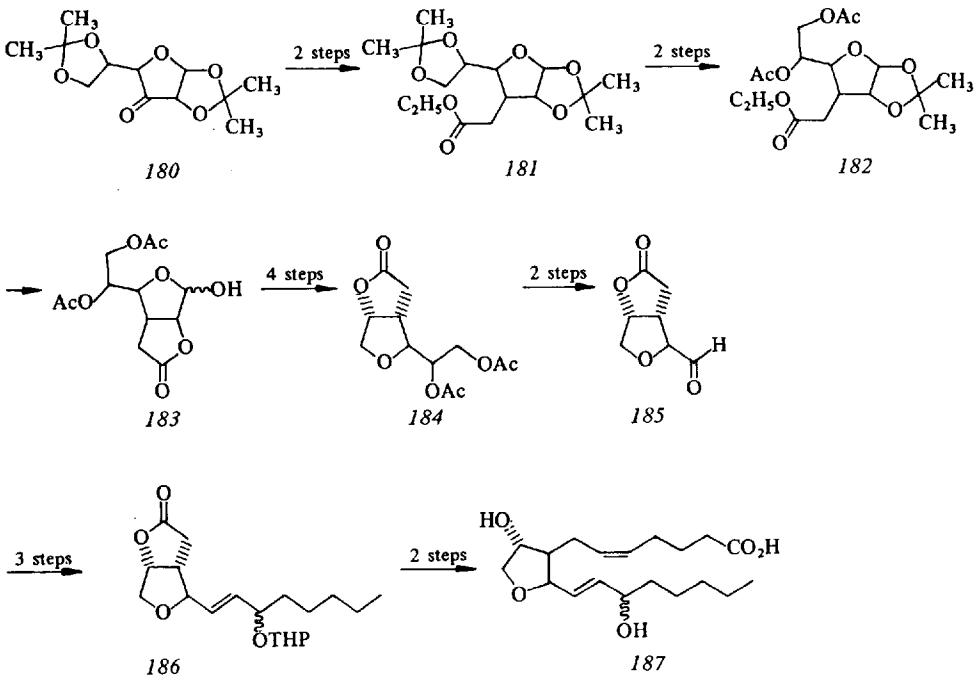
178 *179*

chains as proven by its NMR spectrum. The carboxylic side chain was constructed in several steps by the sequence *173–176*.

The deprotected compound *177* was then used for the introduction of the remaining side chain *178*. Compound *179* was obtained as a mixture of epimeric alcohols and can be regarded as an 11-oxaprostanoid derivative in optically active form.

Another synthetic route published by the same authors[95] started from D-glucose which served for the preparation of the known 1,2,5,5-di-O-isopropylidene-D-ribo-hexofuranos-3-ulose *180*. For compound *181*, which had been synthesized by condensation of *180* with triethylphosphonoacetate and hydrogenation, the allo-configuration was proven by NMR studies. Selective hydrolysis and acetylation of the resulting diol gave the diacetate *182* which was converted under acidic conditions to the lactone *183*. The free additional hydroxy group was then eliminated by acetylation with p-nitrobenzoic acid chloride conversion *via* the furanosyl bromide (HBr in CH_2Cl_2) to the phenylthiofuranoside (C_6H_5SK in EtOH), and desulfurisation to the diacetate *184*. Deacetylation and oxydation ($NaJO_4$) led to the aldehyde *185*. The "lower" side chain was constructed as usual resulting in a mixture of epimeric alcohols which were converted to their tetrahydropyranyl-ethers *186*. The remaining side chain is built up in two steps as in the case of the natural prostanoids *187*. Preparative layer chromatography on silica gel resulted in isolation of the more polar compound in pure form.

This isomer has been tentatively assigned the 15-S-configuration in analogy to the TLC-behaviour of the natural prostanoids.



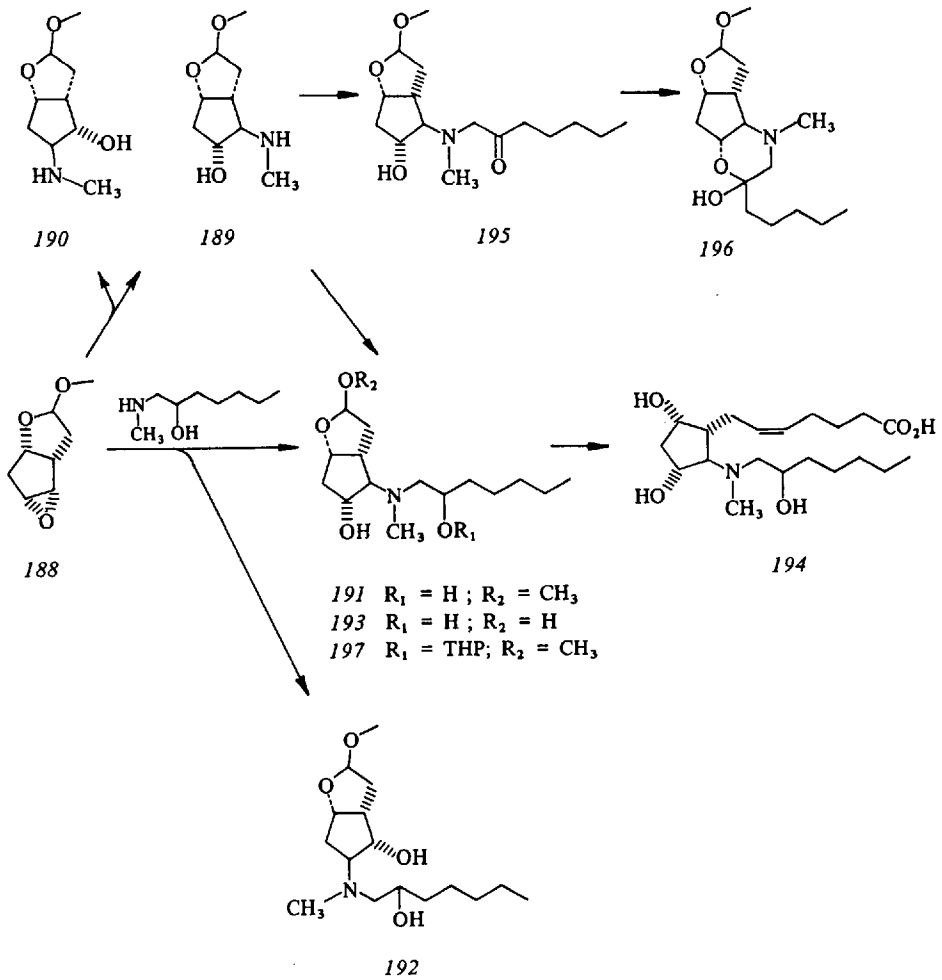
180 181 182 183 184 185 186 187

2.11. 13-Azaprostanoids[97]

The synthesis of these heteroprostanoids in which the 13,14-double bond which is both relevant for activity and metabolic degradation of the natural prostanoids is altered by introduction of a tertiary nitrogen at position 13 started from the well known epoxide *188*[98].

Reaction of this epoxide with methylamine yielded the mixture of position isomers *189* and *190*. The isomers were separated by column chromatography on silica gel, and the structures correlated by studying their NMR spectra.
N-alkylation of *189* with either 1-bromo-2-tetrahydropyranyloxyheptane or 1-bromo-2-heptanone was not suited for the further synthesis.

Alkylation with 1-bromo-2-tetrahydropyranyloxyheptane gave only low yields of *197*. The alkylation with 1-bromo-2-heptanone resulted in formation of the tricyclic compounds *196* instead of the desired compound *195*.
An alternative route, opening of the epoxide with N-methyl-2-hydroxyheptylamine[99] to the mixture of the position isomers *191* and *192* was developed further, because the separation and structural correlation with NMR was possible also in this case. Cleavage of the acetal function to the hemiacetal *193* was achieved by treatment of *191* with perchloric acid. The last step of the synthesis was the introduction of the remaining side chain in analogy to the method developed by E. J. Corey[100] in the case of the natural prostanoids.
The target molecule *194* was obtained as a mixture of the 15-epimeric alcohols which could not be separated further.



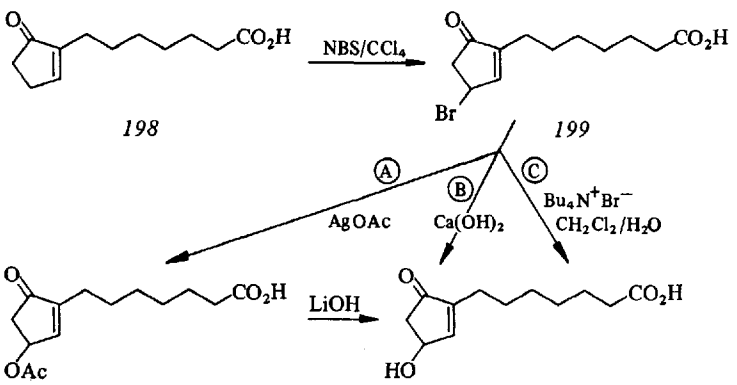
2.12. 13-Thiaprostanoids

We have investigated in our laboratories[105] the total synthesis of heteroprostanoids which contain a sulfur atom in the lower side chain. We assume, that a small enlargement of the distance between the hydroxy groups at positions C_{11} and C_{15} should change the biological activities of these new compounds. It is noteworthy in this connection that from the displacement of the C_{15}-hydroxy group to position C_{16} in the PGF_2-series, R. Pappo *et al.*[101] obtained compounds which are extremely potent gastric antisecretory agents[102]. We have investigated an efficient synthesis of 13-thia-prostanoids, based on the michael addition of corresponding substituted mercaptans to the well known 7-(3-hydroxy-5-oxo-cyclopentenyl)-heptanoic acid *201* in the presence of a basic catalyst. The preparation of the cyclopentenone building block is frequently described in the recent literature[103, 104].

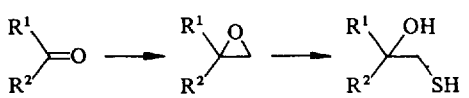
Most syntheses start from 7-(5-oxocyclopentenyl)-heptanoic acid *198* followed by introduction of the hydroxy group at position C_3 in two steps. We have repeated this synthesis and found, that the change of the very sensitive allylic bromide *199* into *201* is possible on different ways. The S_{N2}-reaction succeeds with silver acetate (way A), as well as with calcium hydroxide (way B) or in a phase transfer reaction with $Bu_4N^+Br^-$ (way C) (Scheme 2).

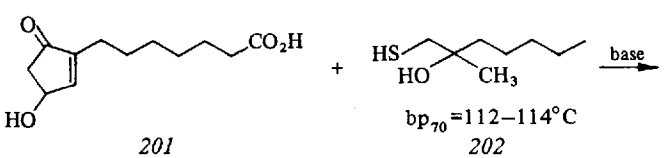
Treatment of *200*, obtained by way A, with lithium hydroxide in methanol, containing a small amount of water, at room temperature[106], gave the expected synthon *201* in pure and excellent yield [m.p. 55–58 °C (ether)].

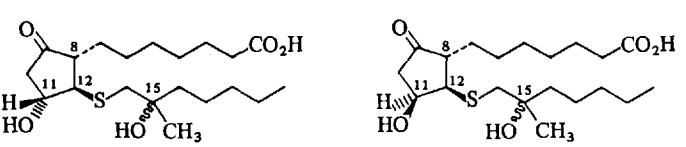
The lower mercapto side chains were mainly prepared by formation of epoxides starting from the corresponding ketones or aldehydes, respectively[107]. Treatment



Scheme 2.



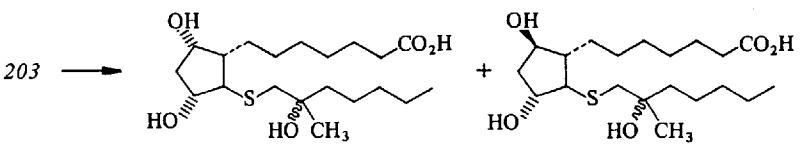




Scheme 3.

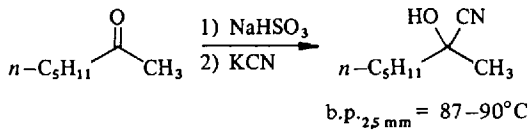
of the terminal oxiranes with H_2S in presence of amines[108] give the desirable β-hydroxymercaptans in near quantitative yields (see Scheme 3).
The anions of these mercaptans, successfully generated with steric hindered amines underwent smooth addition to the α,β-unsaturated five membered ring ketones *201*. Under defined reaction conditions we obtained *203* as a major product when *202* was added to *201*. Column chromatography on silica gel of the crude product gave besides *203* a small amount of the isomeric compound *204*.

The stereochemical structures assigned to the obtained 11-thiaprostanoids *203* and *204* has been supported by the results of IR and MS-spectra, and especially by NMR double resonance technique. The most notable difference in the ^{1}H-spectra of both epimers is the differentiated fine structure of the signals for the protons at positions C_{11}. In isomer *203* this proton appears as quartett, centred at 4,26 ppm (Irradiation experiments show the following coupling constants: $J_{11,\,12}$ = 9,5 Hz, $J_{8,\,12}$ = 11,0 Hz). In contrast, compound *204* shows for the same proton a triplett, centred at 4,36 ppm (Irradiation experiments show as coupling constants: $J_{11,\,12}$= = 3,5 Hz, $J_{8,\,12}$ = 11,0 Hz). These findings are in good agreement with those presented in the NMR spectra of prostanoids E_1 and 11-epi-E_1 by M. Miyano *et al.* [109–111]. In addition, a convenient preparation of 13-thiaprostanoids of the F-series has been generated from the 13-thia-E-prostanoids. *203* was converted into the isomers *205* and *206* by reduction with sodium borohydride or other complexed hydrides.

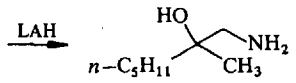


205 *206*

The assignment of the stereochemistry of both compounds based on NMR spectral data and their different behaviour on thin layer plates (silica gel), impregnated with boric acid[112]. Only isomer *205* is able to form a cyclic boric ester, therefore *205* runs faster on impregnated plates than *206*.
An unresolved problem is the differentiation of the C_{15}-isomers. It is impossible to differentiate these epimers whether by NMR-technique nor by thin layer chromatography.
In order to synthesize the enantiomeric forms of the methyl ester of *203* we started from the described methyl ester of 7-(3(R)-hydroxy-5-oxo-cyclopentenyl)-heptanoic acid[113] and the optical active lower side chains.
Scheme 4 presents the route of total synthesis of optical active mercaptans *210* and *211*.
The racemic aminoalcohol *207* has been resolved into both enantiomeric forms with (D)–(-)-mandelic acid. Liberation of the amines, following by quaternization with methyl iodide, treatment with silver oxide, and heating in water gave the optically active oxiranes *208* and *209*.



b.p.$_{2,5\ mm}$ = 87–90°C



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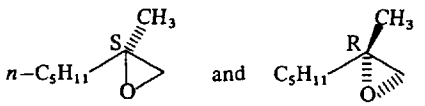
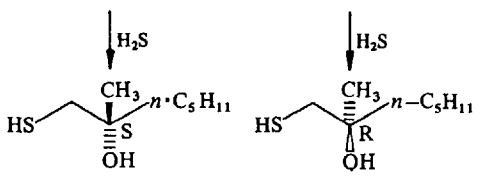


$[\alpha]_{366}^{20}$ = +14,5° (c = 5,5; CH_3OH) *208*

$[\alpha]_{366}^{20}$ = −12,6° (c = 5,0; CH_3OH) *209*

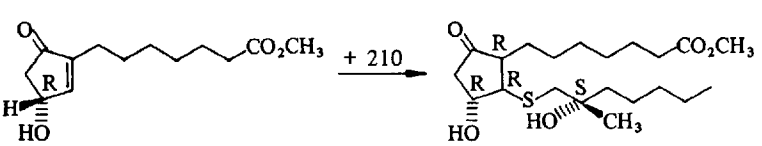


$[\alpha]_{366}^{20}$ = −3,8° (c = 7,8; CH_3OH) *210*

$[\alpha]_{366}^{20}$ = +2,8° (c = 5,0: CH_3OH) *211*

Scheme 4.

These both key intermediates were opened with H_2S in the presence of diisopropylamine. This reaction is known to proceed with full retention of configuration. Therefore we assume, that the obtained thiols *210* and *211* are of the assigned absolute stereochemistry. The optical purity of each enantiomer was directly determined from the relative peak areas and senses of nonequivalence of the resonances of enantiotopic nuclei in chiral solvent, *e. g.* $Eu(TBC)_3$. We observe optical purities for *210* p = 85% and for *211* p = 75%. The addition of *210* to the optically active *212* gave after column chromatography the desired 8 R, 11 R, 12 R, 15 S-13-thiaprostanoid E *213*.



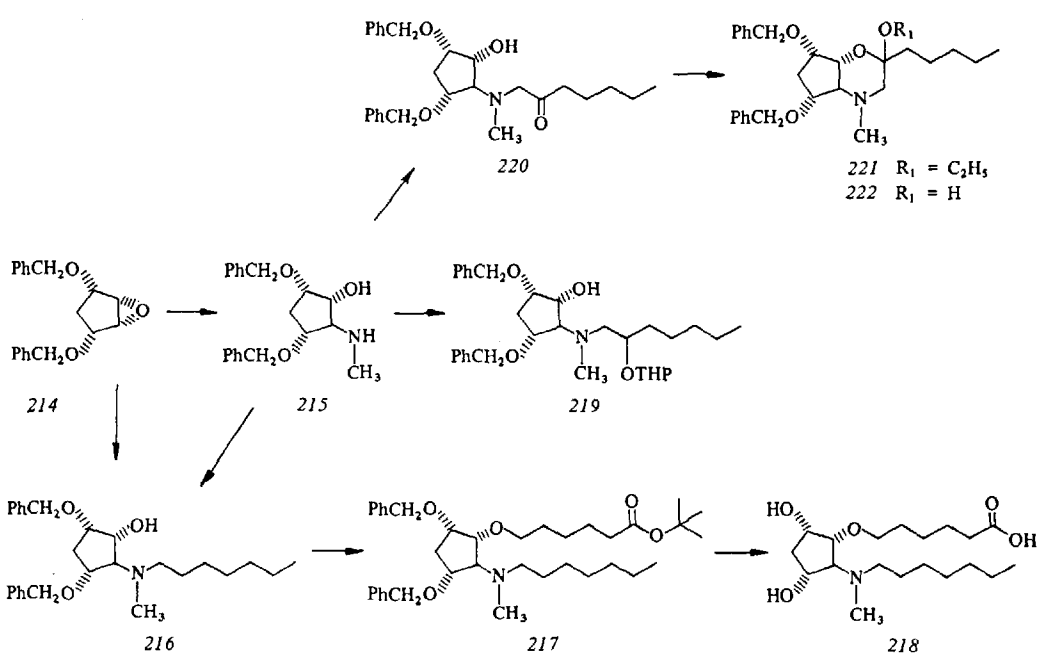
$[\alpha]_D^{20}$ = +16,8°($CHCl_3$) *212*

$[\alpha]_D^{20}$ = −64°($CHCl_3$) *213*

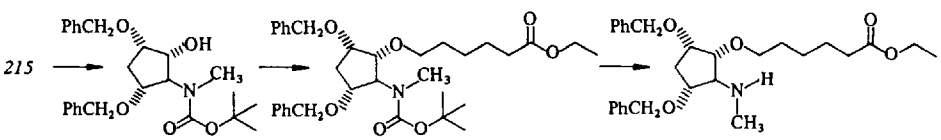
Some of these new heteroprostanoids indicate very interesting biological activities[114]. The E-type analog *203* is a potent vasodilator, its effect in blood pressure (cats i.v.; dogs i.v. or orally) was greater than that of PGE_1. The unnatural configurated analog *204* showed only a small activity on blood pressure in cats and was inactive in dogs. Among the prostanoids studied, *205* as representative compound of the F-series showed remarkable activity in the antifertility-test in intact hamsters.

2.3. 13-Aza-7-oxaprostanoids[115]

These prostanoids were synthesized starting with the well known dibenzyloxy-epoxide[68] *214*. The preparation of 13-aza-13-N-methyl-7-oxa-15-desoxy $PGF_{1\alpha}$ from this starting material was possible by two different routes.
The first route was rather straight forward introducing first the nitrogen containing side chain by reacting the epoxide with methylamine in methanol at 120 °C under pressure to the amino-alcohol *215* and N-alkylation of this intermediate with 1-bromoheptane in ethanol to the tertiary amine *216*. This same product was prepared directly by reaction of the epoxide with N-methylheptylamine in ethanol with a catalytic amount of hydrochloric acid at 120 °C under pressure. O-alkylation was performed according to the method used by J. Fried[69] that is reaction of *216* with *tert.*-butyl-ω-iodohexanoate (NaH in DMSO). Compound *217* was charac-

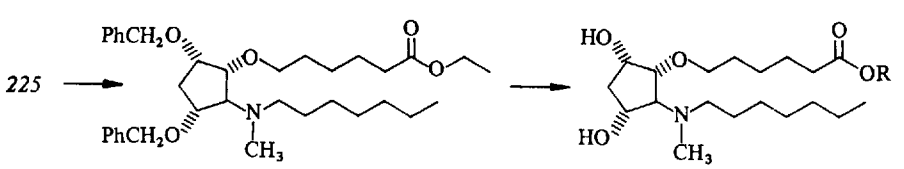


terized as the hydroiodide (m.p. 78–80 °C). The removal of the benzyloxygroups was carried out with the free base by reaction with BF_3-etherate in benzene. This reaction sequence led to the $PGF_{1\alpha}$-derivative *218* as the free acid, because the *tert.*-butylester group was cleaved under the reaction conditions simultaneously. This reaction sequence worked quite well for the synthesis of 15-desoxy-13-aza-7-oxaprostanoids. The synthesis of 15-hydroxderivatives however was not possible by this route. Reaction of *215* with 1-bromo-2-tetrahydropyranyloxyheptan gave the N-alkylated product *219* only in very low yield. N-alkylation of *215* with 1-bromoheptanon-2 resulted in formation of bicyclic compounds *221* when the reaction was carried out in ethanol or *222* in tetrahydrofurane (instead of *220*). Therefore a second route using the N-protected intermediate *223* which was prepared by reaction of *215* with *tert.*-butyloxy-carbonylazide was started. The O-alkylation of this compound was easily possible by reacting it with ethyl-ω-bromohexanoate and silver oxide in DMF to *224*. The *t*-BOC protecting group in *224* was then removed smoothly with trifluoroacetic acid to the unprotected amine *225*.
The amine *225* was the common intermediate for the synthesis of both 15-deoxy- and 15-hydroxy derivatives.



223 *224* *225*

The former prostanoids were synthesized by reaction of *225* with 1-bromo-heptane to *226* and cleavage of the benzylether protecting groups to *227* which was converted to the free acid *228* by alkaline hydrolysis.
The way to the 15-hydroxyderivatives was opened by N-alkylation of *225* with 1-bromo-2-heptanone in ethanol to *229a* in good yield. The reaction product *229a* (free base m.p. 131 °C/hydrochloride m.p. 77 °C) was then used for the preparation of the 15-methyl-15-hydroxy and the 15-hydroxyderivatives.
The latter compounds were obtained as a mixture of the 15-epimers *230* by re-



226

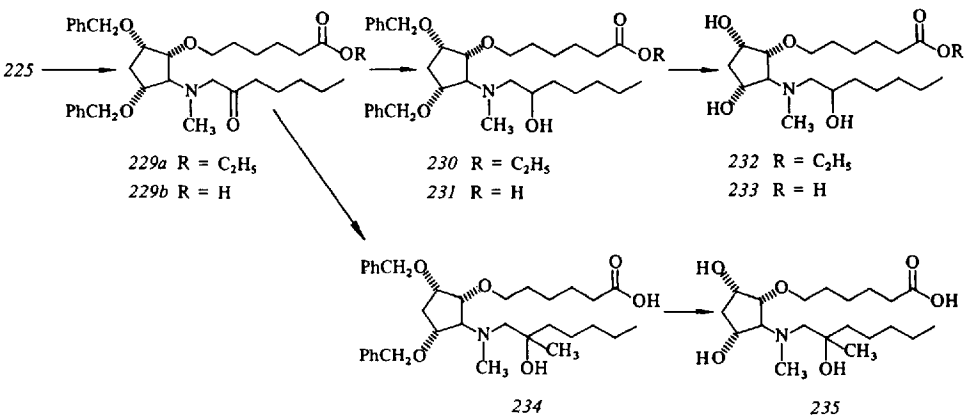
227 R = C_2H_5
228 R = H

duction of the 15-keto-group with $NaBH_4$ in methanol and then cleavage of the benzylethergroups as usual (BF_3-etherate in benzene). Separation of the epimers *232* was possible by chromatography on prepacked columns with silica gel[a]. Designation of the 15-hydroxy compounds to either the natural or epi series was not possible.

