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5 Halogenated natural products in streptomycetes

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Abstract

Biologically active secondary metabolites of microorganisms and plants and their biosynthetic and semi synthetic derivatives belong to the most frequently used therapeutics in human and veterinary medicine. However, even the non-therapeutic applications such as animal husbandry and plant protection are of utmost interest. Among the producers of secondary metabolites streptomycetes play the most important role. Of the huge number of secondary metabolites, halogenated (i.e. chlorinated, brominated and fluorinated) compounds are discussed in the present review. A special attention was devoted to antibiotics of utmost importance, viz. chloramphenicol

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and tetracyclines that are dealt with in more details.

Introduction

It has long been assumed that tlic production of halogenated metabolites by living organisms is only exceptional. Thus for instance in the sixties. only several tens of halogenated organic compounds were known and their number increased quite slowly. During the last two decades, the number of natural halogenated organic compounds increased to up to several thousands. 'This was mainly due to the development of physico-chemical methods (NMR, MS), separation techniques (HPLC) and thanks to the exploitation of new resources such as marine organisms, deep sea organisms and other organisms living in extreme environments. Unfortunately, whereas the number of newly described compounds permanently increases, that of the compounds applied in practice increases only slowly, which holds true particularly for antibiotics [1].

This review is divided into several chapters.

Chlorine derivatives are most frequent halogen metabolites in nature [2]. It is quite logical when considering the distribution of individual halogens on the Earth. Chloramphenicol, the first halogenated metabolite isolated from the soil bacterium *Streptomyces venezuelae*, also contains two chlorine atoms in its molecule (see chapter 10). Additional metabolites, some of them produced on an industrial scale, were subsequently described (chlortetracycline, see chapter 11). Some of them exhibit the antibiotic activity.

Brominated derivatives have not yet been described in streptomycetes, with the exception of cultures to which bromine was intentionally added. Production of bromtetracycline can serve as example.

As far as fluorine is concerned, only two compounds, *viz.* fluoroacetate and 4-fluorothreonine, were isolated after fluoride has been added to the culture medium. The third derivative (nucleocidin) could not be isolated in repeated experiments.

Iodinated metabolites have not yet been described in streptomycetes.

Variability of different compounds isolated from streptomycetes is immense, from the simplest metabolites to compounds containing several tens of carbon atoms. However, it should be stressed here that very simple compounds such as one- or two- carbon derivatives have not yet been identified. As a matter of' fact, they were probably not even looked for, as the main attention was concentrated on the isolation of compounds exhibiting antibiotic activity, *i.e.* non-volatile compounds. In accordance with our hitherto knowledge of the role of enzymes in chlorination and bromination the halogen atom usually binds to an aromatic (heterocyclic) system which can be most readily directly electrophilically substituted.

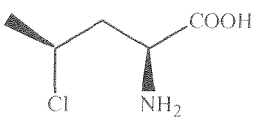
In the present review, we will describe individual compounds according to similarities of their chemical structures, beginning with aliphatic amino acids

and aromatic compounds tip to heterocyclic compounds. Fluorinated compounds are the only exception due to the fact that the presence of fluorine in their molecules is riot common. The size of individual chapters also varies. The main attention was devoted to such halogenated compounds of streptomycetes that are of industrial significance, such as the antibiotics chlortetracycline, chloramphenicol and vancomycin.

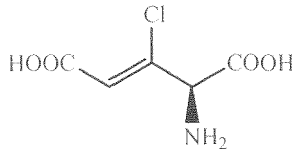
1. Amino acids and peptides

A wide variety of amino acid analogs has been discovered from plants and microorganisms, in particular in the culture broths of *Streptomyces sp.* In the course of screening program for new antibiotics, a *Streptomyces* strain with a code number AL-719 was found to produce an antimicrobial agent that was active against *Pseudomonas aeruginosa* on a synthetic medium, and which was reversed by L-leucine. Chemical study of this agent hereinafter designated as substance Al.-719 **(1)** revealed that it was a chlorine-containing antimetabolite of L-leucine, with one of the two-methyl groups replaced by chlorine.

γ-Chloronorvaline (AL-719) and γ-hydroxynorvalinelactone (AL-719Y) were isolated [3] from the culture broth of *S. griseosporeus* AI -719. Physico-chemical studies led to the structure elucidation of AL-719 2*S*, 1.5 configurations.



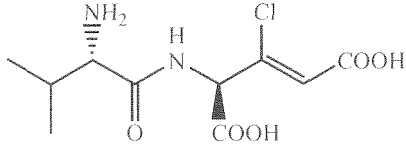
A new unsaturated glutamic acid analog, 4-amino-3-chloro-2-pentenedioic acid (2) (ACPA) was isolated [4] from a fermentation broth produced by a strain of *Streptomyces.* ACPA has a very narrow antibacterial spectrum, which is virtually limited to *Micrococcus luteus.* In the course of screening for new broad spectrum antibiotics, it was found that a new strain of *S. viridogenes* showed wide spectrum antibiotic activity. Paper disc agar diffusion assays with *Micrococcus luteus* and *Bacillus subtilis* were used to follow the isolation o!' the antibiotic activity from fermentation filtrate. It soon became apparent that at least two antibiotics were present, *i.e.* ACPA with antibacterial activity only against the *Micrococcus* and a second compound that had activity against both organisms. After purification, the second compound was found by NMR to be identical to antibiotic FR-900148, a valine dipeptide, see below.



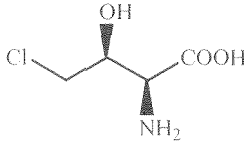
A strain of *Streptomyces*, isolated [5] from a soil sample and identified as *S. xanthocidicus*, was found to produce FR-900148 **(3)**, a new antibiotic containing chlorine in its molecule. The antibiotic inhibits both Gram-positive and negative bacteria. However, it is not effective against wild type of *Pseudomonas aeruginosa*. Its antibacterial action is considered to result from cell wall synthesis inhibition since it causes spheroplast formation from susceptible cells.

The structure of FR-900148 has been established as 1-*N*-valyl-3-chloro-2,5-dihydro-5-oxo-1*H*-pyrrole-2-carboxylic acid on the basis of spectroscopic and chemical methods [6]; however, the structure was riot correct

In the paper of Yasuda and Sakane [7], the revised structure and chemical transformations of FR-900148 were described



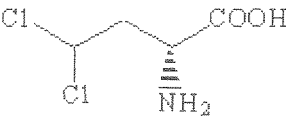
A metabolite of *Streptomyces sp.* OH-5093 from soil sample, which inhibits the growth of radish and sorghum, was isolated [8]. The structure was established to be 4-chlorothreonine **(4)**. It was also found that 4-chlorothreonine is an amino acid antimetabolite with anti-*Candida* activity.



4

A new amino acid, *viz.* 2-amino-4,4-dichlorobutyric acid **(5)** (armentomycin), has been isolated [9] from a fermentation of *Streptomyces*

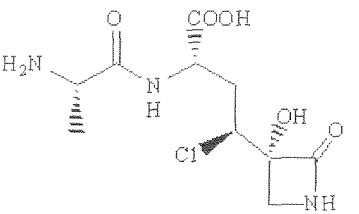
species. The compound, which has the L configuration, has been found to inhibit the growth of several bacteria grown in synthetic media.



5

The biosynthesis of armentomycin [10], a nonprotein amino acid with antibiotic properties is of particular interest, because no mechanism is known for the chloroperoxidase-catalyzed insertion of chlorine substituents remote from other functional groups. The results of investigation of armentomycin biosynthesis in *S. armentosus* var. *armentosus* were described.

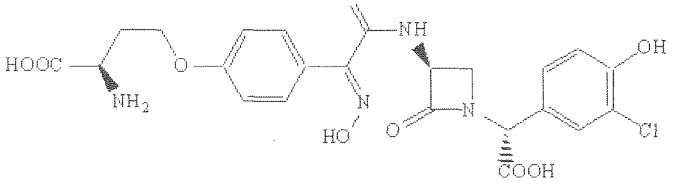
Several di- and tripeptides with antimetabolite activity have recently been isolated from fermentation broths. (*S*)-Alanyl-3-[α-(*S*)-chloro-3(*S*)-hydroxy-2-oxo-3-azetidinylmethyl]-(*S*)-alanine **(6)** was isolated [11] from a fermentation broth of an unidentified *Streptomyces* species 372 *A*. The substance inhibits the growth of several strains of Gram-positive and Gram-negative bacteria in a chemically defined medium but growth inhibition is relieved by addition of L-glutamine to t i medium. The structure was determined by X-ray diffraction analysis.



6

Chlorocardicin **(7)** is a new monocyclic β-lactam produced [12] by a *Streptomyces* sp. It is structurally related to nocardicin A but differs in having a *m*-chloro substituent on the *p*-hydroxyphenylglycine unit. The biological activity of chlorocardicin was similar to nocardicin A but the former showed less antagonism in complex media. Moderate *in vitro* activity was observed against *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Chlorocardicin showed low activity against *Staphylococcus aureus* whereas nocardicin A was inactive. Both compounds were shown to be strongly potentiated by antibiotics

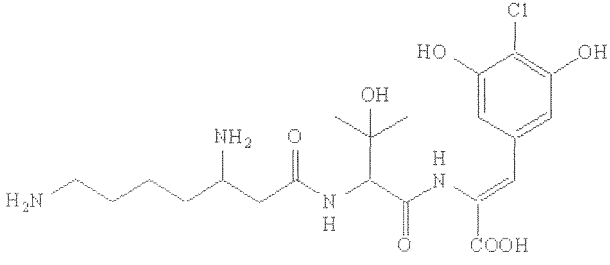
that inhibit peptidoglycan biosynthesis and were antagonized by selected L- and D-amino acids.



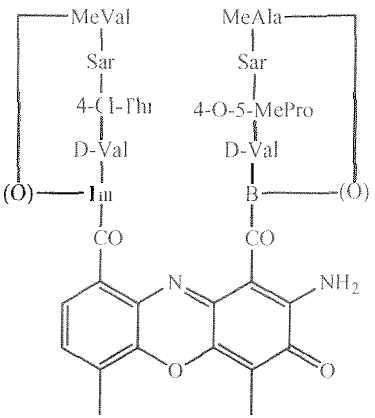
In a further paper [13] chlorocardicin was isolated by tlic use of non-ionic porous resin and reverse phase chromatography. Its physico-chemical characteristics and detailed NMR analysis were described.

A novel herbicidal antibiotic, resormycin (8), was isolated [14] from the culture broth of *C. platensis* MJ953-SF5. The structure of resormycin was determined to be (2Z)-2*N*-[2*N*-[(3*S*)-3,6-diaminoheptanoyl]-(2*S*)-3-hydroxyvalyl] amino-3-(4-chloro-3,5-dihydroxy) phenylpropenoic acid by spectroscopic analyses, degradation studies and X-ray crystallography.

Biological and biochemical activities of resormycin were studied [15] using unicellular green alga *Selenastrum capricornutum* as a test organism. Resormycin inhibited the growth of alga *in vitro*, more strongly in the dark than in the light. A weaker but more photo-stable derivative, (+/-)-2,3-dihydro-resormycin, showed a more long-lasting activity. In the light resormycin started killing of alga, only after exposure for 1 days and longer, even at high concentrations. Resormycin at concentrations near IC_{50} rapidly inhibited incorporation of ^{3}H-Leu, but not ^{3}H-UR or ^{3}H-TdR, into the TCA insoluble fraction of alga. Herbicidal activity of resormycin was confirmed using some crops and weeds.



Structure of five components of the actinomycin Z complex (Z1-Z5) isolated from *S. fradiae* was described [16]. The components were separated by silica gel column chromatography and TLC and/or PLC and analyzed by EIMS, FABMS, LC-MS of derivatized hydrolysates, and 2D NMR techniques. This permitted determination of the complete structures of actinomycins Z1-Z5. In Z3 (9) and Z5 (**10**), right site of the β-depsipeptide is occupied by the rare 4-chloro-L-threonine, an amino acid not previously found in an actinomycin. The structural variants of the actinomycin Z complex have the molecular architecture typical of other actinomycins but possess greater structural diversity resulting from the presence of several highly unusual amino acids. Actinomycins Z3 and Z5, but not Z1, were more potent than actinomycin D in cytotoxicity assays against three tumor cell lines. The complete structure of RP 18,631 (11), a new chlorine containing antibiotic related to novobiocin, has been determined [17] using a combination of degradative and NMR techniques. Of particular interest is the long-range couplings observed in the pyrrole ring present in the molecule.

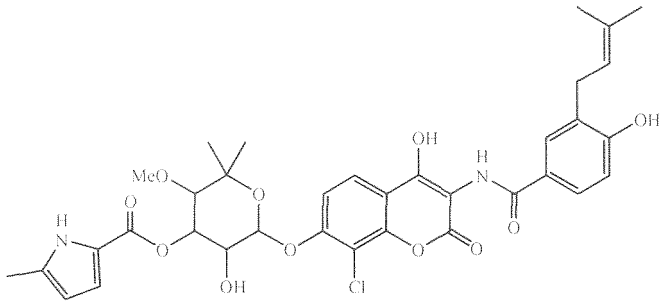


9, 10

	B
9	3-OH-5MePro
10	5-MePro

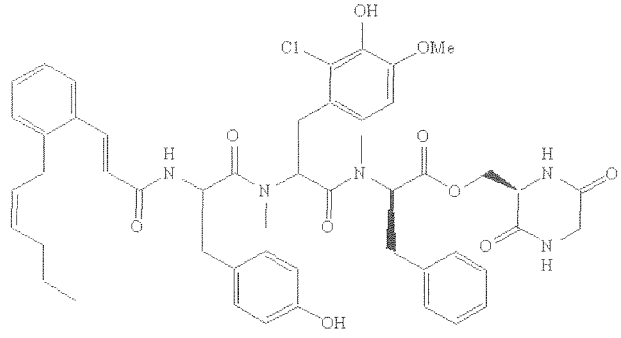
Pepticinnamins A - F, a family of farnesyl-protein transferase inhibitors, were isolated [18] from the fermentation broth of *Streptomyces* sp. OH-4652.

These inhibitors were purified from whole broth by extraction with chloroform, followed by silica gel column chromatography, Sephadex LH-20 chromatography and RP-HPLC. Among these, pepticinnamin C showed the most potent inhibition (IC_{50} 100 nM).



11

The structure of pepticinnamin E (**12**), was elucidated [19] by NMR study and it was found that it is composed of five amino acids and *O*-pentenylcinnamic acid. *C*-terminal glycylserine was in the cyclized diketopiperazine form.



12

More than 45 different strains of *Streptomyces* have been reported to produce peptide antibiotics. All these antibiotics contain a central core heptapeptide. This heptapeptide written in the conventional peptide structure has a high degree of homology in the aromatic amino acids 2 and 4 to 7 for this class of antibiotics. In several glycopeptide antibiotics, the remaining amino acids 1 and 3, are also aromatic amino acids. Vancomycin-type glycopeptide antibiotics [20] are structurally unique in that amino acids, 1 and 3 are aliphatic amino acids.

As is common in microbial secondary metabolites, the glycopeptide-producing organisms elaborate a complex of antibiotics with minor chemical

modifications. For example, tlic HPLC of partially purified vancomycins shows no less than five other structurally closely related factors. if we take such differences in the glycopeptides into consideration, well over 200 glycopeptides are known.

The glycopeptide antibiotics of the vancomycin-type are clinically the most important. Several of those structural analogs of vancomycin have been reported recently. They include many antibiotics, *viz.* A42867, A51568, A82846, A83850, chloroorienticin, decaplanin, eremomycin, MM 45289, MM 477611, OA-7653, orienticin and UK72051.