Chromatographic separation of the 15-epimers was also possible when the free acid derivatives *231* bearing the dibenyloxy protecting groups were used. Separate treatment of the derivatives *231* with BF_3-etherate in benzene gave the two racemic 15-epimers. *233*

Reaction of the 15-ketoderivative *229b* as the free acid with an excess methylmagnesiumbromide in ether gave rise to the mixture of the 15-methyl-15-hydroxy compounds *234*.

Chromatographic separation was obtained also in this series by using prepacked silica gel columns. Removal of the protecting groups yielded the two racemic endproducts *235*. As in the case of the secondary alcohol derivatives correlation of the separated epimers was not possible.

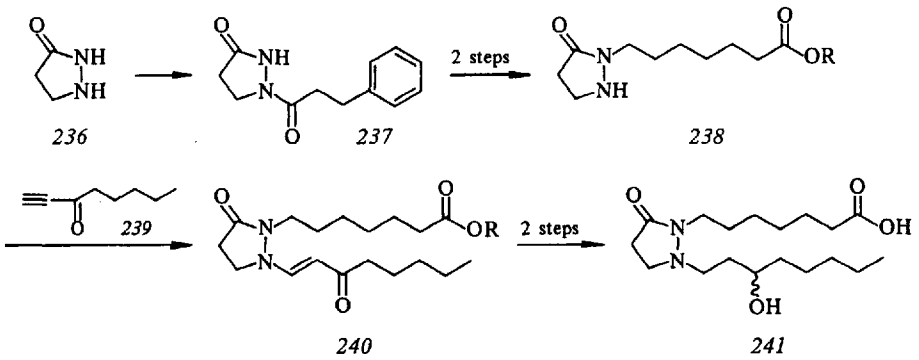


2.14. 8,12-Diazaprostanoids

These compounds have been published in the patent literature[116].

One of the syntheses mentioned there started with 3-pyrazolidinone *236*, which was protected as the benzyloxycarbonyl derivative *237* in order to obtain selective N-alkylation at position 8 yielding compound *238* after removal of the protecting group. Addition of the resulting amine *238* to 1-octyne-3-one *239* formed the enone *240* with the complete diazaprostanoid skeleton. Katalytic

a) Fertigsäule Kieselgel 60, Größe A, E. Merck, Darmstadt.



236 237 238

239

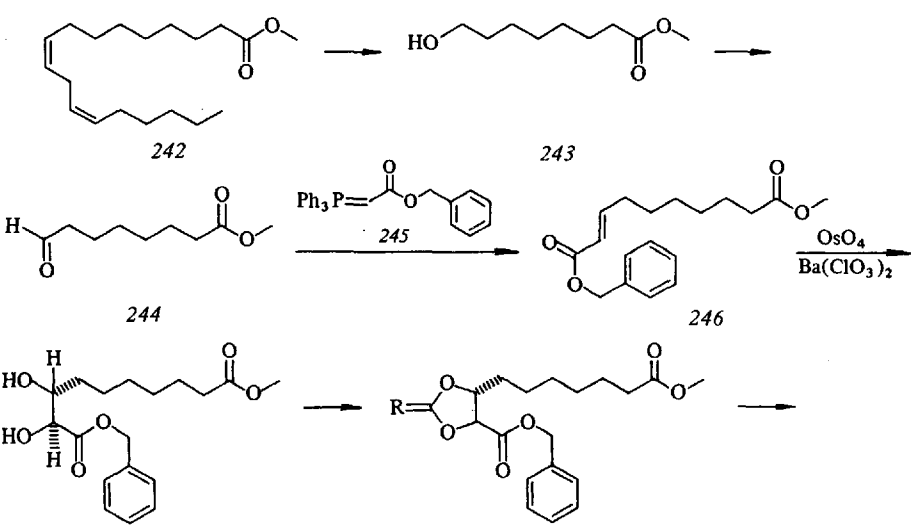
240 241

reduction of the enone and reduction of the ketogroup to the mixture of alcohols *241*, were the last steps on the way to 8,12-diaza-11-deoxy-PGE_0 and its 15-epimer.

2.15. 9,11-Dioxaprostanoids

These compounds with the structure of cyclic acetals or cyclic carbonates have been synthesized by I. T. Harrison and V. R. Fletcher[117]. The compounds represent prostanoids in which oxygen heteroatoms replace hydroxymethine or keto groups in the cyclopentane ring.

The key intermediate for the construction of the dioxacyclopentane moiety was the diol *247* which has been synthesized from the *trans*-olefinic ester *242*. This olefinic ester had been used by the same research group for synthesis of other prostanoids published 1972[118]. The preparation of *246* had been performed by reaction



242 243

244 246

247

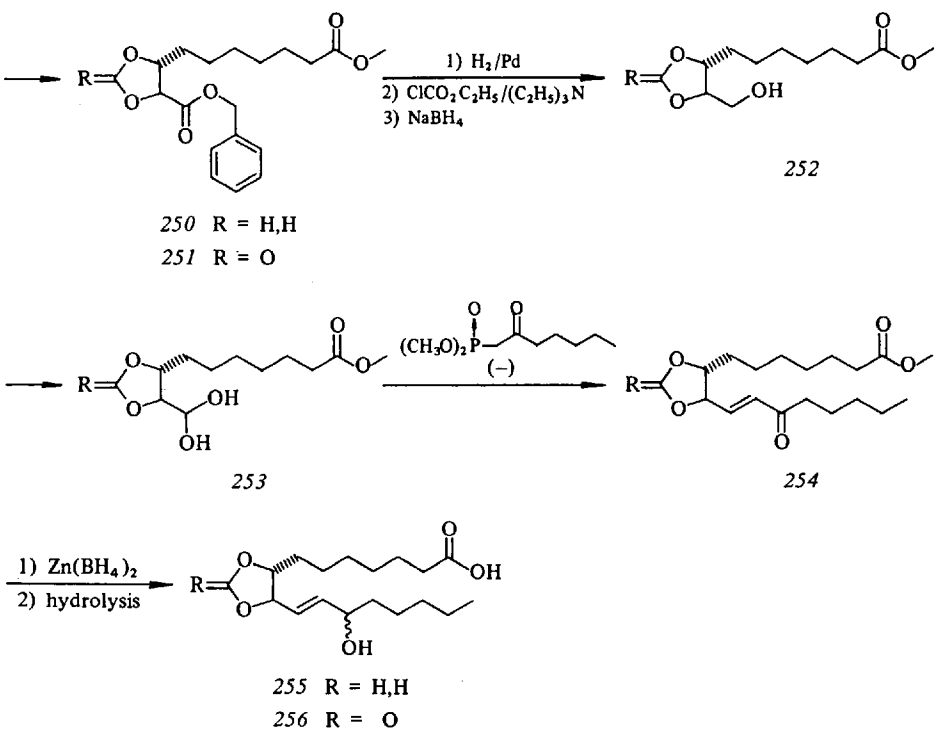
248 R = H,H

249 R = O

of 7-carbomethoxyheptanal *244* with carbobenzyloxymethylidene triphenylphosphorane *245*. The aldehyde *244* was synthesized by first oxydation of methyl linoleate to 7-carbomethoxyheptanol *243*[119] and then oxydation with chromium trioxide pyridinium complex. Reaction with paraformaldehyde converts the diol into the cyclic acetal *248*.

Hydrogenolysis of the benzylester group to the acid, reduction of the acid function *via* the mixed ethyl carbonic anhydride with sodium borohydride led to the alcohol *252*. Construction of the remaining side chain followed well established procedures. The analogous 10-oxo compounds were prepared by first synthesizing the cyclic carbonate by reaction of the diol *274* with phosgene to *249* and then using reactions analogous to those described above. The end products consisted of an inseparable mixture of the 15α- and 15β-epimers.

The products *255* and *256* showed weak activity in the gerbil colon smooth muscle contraction assay.



250 R = H,H
251 R = O

252

253

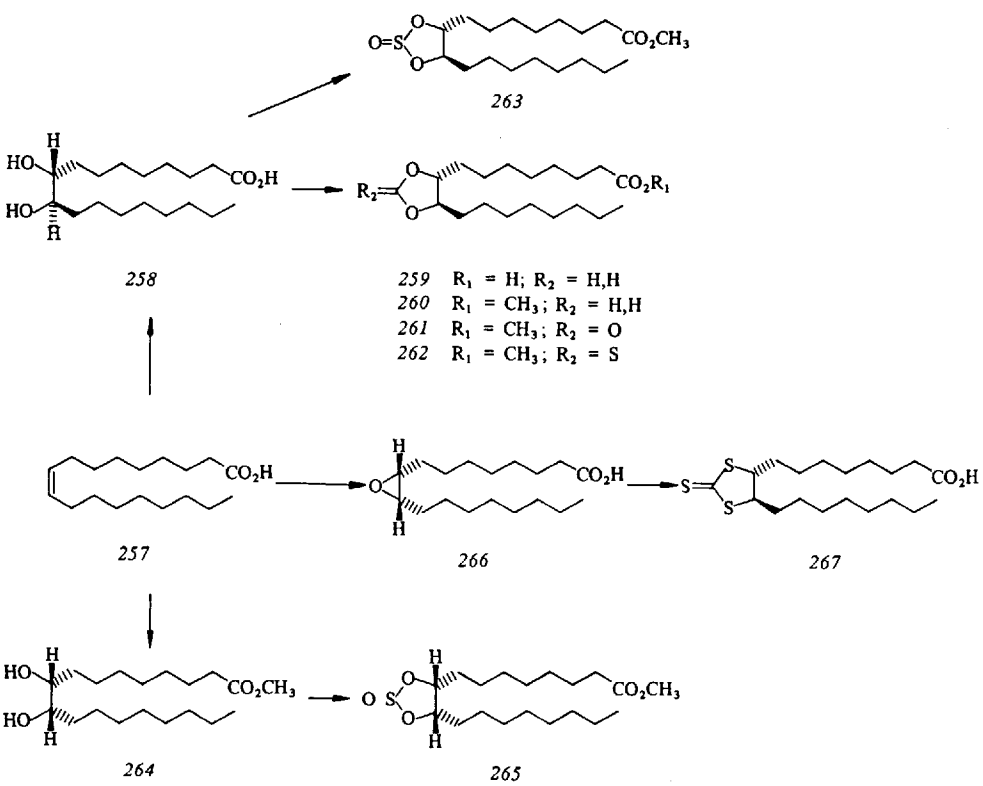
254

255 R = H,H
256 R = O

2.16. 9,11-Dihetero and 9,10,11-Triheterohomoprostanoids

In a recent publication[120], the synthesis of a whole series of 9,11-dihetero and 9,10,11-triheteroprostanoids has been described. The common starting material

for the preparation of these compounds was oleic acid, that means that all prostanoids synthesized had a homoprostanoid structure.
Oleic acid was converted to the erythro diol *264* or the threo diol *258* by reaction with permanganate or hydrogenperoxide respectively. The threo compound *258* was converted to the 9,11-dioxahomoprostanoids. Reaction with paraformaldehyde formed *259* or *260*, phosgene converted the diol into *261* whereas reaction with thiophosgene gave compound *262*.
The corresponding 9,11-dioxa-10-thiahomoprostanoid *263* was synthesized by reaction of the diol *258* with thionyl chloride. When the reaction with thionyl-chloride was carried out with the erythro diol *264* the isomeric 9,11-dioxa-10-thiahomoprostanoid *265* with *cis* side chains was obtained. The 9,11-dithiahomo-prostanoid *267* was prepared by reaction of the epoxide *266* with potassium methyl xanthate.
The compounds *261* and *263* were found to be more potent than PGE_1 or PGE_2 in relaxing the pig tracheal chain. It was interesting to see that *265*, the *cis*-isomer of *263* was inactive in this test. Some of the compounds namely *261*, *263*, *266*, *265* and *259* showed antidiarrheal effects when tested for inhibition of PGE_2-induced diarrhea in mice.
PG-synthetase inhibitory activity was found by testing the compounds *266* and *267*.



3. Conclusions

The publications which were selected for the preceeding review dealt with the synthesis of heteroprostanoids. Patents were added only as an exception.

The amount of publications about the synthesis of heteroprostanoids proves that the introduction of hetero atoms into the structure of prostanoid acid is considered to be a promising starting point.

At the present time it is hardly possible to make a final judgement about the influence of such manipulations in order to promote qualitative and quantitative changes in the biological activity. Specifications are often lacking in this matter. The clarification of the commercial use of the analogues synthesized in the laboratories of some firms is surely one reason for the few results reported so far. On the other hand many of the published results can only be interpreted very carefully, because the biological models used are different.

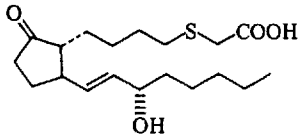
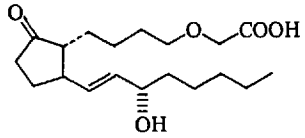
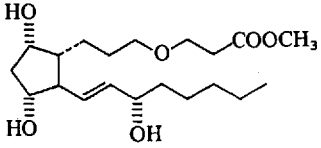
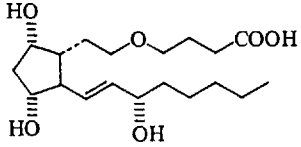
It is remarkable, that most heteroprostanoids are very weak or no substrates for the 15-hydroxy-PG-dehydrogenase. The first metabolic step observed by naturally occurring prostanoids apparently is stopped or slowed down already through the introduction of a hetero atom.

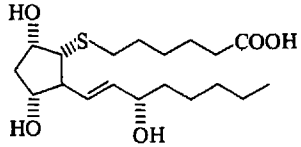
There is a chance therefore to find analogues similar to the 15- or 16,16-alkylated prostanoids of prolonged activity among the heteroprostanoids.

The strength of activity of some compounds is comparable to that of natural prostanoids. Some heteroprostanoids also show a remarkable specifity of activity or antiprostaglandin activity. Nevertheless one must wait for a more precise judgement until further detailed results are published.

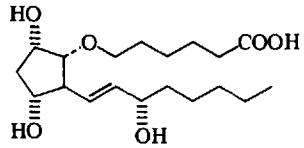
In the following table the heteroprostanoids and their biological activities are summarized (see Table 1). Only those heteroprostanoids were selected which were structurally similar to natural prostanoids.

Table 1. Comparison of biological activities of some E-and F-type heteroprostanoids

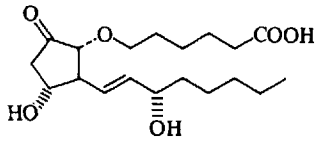
E-type	F-type	Activities
		Not reported
		Not reported
		No substrate for 15-hydroxy-PG-dehydrogenase from rhesus monkey lung), compared to $PGF_{2\alpha}$[121]
		No substrate for 15-hydroxy-PG-dehydrogenase (from rhesus monkey lung), compared to $PGF_{2\alpha}$[121, 122]



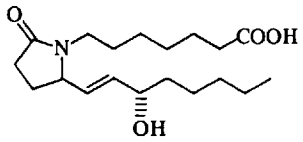
Stimulates c-AMP (mouse ovary) corpus luteum binding 1/10 $PGF_{2\alpha}$ inhibitor of 15-hydroxy-PG-dehydrogenase (from human placenta), $(I)_{50}$ = 5,2 nM[65]



5% of the activity of $PGF_{1\alpha}$ (gerbil colon) 0,1 x $PGF_{1\alpha}$ stimulation of c-AMP (mouse ovary) Substrate for 15-hydroxy-PG-dehydrogenase (swine lung), Km = 0,4 mM[72]

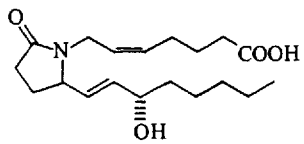
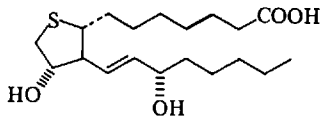
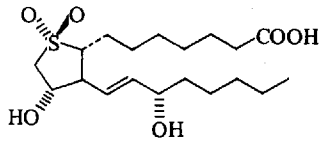
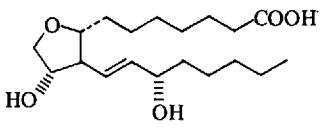
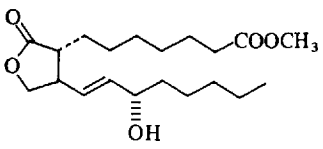


4×10^{-4} PGE_1 smooth muscle stimulating activity (gerbil colon)[123]



Inhibition of gastric ulcers, decrease of blood pressure[74]

Table 1 (continued)

E-type	F-type	Activities
		Substrate for 15-hydroxy-PG-dehydrogenase[74]
		Not reported
		Not reported
		Not reported
		Not reported

0,005 x PGE_2 on smooth muscle (gerbil colon)[86]

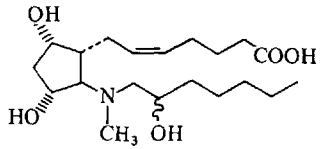
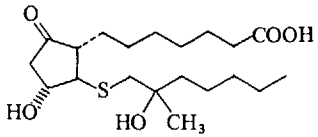
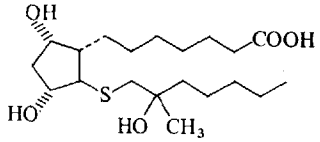
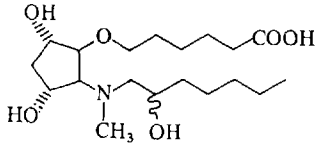
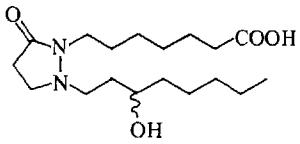
0,005 x PGE_2 on smooth muscle (gerbil colon)[86]

0,05 x PGE_2 on smooth muscle (gerbil colon)[80]

Not reported

Not reported

Table 1 (continued)

E-type	F-type	Activities
		No stimulation of rat uterus (in vitro)[114]
		Blood pressure lowering in cats and dogs (i. v. or orally)[114]
		Remarcable antifertility in hamsters[114]
		Rat uterus antagonistic against $PGF_{2\alpha}$ (in vitro)[114]
		Stimulation of smooth muscle (rat ileum), blood pressure lowering ED_{50} = 0,2 mg/kg, inhibition of gastric ulcers (rat)[116]

4. References

1) Ramwell, P. W., Shaw, J. E.: Rec. Prog. Horm. Res. *26*, 139 (1970)
2) Losert, W.: Arzneimittel-Forsch. *25*, 135 (1975)
3) Russel, P. T., Eberle, A. J., Chemg, H. C.: Clin Chem. *21*, 653 (1975)
4) Bergström, S., Carlson, L. A., Weeks, J. R.: Pharmacological Reviews, Vol. 20, No. 1, p. 1 (1968)
5) Oesterling, T. O., Morozowich, W., Roseman, T. J.: J. Pharm. Sciences *61*, 1861 (1972)
6) The Prostaglandins. Karm, S. M. M. (ed.). MTP Oxford, 1972
7) Andersen, N.: Ann. N. Y. Acad. Sci. *180*, 14 (1971)
8) Nelson, N. A.: J. Med. Chem. *17*, 911 (1974)
9) Corey, E. J., Ravindranathan, T., Terashima, S.: J. Amer. Chem. Soc. *93*, 4326 (1971)
10) For more detailed review see Arch. Intern. Med. *133*, No. 1, 29 (1974)
11) Von Euler, U. S.: Klin. Wochenschr. *14*, 1182 (1935)
12) Bergström, S., Sjövall, J.: Acta Chem. Scand. *14*, 1693, 1704 (1960)
13) Bergström, S., Danielsson, H., Samuelsson, B.: Biochim. Biophys. Acta *90*, 207 (1964)
14) Van Dorp, D. A., Beerthuis, R. K., Nugteren, H. D., Vonkeman, H.: Biochim. Biophys. Acta *90*, 204 (1964)
15) Pike, J. F., Lincoln, F. H., Schneider, W. P.: J. Org. Chem. *34*, 3552 (1969)
16) Behrman, H. R., Anderson, G. G.: Arch. Intern. Med. *133*, 77 (1974)
17) Brenner, W. E.: Am. J. Obstet. Gynecol. *123*, 306 (1975)
18) See Proceedings of the Third Conference on Prostaglandins in Fertility Control on Ja. 17–20, 1972 Organized by WHO at Karolinska Instituted Stockholm, Sweden, Bergstrom, S., Green, K., Samuelsson, B. (eds.)
19) Oxender, W. D., Noden, P. A., Louis, T. M., Hafs, H. D.: Am. J. Vet. Res. *35*, 997 (1974)
20) Classen, M., Ruppin, H.: Z. Gastroenterologie *11*, 217 (1973)
21) Hamberg, M.: Life Sci. *14*, 247 (1971)
22) Samuelsson, B., Granström, E., Gree, K., Hamberg, M.: Ann. N. Y. Acad. Sci. *180*, 138 (1971)
23) Raz, A.: Life Sci. *11* (Part II), 965 (1972)
24) Ferreira, S. H., Vane, J. R.: Nature (London) *216*, 868 (1967)
25) Vane, J. R.: Brit. J. Pharmacol. *35*, 209 (1969)
26) Granström, E.: Prog. Biochem. Pharmacol. *3*, 80 (1967)
27) Lee, S. C., Levine, L.: J. Biol. Chem. *250*, No. 2, 548 (1975)
28) Attallah, A. A., Duchesne, D. J., Lee, J. B.: Life Sci. *16*, 1743 (1975)
29) Lee, S. C., Levine, L.: J. Biol. Chem. *249*, 1369 (1974)
30a) Pike, J. E.: Fortschr. Org. Naturst. *28*, 313 (1970)
30b) Clarkson, R.: Progr. Org. Chem. *1973*, 1
31) Bundy, G. L.: Ann. Reports in Med. Chem. *7*, 157 (1972)
32) Bentley, P. H.: Chem. Soc. Rev. *2*, No. 1, 29 (1973)
33) Bartmann, W.: Angew. Chem. *87*, 143 (1975)
34) Corey, E. J., Weinshenker, N. M., Schaaf, T. K., Huber, W.: J. Amer. Chem. Soc. *91*, 567 (1969)
35) Jones, G., Raphael, R. A., Wright, S.: J. C. S. Chem. Comm. *1972*, 609
36) Corey, E. J., Snider, B. B.: J. Org. Chem. *39*, 256 (1974)
37a) Corey, E. J., Ensley, H. E.: J. Amer. Chem. Soc. *97*, 6908 (1975)
37b) Paul, K. G., Johnson, F., Favara, D.: J. Amer. Chem. Soc. *98*, 1285 (1976)
38) Weeks, J. R., DuCharme, D. W., Magee, W. E., Miller, W. L.: J. Pharmacol. Exp. Therap. *186*, 67 (1973)
39) Hayashi, M., Miyake, H., Tanouchi, T., Iguchi, S., Iguchi, Y., Tanouchi, F.: J. Org. Chem. *38*, 1250 (1973)
40) Binder, D., Bowler, J., Brown, E. D., Crossley, N. S., Hutton, J., Senior, M., Slater, L., Wilkinson, P., Wright, N. C. A.: Prostaglandins *1974*, Vol. 6 No. 1, 87
41) DOS 23 65101, Schering AG, Berlin

42) Jung, A., Schlegel, W., Jackisch, R., Friedrich, E. J., Wendel, A., Rückrich, M. F.: Hoppe-Seyler's Z. Physiol. Chem. *356*, 787 (1975)
43) Rückrich, M. F., Wendel, A., Schlegel, W., Jackisch, R., Jung, A.: Hoppe Seyler's Z. Physiol. Chem. *356*, 799 (1975)
44) König, H.: Klin. Wschr. *53*, 1041 (1975)
45) US Pat. 3 767 695 (Upjohn)
46) Crossley, N. S.: Tetrahedron Lett. *1971*, 3327
47) Muchowski, J. M.: Prostaglandins *1975*, Vol. 10, No. 2, 297
48) Guzman, A., Muchowski, J. M., Vera, M. A.: Chem. Ind. (London) *20*, 884, (1975)
49) Collet, A., Jacques, J.: Chimie Therapeutique *3*, 163, (1970)
50) BE Pat. 774784, Sandoz AG
51) Ambrus, G., Barta, J.: Prostaglandins *1975*, Vol. 10, No. 4, 661
52) Avramenko, V. G., Levinova, N. N., Nazina, V. D., Suverov, N. N.: Khim. Geterotsikl. Soedin. *2*, 204, (1975)
53) Leovey, E. M. K., Andersen, N. H.: J. Amer. Chem. Soc. *97*, 4148 (1975)
54) Leovey, E. M. K., Andersen, N. H.: Prostaglandins *1975*, Vol. 10, No. 5, 789
55) Andersen, N. H., Ramwell, P. W.: Arch. Intern. Med. *133*, 30 (1974)
56) Corey, E. J., Sachdev, H. S.: J. Amer. Soc. *95*, 8483 (1973)
57) Guzman, A., Crabbé, P.: Chem. Ind. (London) *1973*, 635
58) Plantema, O. G., de Konig, H., Huisman, H. O.: Tetrahedron Lett. *1975*, 2945, 4595
59) Hamon, A., Lacoume, B., Pasquet, G., Pilgrim, W. R.: Tetrahedron Lett. *1976*, 211
60) Grieco, P. A., Pogonowski, C. S., Nishizawa, M., Wang, C. L. J.: Tetrahedron Lett. *1975*, 2541
61) Grieco, P. A., Pogonowski, O. S., Miyashita, M.: J. Chem. Soc. Chem. Comm. *1975*, 592
62) Corey, E. J., Shiner, Ch. S., Volante, R. P., Cyr, C. R.: Tetrahedron Lett. *1975*, 1161
63) Bernardy, K. F., Polette, J. F., Weiss, M. J.: Tetrahedron Lett. *1975*, 765
64) US.-Pat. No. 3.836.581 (American Cyanamid Company)
65) Fried, J., Mehra, M. M., Chan, Y. Y.: J. Amer. Chem. Soc. *96*, 6759 (1974)
66) Fried, J., Sih, J. C.: Tetrahedron Lett. *1973*, 3899
67) Jarabak, J.: Proc. Nat. Acad. Sci. U.S. *69*, 533 (1972)
68) Fried, J. Heim, S., Sunder-Plassmann, P., Etheredge, S. J., Santhanakrishnan, T. S., Himizu, J.: Proc. Prostaglandin Symp. of the Worcester Foundation for Experimental Biology (P. W. Ramwell, J. E. Shaw Eds.). Interscience New York 1967, p. 351
69) Fried, J., Mehra, M., Lin, C., Kao, W., Dalven, P.: Ann. New York, Acad. of Sciences *180*, 38 (1971)
70) Schenck, G. O., Dunlap, D. E.: Ang. Chem. *68*, 248 (1965)
71) Sable, H. Z., Anderson, T., Talbert, B., Pasternak, Th.: Helv. Chim. Acta *46*, 1157 (1963)
72) Fried, J., Mehra, M. M., Kao, W. L.: J. Amer. Chem. Soc. *93*, 5594 (1971)
73) Bollinger, G., Muchowski, J. M.: Tetrahedron Lett. *1975*, 2931
74) Bruin, J. W., de Koning, H., Huisman, H. O.: Tetrahedron Lett. *1975*, 4599
75) Vlattas, J., Dellavecchia, L.: Tetrahedron Lett. *1974*, 4459
76) Corey, E. J., Vlattas, J., Andersen, N. H., Harding, K.: J. Amer. Chem. Soc. *90*, 3248 (1968)
77) Vlattas, J., Dellavecchia,: Tetrahedron Lett. *1974*, 4267
78) Vlattas, J., Dellavecchia,: Tetrahedron Lett. *1974*, 4455
79) Ohno, M., Narnse, N., Terasawa, J.: Org. Synthesis *49*, 27 (1969)
80) Harrison, J. T., Fletcher, V. R., Fried, J. H.: Tetrahedron Lett. *1974*, 2733
81) Vlattas, J., Ong Lee, A.: Tetrahedron Lett. *1974*, 4451
82) Ger. Pat. No. 2.229.225 (Tanabe Seijaku)
83) Hauser, F. M., Huffman, R. C.: Tetrahedron Lett. *1974*, 905
84) Mousseron, M., Manon, G., Combes, G.: Bull. Soc. Chim. France *1949*, 396
85) Jones, W. O., Withey, D. S.: J. Chem. Soc. *1954*, 3491
86) Harrison, J. T., Taylor, R. J. K., Fried, J. H.: Tetrahedron Lett. *1975*, 1165
87) Giantureo, M. A., Friedel, P., Giammarino, A. S.: Tetrahedron *20*, 1763 (1964)
88) Zwicky, G., Waser, P. G., Eugster, C. H.: Helv. Chim. Acta *42*, 1177 (1959)

89) Brit. Pat. 568 402 (1945)
90) Corrodi, H., Hardegger, E., Kögl, F.: Helv. Chim. Acta *40*, 2454 (1957)
91) Mantione, R.: Bull. Soc. Chim. France, *1969*, 4523
92) Loebich, F., Krämer, J. M. (E. Merck Research Lab.),: unpublished results
93) Hanessian, S., Dextraza, P., Fougerousse, A., Guidan, J.: Tetrahedron Lett. *1974*, 3983
94) Lourens, G. J., Koekemoer, J. M.: Tetrahedron Lett. *1975*, 3715
95) Lourens, G. J., Koekemoer, J. M.: Tetrahedron Lett. *1975*, 3719
96) Sowa, W.: Can. J. Chem. *46*, 1586 (1968)
97) Orth, D. (E. Merck Research Lab.): unpublished results
98) Corey, E. J., Nayori, R.: Tetrahedron Lett. *1970*, 311
99) Prepared by reaction of 1.2-oxidoheptane with methyl amine in EtOH under pressure
100) Corey, E. J., Weinschenker, N. M., Schaaf, T. K., Huber, W.: J. Amer. Chem. Soc. *91*, 5675 (1969)
101) Bruhn, M., Brown, C. H., Collins, P. W., Palmer, J. R., Dajani, E. Z., Pappo, R.: Tetrahedron Lett. *1976*, 235
102) Collins, P. W., Pappo, R.: Prostaglandins Vol. 10, No. 5, 733 (1975)
103) Heslinga, L., van Gorkem, M., van Dorp, D. A.: Rec. Trav. Chim. Phys.-Bas *87*, 1421 (1968)
104) Heather, J. B., Sood, R., Price, P., Pernzzotti, G. P., Lee, S. S., Lee, L. F. H., Sih, C. J.: Tetrahedron Lett. *1973*, 2313
105) Radunz, H. E., Krämer, J. M. (E. Merck Research Lab.): unpublished results
106) Corey, E. K., Nicolaou, K. C., Shibasaki, M.: J. Chem. Soc. Chem. Comm. *1975*, 658
107) Corey, E. J., Chaykovsky, M.: J. Amer. Chem. Soc. *87*, 1353 (1965)
108) Umbach, W., Mehren, R., Stein, W.: Fette, Seifen-, Anstrichmittel *71*, 199 (1969)
109) Miyano, M., Mueller, R. A., Dorn, C. R.: Intra-Science Chem. Rept. Vol. 6, No. *1*, 43 (1972)
110) Miyano, M., Stealey, M. A.: J. Org. Chem. *40*, 1748 (1975)
111) Miyano, M., Dorn, C. R., Mueller, R. A.: J. Org. Chem. *37*, 1810 (1972)
112) Dünnschichtschromatographie Stahl, E. (Ed.), Springer-Verlag, Berlin, Heidelberg, 2. Auflg., S. 631. New York: 1967
113) Pappo, R., Collins, P., Jung, C.: Tetrahedron Lett. *1973*, 943
114) We thank Dr. Schliep, Dr. Harting (E. Merck, Medical Research Laboratories) for performing the biological tests
115) Orth, D., (E. Merck, Research Lab.) unpublished results
116) DOS 2323193, (E. J. Du Pont de Nemours and Co., USA)
117) Harrison, J. T., Fletcher, V. R.: Tetrahedron Lett. *1974*, 2729
118) Harrison, J. T., Grayshan, R., Williams, T., Semenovski, A., Fried, J. H.: Tetrahedron Lett. *1972*, 5151
119) Schauenstein, E., Esterbauer: Fette, Seifen, Anstrichmittel, *70*, 4 (1968)
120) Bender, A. P.: J. Med. Chem. *18*, 1094 (1975)
121) Sun, F. F., Armour, S. B., Bockstanz, V. R., McGuire, J. C.: Adv. in Prostaglandin and Thromboxane Research (Samuelsson, B., Paoletti, R., eds.), Vol. 1, p. 177 New York: Ravens Press
122) Germ. OS 2423156 (Upjohn Company, USA)
123) Fried, J., Mehra, M. M., Kao, W. L., Lin, C. H.: Tetrahedron Lett. *1970*, 2695
124) Crabbé, P., Cervantes, A., Meana, M. C.: J. Chem. Soc. Chem. Comm. *1973*, 119
125) Crabbé, P., Guzman, A., Velarde, E.: J. Chem. Soc. Chem. Comm. *1972*, 1126
126) Gandolfi, C., Doria, G., Pharmaco, II: Ed. Sc. *29*, 327 (1974)
127) Dikshit, D. K., Kapil, R. S., Anand, N.: Indian J. Chem. *13*, 1353, 1359 (1975)

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Hypolipidaemic Aryloxyacetic Acids

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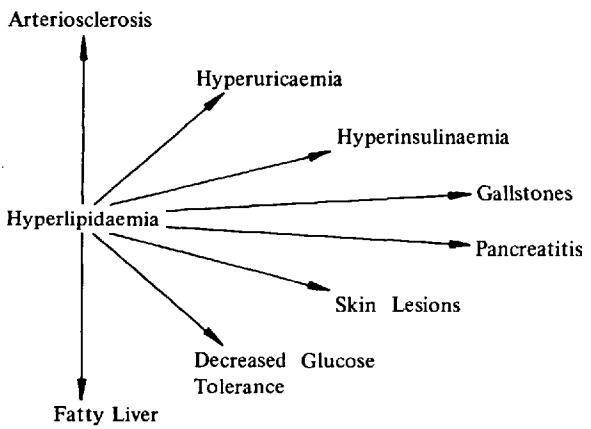
Table of Contents

A. Introduction

The increase of plasma lipids, formerly called hyperlipidaemia, in addition to hypertension, overweight, nicotine abuse, diabetes mellitus and certain environmental effects belongs to the so-called risk factors of arteriosclerosis. It was discovered that the water insoluble lipids (cholesterol, triglycerides and phospholipids) are transported in plasma by forming a macromolecular complex with specific proteins; this disorder is nowaday usually called hyperlipoproteinaemia, which today represents the most frequent metabolic disease. After it was found that cholesterol is the main lipid component of the arteriosclerotic lesions in the vascular wall, the number of investigations dealing with the correlation between plasma cholesterol concentration and the development of arteriosclerosis increased. The coincidence of hyperlipoproteinaemias and coronary heart disease may be considered established on the basis of comprehensive retrospective and prospective studies[1–5]. In a study by Carlson *et al.*[1] it was recently demonstrated that in males up to 60 years an elevated triglyceride serum level, independent of cholesterol, significantly increases the incidence of coronary diseases. The therapy of hyperlipoproteinaemia is an important goal in preventive medicine. Various primary preventive studies have indeed shown that the normalization of pathological values reduces or delays the risk of dying from the complications of arteriosclerosis. Hyperlipoproteinaemia is now, furthermore, recognized to be at least one of the factors involved in the pathogenesis of several other diseases.



This is an adequate basis for a therapy, although the biochemical pathogenetic correlations between increased blood lipids and arteriosclerosis or other diseases are largely unknown. In any case a lipid lowering therapy will be useful only if started early and understood as long-term therapy.

B. Substituted Aryloxyacetic Acids

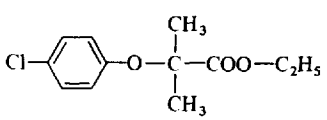
Clofibrate: Clofibrate [ethyl-2-(p-chlorophenoxy)-2-methyl-propionate], *1*, is one of the more frequently prescribed drugs in current use for the management of hyperlipoproteinaemia of various origins. Although the ethyl ester of this acid has been reported by Julia *et al.*[6], it was Thorp and Waring[7] who discovered that this compound and the free acid possess favorable hypolipidaemic activity in the rat. Clofibrate has been shown to be an effective lipid lowering drug in man[8, 9]. It is more effective in lowering triglycerides than serum cholesterol[7, 11]. The drug rapidly undergoes hydrolysis *in vivo*, and the corresponding acid is presumed to be the active drug[10]. It seems that clofibrate may be exerting its effect by multiple modes of actions[12]. Included among the proposed mechanisms of action are a decrease in the synthesis[13–15]and an increase in the catabolism of cholesterol and low density lipoproteins in the liver, decrease in hepatic lipoprotein secretion[16], decrease in plasma unesterified fatty acid concentration[17, 18], increase in the rate of conversion of cholesterol to bile acids in the liver[27], decrease in the rates of synthesis[19] and secretion of triglycerides in the liver and increase of the breakdown of triglycerides and very low density lipoproteins in the peripheral tissues and alterations in thyroid hormone distribution[20, 21]. The hope raised by three more comprehensive investigations[20–24] that clofibrate independent of its effect on raised blood fats might possess still other infarction-preventing properties does not seem to be confirmed[25]. The results of the Coronary Drug Project[25] demonstrate that primary prevention is far superior to secondary prevention, because the prognostic importance of lipid levels is much less in patients after cardiac infarction than before the infarction. The highest risk of infarction is a previous infarction[26]. In addition, the latter study[25] demonstrated only a slight lowering of the cholesterol level by 6.5% under clofibrate medication, while the triglycerides showed a better response with 22.2%. The necessary search for a more potent compound appears confirmed by this study.

Clofibrate derivatives: Several direct derivatives of clofibrate with comparable activities are commercialized or in the last stage of clinical trial, *e. g.* alufibrate as Atherolip (R)[28, 29](hydroxy-aluminium bis-[2-(p-chlorophenoxy)-2-methyl-propionate]), clofibride or MG 46 as Lipenan (R) (4-hydroxy-N-dimethylbutyramide-4-chloro-phenoxy-isobutyrate)[30] or simfibrate or CLY-503 (1,3-propanediol-bis-[2-p-chlorophenoxy-isobutyrate])[31].

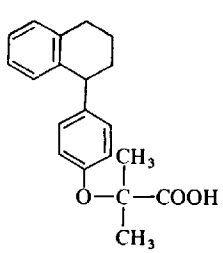
Compounds combining the structural elements of both the proven lipid lowering drugs clofibrate and nicotinic acid or of the corresponding alcohol β-pyridylcarbinol (Ronicol) are more interesting because they are more effective than pure clofibrate. In this context the following products, already on the market, should be mentioned: Etofibrate as Lipo-Merz (R) [2-(p-chloro-phenoxy)-2-methyl-propionic acid-[2-(nicotinoyl-oxy)-ethyl ester][32, 33] and clofenpyride or ATE as Arterium-V (R) [3-hydroxy-methylpyridine-(p-chlorophenoxy)-α-isobutyrate hydrochloride][34]. The potentiation of the lipid lowering activity of clofibrate by combination with low doses of 3-pyridylcarbinol was discovered by Simane and Nowak[35]. A combination of clofibrate plus β-pyridylcarbinol 20:1 (Liapten (R)) was selected, and clinical trials confirmed the synergistic acitivity[36].

1. α-Aryloxyisobutyric Acids

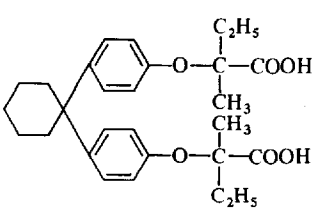
A larger number of aryloxyisobutyric acids have been prepared for hypolipidaemic screening. The reason for this was the great success of clofibrate, but it might also be the ready availability of these compounds. Phenols react readily in acetone with chloroform in the presence of a strong base to furnish α-substituted isobutyric acids[37]. Among these investigational drugs only a few were of some importance, but none obtained the importance of clofibrate. A number of clinical publications on nafenopine or SU-13, 437 [2-methyl-2-[(p-1.2.3.4.-tetrahydro-1-naphthyl)-phenoxy] propionic acid] appeared during recent years[38–42]. In general, results showed greater reduction of serum triglycerides than of serum cholesterol. Early claims for an advantage over clofibrate in treatment of type II hyperlipidaemia have not been confirmed. In a comparative study of the two drugs, Dujovne *et al.* found nafenopine (600 mg per day) slightly, but not significantly superior to clofibrate (2 g per day) in overall reduction of serum lipids, but cholesterol reduction was not superior in type II, and there may have been an "escape" of serum triglycerides in the type IV patients with nafenopine[38]. The detection of liver pathology in long-term, high-dosage studies in rats has resulted in withdrawal of nafenopine from further clinical trials[40]. Methylclofenapate or ICI 55, 695 (methyl-2-[4-(p-chloro-phenyl)-phenoxy]-2-methyl-propionate), a biphenyl derivative of the methyl ester of clofibrate, was slightly more effective than clofibrate in reducing cholesterol in type II patients, and it was much more effective than clofibrate in reducing cholesterol and triglycerides in type III and IV patients[43]. The compound was withdrawn after preliminary trial in humans because it was found to have late hepatotoxic properties in mice and rats[43]. The effect of S-8527 *3* (1.1.-bis-[4′-(1″-carboxy-1″-methyl-propoxy)-phenyl] cyclohexane) on cholesterol metabolism and serum and liver lipids in rats has been studied[44–47]. S-8527 has been reported to possess pronounced hypolipidaemic properties in experimental animals and is considered to be more potent in hypolipidaemic activity and less potent in hepato-



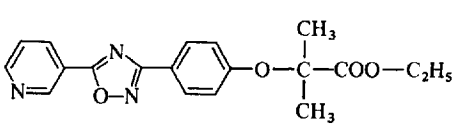
[1] Clofibrate



[2] Su-13,437, Melipan, Nafenopine



[3] S-8527



[4] AT-308

megalic effect than clofibrate[45, 46]. In pharmacological tests on hypercholesterolaemic or normocholesterolaemic rats AT 308 *4* (3-[4-(1-ethoxycarbonyl-1-methyl-ethoxy)-phenyl]-5-(3-pyridyl)-1.2.4-oxadiazole) showed the highest hypocholesterolaemic activity amongst several compounds of a large series[48]. The higher activity of ethyl-2-(dibenzo-furanyl-4-oxy)-2-methyl-propionate against clofibrate in mouse and rat models is reported by a Swedish research group[49, 50]. Another new α-aryl-oxyisobutyric acid BM 15.075 (2-[4-chloro-benzamidoethyl)-phenoxy]-2-methyl-propionic acid) was recently shown to be about 20 times as potent as clofibric acid in rat hypercholesteraemia and hypertriglyceridaemia[51]. Clinical trials have been started with this compound. In Helsinki[52a, b] some pharmacological, toxicological and clinical results of another structural analog of clofibrate, called LF 178 or Lipanthyl (isopropyl-2-[4-(4-chlorobenzoyl)-phenoxy]-2-methyl-propionate, were reported. Depending on the model, Lipanthyl is 6–10 times more active than clofibrate in animal experiments. Since the compound was nontoxic and free of side effects, it has been commercialized.

2. 2-Phenoxyalkylic Acid Derivatives

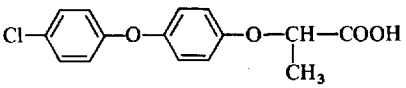
Among a number of 2-phenoxyalkylic acids, which were synthesized and tested[53], only two compounds have obtained greater importance. The first compound, GP-45699 [D,L-2-(p-diphenyloxy)-heptanoic acid] has been prepared by Nardi *et al.*[54], and has been tested in man[55, 56]. The compound gave a greater and more sustained reduction of plasma cholesterol than clofibrate, but the side effects were too common and severe to justify its routine use[55]. The second one, HCG-004 or fenofibric acid *5* (2-[4-(4′-chlorophenoxy)-phenoxy]-propionic acid), has been synthesized and tested by a German research group[57]. In normolipidaemic and hyperlipidaemic animals HCG-004 was a well-tolerated and highly effective oral hypolipidaemic drug. Within the hypolipidaemically interesting dosage range no other pharmacological or chronic-toxicological effects were found[57, 58]. Therefore, clinical trials have been started, but no recent results are available.

3. 2-Phenoxyphenylacetic Acid Derivatives

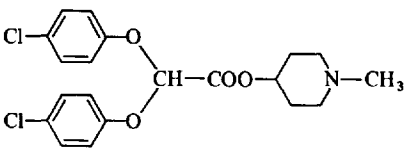
Halofenate or MK-185 *6* [2-acetamidoethyl-(p-chlorophenyl)-(m-trifluoromethyl-phenoxy)-acetate] is the best known compound of the 2-phenoxy-phenylacetic acid type. Its hypolipidaemic activity has been proved by several research groups[59–64]. In rats, halofenate reduced both cholesterol and triglycerides, with a potency 5.7 times that of clofibrate, but in man the changes of cholesterolaemia were minimal, inconsistent and not statistically significant. Halofenate was found, on the contrary, quite effective compared to clofibrate in reducing plasma triglyceride levels. Halofenate is the only drug among the aryloxyacetic acids which has been found to induce a very notable decrease of uricaemia[56, 63]. This may indicate a special rationale for this drug in the not uncommon type IV patient with mild diabetes and hyperuricaemia[64]. In the meantime halofenate is commercialized under the trade name Livipas (R) in Great Britain.

4. Bisaryloxyacetic Acid Derivatives

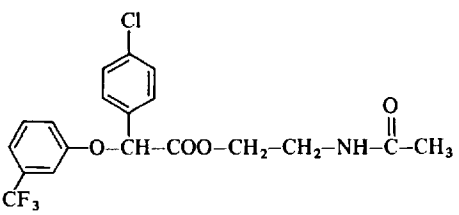
In this structural series SaH 42-348 or lifibrate *7* [1-methyl-4-piperidyl-bis(p-chlorophenoxy)-acetate] is the best studied compound. It has been under investigation for the past 8 years. A lot of publications can be found in the literature [65–71]. SaH 42-348 was reported to be an effective hypolipidaemic agent, 9 times more potent than clofibrate in male rats[65]. Clinical trials have demonstrated its efficacy in the treatment of type II hyperlipoproteinaemic disorders[66]. However, recent observations in one of 4 patients treated with low doses of SaH 42-348 in a pilot study suggested acute hepatotoxicity[69]. It must be supposed that SaH 42-348 has been withdrawn from further clinical trials. Three more, direct derivatives of SaH 42-348, have been described in the literature, but they are not of higher importance than the parent compound[51, 52a, c, 72]. Of more interest in this structural class is treloxinate *8* (methyl-2,10-dichloro-12H-dibenzo[d, g], [1, 3]-dioxocin-6-carboxylate)[73, 74]. The p-chlorophenyl substituents are held in a



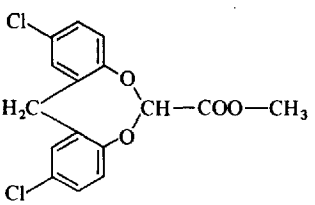
[5] HCG–004, Fenofibric acid



[7] SaH 42–348, Lifibrate



[6] MK–185, Halofenate, Livipas



[8] Treloxinate

fixed conformation as part of the dioxocin ring structure. In rats treloxinate is 8 times as potent as clofibrate in reducing plasma cholesterol, and 30 times as potent in reducing triglycerides. A large number of derivatives of treloxinate have been synthesized and tested for hypolipidaemic activity in rats[74]. The drug is currently undergoing clinical trials, but no recent results could be found in the literature.