Vancomycin *(13)* has been marketed for the past 35 years, as tlic hydrochloride, to treat deep-seated Gram-positive infections. It is the drug of choice for infections caused by *Staphylococcus aureus*, especially those caused hy methicilin-resistant and more importantly the multi-resistant strains. Vancomycin is bactericidal to most Gram-positive organisms, but Gram-negative organisms are resistant. Vancomycin is not absorbed from the gastrointestinal tract and the antibiotic is used to treat enterocolitis, especially that caused by *Clostridium*. in recent years, there is both a medical and scientific resurgence in vancomycin in particular and glycopeptide antibiotics in general. Vancomycin is produced by *Amycolatopsis orientalis* [21] (previously designated as *Norcardia orientalis* and/or *S. orientalis*).

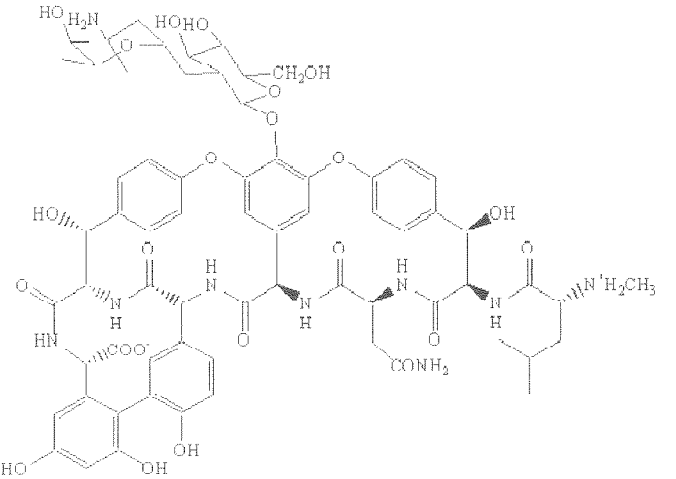
Finally, recent repor-is have described glycopeptides with long chain acylamido side chains on a sugar residue. One of these, teicoplanin, has been claimed to have more favorable antibacterial and pharmacokinetic properties than vancomycin. Synthesis of several *N*-acyl and *N*-alkyl vancomycins and A82846 antibiotics and their- evaluation revealed that the N-alkyl derivatives have substantial advantage over tlic parent antibiotics.

In addition, all the compounds included in this part were evaluated against *Staphylococcus aureus*, *Streptococcus pyogenes* C203 and *Streptococcus pneumoniae* infections in the in *vivo* mice model.

Avoparcin is a commercially important animal feed antibiotic that consists primarily of two closely similar glycopeptides a and β avoparcins, that are structurally related to vancomycin. The published spectral and degradative studies revealed the overall structures of these principal components. The 270-MHz ^{1}H NMR studies which have resolved the site of attachment of the chlorine to the triphenyl diether part and the orientation of the benzylic sugars in terms of the complete structures for α- **(14)**, β-avoparcin **(15)** and aglycone ε-avoparcin **(16)**, respectively, were described.

The strain that produces the glycopeptide antibiotic avoparcin (LL-AV290) has been described previously as a strain of S. *candidus*. Morphological and chemotaxonomic properties of this strain are typical of the genus *Amycolatopsis*. The results of a study of the physiological properties of this organism and its levels of DNA relatedness to previously described *Amycolatopsis* species

supported the decision to describe a new species for this strain, for which the name *Amycolatopsis coloradensis* is proposed. The type strain of *A. coloradensis* is NRRL 3218.



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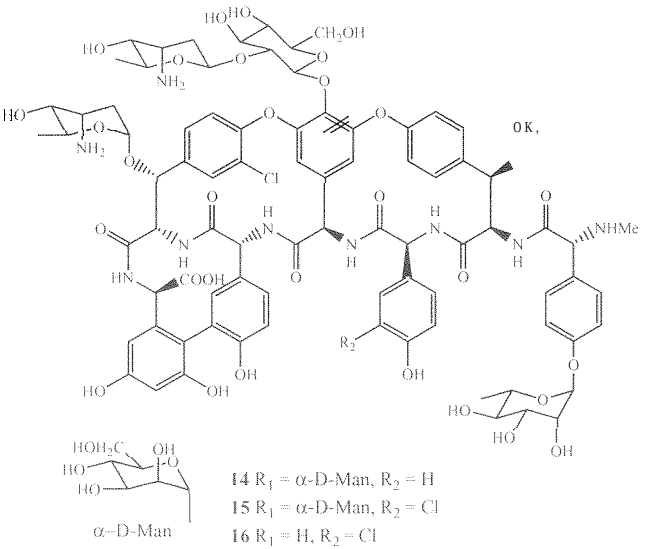


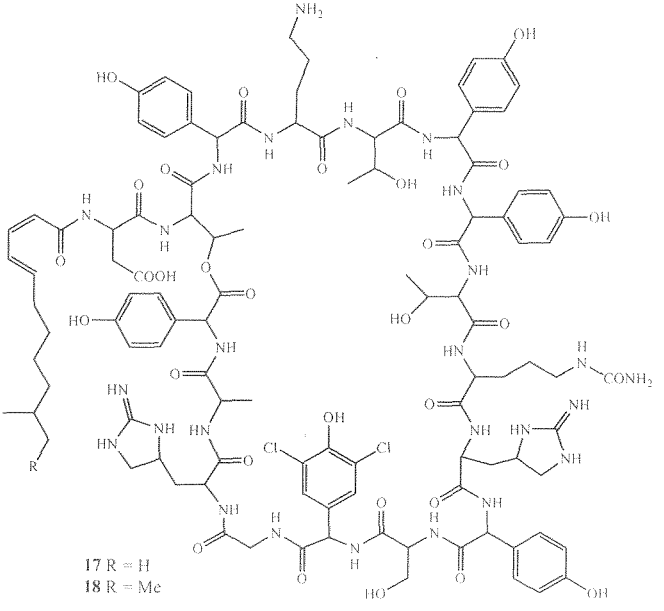
α–D-Man

14 R_1 = α-D-Man, R_2 = H
15 R_1 = α-D-Man, R_2 = Cl
16 R_1 = H, R_2 = Cl

Enduracidin was obtained [22] from the mycelium of *S. fungicidicus* 13-5477 and found to be a novel basic peptide antibiotic containing chlorine in the molecule. Enduracidins showed strong bactericidal activity both *in vitro* and *in vivo* against Gram-positive bacteria including strains resistant to known antibiotics.

From the structural investigation of acidic products obtained by hydrolysis of enduracidins and tetrahydroenduracidins, *viz.* 10-methylundeca-2(*Z*)-4(*E*)-dienoic acid and (+)-10-methyldodeca-2(*Z*)-4(*E*)-dienoic acid, it followed that these compounds are constituents of enduracidins A **(17)** anti B **(18)**, respectively.

Consequently, the structural difference between enduracidins A and B was proved to be present in their unsaturated fatty acid moieties, and their full structures were assumed to be as shown below.

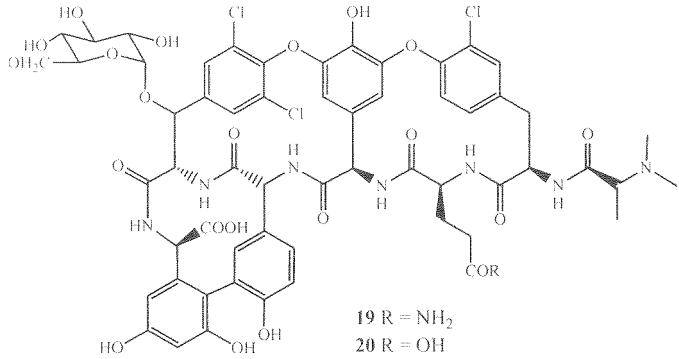


17 R = H
18 R = Me

The structure of vancomycin-type antibiotics, OA-7653A **(19)** and B (20), isolated [23] from *S. hygroscopicus* subsp. *hiwasaensis*, has been elucidated by a combination of classical chemical methods, MS and NMR. The interaction of *Oh-7653* with the peptide cell wall analogues *N*-acetyl-*O*-alanyl-*O*-alanine, and di-*N*-acetyl-L-lysyl-D-alanyl-D-alanine, in solution in aqueous dimethyl sulphoxide, has been examined by NMR and UV spectroscopy. Elucidation of the structure [24] is based primarily on 2D NMR experiments, analogies with

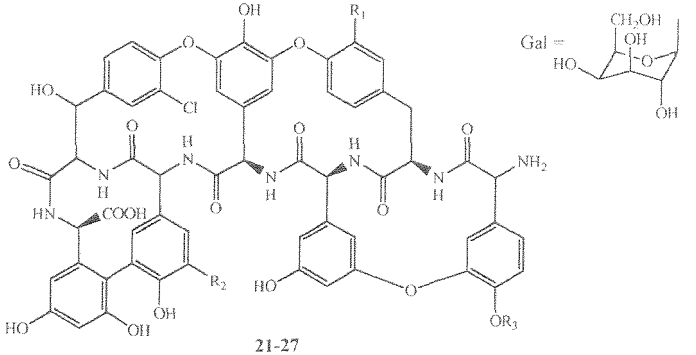
other glycopeptide antibiotics of the vancomycin series, and selective degradation studies. Antibiotic OA-7653 contains a heptapeptide aglycone core in which the amino acids, *N,N*-dimethylalanine and glutamine are encountered as the G and F components of this unit.

Mild acid hydrolysis of the antibiotic effects the conversion of the 8-carboxamide of the glutamine residue to yiciti the carboxylic acid in a reaction that is shown to proceed without rearrangement. The ^{1}H NMR spectra of Oh-7653 and its derivatives in DMSO at pH 4.0 are shown to represent major and minor conformers that are exchanging at rates comparable to the NMR time scale.



A41030 is a complex of novel glycopeptide antibiotics (21-27) produced [25] by a culture isolated from soil. Taxonomic studies identified the microorganism, NRRL 15156, its a strain of S. *virginiae*. The major factor, A41030A (21), and three (B-D) (22-24) of the six minor (B-G) (22-27) factors are unique among glycopeptides in that they arc naturally occurring aglycones, containing no neutral or amino sugars. In contrast to the vancomycin and *N*-demethylvancomycin fermentations, A41030 biosynthesis was stimulated by enriching the medium with K_2HPO_4 at a level of 1 mg/ml. Enrichment with putative precursors of the aglycone, however, did not increase the biosynthesis of A41030.

The neuroprotective agents of microbial origin against excitatory neurotoxins were isolated [26, 27] as two new bicyclohexapeptides, neuroprotectins A (28) and B (29), together with a known compound complestatin, see below, from the fermentation broth of *Streptomyces* sp. Q27107. Neuroprotectins protected primary cultured chick telencephalic neurons from glutamate- and kainate-induced excitotoxicities in a dose-dependent fashion. They are closely related in structure to complestatin, they



21-27

	R_1	R_2	R_3		R_1	R_2	R_3
21	Cl	Cl	H	**25**	H	H	H
22	Cl	H	H	**26**	Cl	Cl	Gal-Gal
23	Cl	Cl	Gal	**27**	Cl	Cl	Gal-Gal
24	H	Cl	H				

also possess an oxoindolylalanine moiety in place of the tryptophan residue present in complestatin.

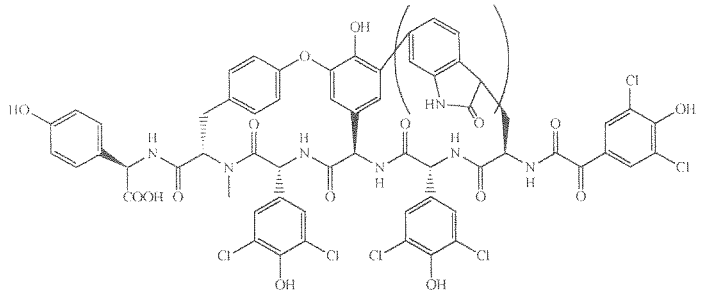
A new potent inhibitor [28] of complement system, named complestatin (30). was isolated from the mycelium of *S. lavendulae* SANK 60477. The structure of complestatin [29], which strongly inhibits the protease activity of complements in the human complement system, has been determined mainly by HMBC NMR. Its structure is closely related to glycopeptide antibiotics.

Complestatin with summary formula $C_{61}H_{45}N_7O_{15}Cl_6$ is a peptide compound having two unusual amino acids, D-(-)-4-hydroxy-phenylglycine and D-(-)-3,5-dichloro-4-hydroxyphenylglycine.

This compound inhibited the hemolysis of sensitized sheep erythrocytes mediated by guinea pig and human complement at concentrations of 0.4 and 0.7 μg/ml, respectively, but did not inhibit trypsin and α-chymotrypsin activities at 200 μg/ml. When complestatin was administered intravenously to the sensitized guinea pigs, it strongly inhibited the systemic anaphylactic shock elicited by the antigen probably by blocking generation of anaphylatoxins.

Two gp120-CD4 binding inhibitors [30], chloropeptins I (31) and II, produced by a soil actinomycete, *Streptomyces sp.* WK-3419, were described. The major chloropeptin I is a new compound, while the second chloropeptin II was identified as complestatin which has been reported to inhibit the hemolysis of erythrocytes sensitized by the complement system.

Chloropeptin (31) was isolated [31] as a pale yellow-brown powder with formula $C_{61}H_{45}N_7O_{15}Cl_6$ from the mycelia of a soil actinomycete, *Streptomyces* sp. WK-3419. Its physico-chemical proper-ties showed that it is a chlorinated



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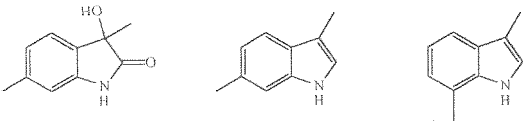


29 30 31

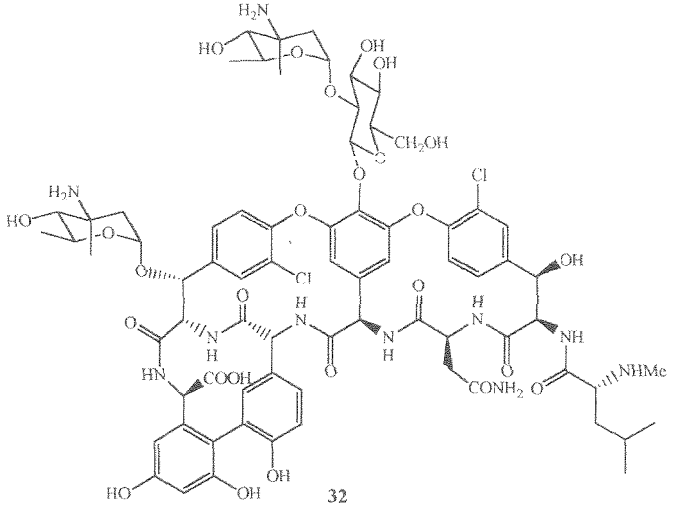
peptide. The structure was elucidated by ^{1}H and ^{13}C NMR experiments and chemical degradation. It was shown to be a peptide antibiotic consisting of six aryi amino acid residues and more aryl acid residues sonic of which have chlorine atoms. The compound inhibited gp120-CD4 binding (IC_{50} 1.3 μM), the cytopathic effect of HIV in MT-4 cells (IC_{50} 1.6 μM) and syncytium formation in co-cultured HIV-l-infected and uninfected MOLT-4 cells (IC_{50} 0.5 μM). Chloropeptin I was synergistic in the inhibition of the cytopathic effect when combined with other anti-HIV drugs.

LY26426 **(32)** is a naturally-occurring glycopeptide antibiotic [32], differing from vancomycin in the stereochemistry of the amino-sugar of the disaccharide function, and the presence of a third sugar attached at the benzylic position of sixth amino acid residue.

Despite these apparent differences, LY264826 is approximately 10 times more active than vancomycin against the enterococci. In the pursuit of new antibiotics active against multiresistant Gram-positive organisms, an extensive side chain changes were developed focusing on tlic reductive alkylation of LY264826 at the amino function of the disaccharide moiety. A new series of derivatives having varying degrees of structural diversity in tlic side chain (e.g. varying lengths and degrees of rigidity) was found to have a potent activity against staphylococci and streptococci as good or better than vancomycin.