5. Miscellaneous Acids

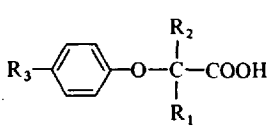
The discovery of the hypocholesterolaemic properties of probucol or DL-581 [4.4-(isopropylidene-dithio)bis(2.6-di-t-butyl-phenol)] led to a structure-activity study among related compounds[75]. The result of this study was the selection of DL

990 [2-(3.5-di-t-butyl-4-hydroxy-phenylthio)-hexanoic acid] for further development. DL-990 possessed better hypocholesterolaemic and especially more potent hypotriglyceridaemic properties than probucol[51]. The clinical examination has been initiated. ICI 59897[2-(4′-chloro-biphenylyl-(4)-methoxy)-2-methyl-propionic acid] must be considered as a drug with slighter hypolipidaemic activity, but with more pronounced beneficial side effects[51]. RMI 14,514 [(5-tetradecyl-oxy)-2-furoic acid] is more effective than clofibrate in reducing plasma cholesterol levels. Plasma triglycerides are reduced to approximately the same degree by both agents[76]. The hepatomegaly was significantly less than that seen after treatment with clofibrate. The synthesis of a new potent antihypercholesterolaemic agent, Wy-14643 [4-chloro-6-(2.3-xylidino)-(2-pyrimidinylthio)-acetic acid], has recently been reported[77]. The activity of this drug depends very much on the animal models used. Wy-14643 had only a slight effect on the serum cholesterol of normal rats, but the compound proved to be more potent than clofibrate by a factor of about 100 in the hypercholesterolaemic rat model[51, 78]. Tibric acid, CP-18.524 or Exirel (R) [2-chloro-5-(*cis*-3.5-dimethylpiperidino-sulfonyl)benzoic acid] is a member of a new class of drugs which produces hypolipidaemic effects in rats[51, 79]. The effects of tibric acid on hyperlipoproteinaemia in man were studied in several different hospitals[51, 80–82]. This novel, generally well tolerated drug is more useful in the treatment of hypertriglyceridaemias than of hypercholesterolaemias. Real advantages over clofibrate could not be noted. The compound was recently commercialized in Switzerland.

It can be seen that several drugs mentioned are useful lipid lowering agents. Generally, however, no drug is known which completely normalizes hyperlipidaemia. Consequently, a search for drugs which are equipotent in affecting the hypercholesterolaemia as well as hypertriglyceridaemia was made in this laboratory. Furthermore, it is necessary to seek for lipid lowering agents which are more specifically effective against various types of hyperlipoproteinaemias. The success of clofibrate derivatives and their acceptance by the medical profession instigated us to synthesize structurally similar compounds.

C. Chemistry

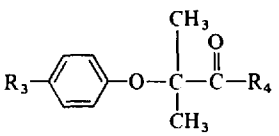
In the present article the syntheses of several substituted aryloxyacetic acids *9* are discussed in short concentrated form. In a later communication the detailed preparative procedures will be reported. The clofibrate structure *1* was modified in the carboxylic acid part by esterification with selected alcohols or by amidation



[9]

with special amines or by reduction to the corresponding alcohols. One or both of the two methyl groups at the α-carbon atom were replaced by hydrogen, aryl or aryloxy groups. In several cases the p-chlorine-atom of the clofibrate structure was substituted by aryl, aryloxy and aryloxymethyl groups, isocyclic and heterocyclic ring systems. The choice of the moieties was not done arbitrarily, but the well-known above mentioned lipid lowering agents formed a structural framework. You will find direct clofibrate derivatives, substituted with heterocyclic groups *22–24*, isocyclic and heterocyclic nafenopine compounds *25, 26, 31–34*, piperidyl substituted methylclofenapate analogs *27–30*, with different heterocyclic moieties substituted halofenate structures *42–46* and substituted lifibrate compounds of various structures *54–57*. Furthermore, a direct structural alteration of the fenofibric acid was performed with the aryloxymethyl types *39–41*. A selection of the prepared compounds will be found in Tables 1–6.

Table 1. α-Aryloxyisobutyric acid derivatives



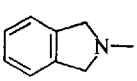
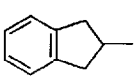
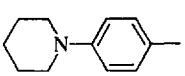
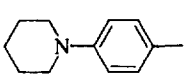
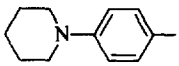
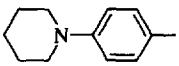
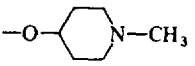
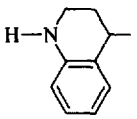
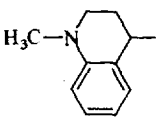
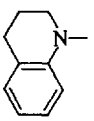
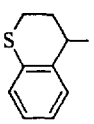
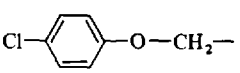
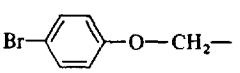
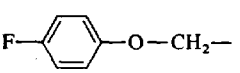
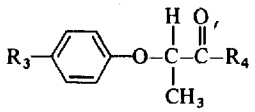
Cmpd.	R_3	R_4	mp. °C[1)]
22		–OH	105–107
23		–OH	203–205
24		–OH	159–160
25		–OH	202
26		–OH	125–127
27		–OH	224–226
28		–O–CH_2–CH(OH)–CH_2–OH	94–96

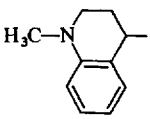
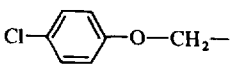
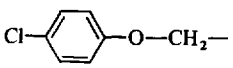
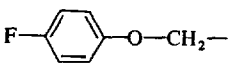
Table 1 (continued)

Compd.	R_3	R_4	mp. °C[1])
29		$-NH_2$	210–212
30			78–82
31		–OH	222–224[2])
32		–OH	221–223[2])
33		–OH	115–117 198–201[2])
34		–OH	220–225[2]) 137–139[3])
35		–OH	153
36		–OH	170–172
37		–OH	123–125

[1]) Uncorrected.
[2]) Cyclohexylamine salt.
[3]) Diisopropylamine salt.

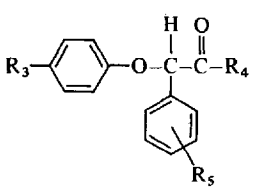
Table 2. α-Aryloxypropionic acid derivatives[88, 91, 92]



[1]) Compd.	R_3	R_4	mp. °C[1])
38		–OH	203–205[2])
39		–OH	158–159
40		$-OCH_3$	70–71
41		–OH	143–144

[1]) Uncorrected.
[2]) Cyclohexylamine salt.

Table 3. α-Aryloxyphenylacetic acid derivatives[88, 89, 92]



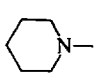
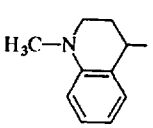
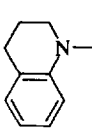
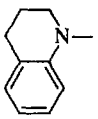
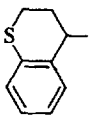
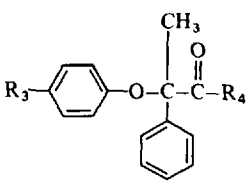
Compd.	R_3	R_4	R_5	mp. °C[1]) (n_D^{20})
42		–OH	p-Cl	173–175
43		–OH	H	165–168[2])
44		–OH	H	169–171[2])

Table 3 (continued)

Compd.	E_3	R_4	R_5	mp. °C[1]) (n_D^{20})
45		$-OC_2H_5$	p-Cl	(1,6129)
46		-OH	H	239–241[3])

[1]) Uncorrected.
[2]) Diisopropylamine salt.
[3]) Cyclohexylamine salt.

Table 4. α-Aryloxyhydratropic acid derivatives[88, 89, 93])



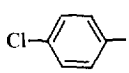
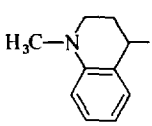
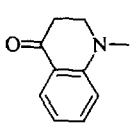
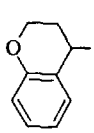
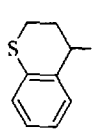
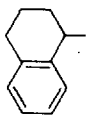
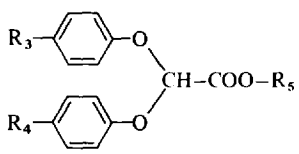
Compd.	R_3	R_4	mp. °C[1])
47		-OH	101–102
48		-OH	127
49		-OH	172; 190–192[2])
50		-OH	178–180[2])
51		$-OC_2H_5$	93–96
52		-OH	187–189[2])

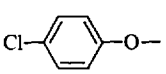
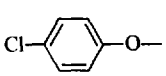
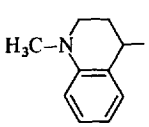
Table 4 (continued)

Compd.	R_3	R_4	mp. °C[1])
53		–OH	195–197[2])

[1]) Uncorrected.
[2]) Cyclohexylamine salt.

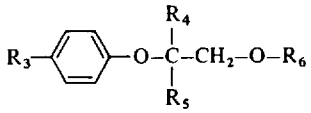
Table 5. Bisaryloxyacetic acid derivatives[95, 96])

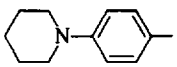
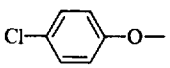
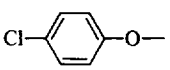
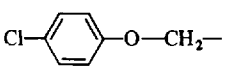
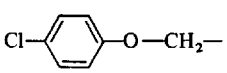
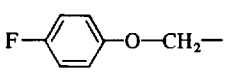
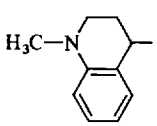


Compd.	R_3	R_4	R_5	mp. °C[1])	(n_D^{20})
54			$-C_2H_5$	208[2])	
55	Cl–		–H	122–123	
56	Cl–		$-C_2H_5$		(1,5796)
57	Cl–		–H	152–155[3])	

[1]) Uncorrected.
[2]) HCl salt.
[3]) Diisopropylamine salt.

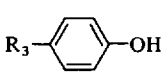
Table 6. Aryloxyalkanol derivatives[97]



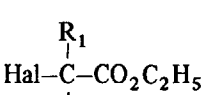
Compd.	R_3	R_4	R_5	R_6	mp. °C[1]	(n_D^{20})
58		$-CH_3$	$-CH_3$	$-H$	158–160	
59		$-H$	$-CH_3$	$-H$	30–32	
60		$-H$	$-CH_3$	$-C(=O)-CH_3$		(1,5550)
61		$-H$	$-CH_3$	$-H$	77–78	
62		$-CH_3$	$-CH_3$	$-H$	67–70	
63		$-CH_3$	$-CH_3$	$-H$	108–109	
64		$-CH_3$	$-CH_3$	$-H$	92–94	

[1]) Uncorrected.

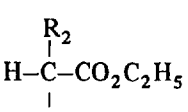
The compounds of structure *9* were obtained by condensing a phenol of formula *10* with a compound of formula *11* and following saponification or in the special case of $R_1 = R_2 = CH_3$ with chloroform and acetone in the presence of potassium hydroxide[37]. The compounds *11* were commercially available or readily prepared by α-halogenation of the corresponding esters *12*.



[*10*]



11



12

The other starting materials, the phenols *10*, were only in part commercially available. Some of these compounds were synthesized by methods described in the literature, but a greater part of them were unknown and new procedures had to be developped for them.

In the course of our synthetic program on hypolipidaemic aryloxyacetic aids all the following phenols have been prepared.

4-(4-Chlorophenyl)-phenol was obtained by the method of Savoy and Abernethy[85], 4-(4-chlorophenoxy)-phenol by the procedure of Granzer and Nahm[57] and 4-(1.2.3.4-tetrahydro-l-naphthyl)-phenol by the route used by Hess and Bencze [86].

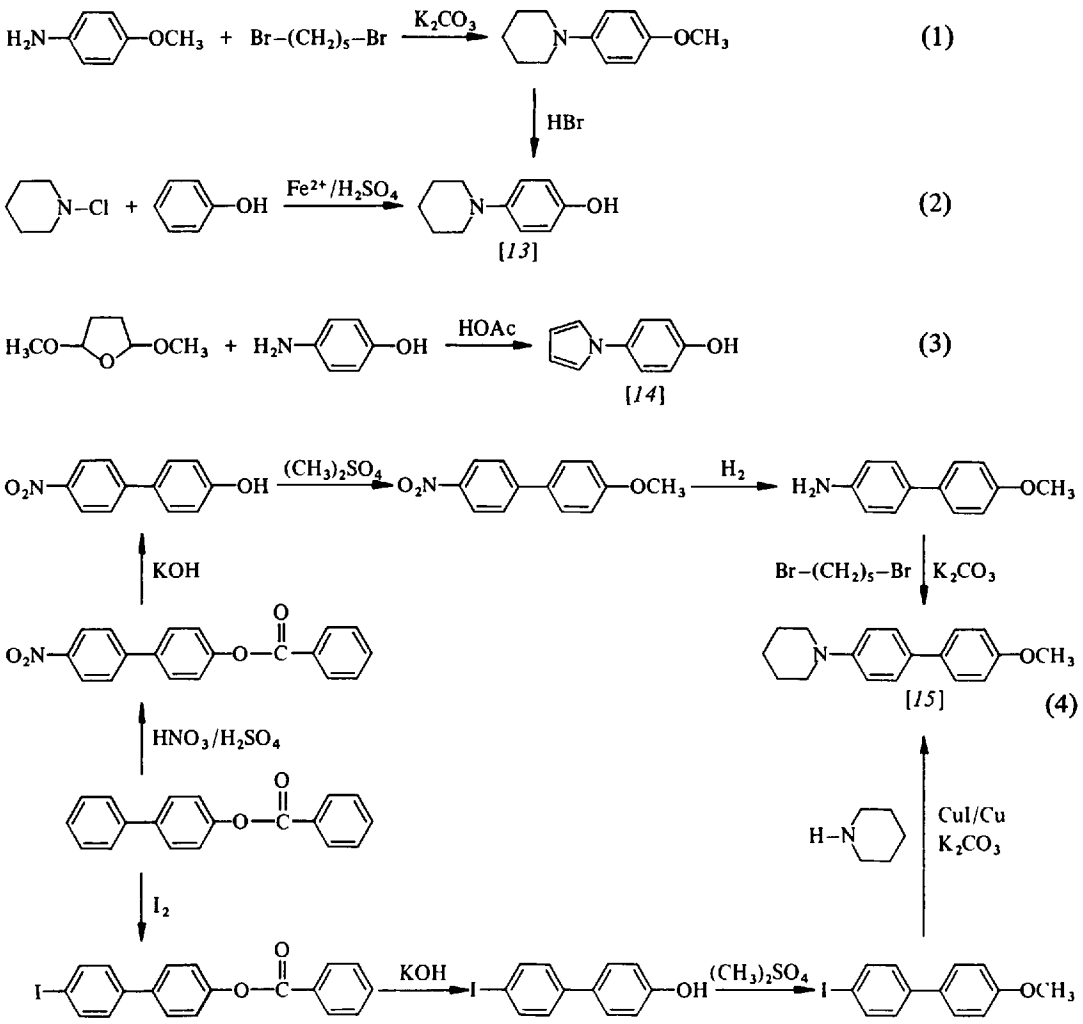
4-Piperidinophenol *13* was prepared by two different ways [Eqs. (1) and (2)]: first by reacting p-anisidine with 1.5-dibromopentane and treating the resulting 4-piperidino-anisole with HBr, and second by the homolytic aromatic amination of phenol with N-chloropiperidine[83].

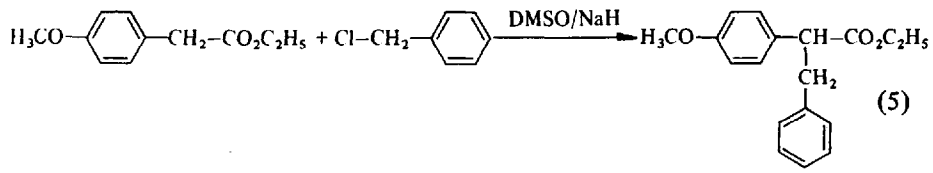
4-Pyrrolidino-phenol was formed analogously from p-anisidine and 1.4-dibromobutane. 4-Isoindolino-phenol was obtained by reacting p-anisidine with o-xylilene dibromide to form 2-p-methoxyphenyl-isoindoline, and splitting the ether with HBr.

4-(1-Pyrryl)-phenol *14* was obtainable by reacting p-amino-phenol with 2.5-dimethoxytetrahydrofuran [Eq. (3)]. 4-(4-piperidinophenyl)-phenol could be easily prepared in two different ways. One of the synthetic possibilities was the reaction of 4-amino-4′-methoxy-biphenyl[84, 94] with 1,5-dibromopentane to form 4-piperidino-4′-methoxy-biphenyl *15* and splitting the ether with HBr. The other possibility to get *15* and the corresponding phenol was the nucleophilic amination of 4-iodo-4′-methoxy-biphenyl[98] with excess piperidine. Starting material for both procedures was 4-phenylphenyl-benzoate [Eq. (4)].

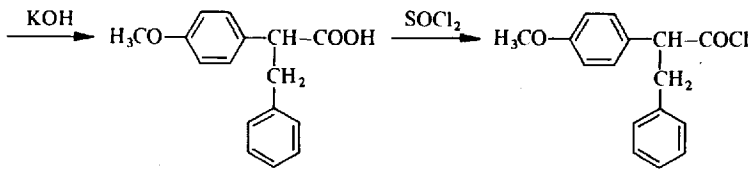
4-(2-Indanyl)-phenol *16* was obtained by reacting p-methoxy-phenyl-acetic acid ethyl ester with benzylchloride to form α-benzyl-p-methoxyphenyl ethyl acetate, saponification into the acid, conversion of the acid with thionylchloride into the chloride, cyclization to 2-p-methoxy-phenyl-l-indanone, $NaBH_4$ reduction to 2-p-methoxyphenyl-l-indanole, dehydration with p-toluene-sulphonic acid in toluene to 2-p-methoxyphenyl-indene, catalytic hydrogenation to 2-p-methoxyphenyl-indene, and treating the ether with HBr [Eq. (5)].

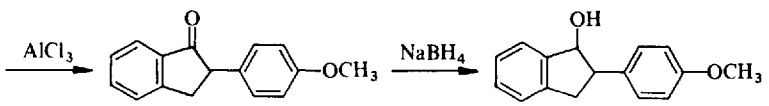
For the preparation of 4-(1.2.3.4-tetrahydroquinolino)-phenol *17* three different procedures were worked out [Eqs. (6)–(8)]. *17* was prepared by reacting N-p-methoxyphenyl-anthranilic acid with acetic anhydride and subsequent saponification to 1-p-methoxyphenyl-4-hydroxy-2-quinolone, reaction with $POCl_3$ to form l-p-methoxyphenyl-4-chloro-2-quinolone, hydrogenation to l-(p-methoxyphenyl)-3.4-dihydro-2-quinolone, splitting the ether with HBr to l-(p-hydroxyphenyl)3.4-dihydro-2-quinolone, and reduction with $LiAlH_4$ [Eq. (6)]. Another synthetic possibility was the reaction of p-anilinophenol with β-propiolactone and subsequent cyclization to l-(p-acetoxyphenyl)2.3-dihydro-4-quinolone *18*. The next step, the Wolff-Kishner reduction, led directly to the desired product [Eq. (7)]. The third way, the direct amination of p-iodoanisole with 1.2.3.4-tetrahydroquinoline and the subsequent splitting of 4-(1.2.3.4-tetrahydro-quinolino)-anisol with HBr was the best one [Eq. (8)]. Saponification of l-(p-acetoxyphenyl)-2.3-

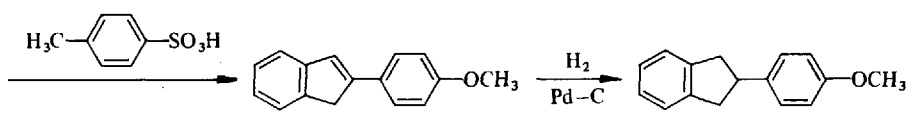

H2N
OCH3
+ Br–(CH2)5–Br
K2CO3
N
OCH3
(1)
HBr
N–Cl
+
OH
Fe2+/H2SO4
N
OH
(2)
[13]
H3CO
O
OCH3
+ H2N
OH
HOAc
N
OH
(3)
[14]
O2N
OH
(CH3)2SO4
O2N
OCH3
H2
H2N
OCH3
KOH
Br–(CH2)5–Br
K2CO3
O2N
O
C
N
OCH3
[15]
(4)
HNO3/H2SO4
H–N
CuI/Cu
K2CO3
I2
I
KOH
I
OH
(CH3)2SO4
I
OCH3

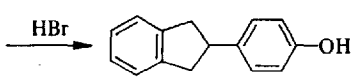


(5)



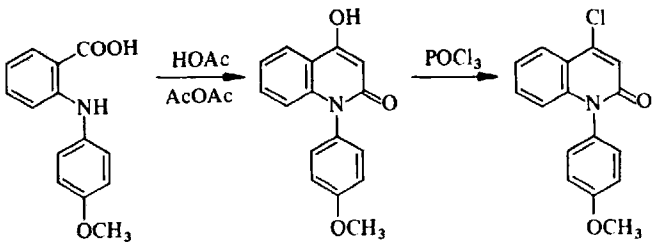




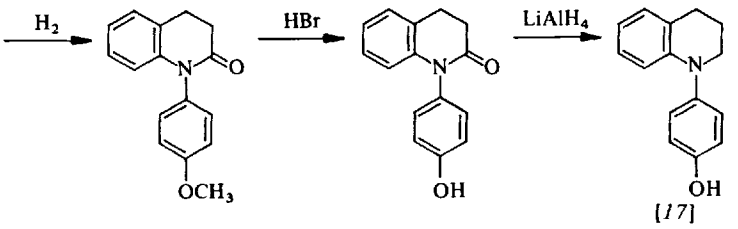


[16]

dihydro-4-quinolone *18* to 1-(p-hydroxyphenyl-2.3-dihydro-4-quinolone was carried out with potassium hydroxide in ethanol. 4-(1.2.3.4-Tetrahydro-4-quinolyl)-phenol *19* was prepared by cyclization of p-methoxy-cinnamic acid anilide with polyphosphoric acid to form 4-(p-methoxyphenyl)-1.2.3.4.-tetrahydro-2-quinolone, reduction with $LiAlH_4$ to 4-(p-methoxyphenyl-1.2.3.4-tetrahydro-quinoline *20*, and splitting the ether with HBr [Eq. (9)]. Catalytic reductive methylation of *20* with formaldehyde (H_2) 5% Pd-C gave l-methyl-4-p-methoxyphenyl-1.2.3.4-tetra-hydro-quinoline. The corresponding phenol, 4-(l-methyl-1.2.3.4-tetrahydro-4-quinolyl)-phenol was obtained by ether splitting with HBr. The attempts to prepare 4-p-hydroxyphenylchromane *21* and 4-p-hydroxyphenyl-thiochromane by an analogous Friedel-Crafts-procedure were successful. 4-Chromanol or 4-thiochromanol reacted with phenol in the presence of $AlCl_3$ to form the above mentioned products [Eq. (10)].



(6)



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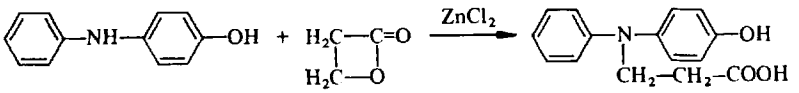

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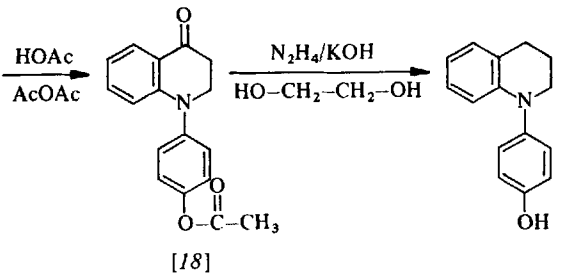
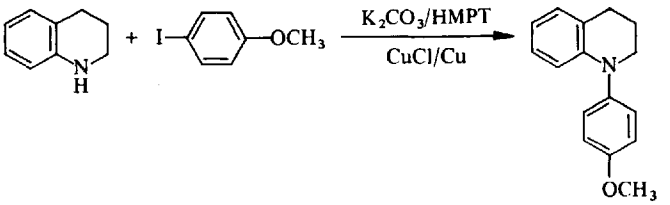
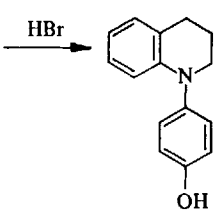
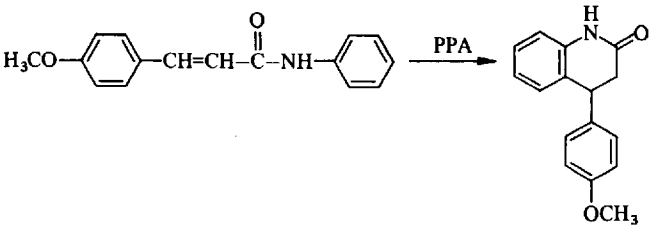


[18]

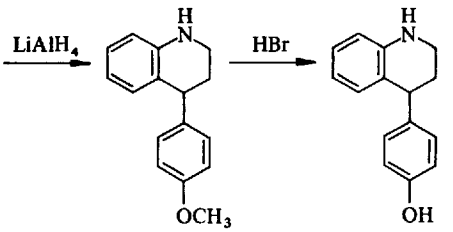


(8)

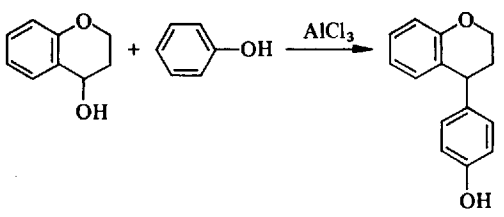




(9)



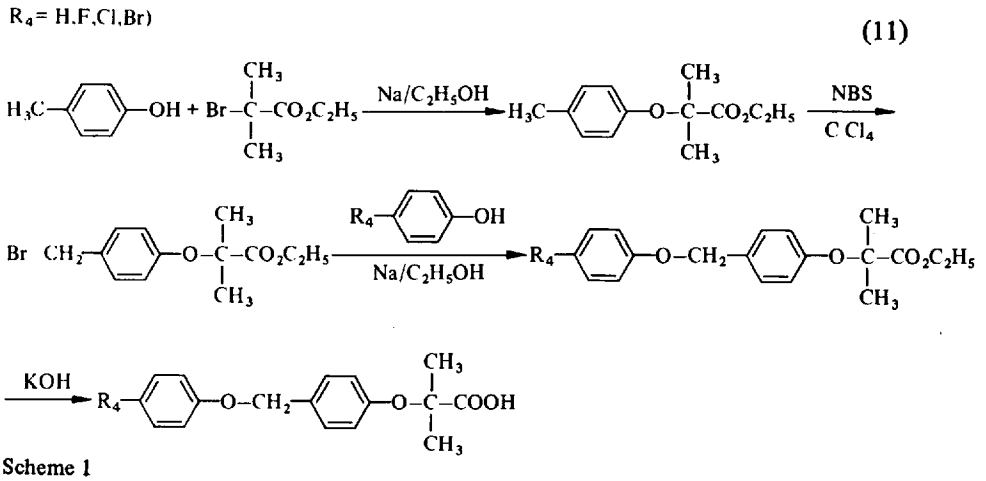
[20] [19]



[21]

(10)

For our structural group (R_1 = H, CH_3; R_2 = CH_3; R_3 = R_4–C_6H_4–O–CH_2–), see formula *9*, special procedures were found to be superior to the normal phenol alkylation as the last step [Eqs. (11) and (12)].

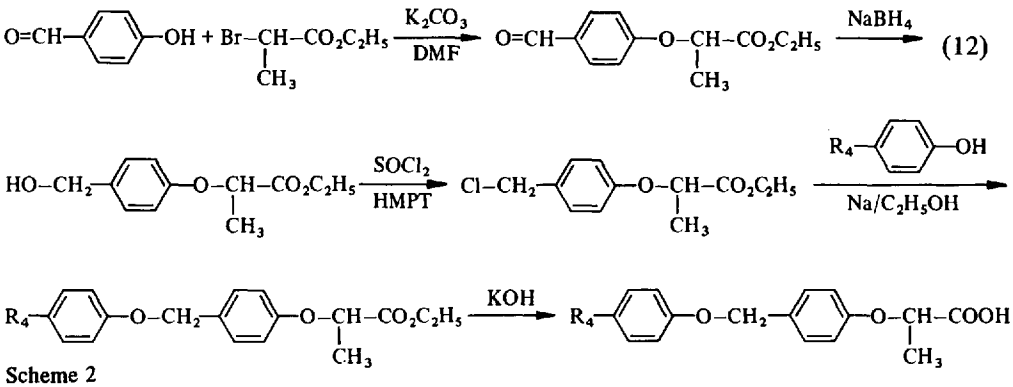


(R_4 = H,F,Cl,Br)

(11)

Scheme 1

R_4= H,F,Cl,Br,)



Scheme 2

D. Hypolipidaemic Activity

The investigation of the hypolipidaemic activity was done by Z. Simane at the Biochemical Research Department of E. Merck, Darmstadt.

1. Methods

Male Wistar rats initially weighing 160–200g were used for the experiments. All animals were kept in macrolon cages in groups of 5, weighed twice weekly and given food (Altromin-R (R)) and water ad libitum. The compounds to be tested were administered orally, suspended in a medium as described by Dorfman *et al.*[99] (0,5 ml per 100 g body weight). The rats of the control groups received the same volume of medium without drugs. The experiments were carried out as follows:

Rats fed basal diet

The drugs were administered for 11 days. Two hours after treatment on the 10th day blood samples were taken retroorbitally[100] and analyzed for total cholesterol [101]. Then the animals received 10% fructose solution in place of drinking water for the next 24 h. After the last administration of the test compound or mixture the rats were killed, blood samples were taken, and the serum obtained was analyzed for triglycerides[102].

PTU-Rats

The animals received drinking water containing 0,01% 6-propylthiouracil (PTU) ad libitum. After 2 days oral administration of drugs to be tested began and was

continued for 11 days. Blood samples for cholesterol estimation were obtained and further treatment of the rats was carried out in the same manner as described for rats fed basal diet. The data obtained in these experiments were statistically evaluated by the t-test according to the method of Dunnett[103].

2. Results

The results of the screening for hypolipidaemic activity of all the synthesized compounds will be presented in a later communication. But the most striking results will be reported in the following discussion.

E. Discussion

1. α-Aryloxyisobutyric Acid Derivatives

Those compounds directly related to clofibrate showed more or less pronounced effects on the lipid levels in experimental animals. Surprisingly, the substitution of the 4-chlorine atom by the isocyclic 2-indanyl moiety *26* or by heterocyclic moieties, for instance pyrrolidinyl *22*, piperidyl *23*, pyrrolyl *24* or isoindolyl *25*, largely maintained the effect. On the other hand, an increase of the lipid lowering activities up to the factor 10 was found if the heterocyclic substitutents were 1.2.3.4.-tetrahydro-4-quinolyl *31*, l-methyl-1.2.3.4-tetrahydro-4-quinolyl *32* or 1.2.3.4-tetrahydro-l-quinolyl *33*. An increase of the lipophilia of the compounds by the introduction of a 4-piperidino-phenyl moiety instead of the 4-chlorine atom into clofibrate elevates the activity additionally. The prototype of these 4′-piperidinobiphenyl-(4)-compounds, the free acid *27*, was 10 to 20 times more active than clofibrate, depending on the experimental model. A number of compounds derived from *27* was made by esterification or amidation of the free carboxylic group to investigate the structure-activity relationship. The amide *29*, the amino ester *30* and the l-glyceryl ester *28* were the most active compounds among the investigated so-called "pro-drugs", especially with regard to their hypotriglyceridaemic effects. Similar results were obtained in other structural classes where the carbocyclic group was modified.

A completely different modification of the clofibrate structure was obtained by synthesis of the phenoxy-methylphenoxyisobutyric acids. The 4′-bromo *36* and the 4′-chloro compound *35* were found to be considerably more active than the reference compound clofibrate. But whether definite advantages of these compounds over clofibrate can be demonstrated will be shown only by the current investigations on the mode of action, of side effects and toxicity.

2. α-Aryloxypropionic Acid Derivatives

One methyl group can be substituted at the α-carbonatom of the clofibrate structure with an hydrogen atom without loss of activity. This could be demonstrated with the identical activity of the compounds *32* and *38*.

Similarly, as in the class mentioned above, the phenoxymethyl derivatives of aryloxy propionic acid derivatives were found to be highly effective. Thus compound *39* on comparing the dose response curves were 23 times more active than clofibrate in lowering the cholesterol level and 48 times more active in lowering the triglyceride level. In comparison to HCG-004 *5*, the direct structural analog, an equal activity in lowering serum lipid levels of rats was found. Unfortunately, compound *39* in acute toxiocological experiments showed only a small safety margin so that development was discontinued. The fluorine derivative *41* showed about the same hypolipaemic effects; its toxicological properties are as yet unknown.

3. α-Aryloxyphenylacetic Acid Derivatives

Investigation of this substance class showed that both methyl groups at the α-C atom of the clofibrate structure may be substituted by the hydrogen atom or the phenyl group without causing a substantial loss of activity. The activity of *42* was directly comparable to that of halofenate *6*, and compounds *43*, *45* and *46* showed the same or a slightly better activity than the corresponding α-aryloxy isobutyric acid derivatives of Table 1. Summarizing, the results are approximately comparable to those described under Section 1.

Compound *43* which was of particular interest, showed a marked inhibition of drug metabolizing enzymes in the liver. The question of whether these structures, similar to halofenate *6*, also possess comparable properties with regard to the lowering of hyperuricaemia, has not yet been elucidated.

4. α-Aryloxyhydratropic Acid Derivatives

The class of α-aryloxy hydratropic acid derivatives (see Table 4) combines the characteristics of the clofibrate and the halofenate structure. Surprisingly, this slight modification of the basic structure caused a striking increase in activity. Compound *47*, structurally most closely related to clofibrate, showed a spectrum of activity, which was superior to that of clofibrate (factor 5–10). Impressive also was the higher activity of compound *53* against Su-13.437 *2*. The advantage of compounds *48–52* was similarly clear. Several representatives selected from this series were prepared for a clinical trial.

5. Bisaryloxyacetic Acid Derivatives

The data already known from the literature showing that compounds of this structural class are 8–10 times more active than clofibrate were confirmed. The results obtained with SaH 42-348 *7*, *54* and *57* showed a comparable activity of the three compounds with slight advantage of SaH 42-348. It is also possible in this structural field to substitute one or both chloroatoms of the Sandoz compound *7* with heterocyclic moieties to preserve the activity. Because of its high activity

and weak acute toxicity the ethyl acetate *56*, substituted unsymmetrically by 4-chlorophenol (clofibrate moiety) and 4-(4-chlorophenoxy)-phenol (HCG-004 moiety), and the corresponding free acid *55* are of particular interest. Their lipid lowering activities were comparable to that of SaH 42-348, but some advantage was found with *55* with regard to the influence on serumtriglyceride levels. More extensive investigations concerning side effects and long-term toxicology are planned with these compounds.

6. Aryloxyalkanol Derivatives

It is a novel finding that in special cases the alcohols and their derivatives, obtained by reduction of the corresponding active carboxylic acid derivatives,also possess antihyperlipaemic activity. These qualities were found with all the compounds listed in Table 6. Compounds *59* and *60* deserve particular mention as their activities are by far superior to clofibrate and equal those of the corresponding acid HCG-004 *5*. There exists some indication that this is an intrinsic effect of the compounds and not the effect of the acid *5* produced by biotransformation. This finding is sufficiently important to deserve more detailed investigation.

Since the development of many promising lipid lowering drugs was interrupted because of lack of clinical efficacy or unfavorable side effects, it will be important to give more and earlier attention than before to the toxicological properties which are of special importance in drugs for a long-term therapy. Beneficial side effects, for instance a fibrinolytic effect or a platelet aggregation inhibition, will become more important in the future. Extensive research has been continued all over the world, and this has increased our knowledge about both genetic and environmental factors inducing hyperlipidaemia. It is a realistic goal for the research programs in the next years to clarify the pathogenesis and etiology and to establish a specific therapy for the various forms of hyperlipidaemia.

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F. References

1) Carlson, L. A., Böttiger, L. E.: Lancet I *1972*, 863
2a) Braunsteiner, H., Herbst, M., Sailer, S., Sandhofer, F.: Schweiz. med. Wschr. *98*, 828 (1968)
b) Kanell, W. B., Castelli, W. P., Gordon, T., Mc Namara, P. M.: Ann. intern. Med. *74*, 1 (1971)
c) Klör, H. U., Ditschuneit, H.: Münch. med. Wschr. *115*, 626 (1973)
d) Klör, H. U., Mertens, H. R., van Eimeren, W., Härich, B., Ditschuneit, H.: Arzneimittel; Forsch. *22*, 1808 (1972)
3) Westlund, K., Nicolaysen, R.: Scand. J. Clin. Lab. Invest. *30* (suppl.), 127 (1972)
4) Nikkilä, E. A., Aro, A.: Lancet I *1973*, 954
5) Berchtold, P., Berger, M., Gries, F. A.: Therapiewoche *26*, 4, (1976)
6) Julia, M., Baillarge, M., Tchernoff, G.: Bull. Soc. Chim. France *23*, 776 (1956)
7) Thorp, J. M., Waring, W. S.: Nature (London) *194*, 948 (1962)
8) Hellman, L., Zumoff, B., Hessler, G., Kara, E., Rubin, I. L., Rosenfeld, R. S.: J. Atheroscler. Res. *3*, 454 (1963)
9a) Green, K. G., Inman, W. H. W., Thorp, J. M.: J. Atheroscler. Res. *3*, 593 (1963)
b) Berkowitz, D.: J. Am. Med. Assoc. *218*, 1002 (1971)
c) Schartzkopff, W., Hoffmann, H., Russ, E.: Herz/Kreisl. *5*, 11, 474 (1973)
10) Thorp, J. M.: Lancet *1*, 1323 (1962)
11) Best, M. M., Duncan, C. H.: J. Am. Med. Assoc. *187*, 37 (1964)
12) Lang, P. D.: Ther. Gegenwart *114*, 2, 204 (1975)
13) Avoy, D. R., Swyryd, E. A., Gould, R. G.: J. Lipid Res. *6*, 369 (1965)
14) Krishnaia, K. V., Ramasarma, T.: Biochem. J. *116*, 321 (1970)
15) Nestel, P. J., Hirsch, E. Z., Conzens, E. A.: J. clin. Invest. *44*, 891 (1965)
16) Azarnoff, D. L., Tucher, D. R., Barr, G. A.: Metabolism *14*, 959 (1965)
17) Rifkind, B. M.: Metabolism *15*, 673 (1966)
18) Barrett, A. M., Thorp, J. M.: Brit. J. Pharm. Chemother. *32*, 381 (1968)
19) Biermann, E. L., Brunzell, J. D., Bagdade, J. D., Lerner, P. L., Hazzard, W. R., Porte, D. L., Jr.: Trans. Assoc. Am. Physns. *83*, 211 (1970)
20) Westerfeld, W. W., Richert, D. A., Ruegamer, W. R.: Biochem. Pharmac. *17*, 1003 (1968)
21) Ruegamer, W. R., Ryan, N. T., Richert, D. A., Westerfeld, W. W.: Biochem. Pharmac. *18*, 613 (1969)
22) Krasno, S. R., Kidera, G. J.: J. Am. Med. Assoc. *219*, 845 (1972)
23a) Dewar, H. A., *et al.*: Brit. Med. J. *4*, 767 (1971)
b) Dewar, H. A.: Arzneim.-Forsch. *22* (10a), 1835 (1972)
24) Oliver, M. F., *et al.*: Brit. Med. J. *4*, 775 (1971)
25) Coronary Drug Project Research Group: J. Am. Med. Assoc. *231*, 360 (1975)
26) Schettler, G.: Dtsch. med. Wschr. *100*, 1611 (1975)
27) Horlick, L., Kudchokar, B. J., Sodhi, H. S.: Circulation *43*, 299 (1971)
28) Stachelin, H. B., Locher, J. T., Maier, R.: Helv. Med. Acta *37*, 5–6, 388 (1974)
29) Stachelin, H. B., Hartmann, G.: Dtsch. med. Wschr. *99*, 1392 (1974)
30) Leutenegger, M., Cloisy, H., Caron, J., Paris, H.: Therapie *29*, 4, 599 (1974)
31) Nakanishi, M., Kobayakawa, T., Okada, T., Gotoh, K.: J. Pharm. Soc. Japan *90*, 8, 926 (1970)
32) Spöttl, F., Motamedi, S., Haase, W.: Herz/Kreislauf *6*, 11, 620 (1974)
33a) Knüchl, F.: Med. Welt *25*, 43, 1766 (1974)
b) Kaffarnik, H., Schneider, J., Haase, W.: Dtsch. med. Wschr. *48*, 2486 (1975)
34) Silingardi, V., *et al.* Minerva med. (Torino) *64*, 751 (1973)
35) Simane, Z., Nowak, H.: Atherosclerosis *20*, 447 (1974)
36) Hernandez, A., Maisenbacher, H. J., Stoll, K. D.: Therapiewoche *36*, 4819 (1975)
37) Melandri, M. M., Galimberti, P.: Chim. Ther. *2*, 9 (1967)

38a) Weiss, P., Dujovne, C. A., Margolis, S., Lasagna, L., Bianchine, J. L.: Clin. Pharm. Therap. *11*, 90 (1970)

b) Dujovne, C. A., Weiss, P., Bianchine, J. R.: Clin. Pharm. Therap. *12*, 117 (1971)

39) Manucci, F. M., Paretti, F., Maggi, C. A., Diaguardi, M.: Atherosclerosis *13*, 1 (1971)

40) Russo, C., Mendlowitz, M.: Clin. Pharm. Therap. *12*, 676 (1971)

41) Fellin, R., Fedele, D., Bagnariol, G., Pagnan, A., Crepaldi, G.: Atherosclerosis *17*, 383 (1973)

42) Schonbeck, H., Forster, G., Hizel, H., Jakob, T., Rosemund, H.: Deut. med. Wochenschr. *95*, 1761 (1970)

43a) Craig, G. M.: Atherosclerosis *15*, 265 (1972)

b) Craig, G. M., Walton, K. W.: Atherosclerosis *15*, 189 (1972)

44) Kritchevsky, D., Tepper, S. A.: Atherosclerosis *18*, 93 (1973)

45) Toki, K., Nakamura, Y., Agatsuma, K., Nakatani, H., Aono, S.: Atherosclerosis *18*, 101 (1973)

46) Sakamoto, Shin-Ichi, Yamada, K., Anzai, T., Wada, T.: Atherosclerosis *18*, 109 (1973)

47) Suzuki, K.: Biochem. Pharmacol. *24*, 1203 (1975)

48a) Imai, Y., Shimamoto, K.: Atherosclerosis *17*, 121 (1973)

b) Imai, Y., Matsumura, H., Tamura, S., Shimamoto, K.: Atherosclerosis *17*, 131 (1973)

49) Bondesson, G., Hedborn, C., Hogberg, T., Magnusson, O., Stjernstrom, N. E., Carlson, L. A.: J. Med. Chem. *17*, 1, 108 (1974)

50a) Bondesson, G.: Pharm. Ztg. *18*, 46, 1875 (1973)

b) Bondesson, G., Hogberg, T., Magnusson, O., Stjernstrom, N. E.: Acta Pharm. Suec. *12*, 4, 361 (1975)

51) 5th Intern. Symp. Drugs Affecting Lipid Metabolism, Milan, 1974

52a) 6th Intern. Congr. Pharmacol., Helsinki, 1975

52b) Gurrieri, B., Le Lous, M., Renson, F. J., Tourne, C., Voegelin, H., Majoie, B., Wülfert, E.: Arzneim.-Forsch. (Drug Res.) *26*, 5, 889 (1976)

c) Maderspach, A., Borsy, J., Elekes, I., Fischer, J., Mikete, G., Rakoczi, J.: Acta Physiol. Acad. Sci. Hung. *44*, 3–4, 367 (1973)

53) Bencze, W. L., Hess, R., Stevens, G., De: Fortschr. Arzneimittelforsch. *13*, 217 (1969)

54) Nardi, D., Mauri, L., Barzaghi, F.: Farmaco (Pavia) Ed. Sci. *20*, 456 (1965)

55) Gustafson, A., Sannerstedt, R.: Europ. J. Clin. Pharmacol. *5*, 4, 259 (1973)

56) Beaumont, V., Buxtorf, J. C., Jacotot, B., Beaumont, J. L.: Atherosclerosis *20*, 141 (1974)

57) Granzer, E., Nahm, H.: Arzneim.-Forsch. (Drug Res.) *23*, 9, 1353 (1973)

58) 5th Intern. Congr. Pharmacol. San Franciso, Abstr. 520, 1972

59) Morgan, J. P., Bianchine, J. R., Hsu, T. H., Margolis, S.: Clin. Pharm. Therap. *12*, 517 (1971)

60) Ryan, J. R., Jain, A., Maha, G. E., Mc Mahon, F. G.: Clin. Pharm. Therap. *12*, 464 (1971)

61) Sirtori, C., Hurwitz, A., Sabih, K., Azarnoff, D. L.: Lipids *7*, 2, 96 (1972)

62a) Aronow, W. S., Vangrow, J., Pagano, J., Khemka, M., Vawter, M., Pagegeorges, N. P.: Current Ther. Res. *16*, 9, 897 (1974)

b) Aronow, W. S.: Clin. Pharmacol. Ther. *15*, 1, 67 (1974)

63) Hutchison, J. C., Wilkinson, W. H.: Atherosclerosis *19*, 417 (1974)

64) Berkowitz, D.: Clin. Pharm. Therap. *14*, 1, 130 (1973)

65) Timms, A. R., Lawrence, A. K., Ho, R. S., Trapold, J. H.: Biochem. Pharmacol. *18*, 8, 1861 (1969)

66a) Berkowitz, D.: J. Am. Med. Assoc. *208*, 8, 1475 (1969)

b) Berkowitz, D.: Circulation *40*, Suppl. 3, 44 (1969)

67) Kelly, L. A., Timms, A. R., Trapold, J. H., Graupner, R. D., Cillo, J. J.: J. Am. Oil Chem. Soc. *48*, 2, 92A (1971)

68) Hartman, H. A., Bagdon, R. E., Pyzin, R. J., van, Tousimis, A. J.: Toxicol. Appl. Pharmacol. *17*, 1, 315 (1970)

69) Bochner, F.: Toxicol. Appl. Pharmacol. *24*, 4, 653 (1973)