By it combination of chemical and spectroscopic (^{1}H and ^{13}C NMR) studies [33], tlic structure of a glycopeptide antibiotic eremomycin (formerly LY 26426) has been elucidated. It is closely related to vancomycin, but differs in sugar and chlorine content. The eremomycin aglycone contains

monodechlorovancomycinic acid; the only chlorine atom is situated in the second amino acid after the N-terminal amino acid residue of the peptide. The sugar part is composed of glucose and two residues of an amino sugar shown to be 2,3,6-trideoxy-3-amino-*C*-3-methyl-L-arabino-hexopyranose (4-epi-vancosamine). One of the amino sugar residues is a component of the disaccharide 2-*O*-(α-L-4-epi-vancosaminyl)-glucopyranose, attached to a triphenyl ether moiety; the position of another one is at the serine oxygen in the C-terminal region of the aglycone.



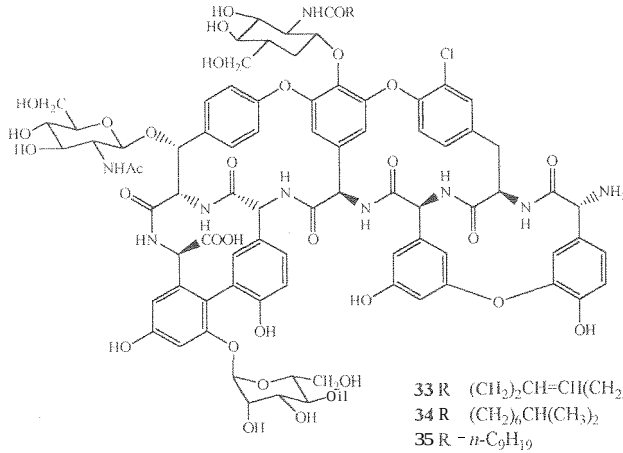
32

Novel glycopeptides **(33-41)** derived from teicoplanin were prepared [34] and evaluated for activity against antibiotic-resistant Gram-positive pathogens. Removal of the fatty acid side chains of teicoplanin was accomplished by enzymatic deacylation. The resulting deacylated teicoplanin was subjected to reductive alkylation resulting in mono- and di-alkylated compounds at the two possible primary amines. Deacylated teicoplanin was less active than teicoplanin against enterococci and staphylococci (MIC greater than or equal to 32 μg/ml).

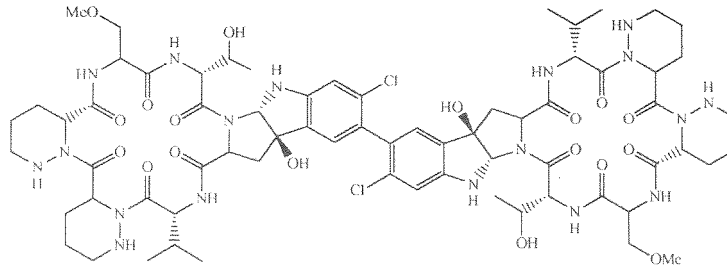
All mono- and di-alkylated products regained some activity, and some had potent activity against both staphylococci and glycopeptide-resistant enterococci. MICs of the most potent di-alkylated compounds ranged from 0.25 to 2 μg/ml against glycopeptide-resistant enterococci.

A new apoptosis-inducing agent chloptosin **(42)** was isolated [35] from the culture broth of *Streptomyces* MK498-98F14 strain. The structure of the dimeric cyclohexapeptide consisting of D-valine, *(3S)*- and (3*R*)-piperazic acids, *N*-methyl-L-serine, D-threonine, and (2*S*,3a*R*,8a*R*)-6-chloro-3a-hydroxy-2,3,3a,8a-

hexahydropyrrolo[2,3-b]indole-2-carboxylic acid was elucidated by spectroscopic and chemical degradation studies. The amino acid components in each cyclohexapeptide domain were presented in alternating *R* and *S* configurations. Chloptosin was found to induce apoptotic activity in apoptosis-resistant human pancreatic adenocarcinoma cell line AsPC-1 and showed a strong antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*.



33 R $(CH_2)_2CH{=}CH(CH_2)_4Cl$
34 R $(CH_2)_6CH(CH_3)_2$
35 R $n\text{-}C_9H_{19}$
36 R $(CH_2)_6CH(CH_3)CH_2CH_3$
37 R $(CH_2)_7CH(CH_3)_2$
38 R $(CH_2)_8CH(CH_3)_2$
39 R $n\text{-}C_{11}H_{23}$
40 R $(CH_2)_4CH(CH_3)CH_2CH_3$
41 R $n\text{-}C_8H_{17}$

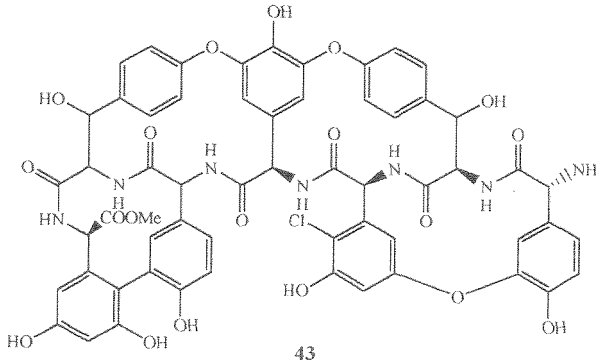


42

The structure of the pseudoaglycone of the glycopeptide antibiotic A35512B **(43)** [36] was described. The structure elucidation was based on ^1H

NMR studies of A3551213 anti A35512B-aglycone; negative nuclear Overhauser effects and analogies between A35512B and other glycopeptides have led to the structural proposals.

The A35512B aglycone differs from the aglycone of ristocetin A only in the structure of the diphenyl ether amino acid. The amino sugar of the ψ-aglycone is linked to the peptide core through an aliphatic hydroxyl group, and intact A3551213 contains neutral sugars attached to the ψ-aglycone at three phenolic sites.



43

2. Polyethers

X-14766A (44) is a novel, chlorine containing polyether antibiotic produced by *S. malachitofuscus* subsp. *downeyi* (NRRL 11335 and/or ATCC 31547, respectively) isolated from a sand sample collected from Playa Bianca, Mexico in 1976. The production in the shake flasks was 64 μg/ml in 5th day. The antibiotic is active *in vitro* against Gram-positive bacteria and is capable of complexing (see Table 1) and transporting monovalent as well as divalent metal cations (K^+>Na^+>Rb^+>Cs^+, Ba^{2+}>>Sr^{2+}, Ca^{2+}>Mg^{2+}> Li^+) [37]. In a further report [38] the isolation and chemical characterization of antibiotic X-14766A

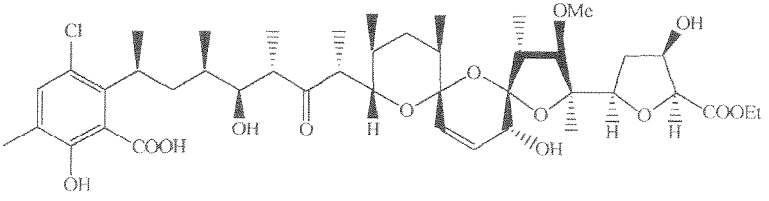


Table 1. The cation selectivity sequence of X-14766A

Displacing cation	Cation radius A	Rb and Ca remaining in complex (%)	
None	-	68	70
Mg^{2+}	0.82	67	55
Ca^{2+}	1.18	68	40
Sr^{2+}	1.12	70	37
Ba^{2+}	1.34	69	5
Li^{+}	0.68	68	64
Na^{+}	0.97	17	0.4
K^{+}	1.33	10	0.3
Rb^{+}	1.47	33	1
Cs^{+}	1.67	52	6

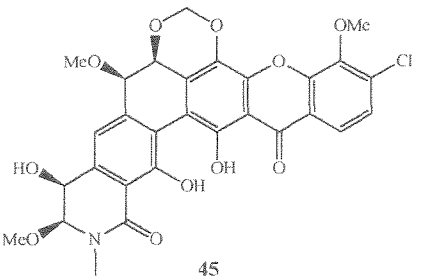
were described. The structure of this novel antibiotic was determined by X-ray analyses of the thallium and rubidium salts. In addition to the chlorine atom, which is attached to a salicylic acid chromophore, the molecule contains a tricyclic di-spiroketal ring system and an ethyl ester group.

3. Quinones

From the cultures of *S. violaceoniger*, strain Tü 96, two new lipophilic antibiotics, lysolipin I **(45)** and lysolipin X **(46)** were isolated [39]. The latter one is chemically unstable and is easily transformed to lysolipin I. The deeply yellow lysolipin I has a molecular formula $C_{29}H_{24}ClNO_{11}$. The IR, UV, ^{1}H and ^{13}C NMR spectra indicate that they are very likely of quinone structure. X-ray analysis was used to determine the structure of lysolipin I [40]. Lysolipin I is active against Gram-positive and Gram-negative bacteria. However, enterobacteria are only inhibited in high concentration, when the membrane permeation is damaged. Lysolipin I acts lytically against bacterial cells. Its activity is decreased by several lipids. The site of action is the biosynthesis of bacterial cell walls, an interaction with the carrier lipid for mureine intermediates being likely.

Feeding experiments [41] with ^{13}C and ^{18}O-labeled precursors revealed that the molecular framework of the polycyclic xanthone antibiotics, the lysolipins X and I, is derived from the polyketide pathway (12 malonate units), the C-1 pool (methionine), molecular oxygen, and the nitrogen pool. Surprisingly, an intact malonate moiety serves as the three-carbon starter unit of the polyketide backbone, and 9 of the 12 oxygen atoms of lysolipin originate from molecular oxygen, including both of the xanthone oxygen atoms. The orientation of the

malonate unit incorporated intact into lysolipin is unique, opposite from those in tetracycline and cycloheximide, i.e., the activated carbon of malonyl-CoA is bound to the nitrogen of the lysolipin isoquinoline ring, and the CO_2-derived carbon serves as the starter of the polyketide chain. From the biogenetic origin of the oxygen atoms, several unusual prearomatic deoxygenation steps early in the biosynthesis have to be postulated.



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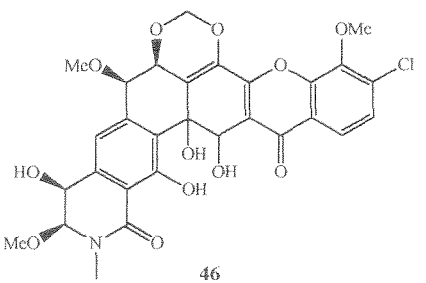
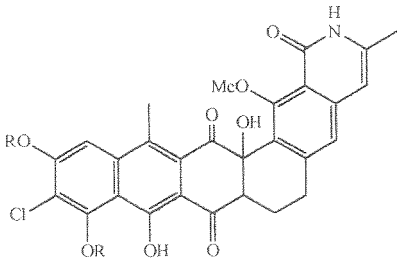


46

Further antitumor substance, designated RE-10412A (47), was isolated [42] from the culture broth of *Streptomyces* sp. A19412. The active principle was extracted from the mycelium by methanol and purified by silica gel and Sephadex LH-20 column chromatography. BE-19412B was prepared by methylation of BE-19412A. BE-19412A and BE-19412B (48) exhibited cytotoxic activity against murine and human tumor cell lines. BE-19412A prolonged the survival of CDF, mice bearing i.p. implanted Ehrlich carcinoma cells.

Angucyclinone-type antibiotic, simocyclinone D8 (50), was detected [43] in the mycelium extract of S. *antibioticus* Tü 6040 *by* HPLC-DAD and HPLC-ES-MS screening. The compound shows antibiotic activities against Gram-positive bacteria and cytostatic effects on various tumor cell lines.

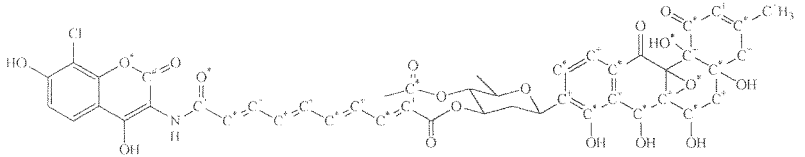
Simocyclinone D8 [44] consists of angucyclinone, deoxysugar, octatetraene dicarboxylate and aminocoumarin structural elements. The structure elucidation



47 R = H
48 R = Me

was done by 1D and 2D NMR experiments and other spectroscopic methods in combination with incorporation experiments using ^{13}C labelled precursors.

Biosynthesis of simocyclinone from *S. antibioticus* was elucidated [45] by feeding experiments using $^{18}O_2$ rich atmosphere. The incorporation of $^{18}O_2$ into molecule of antibiotic is depicted below. The results suggest that the aminocoumarin moiety has a tyrosine precursor and that flavoprotein monooxygenase participates in the biosynthesis of this antibiotic.



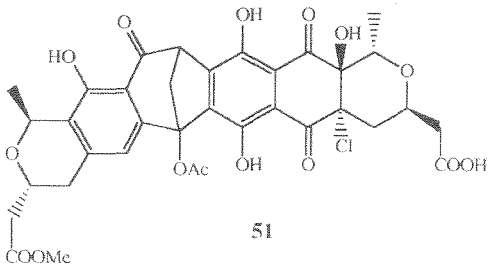
C* [1,3-$^{13}C_2$]malonic acid, C' [2-^{13}C]malonic acid, C# L-[1-^{13}C]tyrosine, * $^{18}O_2$

50

A new naphthocyclinone was isolated [46] from the mycelium of *S. arenae*. Isochromanquinone **(51)** contains the molecular skeleton of β-naphthocyclinone, the chromophore part is changed by the addition of OH/Cl to the double bond and by epoxidation, respectively. Possible regio- and stereo-specific isomers were discriminated by spectroscopic anti chemical methods. The halves of the molecule of γ-iso-naphthocyclinone are connected asymmetrically, which is remarkable with regard to their biosynthesis. The corresponding stereochemistry of the naphthocyclinones has been confirmed by chemical transformation. The chemical screening detected [47] a new naphthoquinone, naphthomevalin **(52)**, in the culture broth of a *Streptomyces sp.* (strain Gö 28) besides a number of already known metabolites. The strain was isolated from a soil sample collected in Strathgordon, Australia. After standard purification, pale yellow oil with yield

2.5 mg/l of culture broth ~ ; i obtained. The structure mas elucidated by physico-chemical methods and stereochemistry mainly by CD spectra.

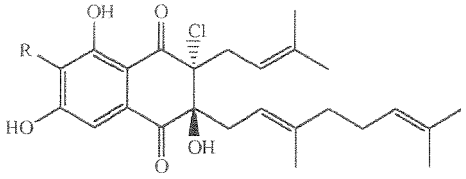
A new species of *Streptomyces* was described [48] for which the name **S. *aculeolatus*** was proposed. The organism produces new antibiotics SF2415A1 (53), A2, A3 (54), B1 (55), B2 and B3 (56) active against Gram-positive bacteria.



51

Table 2. Antimicrobial disc-diffusion assay of naphthomevalin.

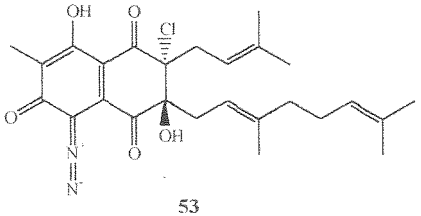
Microorganism	Inhibition diameter (mm) of naphthomevalin		
Concentration	8 mg/ml	4 mg/ml	1 mg/ml
Bacillus subtilis	14	12	8
Escherichia coli	20	17	11
Mucor miehei	-	-	-



52 R = H
55 R = Me

Further, tire structures of these antibiotics were deduced [49] from spectroscopic analyses and degradation studies. The structure of' SF2415A3, which has the highest antibacterial activity, was proposed to be 3,4a-dichloro-9-diazo-3,4,4a,10a-tetrahydro-6-hydroxy-2,2,7-trimethyl-10a-[(*E*)-3,7-dimethyl-2,6-octadienyl]-(2*H*, 9*H*)naphtho-[2,3b]pyran-5,8,10-trione. All the antibiotics SF2415 have a semi-naphthoquinone structure. The antibiotics SI 2415A1, A2,

A3, B1, 132 and 133 have been isolated from the culture filtrate of *S. aculeolatus*, isolated from soil sample collected in the Tottori Prefecture, Japan. The structures of antibiotics SF2415 were elucidated by chemical and 1D and 2D NMR spectral studies (including COSY, HMBC, *etc.*). These antibiotics have *peri*-hydroxy semi-quinone structure and SF2415A1, A2 and A3 contain an additional unique α-diazoketone structure. One of these antibiotics, SF2415B3, is a methyl derivative of napyradiomycin A. From 50 l jar fermentor the following amounts of the antibiotics were isolated: 70 mg yellow needles of A1, 50 mg of A3, 280 mg 131 and 250 mg of B3.



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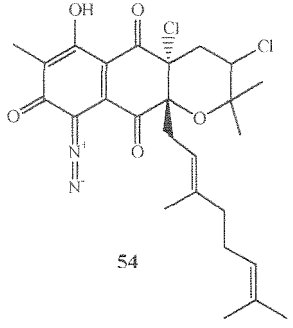

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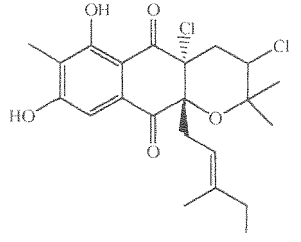
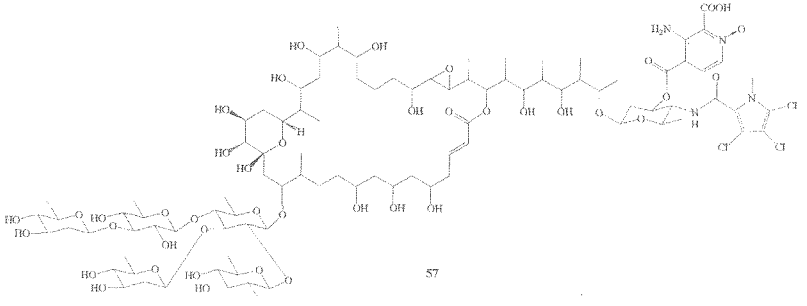


4. Macrolides

The tern? macrolide was originally applied to a group of lipophilic basic antibiotics with a macrocyclic lactone ring in their chemical structure. These antibiotics are highly active against Gram-positive bacteria and mycoplasmas and hence at-c effective, when administered orally. against infectious diseases caused by these microorganisms. When administered to mammals, these drugs are characteristically distributed in the liver. lung, and spleen and effectively halt microbial respiratory or biliary tract infection. They arc therapeutically and economically important drugs.

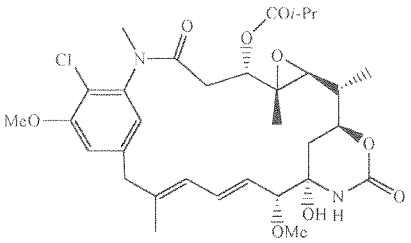
Among the numerous macrolide antibiotics discovered during the past 50 years, erythromycin, oleandomycin, leucomycin, spiramycin, josamycin, midecamycin, and tylosin have been produced industrially. The present chapter will focus mainly on discovery, strain improvement, fermentation, and the more recent work on their biosynthesis and mode of action of halogen (chlorine) containing macrolides.

4 new 34-membered macrolide antibiotic, colubricidin A **(57),** was isolated from the fermentation broth of a new *Streptomyces* species [50]. It was identified as a glycomacrolide with an unprecedented aromatic chromophore. An extract from an unidentified *Streptomyces* culture, designated LL-C 13122, was found to have nematocidal activity *in vitro* against *Caenorhabditis elegans*. Subsequently, colubricidin also demonstrated *in vivo* activity in gerbils infected with *Trichostrongylus colubriformis*. The isolated nematocidal agent, colubricidin A, demonstrated potent activity against Gram-positive bacteria but only weak activity against Gram-negative bacteria. It was slightly more potent than vancomycin against staphylococci but less potent against streptococci and enterococci. The fermentation broth was analyzed by RP-HPLC and colubricidin A was isolated as a yellowish powder. Its molecular formula was determined by HR-MS. Molecular weight of 21511 Da was demonstrated, appropriate for a molecular formula of $C_{96}H_{154}Cl_3N_5O_{42}$. The complete structure was elucidated by 1D and 2D NMR coupled with chemical degradation.



57

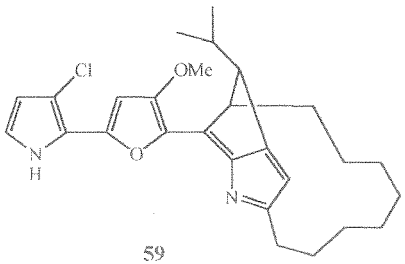
Ansamitocin P-3 **(58)**, a derivative of maytansinoid, was isolated [51] from two members of the moss family *Thuidiaceae*, *Claopodium crispifolium* and *Anomodon attenuatus* in very low yield.



58

From 13 kg of *C. c.* whole air dried moss was isolated 1 mg of pure compound, *i.e.* 1×10^{-5} % yield, or from *A.a. i.e.* 4×10^{-6} % yield, respectively. *C. crispifolium* was collected from rocks on steep slopes in a Douglas fir forest in Oregon, USA in 1981 and *A. attenuatus* was collected from rocks and ti-ccs of very steep slopes in the forest of West Virginia in 1985. This compound is probably the product of unidentified actinomycete associated with the above-mentioned mosses. Ansamitocin P-3 showed a potent cytotoxicity against the human solid tumor cell lines A-549 and HT-29. The compound exhibited a significant activity against P-388 lymphocytic leukemia in mice and both 9PS (murine lymphocytic leukemia) and 9KB (human nasopharyngeal carcinoma) in cell culture systems.

The structure of a new antibiotic, roseophilin **(59)**, was elucidated [52] by NMR spectral analysis including a variety of two-dimensional techniques. Roseophilin was found to possess a novel skeleton containing pyrrole and furan moieties. It was isolated from the culture broth of an actinomycete identified as *S. griseoviridis*. The compound showed cytotoxicity against K562 human erythroid leukemia cells (IC_{50} 0.34 μM) and KB human epidermoid carcinoma cells (IC_{50} 0.88 μM).



59

Roseophilin was efficiently synthesized [53] starting with the known 3-formylpyrrole; the method features intramolecular alkylation to form the desired thirteen-membered carbocycle and base-induced intramolecular acylation to construct the requisite pyrrole-fused cyclopentanone ring system as the key steps.

The ansamycins represent some of the most complex antibiotics. They and their derivatives have aroused considerable interest as antiviral and antimicrobial agents, and as inhibitors of RNA tumor virus reverse transcriptases. Hence, the potential significance of various structural features of these antibiotics and their derivatives in relationship to their biological activity is being investigated. The constitutional structure of naphthomycins was described arid it was demonstrated that they belong to the class of naphthalenic ansamycins. These antibiotics were isolated [54] from the fermentation broth of' an unidentified species of *Streptomyces* and found to exhibit antimicrobial activity against *Bacillus subtilis*. The growth inhibition was relieved by addition of cysteine to a chemically defined minimal medium. The biological and chemical properties (structures were determined by ^{1}H NMR spectra and using lanthanide shift reagent) showed a marked similarity with naphthomycins.

The antibacterial and antifungal ansamycin type antibiotic naphthomycin A (60) was first isolated from *S. diastatochromogenes* (synonym: *S. collinus*) Tü 105. Its structure was determined by ^{1}H NMR investigation and slightly revised upon a chemical degradation study. It has been characterized by analytical, spectroscopic, and microbiological data. Naphthomycin A has been found to antagonize vitamin K.

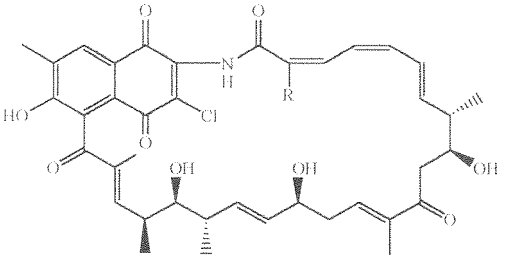
From the fermentation products of a *Streptomyces* culture number Y-8340369 (fi-on? a soil sample collected near Dharchuia, Hjmachai Pradesh, India) was also isolated [55] naphthomycin A as a minor constituent and a further compound designated as naphthomycin H, a light yellow crystalline substance having formula $C_{39}H_{44}ClNO_9$, as the major constituent. Naphthomycin H (61) was shown to be a geometrical isomer of naphthomycin 13 (62) by extensive 'ti NMR double-resonance studies.

Naphthomycins 13 and C were isolated [56] from two different strains of *Streptomyces*. The structures were determined by comparison of the spectra with those of the known naphthomycin A, by spin decoupling experiments and by 211 NMR analyses. Naphthomycin B is 30-chloronaphthomycin C. Strikingly naphthomycin A differs from B and C not only by the presence of an additional methyl group, but also in the configuration of some of the double bonds.

A different strain of *Streptomyces*, strain Tü 353, belonging to the species *S. galbus* subsp. *griseosporeus*, isolated from a soil collected in Kouroussa (Guinea), produced an antibiotic closely related to naphthomycin A, which was called naphthomycin B. A third compound of the same family, naphthomycin C, was obtained during the isolation of ansatrienins from *S. diastatochromogenes*

var. *diastatochromogenes*, strain Tü 1892.

Another *Streptomyces sp.* E/784 produced low levels of the substituted ansamycin antibiotics, including ('-30 chlorinated analogues naphthomycins A and H [57]. The addition of *N*-acetyl-L-cysteine to the fermentation medium substantially increases the production of the thionaphthomycins I and J at the expense of their chloro analogues A and H. The formation of thioansamycins from chloroansamycins and thiols *in vivo* is probably non-enzymic since similar conversions can be effected *in vitro*.



60 R = Me
61 R = H

In the paper of Brufani [58], the structure of naphthomycin was revised. Based on air extensive study including paramagnetically induced chemical shifts it was proposed that the chemical shift of the phenolic proton (9.63 ppm) is not in agreement with the OH group in *peri*-position of a naphthoquinone system. The structure was revised to naphthomycin with the phenolic hydroxyl group in position 6. Chemical degradation of naphthomycin was also carried out. The ozonization of the antibiotic in acetic acid at room temperature, oxidation of the ozonides with hydrogen peroxide in acetic acid and subsequent treatment of the mixture of products with diazomethane yielded different degradation products.

The relative and absolute configurations of naphthomycin A were elucidated [59] by X-ray structural analysis of a methylation product, 25-0-methylnaphthomycin A iminomethyl ether. The absolute configuration was confirmed by degradation (O_3, $NaBH_4$) to (*S*)-butane-1,2,4-triol.

$^{18}O_2$ was incorporated into the molecule of the antibiotic and it was shown [60] that actamycin (compound with the same skeleton but without chlorine at C_{30} position) is labeled at C_{28} and C_{30}.

Chlorothricin **(63)** is an unusual macrolide antibiotic produced [61] by *S. antibioticus*. Its structure consists of three types of components, a modified 6-methylsalicylic acid, two identical 2,6-dideoxyhexose (2-deoxy-rhamnose) moieties and the aglycone, chlorothricolide. Earlier biosynthetic studies have established the biosynthetic origin for most of the carbon frameworks of these components. The modified 6-methylsalicylic acid comes from 4 acetate-

malonate units *via* tlic polyketide pathway, with contribution of the additional *O*-methyl group by methionine, and tiic two 2,6-dideoxyhexose moieties are derived directly from glucose. The aglycone is predominantly of polyketide origin, being made of up to ten acetate and two propionate units which account for all but three carbon atoms of chlorothricolide. While carbon atoms 35 and 26 of the tetronic acid moiety are derived from an intact acetate unit, C-24, C-23 and the adjacent C-22 arc not labeled by either acetate or propionate. The metabolic route by which the propionate units are generated from primary metabolites and stereochemical aspects of the conversion of glucose to the 2,6-dideoxyhexose moieties have been described.

The biosynthesis [62, 63] of chlorothricin, a macrolide antibiotic isolated from *S. antibioticus* Tü 99, has been studied by feeding experiments with ^{14}C and ^{3}H-labeled precursors. Ten acetate and two propionate units, but not methionine and mevalonate, were incorporated into tire macrocyclic aglycone of' the antibiotic. Glucose and the various carbon atoms of tyrosine, except for the carboxyl carbon, also contributed label to the aglycone. Glucose also seems to be a specific precursor of the 2-deoxyrhamnose moiety, probably *via* a process involving a hydrogen shift from C-4 to C-6 of the hexose. The substituted 6-methylsalicylic acid moiety seems to be derived from four acetates and one *O*-methyl group provided by methionine, whereas shikimic acid is not incorporated.

Further feeding experiments [61] with [*U*-$^{13}C_3$] and (2*R*)-[1-$^{2}H_2$] glycerol showed that glycerol is incorporated intact into carbon atoms 22, 23 and 24 of the aglycone of chlorothricin. C-1 of glycerol gives rise to C-22 with retention of one atom of deuterium, which occupies the 11-22!? position. A mechanism for the assembly of the aglycone is proposed which invokes phosphoenolpyruvate as the direct precursor of the 3-carbon moiety and a Baeyer-Villiger oxidation as the mode of formation of the macrocyclic lactone functionality. A feeding experiment with [1,2-$^{13}C_2$] succinate suggests that the propionate units of tlic aglycone polyketide arc formed entirely *via* the methylmalonyl-CoA mutase reaction. The formation of the two 2-deoxy-D-rhamnose moieties of chlorothricin from glucose was shown ro involve replacement of the 2-hydroxyl group of the sugar by hydrogen with inversion of configuration at C-2. The antibiotic mixture was active [64] against Gram-positive bacteria on synthetic, but not on complex media.

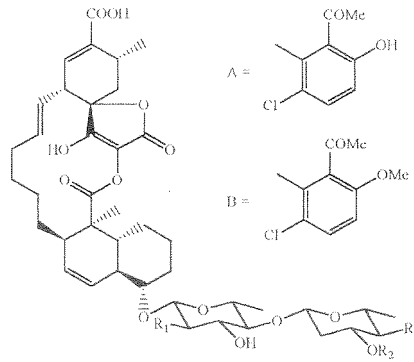
Four new compounds from the culture broth of *Streptomyces* sp. A7361 were isolated [65] from a soil sample collected in Ohmiya city, Saitama Prefecture it? Japan. The fermentation, isolation, structure determination and biological activities were described. The isolation of' four new compounds designated MC-031 **(64)**, 032 **(65)**, 033 (66) and 034 **(67)**, from a culture broth of *Streptomyces* sp. A7361 was described that are structurally related to. but distinct from chlorothricin.

The cultured broth of *Streptomyces* sp. K818 showed [66] antitumor activity and contained a new antibiotic identified as 2-hydroxychlorothricin **(68)**.

The X-ray crystal structure analysis of the cesium salt of chlorothricolide methylester, derived from the aglycone of the antibiotic chlorothricin was described [67].