70) Reddy, J. K., Krishnakantha, T. P., Azarnoff, D. L., Moody, D. E.: Res. Commun. Chem. Path. Pharm. *10*, 3, 590 (1975)

71) Kritchevsky, D., Tepper, S. A.: Arzneim. Forsch. *23*, 6, 858 (1973)
72) Buchanan, R. L., Spranemanis, V.: J. Med. Chem. *16*, 174 (1973)
73) Kariya, T., Blohm, T. R., Grisar, J. M., Parker, R. A., Martin, J. R.: Advan. Exptl. Med. Biol. *26*, 302 (1972)
74) Grisar, J. M., Parker, R. A., Kariya, T., Blohm, T. R., Fleming, R. W., Petrow, V., Wenstrup, D. L., Johnson, R. G.: J. Med. Chem. *15*, 1273 (1972)
75) Wagner, E. R.: Abstr. Papers, Am. Chem. Soc. *16*, 33 (1975)
76) Kariya, T., Parker, R. A., Grisar, J. M., Martin, J., Wille, L. J.: FEBS Meet, Atlantic City; Abstr. 3225 (1975)
77) Santilli, A. A., Scotese, A. C., Tomarelli, R. M.: Experientia *30*, 10, 1110 (1974)
78) Santilli, A. A., Scotese, A. C., Tomarelli, R. M.: Abstr. Papers, Am. Chem. Soc. 167. Meet. (1974)
79) Holland, G. F., Pereira, J. N.: Abstr. Papers, Am. Chem. Soc. 168. Meet. (1974)
80) Sirtori, C. R., Zoppi, S., Quarisa, B., Agradi, E.: Pharmacol. Res. Commun. *6*, 5, 445 (1974)
81) Noseda, G., Sirtori, C. R.: Schweiz. med. Wschr. *104*, 1917 (1974)
82) Bielmann, P., Brun, D., Moorjani, S., Gague, C., Lupien, P., Tetreault, L.: Clin. Pharm. Therap. *17*, 5 (1975)
83) Minisci, F., Galli, R., Perchinunno, M.: Org. Prep. Proced. *1*, 87 (1969)
84) Jones, B., Chapman, F.: J. Chem. Soc. *1952*, 1829
85) Savoy, C. M. S., Abernethy, J. L.: J. Am. Chem. Soc. *64*, 2719 (1942)
86) Hess, R., Bencze, W. L.: Experientia *24*, 418 (1968)
87) Dahm, J., Borck, J., Nowak, H., Simane, Z., Kayser, D.: DOS 2112272
88) Schacht, E., Mehrhof, W., Nowak, H., Simane, Z.: DOS 2319642
89) Schacht, E., Mehrhof, W., Nowak, H., Simane, Z., Kayser, D.: DOS 2325184
90) Schacht, E., Mehrhof, W., Nowak, H., Simane, Z.: DOS 2332801
91) Schacht, E., Lauterbach, G., Mehrhof, W., Nowak, H., Simane, Z.: DOS 2342118
92) Schacht, E., Mehrhof, W., Nowak, H., Simane, Z.: DOS 23 12 344
93) Schacht, E., Mehrhof, W., Simane, Z., Nowak, H., Kayser, D.: DOS 2358789
94) Bach, F. L., Barclay, J. C., Kende, F., Cohen, E.: J. Med. Chem. *11*, 5, 987 (1968)
95) Schacht, E., Dahm, J., Mehrhof, W., Nowak, H., Simane, Z., Kayser, D.: DOS 2163056
96) Schacht, E., Mehrhof, W., Simane, Z., Kayser, D., Nowak, H.: DOS 2362416
97) Schacht, E., Mehrhof, W., Simane, Z., Nowak, H.: DOS 2415867
98) Schmidt, H. R., Savoy, C. M., Abernethy, J. L.: J. Am. Chem. Soc. *66*, 491 (1944)
99) Dorfman, R. J., Klimstra, P. D., Ranney, E. R., Cook, D. I.: Endocrinology *68*, 43 (1961)
100) Cilgner, S., Metzke, H.: Versuchstierkunde *5*, 59 (1964)
101) Levine, J., Morgenstern, S., Vlascelica, D.: Technicon Symp. 1967, Automation in Analytical Chemistry. Whiteplains N. Y.: Mediad INC., 1968, p. 25
102) Noble, R. P., Campbell, F. M.: Clin. Chem. *16*, 166 (1970)
103) Dunnett, C. E.: J. Am. Stat. Assoc. *50*, 1096 (1955)

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Tilorone Hydrochloride: The Drug Profile

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Table of Contents

1. Introduction

Krueger and Mayer[1] made the initial observations that tilorone hydrochloride was a broad-spectrum, orally active antiviral agent in mice. They described activity against several DNA and RNA viruses including Semliki Forest virus, vesicular stomatitis virus, herpes simplex and others. Mayer and Krueger[2] described the stimulation of synthesis of a protein in mice which fulfills the criteria necessary to characterize it as interferon. Tilorone has subsequently been shown to influence the immune mechanisms[3], to have antitumor activity[4–7], and to have *anti*-inflammatory properties[8].

This review is intended to summarize the information currently available in the published literature or information which has been presented at numerous scientific forums. Since the literature is so voluminous, the authors will not attempt to cite every paper but will, instead, document the discussion to the extent that those who have a deeper interest can extend the information presented in this review.

2. Chemistry of Tilorone

The chemistry of tilorone hydrochloride and related compounds have been presented in a series of five papers by Sill *et al.*[9], Andrews *et al.*[10], Albrecht *et al.*[11], Grisar *et al.*[12], and Sill *et al.*[13].

The compounds found to have potent antiviral properties have two features in common[14]:

1. Two side chains containing basic (amine) functions.
2. A lipophilic central ring system of an aromatic or heteroaromatic type.

Monobasic derivatives do not yield potent antiviral agents, nor do they induce interferon when compared to the corresponding bis basic derivatives. Removal of the amine terminus results in loss of antiviral activity. Maximum activity is observed with no less than 3-rings in the central aromatic or heteroaromatic nucleus.

The central ring systems found to yield antiviral compounds have included fluorene (fluorenone), dibenzofuran, dibenzothiophene, fluoranthene, anthraquinone, acenaphthene, xanthene, thioxanthene, phenothiazine, carbazole, phenanthrene and others. The side chains were represented by basic ethers, basic ketones, basic esters plus carboxamides, sulphonamides, alkanols, methylene and others attached to the various ring systems. The amine function was usually substituted to the tertiary amine with various alkyl substituents although a few ring types (*e.g.*, pyrrole or piperidino) were synthesized.

Tilorone hydrochloride, 2,7–bis [2-(diethylamine) ethoxy]-9H-fluorene-9-one dihydrochloride, is an orange solid which is highly water soluble at neutral and acidic pH. The compound is anhydrous and melts between 234-234.5 °C with decomposition. The molecular weight is 483.47. Tilorone·HCl has an intense absorption band at 270 nm in water. The pKa of the amine functions are 8.64 and 9.27, respectively; the compound is stable in acid or base. The synthesis of tilorone hydrochloride was outlined by Andrews *et al.*[10], and Gaur and Wacker[15].

3. Toxicological Evaluation of Tilorone Hydrochloride

Preliminary toxicologic evaluation of tilorone hydrochloride has been reported by Rohovsky, Newberne and Gibson[16] in mice, rats, dogs and monkeys. The 24 hr oral LD_{50} was 959 mg/kg for mice and 852 mg/kg for rats. In subchronic studies, oral doses up to 180 mg/kg/day in rats and mice produced a dose-related depression of body weight gain and food consumption. In dogs and monkeys oral administration of $\geq$ 20 mg/kg/day produced clinical signs of anorexia, emesis, ptosis, salivation, ataxia and tremors. Oral doses of 3, 10 or 30 mg/kg/day in pregnant rabbits during days 9 through 16 of gestation produced no teratologic effects.

Rohovsky, Newberne and Gibson[17] reported the appearance of vacuolation and granulation of peripheral leucocytes in the mouse, rat, dog and monkey. A concomitant alteration in the reticuloendothelial system also occurred which consisted of intracytoplasmic accumulation of ovoid structures with vacuolated centers in Kupffer cells of the liver and mixed macrophages of the spleen and lymph nodes. Smears of bone marrow revealed abnormal granules in myeloid cells as primitive as neutrophilic myelocytes, but the myeloid:erythoid ratio was not altered. The post-treatment regression of these effects was species dependent.

Zbinden and Emch[18] extended the observations of Rohovsky *et al.*[17], on the effect of tilorone hydrochloride on the peripheral blood of laboratory animals. They described in rats, a marked, but transient, lymphopenia after single oral doses of 100 mg/kg. Recovery of the lymphopenia started after 12 hr and was accompanied by depletion of small lymphocytes in lymph follicles of the spleen and the appearance of young lymphocytes with vacuoles and basophilic granules in smears of lymph nodes and spleen. Abnormal mononuclear cells whose protoplasm often contained vacuoles and basophil granules appeared in the blood after 48 hr and disappeared in the course of 2–3 weeks. Repeated dosing caused only minor hematologic changes and affected no other organs.

Levine, Gibson and Megel[19] studied the depletion of the lymphocyte population caused by tilorone hydrochloride given orally or subcutaneously to rats and mice in spleen, lymph nodes and Peyer's patches. Although the thymus itself was not affected, thymus-dependent areas were affected similarly to that caused by neonatal thymectomy, thymic aplasia, or antilymphocytic serum. The effects of tilorone were not dependent on the presence of adrenal, thymus or spleen, and the depleted tissues were rapidly repopulated. The previously sensitive areas were resistant to further doses of the same drug.

The effect of tilorone hydrochloride on the ability of platelets to aggregate was studied by MacKenzie and Schatzman[20]. *In vitro*, tilorone hydrochloride did not inhibit the first phase of human platelet aggregation induced by ADP but was a potent inhibitor of the second phase of platelet aggregation (release reaction) caused by epinephrine. In rats given two 100 mg/kg 24 hr apart, collagen-induced aggregation was inhibited in platelets obtained 24 hr after the last dose. These results are consistent with inhibition of the second phase platelet aggregation (release reaction) similar to that seen with other non-steroidal *anti*-inflammatory compounds. With the exception of aspirin, the effect from tilorone is of longer duration.

4. Disposition and Pharmacokinetics of Tilorone Hydrochloride

Wacker *et al.* [21], reported the distribution of ^{14}C-tilorone in mice 16 hr after intraperitoneal injection of 1.5 mg in phosphate buffered saline. Higher concentrations of radioactivity were found in liver followed by spleen, kidney and lung. Relatively lower concentrations were found in thymus, heart, adipose tissue, skeletal muscle and blood. A large proportion of the radioactivity isolated from liver homogenates and urine was unchanged tilorone. Two unidentified products were seen in addition to tilorone. A significant amount of the radioactivity was associated with the sediment following centrifugation. Since Triton X-100 was needed to release some of the radioactivity, binding to membrane particles was suggested. Nucleic acids extracted by phenol contained no radioactivity.

The metabolic disposition of ^{14}C-tilorone hydrochloride in mice, rats and dogs has been studied by Hook *et al.*[22]. In contrast to the studies reported by Wacker *et al.*,[21], all doses were administered orally. In the Long-Evans rat, elimination of a radioactive dose (100 mg/kg) of tilorone hydrochloride was about evenly divided between urine and feces. Elimination was biphasic with the $t_{1/2}$ of the first phase 1.3 days and the $t_{1/2}$ of the second phase 4.2 days.

Six days after dosing, 40–45% of the dose remained in the rats. Tissue radioactivity was located principally in the liver (8%), lung (1–2%), spleen (1–2%), gastrointestinal tract and contents (4–5%) and the carcass (20–24%). Tissues having the highest concentrations of radioactivity six days post-dose were spleen, liver, lung, eyes, adrenals and lymph nodes. Concentrations in heart, skeletal muscle and brain were relatively low, while blood and plasma concentrations were very low. Concentrations of radioactivity in the liver reached a maximum at 3 hr post-dose, in the lung at 4 hr and was still increasing in the spleen at 6 hr. These results imply rapid absorption with subsequent redistribution.

Elimination of radioactivity and of material with similar UV absorption properties to tilorone occurred with a half life of 3.5–4 days in mice after a dose of 250 mg/kg.

In beagle dogs, urinary excretion accounted for 17–21% of the dose (20 mg/kg) during 7 days following dosing. Excretion by way of feces accounted for 13–14% of the dose. Only 8–9% of the dose appeared in feces during the first 3 days, which suggested that absorption was at least 90%, if not 100%, complete. Seven days post-dose, radioactivity was located primarily in the liver (22–24%), small intestine and contents (5–6%), lung (2.7–3.0%) and spleen (1.5–1.7%). Highest concentrations of radioactivity were present in liver, spleen and adrenals. Lower levels were found in heart, brain and skeletal muscle. Low plasma concentrations of radioactivity, in comparison with tissue concentrations, resulted in extremely high tissue-plasma ratios. Ratios exceeding 1000-to-1 were found for liver, spleen, adrenals and pancreas. High concentrations of radioactivity occurred in the pigmented structures of the eye, the choroid and iris, and appreciable concentrations in the retina. Concentrations in all other eye substructures were quite low suggesting that binding to melanin containing substructures had occurred.

Tissue metabolites from the rat were examined and identified. Thin-layer chromatography and autoradiography revealed the presence of 6–8 radioactive

compounds. Unchanged tilorone represented the major portion of the radioactivity in rat tissues, even at 6 days. Mono-de-ethylated tilorone was found in the highest quantities of any of the metabolites and represents the major metabolite. Di-de-ethylated tilorone with one ethyl group removed from each side chain was found in considerably lower amounts than the mono-de-ethylated product. Reduction of the carbonyl resulted in the fluorenol analog of tilorone. Polar metabolites were found to be the mono-N-oxide and di-N-oxide analogs of tilorone. Metabolism of tilorone proceeds by N-dealkylation, N-oxide formation and carbonyl group reduction.

Gaur and Chandra[23] extended their earlier studies in mice with ^{14}C-tilorone. Following an intraperitoneal injection of 20 mg/kg in male AKR-mice, the half-life of elimination was about 72 hr with the major excretory route by way of the kidney. In confirmation of earlier findings, spleen and kidney had the highest specific activity. Distribution of radioactivity 24 hr after the dose was: liver, 25%; spleen, 2.5%; kidney, 2.3%; lungs and pancreas, about 1.5% each; and less than 0.5% of the administered dose in each of the remaining tissues.

Subcellular distribution of tilorone hydrochloride has been examined. Gaur and Chandra[24] studied the subcellular distribution of radioactivity in liver, spleen, brain, lungs and kidney of mice and liver, spleen, brain, kidney and heart of rats after a dose of 50 mg/kg of ^{14}C-tilorone by intraperitoneal injection. A substantial proportion of the radioactivity was found in the fraction which sediments at 700 x g and contains, in addition to cell debris, the nuclei. The remaining radioactivity was distributed between the mitochondrial, microsomal and supernatant fractions. The authors suggested that the primary target of tilorone hydrochloride may indeed be the molecular species localized in the nuclei; *i.e.*, the DNA.

The studies of Gaur and Chandra[24] were extended by Leeson *et al.*[25]. Since Chandra *et al.*[26–28], had described the *in vitro* binding of tilorone to DNA and later extended these observations, the studies of Leeson *et al.*[25], were specifically designed to study the localization of tilorone and one of the metabolites in the nucleus of rat liver. Equilibrium dialysis studies showed that binding occurs with both plasma and tissue homogenates with stronger binding by the tissues. In confirmation of the findings reported by Gaur and Chandra[24], the 700 x g ppt. of rat liver homogenate contained the major fraction of drug related material. However, intact nuclei isolated from the liver homogenate contained proportionately less drug related material even though the DNA was relatively concentrated. These *in vivo* findings suggest that tilorone does not selectively localize in the nuclei. Instead, the concentration of tilorone in the various subcellular fractions appeared to relate better to protein concentration suggesting a relatively non-specific binding throughout the cell.

5. Effect of Tilorone Hydrochloride on the Drug Metabolizing Enzymes of Rat Liver

In companion papers, Renton and Mannering[29] and Leeson *et al.*[30], described the decrease in activity of the hepatic cytochrome P450 mono-oxygenase system following

oral administration of tilorone hydrochloride (doses ranged from 20–250 mg/kg.) Hexobarbital sleeping times were prolonged and blood levels of hexobarbital were elevated after doses of tilorone. Zoxazolamine paralysis times were prolonged after four doses of 100 mg/kg/day but not after a single dose. Concentrations of cytochrome P450 and NADPH-cytochrome c-reductase were reduced as were various enzyme activities related to the mono-oxygenase systems. Microsomal protein concentrations were initially reduced but had recovered to control levels with 21 days of continuing treatment with 100 mg/kg/day. Aminopyrine demethylase and hexobarbital oxidase remained decreased with 21 days of continuing treatment. Similarly, cytochrome P450 concentrations remained decreased. Electron micrographs of rat liver, after treatment with tilorone hydrochloride 100 mg/kg/day for 21 days, revealed many membranous structures in the form of whorls in the cytoplasm of the cell. It was postulated that the whorls originated from the endoplasmic reticulum in an attempt by the liver to restore full enzymatic capabilities in the face of continued drug administration.

Tilorone hydrochloride was not a direct inhibitor of the mono-oxygenase system of rat liver. *In vitro* addition of tilorone hydrochloride did not affect microsomal drug metabolism nor did it affect cytochrome P450 contents of the microsomes. The rate of incorporation of S-Amino (^{3}H) levulinic acid into cytochrome P450 was not affected by tilorone · HCl.

6. Anti-Viral Activity of Tilorone Hydrochloride

Krueger and Mayer[1] first described tilorone as a broad spectrum, orally active antiviral agent. In the first publication, activity against Semliki Forest, vesicular stomatitis, encephalomyocarditis, Mengo, vaccinia, herpes simplex and three strains of influenza viruses in mice was described. These authors[2] attributed the antiviral activity of tilorone largely to the induction of interferon in the treated mice. Mayer and Krueger[31] later described antiviral activity against Semliki Forest virus in rats and eye lesions caused by herpes simplex in rabbits. Activity of tilorone against *herpesvirus hominis*, type 1 and 2, in a rat and mouse tail lesion test was described by Yoshimura *et al.*[32].

In subsequent reports, tilorone hydrochloride prolonged the survival time of mice infected with Friend leukemia virus[33], protected Swiss mice against 6 intracerebral or 6 subcutaneous LD_{50}'s of the CVS strain of rabies virus infected 19 h after treatment with drug[34], and prolonged the survival of mice infected subcutaneously by the "slow virus" scrapie[35]. Hofmann and Kunz[36] described the protective effect of tilorone hydrochloride on experimental tick-borne encephalitis in mice. Mice infected with 80 LD_{50} foot-and-mouth disease virus, type 0, were protected by 250 mg/kg of tilorone given orally[37]. Tilorone hydrochloride prevented the production of serum antibody to live Venezuelian equine encephalomyelitis vaccine in mice presumably because of inhibition of viral replication by interferon production[38]. Mayer, Bray and Camyre[39] suggested, however, that not all of the activity against viruses can be attributed to the induction of interferon production. Direct virus

inactivation was demonstrated and topical activity against herpes-induced lesions on mouse skin or in the rabbit eye was found.

7. Induction of Interferon by Tilorone Hydrochloride

Mayer and Krueger[1] first described the appearance of an antiviral serum component in mice treated orally with tilorone hydrochloride which fulfilled sufficient biological criteria to be classified as an interferon. Krueger *et al.*[40], described the interferon induction properties of a number of analogs of tilorone and described the hypo-responsiveness which occurs with tilorone as well as other antiviral agents. The interferon produced by treatment of mice and rats has been characterized by Camyre *et al.*[41], and Camyre and Groelke[42]. Serum from tilorone or poly I:C treated mice was found to possess an interferon with a single molecular species with a molecular weight by Sephadex G-100 chromatography of 34,000. Serum interferon from tilorone-treated rats was associated with two distinct molecular species of 27,000 and 80,000. The interferons were resistant to ribonuclease and to heat. Some differences between rat and mouse interferons were described; *e.g.*, sensitivity to pH 2.5.

Administration of cycloheximide (60 mg/kg) 1 hr prior to tilorone administration inhibited the interferon response[43,44]. The inhibition by cycloheximide suggested that protein synthesis was involved in the appearance of interferon in the serum. For a more complete discussion of interferon induction, see the review on synthetic interferon inducers written by DeClercq[45].

In discussing the mechanism of antiviral protection and stimulation of interferon production in the mouse, DeClercq and Merigan[46] concluded that there was a direct relationship between the extent of protection against vesicular stomatitis virus, the titers of interferon produced and the doses of tilorone. Giron *et al.*[47], however, found no correlation between interferon induction and protection against MM virus in mice. Protection was achieved at doses far below the doses at which detectable interferon was found in the serum. Both findings may be consistent with differing mechanisms of viral inactivation for the two viruses under study.

Although interferon could be readily induced in mice and rats by tilorone, attempts to induce interferon in monkey and humans have not been successful[48,49].

Interferon induction in normal and leukemic lymphocyte cultures with tilorone has been observed[50]. The interferon response observed in normal lymphocyte cultures appeared to be correlated with the toxic effect of tilorone. The effect observed in leukemic cultures required higher concentrations of tilorone, but, similarly, appeared to be related to cell viability. Tilorone has been reported to stimulate production of interferon by itself in mouse embryo fibroblasts and, in combination with poly rI:poly rC/DEAE-dextran in mouse L929 cells[51]. Human foreskin fibroblasts were not stimulated. The degree of synergism between tilorone and the nucleotide-dextran complex was proportional to the concentrations of tilorone and poly rI/poly rC and was influenced by the times of addition of the compounds relative to each other.

8. Effects of Tilorone Hydrochloride on Humoral and Cell-Mediated Immunity

Hoffman *et al.*[52], presented evidence that a single oral dose of tilorone enhanced the primary immune response to sheep red blood cells (SRBC) in mice as measured by the Jerne Plaque technique. They also reported an increase in hemolysin titer after tilorone administration. To further evaluate the action of tilorone on humoral antibody responses, Megel *et al.*[3] have studied its effect on 19S and 7S production in the primary and secondary immune responses in mice. It was found that tilorone elevated 19S antibody titer on days 3 and 4 after immunization. After 9 days of continuous drug administration, the 19S response for both groups was diminished compared to days 3 and 4; however tilorone was found to cause a significant increase in the 7S antibody production compared to controls. Tilorone also stimulated the 19S response to *E. coli* endotoxin, a thymus-independent antigen, on days 3 and 4 after immunization.

The effect of tilorone on 19S and 7S antibodies was also measured in the secondary immune response[3]. In this study the mice were immunized with SRBC at day zero. Tilorone was administered at a dose of 50 mg/kg subcutaneously starting on day 20. A second immunization was given at day 21 and tilorone was given daily until day 23 (3 days after challenging). Both 19S and 7S responses were significantly increased compared to control.

Besides IgG (7S) and IgM (19S) production, tilorone was also found to elevate IgE levels. Using a parallel line assay, as described by Finney[53], Megel *et al.*[3] found that tilorone elevated IgE-like antibody levels 3.2 times with relation to saline control.

The data show that the drug serves as an adjuvant for a variety of immunoglobin classes (IgG, IgM and IgE antibody production). The effects on IgA responses to antigenic stimulation remain still to be determined.

In contrast to the effects of tilorone on humoral antibody production, the drug suppressed cell-mediated immune responses as evidenced by the significant decrease in paralysis in the EAE (experimental allergic encephalomyelitis) model, the inhibition of the tuberculin skin reaction, and the reduction in the secondary swelling in adjuvant arthritis[3].

Tilorone appears to be very selective in its action in that, it enhances humoral antibody production while suppressing delayed hypersensitivity response. It differs from the well-known immuno-suppressive compounds (*e.g.* glucorcorticoids, anti-metabolites etc.), which are capable of suppressing both. More recently, other synthetic compounds having selective effects on the immune systems have been reported. Freedman *et al.*[54] and Fox *et al.*[55] have reported that oxisuran suppresses skin-graft rejections in mice, rats and dogs but has no effect on antibody production. Renoux and Renoux[56] have shown that levamisole enhances cell-mediated immunity as evidenced by its effect on graft vs. host reactions. Thus, the results with tilorone and some other drugs suggest that, the immune system can be modulated selectively by pharmacologic manipulating.

9. Macromolecular Interactions and Their Biological Consequences

9.1. Influence of Tilorone Hydrochloride on the Secondary Structure of DNA

The possibility that this compound may react directly with DNA was indicated by the cytogenetic studies of Green and West[57]. Tilorone was found to inhibit mitosis significantly at 3.0 μg/ml, and produced chromosomal abnormalities at 1.5 μg/ml. Soon it was discovered by Chandra *et al.*[26–28] that tilorone does form molecular complexes with DNA and polydeoxynucleotides. Some of these studies will be described here.

Interaction between nucleic acids and biologically active compounds may induce changes in the electronic spectra of the components. Tilorone hydrochloride in water shows two absorption maxima, in the ultraviolet region around 271 nm, and in the visible region around 470 nm. Thus the investigation of the long wavelength band, where DNA and RNA do not absorb, should provide some evidence whether or not the chromophore of tilorone hydrochloride is involved in the binding process.

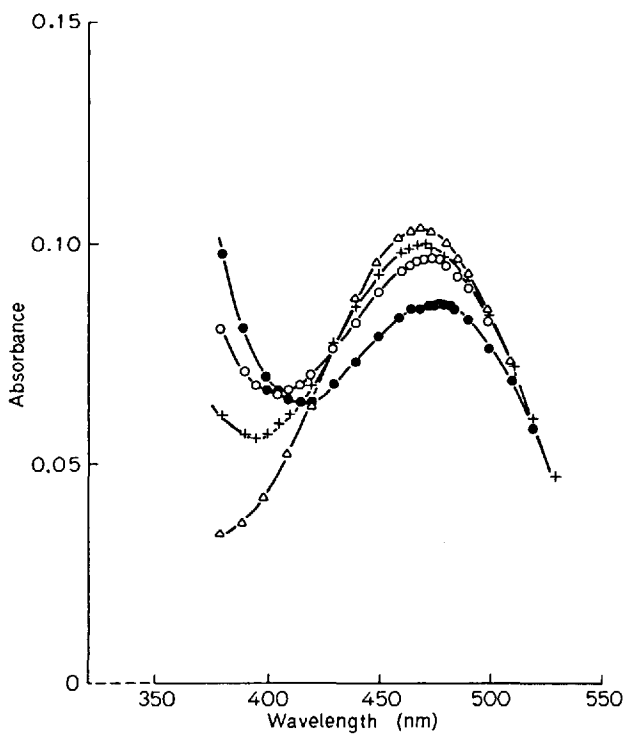


Fig. 1. Effect of calf thymus DNA on the visible absorption spectrum of tilorone hydrochloride. Samples contained 4.25×10^{-4} M of tilorone hydrochloride, 0.01 M Tris-HCl (ph 7.0) and DNA at 0.5×10^{-3} M (+ —— +); 1×10^{-3} M (o —— o); 2×10^{-3} M (• —— •). No DNA was added to the sample (Δ —— Δ)

Figure 1 depicts the absorption spectrum (350–500 nm) of tilorone hydrochloride alone (continuous line with triangles) or in the presence of various amounts of calf thymus DNA. There is a characteristic change in tilorone spectrum in the presence of DNA. In the presence of calf thymus DNA the visible absorption spectrum of tilorone hydrochloride is depressed and red shifted. This hypochromic effect of DNA on the absorption of tilorone chromophore is dependent on DNA concentration. The largest hypochromic effect is observed at 2×10^{-3} M DNA-P in a 4.25×10^{-4} M solution of tilorone hydrochloride.

The concentration-dependent effect of calf thymus DNA on the visible absorption spectrum of tilorone hydrochloride indicates that the tilorone chromophore interacts with DNA. Figure 2 depicts the visible absorption spectra of tilorone alone (curve one), or in the presence of yeast RNA (curve two), denatured DNA (curve three) and native double-stranded DNA (curve four). The visible spectra indicate that at equimolar concentrations, DNA in its double helical state produced largest changes

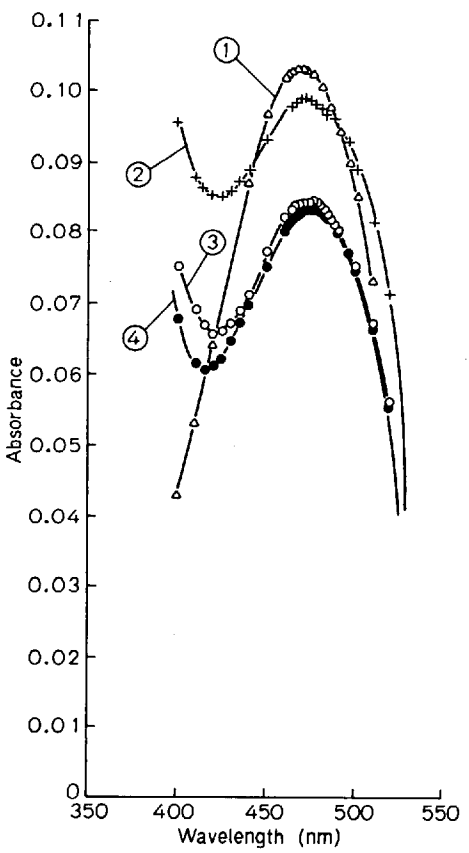


Fig. 2. Effect of native calf thymus DNA, denatured calf thymus DNA and yeast RNA on the visible absorption spectrum of tilorone in 0.01 M Tris-HCl (pH 7.0). Curve 1 is the spectrum of free tilorone (4.25×10^{-4} M). Other curves depict the spectra of tilorone in the presence of yeast RNA (curve 2), denatured DNA (curve 3) and native DNA (curve 4). Molar concentrations of nucleic acids (2×10^{-3} M) refer to phosphorous content of the polymer

in the absorption spectrum of tilorone, whereas the effect of single-stranded DNA is slightly weaker. In contrast, the yeast RNA exerts only a slight effect on the visible spectrum of tilorone hydrochloride. These data indicate a specificity of the tilorone chromophore towards DNA.

Further information on the binding of tilorone with DNA was derived by studying the thermal melting of the complex[27,28]. In order to characterize the stability of DNA secondary structure in the presence of tilorone, temperature profiles were run at tilorone/DNA-P molar ratio of 1 : 5. Tilorone hydrochloride shows a large increase in the thermal transition temperature (T_m) of native DNA; the T_m of calf thymus DNA was raised from 71.6 to 85.2 °C under these conditions.

9.2. Mode of Tilorone Hydrochloride Interaction to DNA

Hypochromic effect of native DNA on the absorption of tilorone chromophore is partially reversible by Mg^{2+} ions. Figure 3 depicts the absorption spectra (350–550 nm) of tilorone hydrochloride alone, 4.25×10^{-4} (curve one), in the presence of 4×10^{-3} M DNA-P (curve four) containing 0.01 $MgCl_2$ (curve two) or 0.1 M NaCl (curve three). It follows from these results that the DNA-drug interaction is

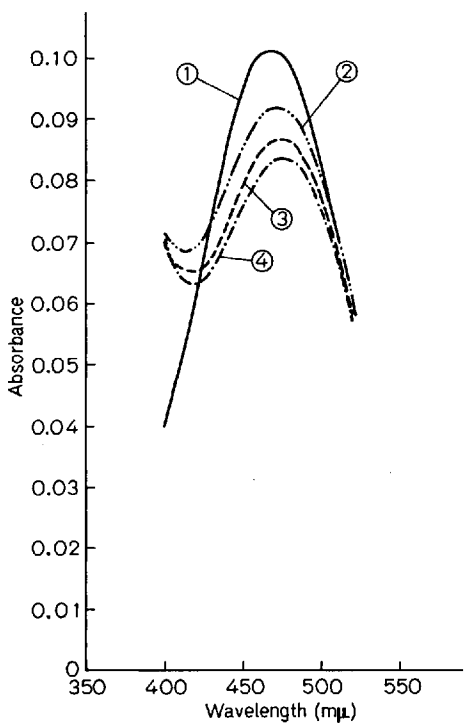


Fig. 3. Effect of Na^+ and Mg^{2+} on the visible absorption spectrum of the tilorone-DNA complex. Samples contained 4.25×10^{-4}M tilorone, 4×10^{-3}M DNA-P, 0.01 M Tris–HCl (pH 7.0) and 0.01 M $MgCl_2$ (curve 2) or 0.1 M NaCl (curve 3). Curve 1 is the spectum of free tilorone; curve 4 is the spectrum of the tilorone-DNA complex in the absence of Na^+ and Mg^{2+}

very sensitive to magnesium ions. The effect of magnesium ions on tilorone binding to DNA was confirmed by density-gradient studies using labeled tilorone hydrochloride[28].

These studies indicate that electrostatic forces contribute greatly to the binding process. The interaction between tilorone and DNA may, however, involve other kinds of forces. Tilorone forms a reversible complex with DNA, since the drug could be completely dissociated from a DNA-cellulose column. Interaction of apurinic and apyrimidinic DNA's with tilorone hydrochloride also gave spectral changes. However, only with the apyrimidinic DNA, the spectrum of the bound drug was similar to that found with native DNA.

The absorption spectrum studies presented above merely reflect the electronic environment of the molecule and do not give specific information about the type of interaction. The data which must be accounted for in considering a physical mode for the binding process can be derived from several different approaches. Hydrodynamic measurements on the DNA-drug complex are of interest, since Lerman[58, 59] has established that an increase in the intrinsic viscosity of DNA and a decrease in the sedimentation coefficient of the polymer are two criteria for intercalation of ring systems between base pairs of a double-helical DNA.

The relationship between the intrinsic viscosity of DNA and the amount ("r") of bound tilorone was studied[28]. The intrinsic viscosity of the complex increases with r up to a limiting value of about 0.05. The maximum relative enhancement of viscosity was about 1.7. In addition, at the same ionic strength and at a ligand to DNA-P molar ratio of 0.1, the sedimentation rate of DNA was decreased to 78% of the value in the absence of ligand.

These observations are consistent with an intercalative mode of binding in the interaction of tilorone hydrochloride with double-helical DNA. These results were not examined in an attempt to verify whether they agree with measurements of the length increase on sonicated DNA. For this reason, the intercalation model of the DNA complex remains tentative.

The interaction of tilorone hydrochloride with native DNA stabilizes the double helical structure of the macromolecule towards thermal denaturation. The effect of tilorone hydrochloride on the thermal denaturation of DNA's from various sources having different base composition has been studied[28]. At a drug to DNA-P molar ratio of 0.21, the ΔT_m increased with increasing AT content of the DNA. This observation indicates that tilorone hydrochloride perferentially binds to the dAT portions of the DNA molecule. This is confirmed by the strong effect of tilorone hydrochloride on the thermal transition temperature of poly d (A-T), $\Delta T_m = 29\,^{\circ}C$.

An intercalative mechanism for binding of a ligand to DNA is consistent with a stabilization of the double helix. Such a stabilization, however, does not constitute proof of intercalation. But, when considered with the evidence of the results reported here, showing increased viscosities and decreased sedimentation rate of DNA, one may conclude that the large increase of T_m points to an intercalative mode of binding.

The AT-specificity in the binding of tilorone to DNA was also observed in the quantitative equilibrium binding measurements. The equilibrium binding data were plotted as $r/(u)$ *vs.* r, where r is the moles of bound tilorone divided by the DNA concentration in base pairs[60], and (u) is the concentration of unbound tilorone.

From this plot binding parameters were obtained appropriate to a model in which all DNA binding sites are considered to be independent of each other[61]. The equation from this model is:

$$r/(u) = K_{app}\ (B_{app} - r),$$

where K_{app} and B_{app} are the apparent binding constant and number of binding sites per base pair, respectively; K_{app} is the negative of the slope, and B_{app} the $r/(u) = 0$ intercept of the linear region of the $r/(u)$ *vs.* r plot.

The Scatchard plots for the binding of tilorone to calf thymus DNA (Fig. 4a), *Mic. lys.* DNA (Fig. 4b), poly (dA-dT)·poly (dA-dT) (Fig. 4c), and poly (dG-dC) · poly (dG-dC) (Fig. 4d) are shown in Fig. 4. As follows from the Scatchard plots for natural DNAs, (Fig. 4a and 4b), the independent site model does not fit for DNA-ligand interaction; however, we employed the binding parameters of this model to analyse the Scatchard plots. The binding parameters, K_{app} and B_{app} derived from the Scatchard plots (Fig. 4a-d) are presented in Table 1. This table summarizes the results of several measurements.

As seen in Table 1 the apparent binding constants for both the natural DNAs are of the same magnitude, whereas the K_{app} for poly (dA-dT) · poly (dA-dT) is higher by a factor of two. The K_{app} value for poly (dG-dC) · poly (dG-dC) is less by a factor of 10^{-2}, compared with those of natural DNAs and the synthetic polydeoxynucleotide poly (dA-dT) · poly (dA-dT). The data on the number of binding sites per base pair (B_{app}) for various DNAs show a strict correlation with the AT-content of the biopolymer. On the basis of the B_{app} data, the closest distance between bound tilorone molecules is four base pairs. The values shown in Table 1 give almost a linear curve, showing a linear dependency of B_{app} on the AT

Table 1. Binding constants for the interaction of tilorone hydrochloride with DNAs and synthetic polydeoxynucleotides

Source of DNA		B_{app}	K_{app} M^{-1}
A.	Natural DNAs		
	Calf thymus	0.16	2.9×10^5
		0.12	4.1×10^5
	Mic. lysodeikticus	0.066	5.5×10^5
		0.062	6.0×10^5
B.	Synthetic polymers		
	Poly (dA-dT)·poly (dA-dT)	0.25	1.02×10^6
		0.25	1.04×10^6
	Poly (dG-dC)·poly (dG-dC)	---	6.9×10^3

All experiments were carried out at 20° in 0.1 M Tris-HCl (Ph 7.0). The binding parameters were derived from the Scatchard plots (Fig. 4, a-d); the experiments were carried out by equilibrium dialysis as described under Fig. 4. K_{app} = apparent binding constant; B_{app} = number of binding sites per base pair.

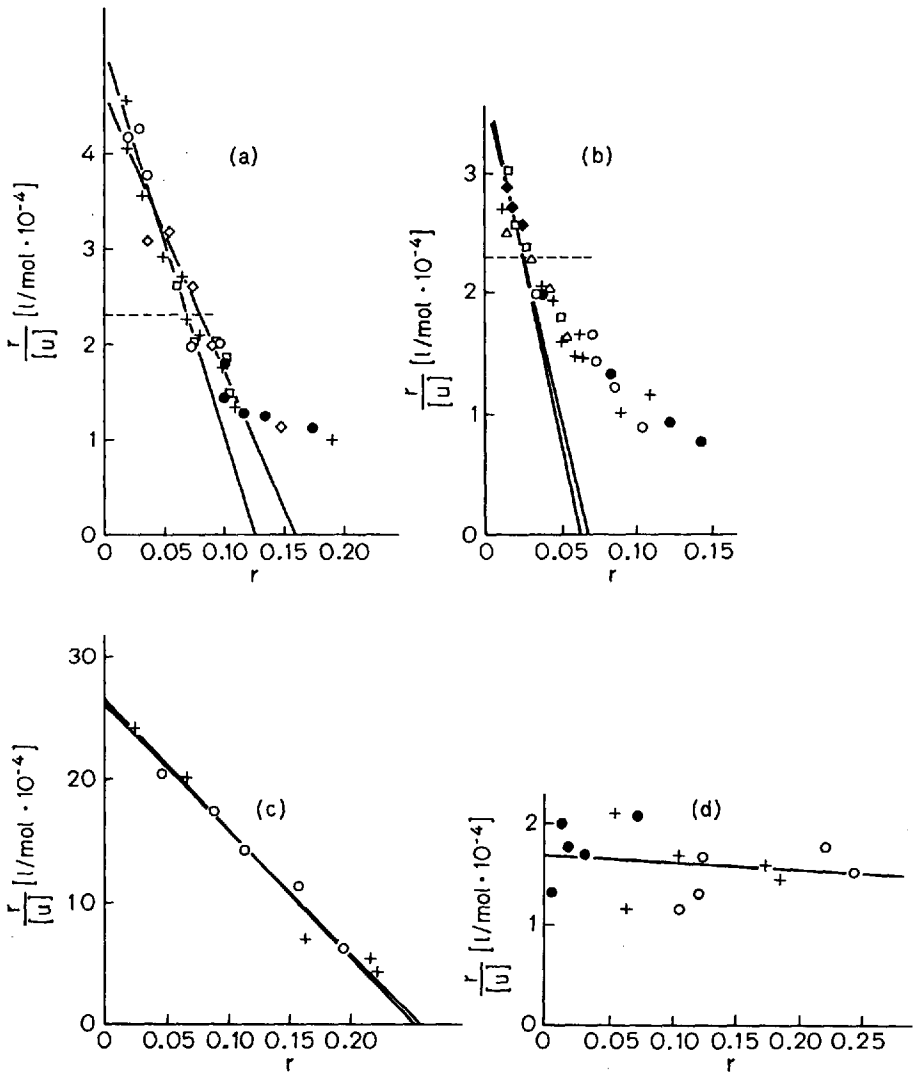


Fig. 4. Scatchard plots for the binding of tilorone hydrochloride to calf thymus DNA (a); *Mic. lysodeikticus* DNA (b); poly (dA-dT) · poly (dA-dT) (c); and poly (dG-dC) · poly (dG-dC) (d). Each different symbol corresponds to a separate experiment. Thus, each figure represents a set of 4 or 5 separate experiments. r is moles of bound tilorone/base pair concentration and (u) is the concentration of unbound tilorone. Equilibrium dialysis was carried out by a procedure and an apparatus (Dianorm, supplied by Dr. Virus KG, Bonn, Germany) described by Weder *et al.*[67]. Dialysing membrane (0.025 mm thick) was sandwiched between two halves of a Teflon (round) macro-cell (dialysable volume = 1 ml). The DNA, or labelled tilorone solutions were introduced by separate micro syringes on either side of the membrane through the side valves. The valves were closed air tight and the macro-cells were fixed into a rotating machine. All equilibrium dialysis studies were carried out at 20°, and at 10 rotations/min. Under these conditions equilibrium was attained in 4–5 hr. After the equilibrium was reached 0.8 ml of the solution from either side of the membrane was withdrawn by microsyringes and the radioactivity was determined using dioxan scintillation fluid

content of DNAs. The value for poly (dG-dC). poly (dG-dC), was extrapolated to zero, since the Scatchard plot (cf. Fig. 4d) does not indicate any specific binding of tilorone to this polymer.

9.3. Effect of Tilorone on the Template Activity of Nucleic Acids

The interaction of tilorone hydrochloride to DNA encouraged us to study the template activity of the complexes in DNA- and RNA- polymerase systems from *E. coli.* Both the activities were found to be inhibited by tilorone; the DNA polymerase reaction being more sensitive towards tilorone. The inhibition of the RNA-polymerase reaction by tilorone was dependent on the A-T content of DNA-template. The template activity of poly (dA-dT) in the RNA polymerase reaction is distinctly more sensitive towards tilorone than that of DNA; particularyly, at low drug concentrations the poly (dA-dT)-catalyzed reaction was three times more sensitive than the DNA-catalyzed activity of RNA polymerase reaction[60].

The nature of the inhibitory response, as depicted in Fig. 5, indicates that these compounds compete for the binding sites on DNA. This is clearly shown in the

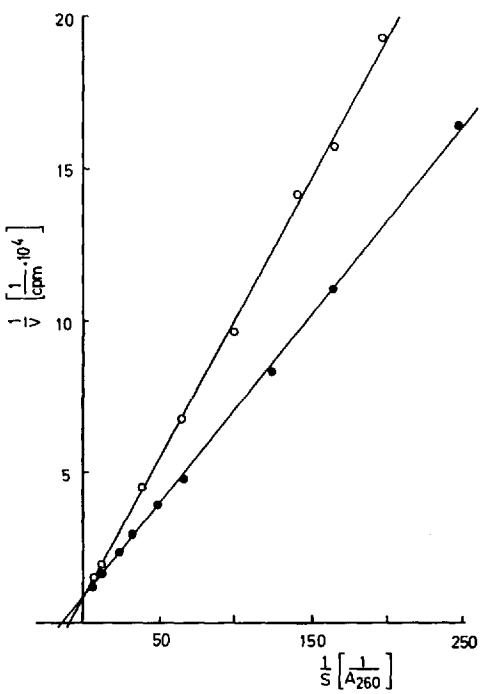


Fig. 5. Lineweaver-Burk plot of the effect of increasing concentrations of template DNA on the incorporation of ^{3}H-AMP into RNA by RNA polymerase of *E. coli* K-12, in the absence of tilorone (• —— •), or in the presence of tilorone, 3.2×10^{-5}M (•——o). Abscissa: $1/S$ = (DNA template, A_{260} per reaction mixture)$^{-1}$. Ordinate: $1/V$ = (^{3}H-labelled AMP incorporated into RNA)$^{-1}$

Lineweaver-Burk plot of the kinetic data obtained by measuring the RNA polymerase activity at various concentrations of template (Fig. 5). Figure 5 depicts the kinetic curves of the reactions in the absence of the inhibitor (● —— ●), and in the presence of 3.2×10^{-5} M tilorone (○ —— ○). This shows that tilorone is a competitive inhibitor of DNA template activity in the RNA polymerase reaction.

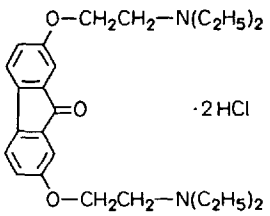
9.4. Studies with Tilorone Congeners

The interaction of tilorone with DNA and synthetic polydeoxynucleotides can be influenced by modifying tilorone structure. Such studies are indeed, important to elucidate the role of structural entities in their complex formation with DNA. Structure-activity relationship of tilorone derivatives (Fig. 6) has recently been studied.[7,60,62]

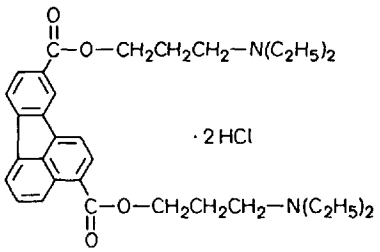
The effect of tilorone and congeners (Fig. 6) on the DNA-dependent RNA polymerase reaction is shown in Fig. 7). The template activity of native DNA is strongly inhibited by DEAP-fluoranthene, showing an 80% inhibition at a concentration 8×10^{-6} M. Other derivatives, at this concentration do not show any significant inhibition of the template activity of DNA. However, at higher concentrations one observes a dose-dependent inhibition of DNA-template activity by DEAE-fluorenone, DMAA-dibenzothiophene, DEAA-fluorene and DMAA-dibenzofuran. The monosubstituted derivative, MEAA-fluorene does not show any activity, even at higher concentrations.

The inhibition of DNA template activity by tilorone and its congeners is strongly influenced by substitutions in the ring (*e.g.* thiophene, furan, etc.), as well as in the side chains. It was, therefore, of interest to study whether such substitutions influence their interaction to DNA. Figure 8 depicts the melting curves of calf thymus DNA alone (curve one), or in the presence of MEAA-fluorene (curve two), DEAA-fluorene (curve three), DMAA-dibenzothiophene (curve four), DMAA-dibenzofuran (curve five) and DEAE-fluorenone (curve six). These studies were carried out at a drug/DNA-P ratio (r) of 0.1. Tilorone hydrochloride (DEAE-fluorenone) shows a large increase in the thermal transition temperature (T_m) of native DNA (curve six). The congener DEAP-fluoranthene, under these conditions, showed a very similar response (curve not shown). It is interesting to note that DMAA-dibenzofuran, though less active than DMAA-dibenzothiophene and DEAA-fluorene in the RNA polymerase reaction, has a higher effect on the T_m of calf thymus DNA, than exhibited by these two derivatives.