Table 3. Antimicrobial activity (MIC μg/ml)

Microorganisms	**63**	**68**
Staphylococcus aureus	25	100
Streptococcus pyogenes	>100	>100
Bacillus subtilis	25	>100
B. cereus	25	100
Micrococcus luteus	50	>100
Staphylococcus aureus	25	100
Escherichia coli	>100	>100
Klebsiella pneumoniae	>100	>100
Serratia marcescens	>100	>100
Pseudomonas aeruginosa	>100	>100
Aspergillus fumigatus	>100	>100
Penicillium chrysogenum	>100	>100
Trichophyton mentagrophytes	>100	>100
Candida albicans	>100	>100



63 R_1 = H, R_2 = B, R_3 = OH (may be also Br)
64 R_1 = H, R_2 = A, R_3 = OH
65 R_1 = OH, R_2 = A, R_3 = OH
66 R_1 = H, R_2 = H, R_3 = OA
67 R_1 = OH, R_2 = H, R_3 = OA
68 R_1 = OH, R_2 = B, R_3 = OH

5. Orthomycins

The name orthosomycins is proposed for a family of antibiotics, which are characterized by the presence, in their structure, of one or more orthoester linkages that are associated with carbohydrate residues. The natural occurrence of orthoesters is rare, but within recent years, several examples of antibiotics possessing this common structural feature have been described [68]. It now seems desirable to group them together under the generic name of orthosomycins.

Flambamycin, curamycin and avilamycin belong to this class of orthosomycin antibiotics which possess a terminal ester residue derived from 3,5-dichloroisoeverninic acid (3,5-dichloro-4-hydroxy-6-methoxy-2-methyl-benzoic acid).

Flambamycin exhibits a very low toxicity and shows an interesting activity against Gram-positive bacteria, *Neisseria*, Gram-negative bacteria and some Gram-positive bacilli, see Table 4. It is practically inactive against Gram-negative bacilli, yeasts and filamentous fungi. This selectivity coupled with excellent therapeutic *in vivo* activity in mice infected experimentally with *Staphylococcus aureus*, *Streptococcus pyogenes haemolyticus*, or *Neisseria meningitidis* encouraged structural investigation of flambamycin.

Table 4. Bacteriostatic activity of flambamycin (MIC in μg/ml)

Test organism	MIC
Staphylococcus aureus	0.85
Sarcina lutea	0.2
Streptococcus faecalis	2
S. viridans	15
S. pyogenes hemolyticus	0.25
Diplococcus pneumoniae	0.1
Neisseria catarrhalis	1.25
N. meningitidis	0.6
N. gonorrhoeae	1.25
Bacillus subtilis	15
B. cereus	9
Mycobacterium sp.	>150
Escherichia coli	>150
Shigella dysenteriae	>150
Salmonella paratyphi	>150
S. schottmuelleri	>150
Proteus vulgaris	>150
Klebsiella pneumoniae	>150
Pseudomonas aeruginosa	>150
Brucella bronchiseptica	35
Pasteurella multocida	0.2
Mycoplasma gallisepticum	3

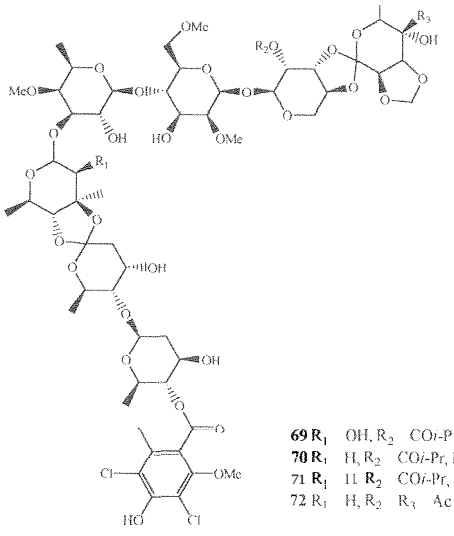
in the course of studies [hi)-71] on the production of antimicrobial agents by microorganisms, a new antibiotic flambamycin (69) (also known as 21190 RP), was discovered in tlic culture hi-oilis of *S. hygroscopicus* DS *23230.* The strain was isolated from a sample of soii collected in Great Britain and its antibiotic properties were demonstrated by classical methods. It exhibits all the main morphological and biochemical characteristics of the species S. *hygroscopicus*, especially the tight spiral sporophores and their clustered insertion upon the main filament, the dark grey color of the normally sporulated mycelium, and also in ageing cultures ilic production of dark patches and of an exudate on the surface of the colonies on agar medium.

The major degradation product of flambamycin, *i.e.* desdichloroisoeverninoyl-desisobutyroyl flambamycin was found to be devoid of antibacterial activity. This result suggests that the antibacterial activity of flambamycin is concerned with the antibiotic molecule as a whole and riot with any particular sequence of residues present in tlic antibiotic.

From culture of iiic avilamycin A **(70)** producing organism, *S. viridochromogenes*, strain ETH 33575 (=NRRL *2800)* obtained from a Venezuelan soil sample, a second antibiotic, avilamycin C, $C_{61}H_{90}Cl_2O_{32}$, was isolated [72] in crystalline form. Third avilamycin, *i.e.* avilamycin B was also obtained, but its molecule does not contain any halogen atom(s). Both avilamycins belong to tlic group of the orthosomycins. By IR, ^{1}H and ^{13}C NMR spectroscopy and by transformation to a common derivative it could be proven that avilamycin A is a methyl ketone and avilamycin C the corresponding methyl carbinol.

Hydrolysis and methanolysis of avilamycin C **(71)** yielded [73] a series of mono- and oligosaccharide-like products, which wcr-o. with one exception, identical with degradation products of flambamycin. Instead of evalose (6-deoxy-3-methyl-D-mannose) a building stone of flambamycin and everninomicin B, evermicose (2,6-dideoxy-3-methyl-D-mannose) was identified as a constituent of the avilamycins. This sugar was known as a degradation product of the everninomicins C and D. From the degradation results, a structure of avilamycin A was postulated which differs from that of flambamycin only by the lack of a hydroxyl group in position 2 of the evalose residue. This hypothesis was confirmed by ^{1}H and ^{13}C NMR studies.

From cultures of *S. curacoi*, a new *Streptomyces* species obtained from a soil sample from Curacoa, Argentina a chlorine containing glycosidic antibiotic, named curamycin **(72)**, has been isolated [74]. Curamycin on acid hydrolysis yields a crystalline product, curacin, and a mixture of monosaccharides. Curacin, which contains all of the chlorine of curamycin, is the ester of dichloroisoeverninic acid with a di-desoxyaldose. Three other monosaccharides are also present, two of which were identified as L-lyxose and 4-*O*-methyl-D-fucose.

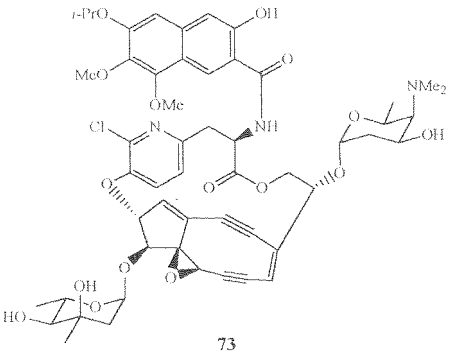


69 R_1 OH, R_2 CO*i*-Pr R_3
70 R_1 H, R_2 CO*i*-Pr, R_3 Ac
71 R_1 H, R_2 CO*i*-Pr, R_3 CH(OH)Me
72 R_1 H, R_2 R_3 Ac

6. Enediynes

A novel chromoprotein antitumor antibiotic kedarcidin **(73)** was isolated [75] from the culture supernatant of an actinomycete strain L585-6 (ATCC 53650). Strain L585-6 wits isolated from a soil sample collected in Maharastra State, India. A taxonomic study showed that strain L585-6 is an unidentified and unknown actinomycete. Production titers of kedarcidin in shake flask and fermented cultures were 1300 μg/ml and 1050 μg/ml, respectively. The antibiotic was recovered from the culture filtrate by adsorption to QAE ion exchanger and purified by successive application of gel filtration and ion exchange chromatography with Sephadex G-50 and DEAE-Sephadex, respectively. Kedarcidin is air acidic complex (pI ~ 3.65) with an apparent molecular weight of' 12,400. The complex consists of a highly unstable, solvent extractable chromophore arid a water soluble peptide. The apoprotein is a single chain polypeptide of 114 residues [76]. The 9-membered enediyne chromophore is a new member of the enediyne class of antitumor agents. Details of the structure elucidation of kedarcidin chromophore by HRFABMS ($C_{53}H_{60}N_3O_{16}Cl$), using a combination of NMR techniques, mass spectrometry, chemical degradation, derivatization, and sodium borohydride reduction experiments, were reported [77]. Acidic methanolysis of kedarcidin afforded three products: methyl α-L-mycaroside, the methyl α-glycoside oi' the novel amino sugar kedarosamine ($C_9H_{19}NO_3$), and a 2'-chloroazatyrosyl naphthoamide fragment. The presence of a reactive 8,9-epoxybicyclo[7.3.0]dodeca- dienediyne

core nab established I;-oiii heteronuclear NMR ;inti sodium borohydride reduction experiments [78]. The nine-membered enediyne core underwent rapid aromatization upon reduction with sodium borohydride to a cyclopentyl-indene ring system. This reduction was shown to be stereospecific when using sodium borodeuteride. The absolute stereochemistry of the aglycone was inferred through a combination of NOE NMR and molecular data modeling.



73

It is active against implanted P388 leukemia and B16 melanoma in mice at a dosage of 3.3 and 2 μg/kg, respectively. The potency of kedarcidin against murine tumor is similar to a class of antibiotics with the unique 1,5-diyn-3-ene structure. Kedarcidin also shows potent activity against Gram-positive bacteria but no activity against Gram-negative bacteria [79].

Cytotoxicity assays on the HCT116 colon carcinoma cell line result in an IC_{50} value of 1 nM. *In vitro* experiments with ΦX174, pM2 DNA, and ^{32}P-end-labeled restriction fragments demonstrate that this chromophore binds and cleaves duplex DNA with a remarkable sequence selectivity producing single-strand breaks. The cleavage requires reducing agents and oxygen similar to the other naturally occurring enediynes. Certain cations (('a^{2+} anti Mg^{2+}) prevent sir-ailti cleavage. High-resolution 'it NMK studies on the chromophore in the presence of calcium chloride implicate the 2-hydroxynaphthoyl moiety in DNA binding. Interestingly, the kedarcidin chromophore appears to be structurally related to neocarzinostatin yet recognizes specific DNA sequences in a manner similar to calicheamicin γ_1^I, an enediyne with a significantly different structure. Moreover, kedarcidin and calicheamicin share a DNA preferred site, the 10 CTN-mer. These observations indicate that the individual structural features of these agents are not solely responsible for their DNA selectivity. Rather, a complementarity between their overall tertiary structure and the local conformation of the DNA at the binding sites must play a significant role in the recognition process [80].

Strain C-1027, an actinomycete isolated [81] from a soil sample collected in China, was found to produce the new antibiotic C-1027 (74). From taxonomical studies on its morphological, cultural and physiological characteristics, this antibiotic-producing strain was identified as *S. globisporus* C-1027.

The antibiotic C-1027 was obtained [82] by precipitation with ammonium sulfate, DEAE-cellulose column chromatography and gel filtration chromatography on a Sephadex G-75 column. This antibiotic, prepared as a white powder, is an acidic polypeptide having an isoelectric point of pH 3.5 - 3.7 and a molecular weight of 15,000 as determined by SDS-polyacrylamide gel electrophoresis and gel filtration chromatography. The acid hydrolysate of the purified antibiotic 4'-I027 contained no methionine or tryptophan. From the physicochemical data, it inay be considered to possess a very labile non-protein chromophore and acidic protein.

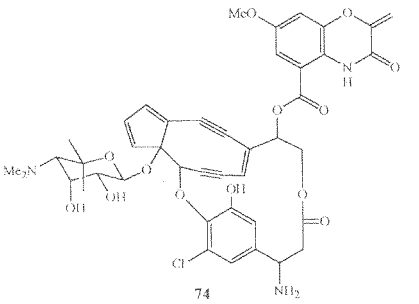
The structure and cycloaromatization mechanism of a novel enediyne, C-1027 chromophore, were elucidated [83]. The C-1027 chromophore has a 9-membered 1,5-diyn-3-ene core structure in the 16-membered macrocyclic ring. The protein-free C-1027-Chr is too labile for the determination of its structure only by spectroscopic methods, and, therefore, tlic structure was elucidated by using its more stable reaction product. its benzodihydropentalene core structure suggested the presence of an enediyne in the native C-1027-Chr. Stereocontrolled synthesis and absolute stereochemistry [84] of the aminosugar moiety of C-1027 chromophore has been disclosed. The structures of the labile C-1027 choromophore and its cycloaromatized product have been determined by detailed 2D NMR studies [85]. While the novel aminosugar moiety is supposed to play an important role in DNA strand scission. its absolute stereochemistry has not been elucidated. By the application of the CD exciton chirality method directly to the bis-p-bromobenzoate the structure described below was determined.

C-1027 showed an extremely potent cytotoxicity against cultured cancer cells [82]. Compared in terms of IC values, the antibiotic C-1027 showed a much more potent cytotoxicity than doxorubicin, mitomycin C and neocarzinostatin. Spermatogonial assay, a prescreen for anticancer drugs, was highly sensitive for detection of C-1027. At tolerable doses, C-1027 exhibited marked inhibition on a panel of transplantable tumors in mice, which included leukemia 1.1210, P388, ascites hepatoma H22, sarcoma 180 in mice and melanoma Harding-Passey [86].

Antibiotic C-1027 had a moderate antimicrobial activity against Gram-positive bacteria but was shown to be inactive against *Mycobacterium* sp. and Gram-negative bacteria except for some strains of *Escherichia coli* and tested fungi.

Further, the presence of another protein [87], designated as C-1027-AG, chemically similar to C-1027, was noticed in culture filtrates. Both proteins

were similar in molecular weight, isoelectric point and amino acid composition but different in absorption specti-a, with one showing the absorption shoulder at 340-360 nm. C-1027-AG had no antimicrobial effect on Gram-positive bacteria. and antagonistically suppressed the inhibitory activity of('-1027 against Gram-positive bacteria.



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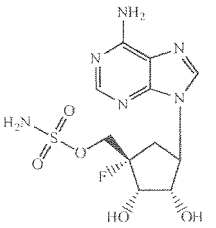

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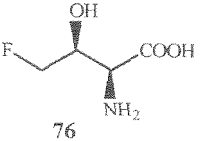
7. Fluorinated compounds

Nucleocidin (75) is an intriguing organo-fluorine natural product with a broad spectrum of antibiotic activity, although it has proved too toxic for clinical use. The nucleocidin was first isolated in 1957 from S.*calvus* which was obtained from an Indian soil sample [88]. However, it was not until 1969 that it was appreciated that the compound contained a fluorine atom and the correct structure. 4'-fluoro-5'-*O*-sulphamoyladenosine, was deduced [89]. The total synthesis was published [90]. It is therefore particularly frustrating that recent attempts in several laboratories [88, 91] have failed to reisolate this metabolite from cultures of *S. calvus* grown from freeze dried organisms obtained from several culture collections and to identify the mechanism by which fluorination occurs in the bacterium. The gene encoding nucleocidin biosynthesis appears to have been lost. The biosynthesis of fluorometabolites by *S. cattleya* using ^{19}F NMR was examined [92] and demonstrated production of both 4-

fluorothreonine **(76)** and fluoroacetate **(77)** in millimolar concentrations when the organism was grown on a chemically defined medium with glycerol and glutamate as the C-sources.