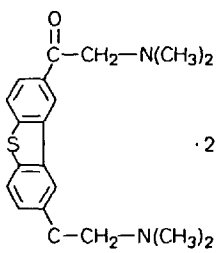
The structure-activity relationship observed in the RNA polymerase reaction, is not strictly exhibited by the melting curves of DNA and congener complexes. The latter studies were done in the absence of magnesium ions whereas, the RNA polymerase reaction requires magnesium ions for its enzymatic activity. The fact that compounds of tilorone type form complexes with Mg^{2+} may explain differences in the structure-activity relationships of congeners in the RNA polymerase reaction, and their effects on the melting behavior of DNA. Thus, the dibenzofuran congener, though less active than DEAA-fluorene in the RNA polymerase reaction, has a higher affinity for DNA than DEAA-fluorene. The dibenzofuran derivative should have a



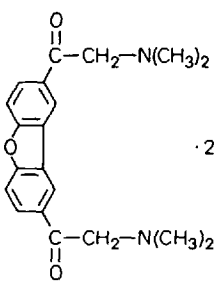
DEAE-Fluorenone
Tilorone



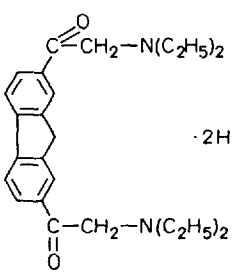
DEAP-Fluoranthene
RMI-9563 DA



DMAA-Dibenzothiophen
RMI-11877 DA



DMAA-Dibenzofuran
RMI-11567 DA



DEAA-Fluorene
RMI-11002

MEAA-Fluorene
RMI-11829 A
monosubstituted RMI 11002

Fig. 6. Chemical structures of tilorone and congeners

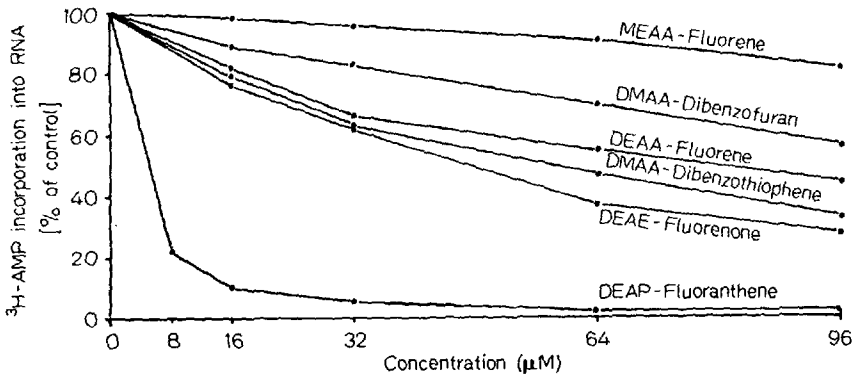


Fig. 7. Inhibition of DNA-dependent RNA polymerase reaction (*E. coli* K-12) by tilorone and congeners

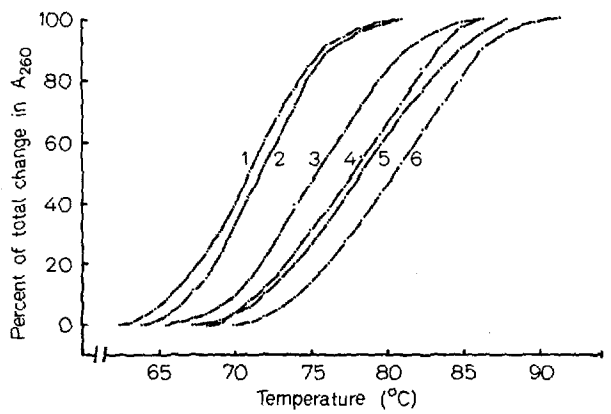


Fig.8. Effect of tilorone and congeners on the thermal transition temperature (T_m) of calf thymus DNA. Solvent is 0.01 M Tris-HCl pH 7.0 and the concentrations of DNA-P and congeners are 5×10^{-6}M, respectively. Curve 1 = DNA; 2 = DNA + MEAA-fluorene; 3 = DNA + DEAA-fluorene; 4 = DNA + DMAA-dibenzothiophene; 5 = DNA + DMAA-dibenzofuran and 6 = DNA + DEAE-fluorenone

higher tendency for chelating with magnesium ions, which leads to its partial inactivation in the RNA polymerase reaction.

To measure the effect of various tilorone congeners on the oncogenic activity of MSV (M), viral suspensions were incubated with 5×10^{-5} moles/ml of each compound at 37 °C for 1 hour. In the control group, where no compound was used, virus was preincubated with the solvent. Tris-buffer, 0.01 M, pH 7.4, 0.2 ml of this mixture containing 1×10^{-7} moles of the drug, was injected intraperitoneally. The amount of compound introduced this way had no direct physiological effect on the host (unpublished results). The mortality and the survival period were significantly influenced by tilorone and two of its congeners, DEAP-fluoranthene showed a

significant inhibition of splenomegaly induced by FLV. DEAA-fluorene showed only slight activity. It is interesting to note that MEAA-fluorene did not show any activity in this system[7,62].

Since none of the compounds at the concentrations used showed a complete supression of splenomegaly, one would expect a residual viral activity in spleen extracts of mice, which received FLV suspensions preincubated with these compounds. Studies are now in progress to evaluate the leukemogenic activity of cell-free spleen extracts, prepared from mice inoculated with pretreated suspensions. Wu *et al.*[63], have carried out such studies with RLV and rifamycin derivatives. They reported that the inoculation of mice with inocula from mice infected with RLV pretreated with AF-ABDP and AF/DNF1 did not cause splenomegaly.

If the supression of biological activity of RNA tumor viruses is due to a block of some molecular event (s) involved in oncogenesis, one would expect an inhibition of DNA polymerases by these compounds. That tilorone does inhibit the DNA polymerase activity of oncornaviruses has been shown earlier[26]. An attempt was made to correlate the biological response of various congeners with their inhibitory activity in the DNA-polymerase system of oncornaviruses. These studies were done using purified FLV, since in our hands this system showed a good endogenous activity.

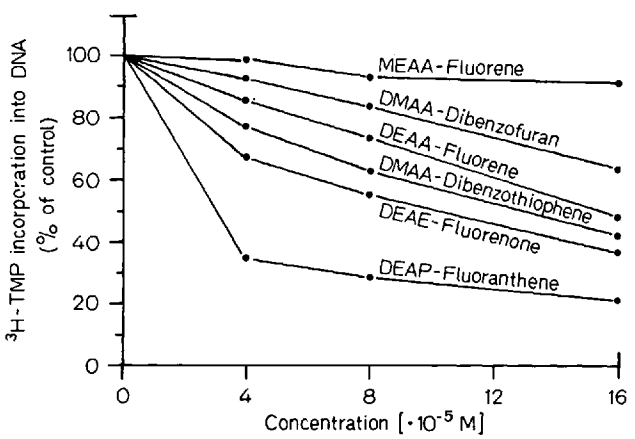


Fig. 9. Inhibition of endogenous RNase-sensitive FLV-DNA-polymerase activity by tilorone and congeners

The inhibition of the endogenous activity of FLV-DNA polymerase by tilorone and congeners is shown in Fig. 9. A maximum inhibition was obtained with DEAP-fluoranthene. The inhibitory responses of tilorone (DEAE-fluorenone), DMAA-dibenzothiophene and DEAA-fluorene were of the same magnitude; whereas, DMAA-dibenzofuran showed a weak response. It is interesting to note that the mono-substituted congener MEAA-fluorene did not inhibit the endogenous reaction at any concentration.

A similar inhibitory response by the tilorone congeners was exhibited[7] in the DNA-polymerase system of FLV, catalyzed by the template poly rA·$(dT)_{12}$. The

effect of tilorone and congeners on the FLV-DNA-polymerase reaction, catalyzed by poly (dA-dT), was also studied[7]. This reaction was more sensitive towards tilorone and its congeners than the endogenous, or poly rA·$(dT)_{12}$-catalyzed reactions. This is in accordance to our findings on tilorone action, reported earlier[26].

The data reported above, or elsewhere[7, 26,62] show that the poly (dA-dT)-catalyzed reaction of viral DNA-polymerase is most sensitive to these compounds. It is possible that the biological activity of these compounds is due to their interaction with the hybrid RNA-DNA (hy-DNA), single-stranded DNA (ss-DNA), or the DNA-DNA duplex (ds-DNA). It was therefore, interesting to locate the site of action of tilorone in the viral DNA-polymerase system. It is still not clear whether a particular site or target in the DNA-polymerase system, other than the true RNA-dependent reaction, can be correlated with the biological role of the oncornaviruses. The key role of the RNA-directed reaction in *in-vivo* leukemogenesis using purified enzyme and rifamycin derivatives, has been nicely demonstrated by Wu *et al*[63].

We have conducted some model studies to analyze the products of the FLV-DNA-polymerase reaction under the influence of tilorone. The procedure we adopted was based on a recent report by Kotler and Becker[64] on distamycin A, which has been shown by us to react with ss-DNA and ds-DNA[65,66].

The product analysis of the DNA-polymerase reaction (FLV) in the absence and in the presence of tilorone (1×10^{-4} M) is depicted in Fig. 10. The products of the viral DNA-polymerase reaction were, under these conditions, eluted in three species. The first species to be eluted from the column contained ss-DNA, the second contained the RNA-DNA hybrid molecules (hy-DNA) and finally, the ds-DNA, eluted in the last species. Analysis of products synthesized in the presence of tilorone showed that the ss-DNA and the hybrid species, but not the ds-DNA species were synthesized. This indicates that tilorone has a low affinity to viral RNA, but can block the synthesis of ds-DNA by interacting with ss-DNA or hy-DNA.

10. Some Future Prospects

The data reported here show that tilorone, a compound endowed with many interesting biological activities, requires very specific structural parameters for its biochemical action; and moreover, the same structural entities are also involved in its interaction with DNA. Using the molecular models of double-stranded helical DNA, one can examine the intercalation of tilorone at the same molecular dimensions. This approach could help to find out the groups or substitutions which will anchor best in A-T rich sites of DNA, thus leading to active derivatives of tilorone.

11. Addendum

Intercalation binding as a molecular mechanism of R factor elimination has been proposed by Hahn and Ciak[68]. Based on earlier data[26–28], that tilorone intercalates

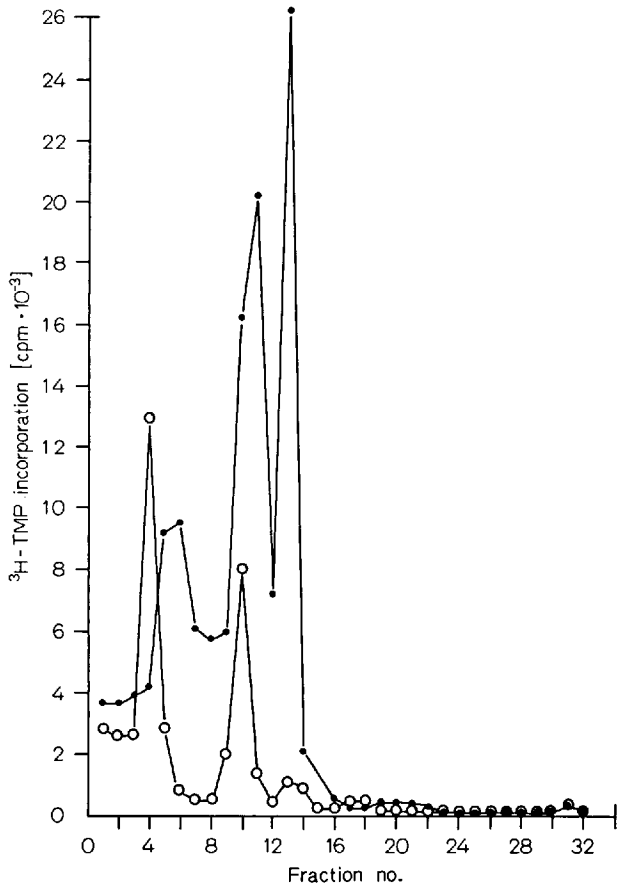


Fig. 10. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxylapatite column. Each column was filled with 1 g. of hydroxylapatite and carefully washed with 0.05 M sodium phosphate (approx. 50 ml). The columns were loaded with the reaction products, as described in text. The columns were washed with 0.05 M sodium phosphate buffer, pH 6.8, until equilibrium was reached. Macromolecules were eluted from the columns by a linear gradient of sodium phosphate (0.05–0.4 M). • —— •, DNA species synthesized in the absence of tilorone; ○ —— ○, DNA species synthesized in the presence of 10^{-4} M tilorone

into DNA, Hahn[69] studied the effect of tilorone on the elimination of resistance determinants in S. *typhimurium*. He reported that tilorone at 10^{-4}M was able to eliminate 78–85% resistance determinants tested against Kanamycin, Chloramphenicol, Streptomycin and Ampicillin. Similar observations were made by De Bary[70] who compared the elimination of four different plasmids (F' lac$^+$, Ra-1, R1 and Rs-a) by five different compounds (ethidium bromide, sodium dodecyl sulfate, nalidixic acid, acridine orange and tilorone). Of all these compounds, tilorone hydrochloride was reported to be most effective eliminator of plasmids.

Another biological effect of tilorone, based on its specific interaction to DNA is a selective inhibition of sporulation of *B. subtilis* strain 60015. At a drug con-

centration of 100 μg/ml the vegetative growth was not inhibited but, the sporulation was completely blocked[71]. The tilorone resistant mutants were asporogenous. The author has suggested that the selectivity in tilorone action may be due to the fact that A-T rich regions are involved in the sporulating phase.

12. References

1) Krueger, R. F., Mayer, G. D.: Science *169*, 1213 (1970)
2) Mayer, G. D., Krueger, R. R.: Science *169*, 1214 (1970)
3) Megel, H., Rychaudhuri, A., Goldstein, S., Kinsolving, C.R., Shemano, I. Michael, J. G.: Proc. Soc. Exp. Biol. Med. *145*, 513 (1974)
4) Adamson, R. H.: J. Nat. Cancer Inst. *46*, 431 (1971)
5) Munson, A. E., Munson, J. A., Regelson, W.: Int. Collog. Interferon Inducers. Leuven, Belgium Abstr. No. 27, 1971
6) Munson, A. E., Munson, J. A., Regelson, W., Wampler, G. L.: Cancer Res. *32*, 1397 (1972)
7) Chandra, P., Will, G., Gericke, D., Götz, A.: Biochem. Pharmacol. *23*, 3259 (1974)
8) Megel, H., Raychaudhuri, A., Goldstein, S., Kinsolving, C. R., Shemano, I. Michael, J. G.: Med. *149*, 89 (1975)
9) Sill, A. D., Albrecht, W. L., Andrews, E. R., Fleming, R. W., Horgan, S. W., Roberts, E. M., Sweet, F. W.: J. Med. Chem. *16*, 240 (1973)
10) Andrews, E. R., Fleming, R. W., Grisar, J. W., Kihm, J. C. Wenstrup, D. L., Mayer, G. D.: J. Med. Chem. *17*, 882 (1974)
11) Albrecht, W. L., Fleming, R. W., Grisar, J. W., Kihm, J. C. Wenstrup, D. L., Mayer, G. D.: *17*, 886 (1974)
12) Grisar, J. M., Hickey, K. R., Fleming, R. W., Mayer, G. D.: J. Med. Chem. *17*, 890 (1974)
13) Sill, A. D., Andrews, E. R., Sweet, F. W., Hoffman, J. W. Tiernan, P. L, Grisar, J. M., Fleming, R. W., Mayer, G. D.: J. Med. Chem. *17*, 965 (1974)
14) Albrecht, W. L.: Conf. Modul. Host Immune Resist. Preven. Treat. Induced Neoplasias, National Cancer Inst., Bethesda, Maryland 1974
15) Gaur, V., Wacker, A.: J. Labelled Compounds *IX*, 281 (1973)
16) Rohovsky, M. W., Newberne, J. W., Gibson, J. P.: Toxic. Appl. Pharmacol. *19*, 415 (1971)
17) Rohovsky, M. W., Newberne, J. W., Gibson, J. P.: Toxic. Appl. Pharmacol. *17*, 556 (1970)
18) Zbinden, G., Emch, E.: Acta Haemat. *47*, 49 (1972)
19) Levine, S., Gibson, J. P., Megel, H.: Proc. Soc. Exp. Biol. Med. *146*, 245 (1974)
20) MacKenzie, R. D., Schatzman, G. L.: Scan. J. Haematol. *15*, 58 (1975)
21) Wacker, A., Lodemann, E., Gaur, V., Diedereich, J.: Naturwissenschaften *59*, 520 (1972)
22) Hook, R. H., Williams, J. M., Brunzie, G., Wright, G. J.: Toxic. Appl. Pharmacol. *29*, 149 (1974)
23) Gaur, V., Chandra, P.: Proc. 9th Int. Congr. Chemother. Vol *8*, 43. New York: Plenum Press 1976
24) Gaur, V., Chandra, P.: Naturwissenschaften *60*, 263 (1973)
25) Leeson, G. A., Biedenbach, S. A., Chan, K. Y., Gibson, J. P., Wright, G. J.: Drug Metab. Dispos. *4*, 232 (1976)
26) Chandra, P., Zunino, R., Götz, A.: FEBS-Letters *22*, 161 (1972)
27) Chandra, P., Zunino, F., Zaccara, A.: FEBS-Letters *23*, 145 (1972)
28) Chandra, P., Zunino, F., Gaur, V., Zaccara, Y.: FEBS-Letters *28*, 5 (1972)
29) Renton, K. W., Mannering, G. J.: Drug Metab. Dispos. *4*, 223 (1976)
30) Leeson, G. A., Biedenbach, S. A., Hook, R. H., Wright, G. J.: Proc. 9th Int. Congr. Chemother. London 1975 (in press)
31) Mayer, G. D., Krueger, R. F.: Proc. 5th Int. Congress Infect. Diseases, p. 245, Vienna 1970
32) Yoshimura, S., Christian, R. T., Camyre, K. P., Krueger, R. F.: Proc. 8th Int. Congr. Chemotherap. Athens, Greece, Sept. 1973
33) Rheins, M. S., Wilson, H. E.: Bact. Proc. p. 188. Abstr. from: Amer. Soc. Microbiol. Minneapolis 2–7 May, 1971
34) Fornosi, F., Talas, M., Weiszfeiler, G.: Acta Microbiol. Acad. Sci. Hung. *18*, 327 (1971)
35) Cochran, K. W.: Abstr. Amer. Soc. Microbiol. Philadelphia, 23–28 April, 1972
36) Hofmann, H., Kunz, C.: Arch. Ges. Virusforschg. *37*, 262 (1972)
37) Richmond, J. Y., Campbell, C. H.: Arch. Gesamte Virusforschg. *42*, 102 (1973)

38) Mayer, G. D., Hagan, A. C. Bray, F.: Abstr. in Fed. Amer. Soc. Biol. Atlantic City 15–20 April, 1973
39) Mayer, G. D., Bray, F., Camyre, K. P.: Fourteenth Intersc. Conf. Antimicrobiol. Agents and Chemother. San Francisco 11–13 Sept., 1974
40) Krueger, R. F., Mayer, G. D., Camyre, K. P., Yoshimura, S.: Int. Colloq. Interferon and Interferon Induction, Sept. 14, Leuven, Belgium 1971
41) Camyre, K. P., Groelke, J. W., Mayer, G. D., Krueger, R. F.: Abstr. of the 71st Ann. Meeting of Amer. Soc. Microbiol. May 4, 1971
42) Camyre, K. P., Groelke, J. W.: Abstr. 72nd Ann. Meeting Amer. Soc. Microbiol. April 24, 1972
43) Mayer, G. D., Krueger, R. F., Camyre, K. P., Bray, F., Hull, C. R.: Abstr. 71st Ann. Meeting Amer. Soc. Microbiol. May 5, 1971
44) DeClercq, E., Merigan, T. C.: Virology *42*, 799 (1970)
45) DeClercq, E.: Topics in Curr. Chem. *52*, 173 (1974)
46) DeClercq, E., Merigan, T. C.: Med. J. Infect. Dist. *123*, 190 (1971)
47) Giron, D. J., Schmidt, J. P., Ball, R. J., Pindak, R.: Antimicrobiol. Agents and Chemother. *1*, 80 (1972)
48) Kaufman, H. E., Centifanto, Y. M., Ellison, E. D., Brown, D. C.: Proc. Soc. Exptl. Biol. & Med. *137*, 357 (1971)
49) Kaufman, H. E., Ellison, E. D., Centifanto, Y. M.: Amer. J. Ophthal. *74*, 89 (1972)
50) Dennis, A. J., Wilson, H. E., Barker, A. D., Rheins, M.S.: Proc. Soc. Exptl. Biol. & Med. *141*, 782 (1972)
51) Groelke, J. W., Camyre, K. P., Mayer, G. D.: Proc. Soc. Exptl. Biol. & Med. *148*, 1044 (1975)
52) Hoffman, P. F., Ritter, H. W., Krueger, R. F., in: Advances in Antimicrob. and Antineop. Chemother. M. Hejzlar, M. Semonsky, and S. Masek, eds.) p. 217. München: Urban and Schwarzenberg 1972
53) Finney, D. J.: Statistical Methods in Biological Assay, p. 99. New York: Hafner Publ. Co. 1964
54) Freedman, H. H., Fox, A., Shavel, Jr., J., Morrison, G. C.: Proc. Soc. Exptl. Biol. & Med. *139*, 909 (1972)
55) Fox, A. E., Gawlak, D. L., Ballantyne, Jr., D. L., Freedman, M. M.: Transplantation *15*, 389 (1973)
56) Renoux, G., Renoux, M.: Nature New Biol. *240*, 217 (1972)
57) Green, S., West, W. L.: Pharmacologist *13*, 260 (1971)
58) Lerman, L. S.: J. Molec. Biol. *3*, 18 (1961)
59) Lerman, L. S.: J. Cell. Comp. Physiol. *64*, 1 (1974)
60) Chandra, P., Woltersdorf, M.: FEBS- Letters *41*, 169 (1974)
61) Crothers, D. M.: Biopolymers *6*, 1575 (1968)
62) Chandra, P., Ebener, U., Steel, L. K., Laube, H., Götz, A.: Approaches for designing inhibitors of oncornaviral DNA Polymerases, in: Cancer Research, (R. C. Gallo, ed.) Ohio, USA: C. R. C. Press Inc. (in press)
63) Wu, A. M., Ting, R. C. Y., Gallo, R. C.: Proc. Nat. Acad. Sci., USA *70*, 1298 (1973)
64) Kotler, M., Becker, Y.: FEBS-Letters *22*, 222 (1972)
65) Chandra, P., Zimmer, C. H., Thrum, H.: FEBS-Letters *7*, 9 C (1970)
66) Chandra, P.: Molecular approaches for designing antiviral and antitumor compounds, in: Topics in Curr. Chem. *52*, 99 (1974)
67) Weder, H. G., Schildknecht, J., Kesselring, P.: Am. Lab. *10*, 15 (1971)
68) Hahn, F., Ciak, J.: Ann. N. Y. Acad. Sci. *182*, 295 (1971)
69) Hahn, F., in: Antibiotics and Chemotherapy. Vol. *20*, 211 (1976)
70) DeBary, E.: Eliminierung von Resisten-Plasmiden, M.D.-Thesis of Frankfurt University (1976)
71) Rhaese, H. J.: Personal communication (1977)

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