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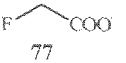


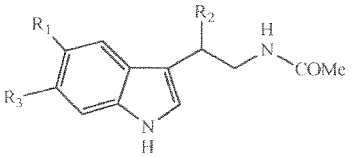
77

This development opened the way for experiments on the incorporation of isotopically labeled precursors into the fluorometabolites to delineate the metabolic pathway prior to the fluorination reaction. Particularly high incorporation from glycine was observed with C-2 of glycine contributing both carboxyl atoms of fluoroacetate and C-3 and C-4 of 4-fluorothreonine [93, 94]. Serine is subsequently converted to pyruvate by serine dehydratase. Tamura *et al.* [95] demonstrated that label from [2-^{13}C]-glycerol was incorporated into C-1 of fluoroacetate by *S. cattleya*. The entry of glycerol into the glycolytic pathway is mediated by glycerol kinase, an enzyme which phosphorylates the *pro*-(*R*) hydroxymethyl group of glycerol (but not the *pro*-(*S*) hydroxymethyl group) to generate *sn*-glycerol-3-phosphate. The enantiotopically labelled (*R*) [1-$^{2}H_2$]- and (*S*) [1-$^{2}H_2$]-glycerols were prepared [96], and their incorporation into the fluorometabolites of *S. cattleya* was compared. Both deuterium atoms from the *pro*-*R* arm of glycerol but neither from the *pro*-*S* arm were incorporated into the fluoromethyl groups of the fluorometabolites. The best studied system for biosynthesis of fluoroacetate and 4-fluorothreonine is *S. cattleya*.

Another part of this chapter deals with fluorinated compounds; however, it should be kept in mind that these compounds were produced either by a partial chemical synthesis or by incorporation of fluorinated derivatives into the molecule of a biologically active compound. In most cases, a significant increase of the biological activity was expected but the results obtained were, with a few exceptions, highly disappointing, although the significance of some of these compounds was described in several industrial patents.

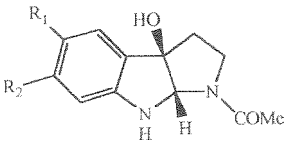
S. staurosporeus (AM-2282) was found to produce the potent protein kinase C inhibitor staurosporine. Some staurosporine derivatives are of a high pharmaceutical significance and precursor-directed biosynthesis is of some interest. Tryptophan, methionine, and glucose have been identified as precursors of staurosporine. Consequently, metabolic studies of indole and sugar derivatives may permit a deeper understanding of staurosporine biosynthesis or may directly produce novel staurosporine derivatives *via* precursor-directed biosynthesis. The 5- and 6-fluorotryptamines could be transformed to 5-fluoro-N_b-acetyltryptamine **(78)** and 6-fluoro-N_b-acetyltryptamine **(79)** and the novel

metabolites 5-fluoro- and 6-fluoro-substituted alkaloids **(80)** and **(81)**, respectively [97]. Also the report about the isolation of additional novel metabolites, 5-fluoro-β-hydroxy-N_b-acetyltryptamine **(82)** and 6-fluoro-β-hydroxy-N_b-acetyltryptamine **(83)** from tlic ethyl acetate extract of S. *staurosporeus* cultures after tryptamine HCl, 5-fluorotryptamine HCl, and 6-fluorotryptamine feeding, respectively, was published [98].



78 $R_1 = F, R_2 = R_3 = H$
79 $R_1 = R_2 = H, R_3 = F$

82 R_1 I R_2 OH, R_3 H
83 R_1 H, R_2 = OH, R_3 I



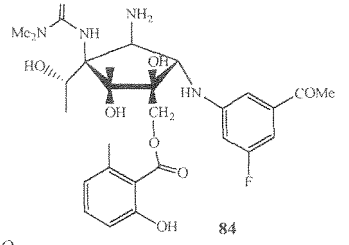
80 R_1 = F, R_2 H
XI R_1 H, R_2 F

\ new pactamycin analogue, 5"-fluoropactamycin **(84)**, was prepared [99] by directed biosynthesis. Supplementation of tiic fermentation medium of S. *pactum*, var. pactum with 3-amino-5-fluorobenzoic acid, an advanced precursor in pactamycin biosynthesis, resulted in co-production of pactamycin and the new pactamycin analogue. A similar feeding experiment with 3-amino-5-methylbenzoic acid did not result in formation of the corresponding methylated pactamycin analogue, but only in inhibition of pactamycin production. Comparison of antimicrobial and cytotoxic activities of pactamycin and 5"-fluoropactamycin showed no significant differences.

5''-Fluoropactamycate **(85)**, another fluorinated metabolite, was isolated from a cultivation medium.

The macrolides FK-506 and FK-520 were obtained [100] by biological fermentation of two streptomycetes. The parent compounds were obtained from S. *tsukubaensis* No 9993 and from *S. hygroscopicus* subsp. *ascomyceticus*

ATCC 14891 The following derivatives (86) [100] wctc semisynthetically prepared, where the sugar ıs fluoro substituted β-D-galactopyranose or α-D-arabinofuranose



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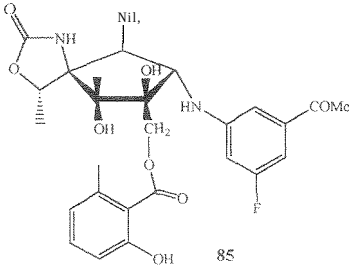

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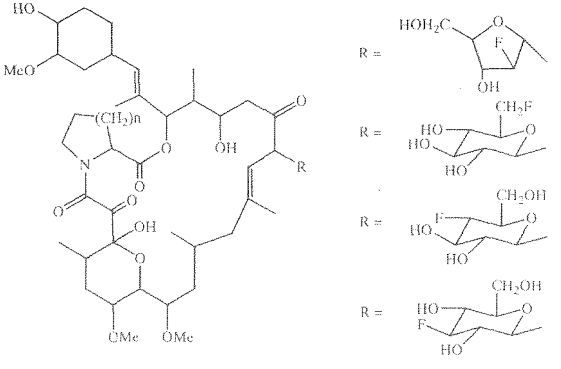
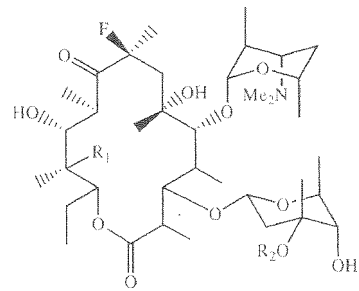
These new compounds arc extremely useful in the treatment of' graft rejection following skin or organ transplant surgery and in the prevention or treatment of autoimmune diseases such as rheumatoid arthritis and psoriasis. Additionally, these macrolide derivates will find use in preventing or treating infectious diseases caused by fungi.



The new aglycones (8*S*)-fluoro erythronolide A, (8*S*)-fluoroerythronolide B and the monoglycoside 3-*O*-mycarosyl-(8*S*)-fluoroerythronolide B obtained by semisynthetic preparation were converted to fluoro-compounds by mutational biosynthesis [101], in *Saccharopolyspora erythraea* ATCC 31772, a mutant blocked in the biosynthesis of erythromycin. Four new antibiotics **(87)**, (8*S*)-fluoroerythromycin A, (8*S*)-fluoroerythromycin B, (8*S*)-fluoroerythro- mycin C and (8*S*)-fluoroerythromycin D were obtained. The biosynthesis of erythromycins has also been discussed in terms of the substrate specificity of the enzymes involved. The new antibiotics exhibited promising biological properties.

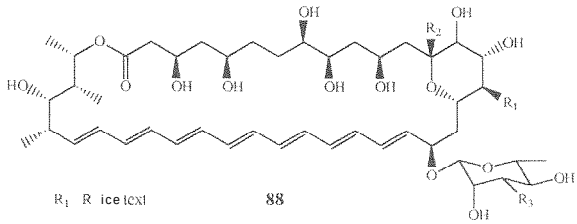


R_1 = H or OH
R_2 = H or Me

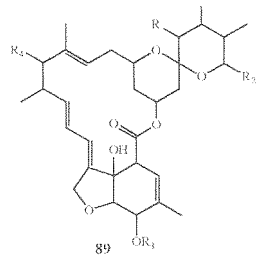
87

The polyene macrolide amphotericin B **(88)**, produced by *S. nodosus*, is widely used for the treatment of fungal infections. Amphotericin B is the only complex polyene macrolide antibiotic. Compounds with substitution on C-13, *e.g.* fluorine, have been described in the patent literature. The patent [102] included practically all possible arid impossible (these especially) substituents (see below). The USA invention includes pharmacological significance of these novel compounds, their preparation, and their use in the treatment of fungal infections in animals including humans. Unless otherwise specified, the term halogen includes fluorine, chlorine, bromine and iodine, *etc.* For example, the R_1 is a carboxylic acid group or a derivative thereof, a ketone residue, an aldehyde function or optionally substituted methyl, R_2 is hydroxyl, $C_{1\,8}$ is an alkoxy group or a fluorine atom, and R_3 is an amino group or a derivative thereof were prepared.

In the next invention [103] antiparasitic agents **(89)**, in particular compounds related to avermectins and milbemycins but having a novel substituent group on C_{25}, and the procedure of their preparation have been described. Substituent on C_{25} was designated R_2. Any group (alkyl, alkenyl, alkinyl, cycloalkyl), not necessarily substituted by a halogen, can be involved.



R_1 R ice text 88



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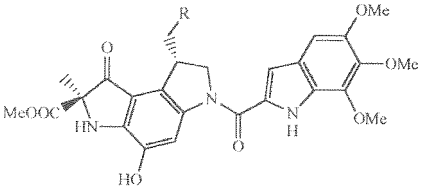
8. Indoles

The structures of new antitumor antibiotics [104], duocarmycin C_1 (=pyrindamycin 13) **(90)** and C_2 (= pyrindamycin A) **(91)** isolated from the culture broth of *Streptomyces sp.*, have been determined by chemical and physicochemical evidences. The producing organism was isolated [105] from a soil collected at the foot of Mt. Fuji in Shizuoka, Japan, and was taxonomically classified as *Streptomyces sp.* DO-88 (FERM BP 1002).

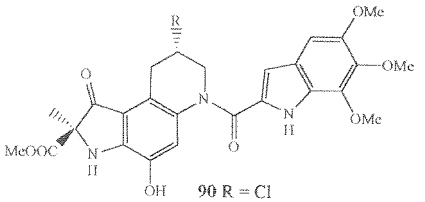
The MICs against Gram-positive bacteria were under 0.01 μg/ml, while values for Gram-negative bacteria and yeast were between 1 10 μg/ml. Duocarmycins C_1 arid C_2 were produced by *Streptomyces sp.* as novel antitumor antibiotics that are effective against murine lymphocytic leukemia P388 and murine sarcoma 180 in mice [106, 107].

Further, two new antitumor antibiotics, *i.e.* duocarmycins B_1 **(92)** anti B_2 (93) were produced in the medium modification (addition of bromide) fermentation broth of *Streptomyces sp.* DO-80. The production, isolation, physico-chemical properties and biological properties of duocarmycins B_1 and B_2 were reported by Ogawa *et al.* [10X].

The all seven duocarmycins A, C_1 (Y0), C_2 (91). D, B_1 **(92)**, B_2 **(93)** and SA (only B and C contained halogen), were isolated [I 09] from three independently collected *Streptomyces* sp. The complete structures by HRMS, UV, IR, ^1H and ^{13}C NMK (2D NMR, *i.e.* COLOC. NOEs, etc.), including absolute stereochemistry by MTPA esters and CD spectra of benzoates. were determined by spectral and chemical studies of those duocarmycins and several derivatives.



91 R = Cl
93 R = Br



90 R = Cl
92 R = Br

Further, duocarmycin C_1 (=pyrindamycin 13) **(90)** and C_2 (=pyrindamycin A) **(91)** have been found in the culture broth of *Streptomyces* sp. SF2582, which was isolated from a soil sample collected at Sagamihara, Kanagawa Prefecture, Japan. The antibiotics arc active against (;sari?-positive and Gram-negative bacteria. In a paper of Ohba *et al.* [110], the fermentation, isolation, physico-chemical properties and structural elucidation, including X-ray analysis of pyrindamycin A, were reported.

Duocarmycin C_1 (=pyrindamycin B) (90) and C_2 (=pyrindamycin A) (9I) exhibited stronger cytotoxic activities than doxorubicin towards murine and human tumor cell lines and especially towards doxorubicin-resistant cells [111]. Pyrindamycins A and B were also active *in vivo* against P388/ADR, a multidrug-resistant tumor cell line. Intracellular accumulation of pyrindamycins A and 13 in P388/ADR was the same as in P388. These antibiotics strongly inhibited DNA synthesis compared with RNA or protein synthesis. They showed significant therapeutic effects towards murine leukemia, but not to solid tumors.

Pyrroindomycins A (without Cl) and B (with Cl) **(94)** arc the two principal components of the antibiotic complex isolated from fermentations of culture LL-42D005, a strain of *S. rugosporus* [112] during the course of a screening program for agents active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. Both structures were determined on the basis of 1D and 2D NMR, MS and UV analyses of the intact molecules and chemical degradations. They are composed of a novel pyrrolindole entity linked *via* an ester bond to an unbranched deoxytrisaccharide. A polyketide macro-ring system containing a tetramic acid functionality is connected to the other end of

Table 5. Antitumor activity of duocarmycins against murine sarcoma 180.

Compound-	Dose (mg/kg)	TIC —
A	0.0075	0.26
C_1	6	0.28
C_2	3	0.19
B_1	0.5	0.22
	0.25	0.28

Single dose given *iv* on day-I after tumor inoculation

[a] T/C represents the ratio median tumor volume of the treated group divided by that of the control group

the trisaccharide through a glycosidic linkage. Pyrroindomycins are the first natural products that contain the highly unsaturated pyrrolindole moiety.

The pyrroindomycins demonstrated an excellent *in vitro* activity against Gram-positive bacteria [113]. The semisynthetic diacetyl derivative of pyrroindomycin B (pyrroindomycin B-Ac-2) had bactericidal effect on exponential-phase cells, but not on stationary-phase cells. This compound also exhibited marginal protection against a lethal *Staphylococcus aureus* challenge in mice. The poor *in vivo* activity of this antibiotic complex may he related to binding io blood components, as suggested by elevated MICs observed in blood-containing media. Incorporation of radiolabeled precursors into DNA, RNA, and protein was inhibited in an exponential-phase culture of *Bacillus subtilis* within ten minutes of exposure to pyrroindomycin t3-Ac-2. Microscopic examinations of drug-treated cells revealed lysis within the same ten minute period. These data arc consistent with an effect of pyrroindomycin B-Ac-2 (95) on the integrity of the bacterial membrane.

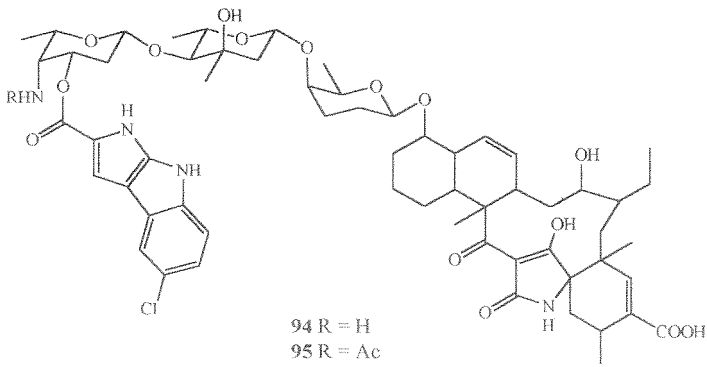
Production of pyrroindomycin A was characterized in a semi-defined fermentation medium containing glucose, casein, phosphate, vitamins anti minerals [114]. Accumulation trf pyrroindomycin B increased with increasing concentrations of glucose, reaching maximum titers at approximately 5 g/l glucose. Glucose concentrations greater than 7.5 g/l decreased yields of pyrroindomycin B. Inhibition of pyrroindomycins accumulation at higher glucose concentrations could be reversed by increasing the casein concentration. Ammonium chloride, arginine or glutamine could replace casein as the sole nitrogen source for growth and pyrroindomycins production. Glucose, fructose or mannitol were utilized as the sole carbon source, while sucrose, maltose, glycerol, corn oil and starch were poorly metabolized. Incubation of this isolate

in a vitamin-deficient medium resulted in a delay in growth and pyrroindomycins production; this delay was eliminated by the addition of biotin. Addition of L-tryptophan to the medium resulted in the production of pyrroindomycin A as the major species.

9. Different heterocycles

The antibiotic virantmycin (96) was isolated from S. *nitrosporeus* in 1981 [115]. The planar structure of virantmycin has been established by chemical and spectroscopic studies and the synthesis of (±)-virantmycin was reported. The determination of the absolute configuration of virantmycin through the synthesis of its (+)-form was also described.

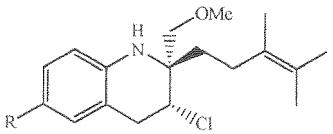
The unusual metabolite (-)-virantmycin isolated [115] by Japanese workers, from *S. nitrosporeus* No. AM-2722 has been found to possess potent antiviral activity. it was isolated from the putrefied tissue portion of a pumpkin and contained an antibiotic possessing a potent inhibitory activity against both RNA and DNA viruses [116]. The antibiotic also shows a weak antifungal activity. The active substance in culture broth was isolated [117] as colorless needles by solvent extraction followed by NP-HPLC. The molecular formula is $C_{19}H_{26}NO_3Cl$ from the elemental analysis and mass spectrum. Its gross structure was elucidated mainly by NMR studies and the relative and absolute stereochemistry at the two chiral centers (C-2 and C-3), have been determined using NOE difference experiments. The stereochemistry has been wrongly determined [115] by NOE difference spectroscopy to be 2*R*. 3*R*. NOEs from a slowly exchanging hydroxy proton in a synthetic intermediate provided the key stereochemical information. A total synthesis of antipodal virantmycin was accomplished [118] starting from p-aminobenzoic acid. The absolute stereochemistry of the natural compound was corrected to be 2*S*, 3*R*.



94 R = H
95 R = Ac

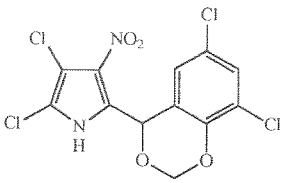
Rare metabolites, benzastatins C and D **(97)**, having tetrahydroquinoline skeleton, were isolated [119] from the culture broth of *S. nitrosporeus 30043.* They showed inhibitory activity against lipid peroxidation in rat liver microsomes. In the cell assay, benzastatins C and D inhibited glutamate toxicity in N18-RE-105 cells with EC_{50} values of 2.0 and 5.4 μM, respectively.

The structures of benzastatins A, B, C, and D, new free radical scavengers and unique alkaloids related to virantmycin were determined [120] by spectroscopic studies.



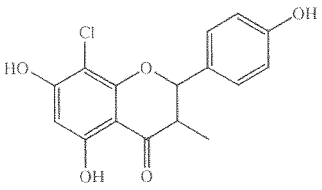
96 R = COOH
97 R = $CONH_2$

The strain designated as *Streptomyces sp.* 546506 was found [121] to produce a new antibiotic named pyrroxamycin **(98)**. The chemical structure was determined to be 4,5-dichloro-2-(6′,8′-dichloro-4′H-1′,3′-benzodioxon-4′-yl)-3-nitropyrrole by its chemical character and ^{1}H and ^{13}C NMR spectral analysis. Pyrroxamycin was active against Gram-positive bacteria and dermatophytes.



98

New flavanones were isolated from culture broth and mycelium of *S. graminofaciens HA* 14348. This *Streptomyces* was isolated [122] from a soil sample and was found to produce new specific inhibitors of estrogen binding to its receptor. Five related substances, BE-14348A–E were isolated and their structures were determined by analyses of spectral properties. Of these substances, A was identical with the known flavanone, naringenin. On the other hand, B, C, D and E were all new compounds; the structure of B was determined to be 2(*S*),3(*S*)-3-methyl-4',5,7-trihydroxy-flavanone, C was a racemic mixture of 2(*S*),3(*R*) and 2(*R*),3(*S*)-3-methyl-4',5,7-trihydroxyflavanone; D **(99)** and E **(100)** were 8-chloro derivatives of B and C, respectively.

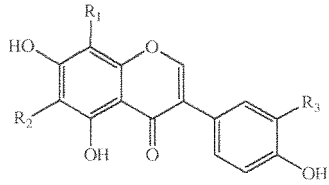


99 2(*S*), 3(*S*)
100 racemic mixture of 2(*S*), 3(*R*) and 2(*R*), 3(*S*)

Fermentations conducted [123] with aureolic-acid producing and non-producing *S. griseus* strains in soybean-meal-containing medium yielded phenolic metabolites which wcrc chomatographically and spectrally unrelated to either aureolic acid or its aglycone, chomomycinone. The yellow, phenolic compounds were isolated and characterized by ^{1}H NMR, ^{13}C NMR. and MS as the isoflavonoids gcnistcin (5,7,4'-trihydroxyisoflavone), 8-chlorogenistein **(101)**, and 6,8-dichlorogenistein **(102)**. The presence of gcnistcin in the soybean meal used in these fermentations was confirmed by extraction, isolation, and spectral identification. *S. griseus* grown in medium without soybean meal produced no isoflavonoids. Biotransformation of genistein yielded both chlorinated metabolites, demonstrating that these isoflavonoids are products of microbial halogenation. Labelled isoflavonoids wcrc not obtained when *S. griseus* was incubated with radiolabelled acetate or phenylalanine. These results demonstrate that isoflavonoids isolated from streptomycetes cultivated in media containing plant-derived nutrients such as soybean meal or cotton seed meal likely originate from the medium components and arc not of microbial biosynthetic origin.

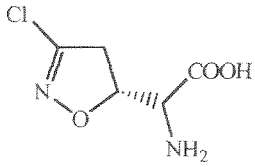
A strain of *S. griseus* produced [124] chlorinated metabolites in media containing soybean meal. Two of these metabolites wcrc isoiatcd together wit11 a chlorine free compound, which could be identified as the isoflavone genistein by its spectral properties and by comparison wit11 an authentic sample. By NMR and MS the chlorinated compounds were shown to be 6-chloro-genistein (103) and 6,3′-dichlorogenistein **(104)**. The 7-glucoside of genistein is a constituent of soybean meal; it is hydrolyzed and chlorinated by *S. griseus*.

Three antioxidant isoflavonoids characterized [125] as 7,8,4'-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone and 5,7,3',4'-tetrahydroxy-8-chloro-isoflavone **(105)** wcrc characterized from the cultured broth of" *Streptomyces sp.* OH-1049 which had been isoiatcd from a soil sample collected in Kanagawa Prefecture, Japan. Among them, 5,7,3',4'-tetrahydroxy-8-chloro-isoflavone is a novel isoflavonoid possessing a chlorine atom in the molecule. In *in vitro* studies, these antibiotics were found to possess antioxidant activity whereas showed almost no cytocidal activities against HeLa S_3cells.



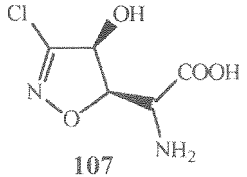
101 R_1 Cl, R_2 H, R_3 H
102 R_1 Cl, R_2 Cl, R_3 H
103 R_1 H, R_2 Cl, R_3 H
104 R_1 H, R_2 Cl R_3 Cl
105 R_1 Cl, R_2 H, R_3 OH

S. *sviceus* produces [126] an antimicrobial agent active *in vitro* against bacteria cultivated in synthetic media and fungi. The isolation and purification of the active agent, formed in minute amounts, was greatly facilitated by bioassay and bioautography techniques. Approximately 250 l of clarified fermentation broth containing, after further purification steps, 244 g of antibiotic U-42,126 (106). The antimetabolite antibiotic U-42,126, which significantly increased the life span of tumor-bearing L1210 leukemia mice at low drug levels with no demonstrable signs of toxicity to the hosts. was described.



106

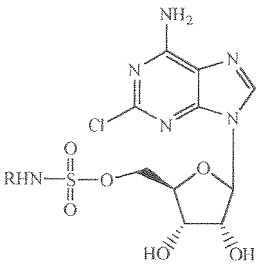
Improved purification procedures [127] and greatly improved fermentation titers have allowed preparation of relatively large quantities of U-43,795 (**107**) required for evaluation in other tumor systems; the successive processing of 16 000 liters of fermentation broth with Dowex and then Amberlite afforded after purification over 150 g of pure antitumor agent.



107

A novel, chlorine containing sulfamoyl nucleoside antibiotic, AT-265 **(108)**, was found [128] from *S. rishiriensis* subspecies No. 265 and its structure and biological activities were determined. The antibiotic is active *in vitro* against some Enterobacteriaceae and some Gram-positive bacteria.

The ill'-265 represents a highly cytotoxic 5′-*O*-sulfamoyl nucleoside whose sulfamoyl group is believed to simulate a phosphate group. Because of its unionized nature, the molecule can cross cellular membranes. The structure was proposed as 5′-*O*-sulfamoyl-2-chloroadenosine based on spectral evidence. However, no direct proof was given regarding the configuration of this compound. Further the isolation of two nucleoside antibiotics from a fermentation broth was realized [129]. The organism was isolated from a soil sample collected in Hamamatsu-shi, Shizuoka-ken, Japan and taxonomic studies indicated that it belongs in *Streptomyces*. One of them was found to be identical with antibiotic AT-265, however. the other one was the structurally remarkable alanyl derivative of AT-265 and was designated ascamycin (109). Tire structures were deduced from the spectral data inducing mass spectrometry, and the absolute configuration of ascamycir-1 as well as antibiotic AT-265 was unambiguously established by their conversion to 2-chloroadenosine.



108 R = H
109 R = $COCH(CH_3)NH_2$

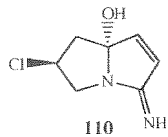
Two chlorine-containing antibiotics, clazamycins A (**110**) and B (**111**) have been isolated [130] from the culture broth of *Streptomyces* No. MF990-BF4 that is closely related to S. *violaceorectus* and *S. cinereoruber*. From *S. puniceus* subsp. *doliceus* NRRL 11160 was isolated [131] also the antibiotic clazamycin B.

The crystal structure of clazamycin A hydrochloride has been determined [132] by the X-ray diffraction methods which resulted in the elucidation of the chemical structure of clazamycins A and B.

The isolation and characterization of two new antimicrobial (antifungal) substances with broad spectra, pyrrolomycin A **(112)** and B **(113)** (SF 2080 A and B) from the culture broths of a strain of actinomycetes was described. The

Table 6. Antimicrobial spectrum of AT-265

Organism	MIC (μg/ml)
Bacillus subtilis	25
Staphylococcus aureus	50
Corynebacterium fascians	1.5
C. equi	1.5
Anthrobacter simplex	1.5
Mycobacterium smegmatis	50
M. smegmatis	25
M. phlei	25
Escherichia coli	3.1
Vibrio metschnikovii	0.2
Aerobacter aerogenes	1.5
Enterobacter aerogenes	100
Proteus vulgaris	1.5
P. mirabilis	50
Klebsiella pneumoniae	>100
Serratia marcescens	50
Xanthomonas oryzae	100
Erwinia aroideae	1.5
Salmonella anatum	50
Pseudomonas aeruginosa	>100



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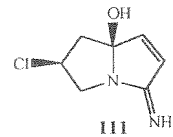


111

strain belongs to the genus *Streptomyces* (described as SF-2080);it was isolated [133] from a soil sample collected at the Chikuma River of Nagano City in Japan. A taxonomic study was undertaken by a detailed examination on agar and submerged culture, but the substrate mycelium of strain SF-2080 did not yield any morphologically important items so far, such as aerial mycelium, spore, nocardioform, synnemata, *etc.* However, L,L-diaminopimelic acid detected from the hydrolyzate of wliole cells suggests that it is most probably some genus *Streptomyces.*

Two antibiotics named pyrrolomycins A and B (SF-20x0 A and B) were discovered [134] in the fermentation broth of an actinomycete strain. The structure of pyrrolomycin A was elucidated by spectroscopic and synthetic studies. Pyrrolomycin A has a molecular formula $C_4H_2N_2O_2Cl_2$. its structure was

Table 7. The antimicrobial spectra of clazamycins

Test organisms	Clazamycin MIC	
	A	B
Staphylococcus aureus	100	100
S. aureus Smith	100	100
Micrococcus flavus FDA 16	25	25
Sarcina lutea PCI1001	100	100
Bacillus anthracis	6.25	12.5
B. subtilis PCI219	100	100
B. subtilis NRRL B-558	100	100
*Corynebacterium bovis*1810	100	100
Escherichia coli NIHJ	100	100
E. coli K-12	50	50
E. coli ML 1629	50	50
E. coli ML 1410	50	50
E. coli ML 1410 R81	50	50
E. coli W677	50	50
E. coli JR66/W677	50	50
Klebsiella pneumoniae PC1602	25	50
K. pneumonie 22#3038	50	100
Shigella dysenteriae JS11910	25	25
S. flexneri 4b JS11811	12.5	25
S. sonnei JS11746	25	25
Salmonella typhi T-63	25	12.5
S. enteritidis 1891	25	50
Proteus vulgaris OX 19	25	25
Pseudomonas aeruginosa A3	25	12.5
P. aeruginosa No. 12	100	100
P. aeruginosa TI-13	50	50
*P. aeruginosa GN*315	50	25
P. aeruginosa K-Ps102	25	50
P. maltophilia GN907	50	50

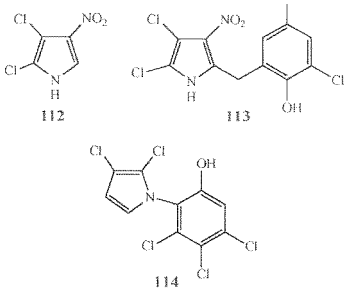
determined to be 2,3-dichloro-4-nitropyrrole by spectroscopic analysis and synthetic studies. Structure-activity relationships among the analogs of pyrrolomycin A were also discussed.

The structure of pyrrolomycin B was examined by spectroscopic means and X-ray crystallographic analysis [135].

The isolation of a new antibiotic, neopyrrolomycin (114), which inhibits the growth of Gram-positive bacteria, Gram-negative bacteria and some fungi, from cultured broth of streptomycete, was described [136]. The production, isolation, physico-chemical properties, structure and biological properties of neopyrrolomycin were elucidated. The strain was isolated from a soil sample collected at Bunkyo-ku, Tokyo, Japan and classified as *Streptomyces sp.* M1424-38F1.

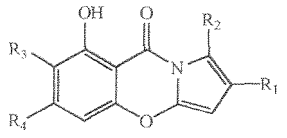
Table 8. MIC values of A and B pyrrolomycins

Test organisms	pyrrolomycin MIC (μg/ml)	
	A	B
Staphylococcus aureus	3 13	0.20
S epidermidis	6.25	0.10
Streptococcus faecalis	6.25	0 39
Bacillus anthracis	1 56	0.10
Escherichia coli	6.25	12.5
Citrobacter freundii	6.25	12.5
Salmonella typhi	3.13	12 5
Shigella sonnei	6.25	12.5
Klebsiella pneumoniae	12.5	12.5
Proteus vulgaris	6.25	12.5
morganii	6.25	*12.5*
P. mirabilis	6.25	12.5
Serratia marcescens	6.25	25
Pseudomonas aeruginosa	12.5	12.5
Candida albicans	100	*100*
Trichophyton mentagrophytes	6.25	*100*
T. interdigitale	3.13	100
Aspergillus fumigatus	25	100



A series of halogenated pyrrolo [2,1-b] [1,3] benzoxazines (**115** to **123**) was isolated [137] from fermentations of an actinomycete strain X10/78/978 (NCIMB40808), identified as *S. rimosus*, during a microbial extract screening programme to identify inhibitor; of bacterial histidine kinase. The structures of these compounds were elucidated by 2D NMR experiments. The structure of one streptopyrrole **(115)** was confirmed by X-ray crystallographic studies. Compounds (119) and **(120)** were produced in fermentations in the presence of NaBr anti NaI, respectively. The most abundant member of the series, streptopyrrole (**115**), inhibited the nitrogen regulator II histidine kinase from

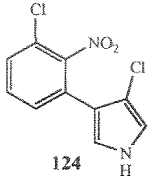
Escherichia coli with an IC_{50} of 20 μM and exhibited antimicrobial activity against a range of bacteria and fungi.



115 - 123

	R_1	R_2	R_3	R_4
115	Cl	H	Pr	OH
116	Cl		Et	OH
117	Cl	Cl	Pr	OH
118	Cl	H	Pr	OMe
119	Br	H	Pr	OH
120	Cl	Cl	Et	OH
121	Cl	H	Bu	OH
122	Cl	H	Et	OMe
123	Cl	Cl	Pr	OMe

The pyrrolnitrin **(124)**, an antifungal antibiotic isolated from *S. pyrrocinia* was tested [138] against *Mycobacterium tuberculosis* and *M. avium*. Synthetic studies well correlate antimycobacterial potency with the presence of halogen and nitro group in the phenyl ring and chloro group in pyrrole.



124

10. Chloramphenicol

Cultivation of soil from Venezuela in 1947 resulted in the isolation of a streptomycete strain that produced an antibiotic with a broad antimicrobial spectrum 1\39. 140]. The antibiotic was chloramphenicol **(125)**, and the organism that produced it was *S. venezuelae* [141, 142].

S. venezuelae readily develops on common nutrient media. Its colonies are usually colorless; sometimes the underlying side is yellowish brown or brown (depending on the composition of the medium). Best yields of the antibiotic arc obtained with submerged cultivation of *S. veneauelae* at *27-28* °C on media containing peptone, glycerol, and sodium chloride. The antibiotic can also be

formed by cultivating tlic streptomycete on synthetic media contaiming seiine as the source of nitrogen, glycerol, and sodium lactate as the source of carbon Ions of iron, zinc, and magnesium are necessary foi the biosynthesis of the antibiotic

The yield of chloramphenicol increases if *p*-nitrophenylserine oi phenylalanine [143] arc added to the synthetic medium The stimulating effect is the highest if phenylalanine is added 10 the medium before the growth of the streptomycete enters the active phase Some amino acids such as leucine isoleucine, methionine. tryptophan glutamic acid, norvaline, and threonine also have a certain stimulating effect orl the biosynthesis of tlic antibiotic

Chloramphenicol was produced [144] by cultures of *Streptomyces* species 3022a supplemented with sodium [1,2-^{13}C] It was labeled with ^{13}C exclusively in the dichloromethine (2 6 ± 0 1%) and carbonyl (0 59 ± 0 05%) carbon atoms Since [2,3-^{13}C] succinic acid enriched only the carbonyl carbon atom of chloramphenicol, these results suggest that neither acetate oi a Krebs cycle intermediate is a direct precursoi of the dichloroacetyl group Cultures supplemented with [2-^{3}H]- or [^{2}H2]-dichloroacetic acid incorporated negligible amounts of isotope into the antibiotic on the basis of this evidence it was concluded that the free acid is not an intermediate in chloramphenicol biosynthesis and that the acylation step may precede chlorination

When bromide ion was added to the fermentation to antagonize chloramphenicol synthesis, bioactivity was depressed, but chromatography revealed the appearance of three unidentified antibiotic activities. These compounds were characterized as tlic 2-butyramido-, 2-bromochloroacetamido-, and 2-dibromoacetamido- derivatives of D-*threo*-*p*-nitrophenyl-1,3-propanediol. The effects of limiting chloride ion concentrations and various levels of bromide. iodide, and fluoride ions on tlic chloramphenicol fermentation were investigated and the results were reported [145]

I he biosynthesis of chloramphenicol and metabolism of the streptomycete during its development were studied and it was shown that S *venezuelae* grows in two stages By the 72nd hour of cultivation. the weight of the mycelium attains its maximum; then the lysis of the mycelium prevails over its growth and the total weight of the mycelium slowly decreases (to 60-70 per cent) In 73 hours of cultivation, glycerol is no longer consumed by the streptomycete.

Chloramphenicol is produced by S. *venezuelae via* the following pathway: chorismic acid → *p*-aminophenylalanine → *p*-aminophenylserine → α-N-dichloroacetyl- *p*-aminophenylserine → chloramphenicol. Chloramphenicol biosynthesis genes form a tight cluster on the chromosome of S. *venezuelae* ATCC 10712 [146].

During the growth of S. *venezuelae*. the main quantity of chloramphenicol is liberated into the medium and only its small part remains in the mycelium. Only 0.6 μg of the antibiotic can be extracted from a 36-hour old mycelium contained in 100 ml of the culture, while the filtrate contains 4000 μg of chloramphenicol.

If the streptomycete is cultivated in the medium free from the chloride ions. analogues of chloramphenicol are produced rather than chloramphenicol. The dichloroacetyl group is replaced with the residues of acetic, propionic, butyric or valeric acid.

The chloramphenicol producing [147] streptomycete modifies the primary C-3 hydroxyl of the antibiotic by a novel chloramphenicol-inactivating enzyme, chloramphenicol 3-*O*-phosphotransferase. The quaternary structure has ATP binding pockets and suggests that an aspartate acts as a general base to accept a proton from the 3-hydroxyl of chloramphenicol, concurrent with nucleophilic attack of the resulting oxyanion on the gamma-phosphate of ATP. The gene disruption results suggest that *S. venezuelae* may have a third set of genes encoding *p*-aminobenzoic acid synthase [148].

The chemical structure of chloramphenicol was studied and its structural formula and total synthesis were established [149, 150]. Its structure is simple: D-*threo*-1-(*p*-nitrophenol)-2-dichloracetamido-1,3-propanediol. This was the first example of chemical synthesis of antibiotics on an industrial scale. Now, the antibiotic chloramphenicol is produced only by chemical synthesis. However, the synthesized product is racemic containing the L-*threo* and D-*threo* forms of chloramphenicol.

The two forms can be separated to the L-*threo* and D-*threo* isomers using appropriate techniques. The D-*threo* isomer of chloramphenicol was given the name levomycetin.

Chloramphenicol has a bacteriostatic effect on marly Gram-positive and Gram-negative bacteria. It inhibits the growth of bacteria of the genera *Aerobacter, Staphylococcus, Streptococcus, Diplococcus, Proteus, Bacillus, Vibrio, etc.* It also inhibits rickettsia. those causing louse-borne typhus included, and some large viruses (causing trachoma, veneral lymphogranuloma, atypical pneumonia, and sonic other diseases). The antibiotic is a specific drug for the treatment of typhoid and paratyphoid fevers, dysentery, brucellosis, toxic dyspepsia, trachoma, and other diseases. Chloramphenicol is very effective against bacillary dysentery in children. When given *per os*, it is well absorbed and is carried by blood to tissue and tissue fluids.

Sonic microorganisms develop resistance to chloramphenicol but very slowly, and thus the resistance car? practically be disregarded. Chloramphenicol inhibits bacteria stable to penicillin and streptomycin.

The easy availability of chloramphenicol **(125)** produced synthetically [151] enabled the study of' structure -- function relationships. in general, none of the obtained chloramphenicol derivates is more active against bacteria than chloramphenicol itself; in fact, most of them are less active or completely inactive.

I structure function relationship can be summarized as follows:

1. I ire nitro group is riot essential for the biological activity.

2. Substitution of phenyl group by other aromatic or heterocyclic residues always leads to a decreased biological activity.
3. In tlic amide side chain, nitrogen with single hydrogen is essential. Substitution of this hydrogen by methyl group results in a complete loss of the biological activity. An acyl residue bound in this position affects the biological activity depending on its volume and electronegativity. The dibromacetyl analog exhibits about X0 % activity as compared with chloramphenicol.
4. Esterification of the primary alcohol group yields compounds that are inactive or little active *in vitro*.
5. Steric configuration of the side chain is absolutely essential for tlic biological activity. Only the D-*threo* isomer **(125)** exhibits a high antibacterial activity. The other three optically active forms (126 - **128)** are only little active or fully inactive. If the activity of the D-*threo* form is set 100, the L-(+)-*erythro* form and the D-*threo* isomer exhibit the activity index of < 0.4 and that of the L-(+)-*erythro* form is 1-2. The fact that the D-*threo* form and the L-*erythro* isomer have the same configuration on the asymmetric carbon with the secondarily bound alcohol group indicates that the spatial arrangement of substituents on this atom is more important than that on the second asymmetric carbon atom. The changed configuration on tlic former atom leads to a complete loss of tlic biological activity, whereas the changed spatial configuration on the second asymmetric carbon atom decreases the biological activity 60- 100-times but does not abolish it completely.

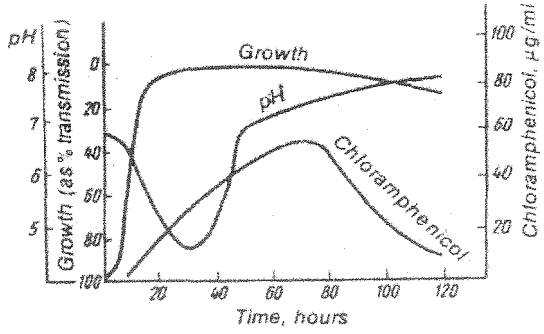
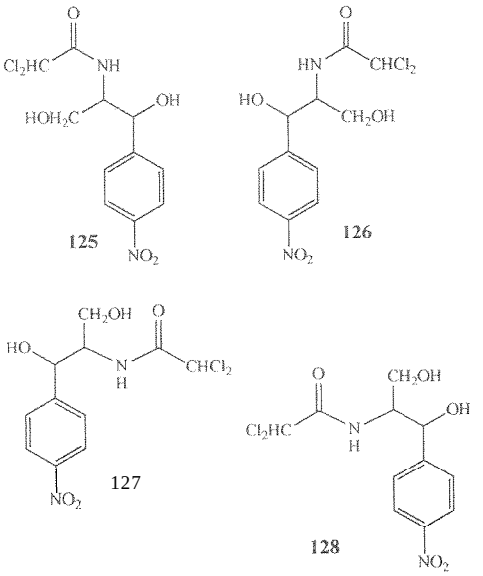


Figure 1. Production of chloramphenicol

II. Tetracyclines

The group of antibiotics of the tetracycline series includes substances of similar chemical structure. The tetracyclines have interested many investigators

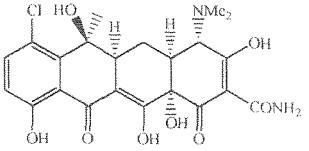


[152-157] because of their great practical importance. Owing to the broad antibiotic activity of the tetracyclines (they are effective against Gram-positive and Gram-negative bacteria, rickettsia, and some large viruses) these compounds are widely used in medicine. Moreover, some antibiotics are also used in animal husbandry as growth factors. The tetracyclines are also valuable for their relatively low toxicity.

Tetracyclines are produced mainly by microorganisms *S. aureofaciens, S. rimosus,* and some others, see below. They include not less than 100 compounds, only sonic important compounds are quoted (129 - **161**). Furthermore, several thousands tetracyclines were produced semisynthetically. Some of them are used in human and veterinary medicine.

Tetracycline antibiotics constitute a group of highly similar compounds with a partially hydrogenated naphtacene ring. They belong to the most commonly used wide spectrum antibiotics.

Tetracycline antibiotics described so far are structurally related. As a result, they also exhibit a highly similar antibacterial spectrum, crossed resistance, similar mechanisms of resistance development *etc.* The tetracycline ring consists of four linearly annealed six-membered rings forming 1,4,4a,5,5a,6,11,12a-octahydronaphtacene with a characteristic arrangement of double bonds. The D ring has an aromatic structure; the remaining three cycles are hydroaromatic. Each ring contains at least one hydroxyl group, phenolic hydroxyl groups being



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responsible for the acidic character of these compounds. Among acidic hydroxylic groups in positions 3, 10, 12, and neighboring carbonyl groups there are strong intramolecular exchange effects resulting in a highly decreased reactivity of these groups with typical chemical agents. On the other hand, there is a basic dimethylamino group in position 4 of the A ring and an amide group in position 2 of the same ring.

Tetracycline antibiotics are thus amphoteric compounds forming salts with both acids and bases. Their salts with inorganic acids (hydrochlorides, phosphates) are most commonly used in practice. Ions of divalent or trivalent metals (*e.g.* iron, aluminum, copper., nickel, magnesium, manganese, calcium) antagonize the effect of tetracycline antibiotics by forming biologically inactive chelates. With a corresponding substitution, carbon atoms 4,4a,5,5a,6 and 12a are asymmetric. Tetracyclines are yellow, crystalline, bitter. light sensitive compounds.

More than thirty years ago. *i.e.* before the vigorous boom of genetics. our colleagues published a fundamental paper [158] in the field of chlortetracycline biosynthesis and biosynthesis of other antibiotics of the cycline group. In the above review, the complete biosynthesis of tetracyclines was described in three chapters. Tetracycline biosynthesis begins with glucose and proceeds *via* acetyl-CoA, a tricyclic nonaketide and pretetraamide (methylpretetraamide) all the way to many tetracyclines, of which more than thirty contain chlorine atom in their molecule.

First discovered was 7-chlorotetracycline and we shall start our discussion of the tetracyclines with this substance.

A new actinomycete *S. aureofaciens* was isolated from the Missouri soil in 1948. This streptomycete produces aureomycin. Aureomycin owes its name to the species name of the streptomycete producing this antibiotic, *aureofaciens*, and also to the golden color of its crystals. Now to this antibiotic is given the name chlortetracycline. The names of the commercial products are *e.g.* biomycin, aureomycin, and duomycin. *S. aureofaciens* is an aerobic organism growing well in liquid media or on solid agar at 26-18 °C. This microorganism also produces vitamin B_{12} (up to 0.5-0.7 μg/ml), tetracycline, and also some other antibiotics. Sucrose, maltose, glucose, starch, and other water-soluble carbohydrates miry be used as carbon sources in media for the production of chlortetracycline.

It was recommended that potato wastes (starch production wastes) hydrolyzed for an hour may be used as the source of carbon in the manufacture of fodder chlortetracycline. Glucose is the best source of carbon for the cultivation of ihc streptomycete and for the biosynthesis of chlortetracycline. It is completely utilized by the microorganism within 40-45 hours of its growth. Fats arid sonic organic acids (*e.g.* lactic acid) arc also used as the source of carbon for cultivation of *S. aureofaciens*. Their addition to corn-steep liquor intensifies the biosynthesis of chlortetracycline. The date-seed lipids from date palm (*Phoenix dactylifera*) were used [159] as carbon source in the fermentation medium for production of tetracycline arid chlortetracycline. The high titers of' both compounds were obtained when date-seed lipid was at 30 mg/ml. Date-seed lipids were more efficient than dextrin when used together as a carbon source. Starch anti glycerol arc also used, while lactose, sucrose, galactose, and arabinose are not assimilated by some strains of the streptomycete.

Ammonium salts, some amino acids, and also various nitrogen compounds of plant and animal origin (corn-steep liquor, meat extract, *etc.*) arc used (Table 9) ins the source of nitrogen to stimulate the growth of the streptomycete and to intensify the biosynthesis of the antibiotic. The amount of nitrogen is 4.0 mg/ml and higher in media containing 50 mg/ml glucose and exhibits a toxic effect on the streptomycete.

Table 9. The medium normally used for the manufacture of chlortetracycline (in percentage, water up to 100 %).

Sum	30
Corn-steep liquor	90
$CaCO_3$	1.0
$(NH_4)_2SO_4$	0.2
NH_4Cl	0.1

The best initial pH of the medium is 6.0-7.0. The time of cultivation of the streptomycete, during which the maximum quantity of the antibiotic is produced, is 30-40 hours. If *S. aureofaciens* is cultivated on a medium containing soy meal, corn-steep liquor, and carbohydrates, the synthesis of chlortetracycline is stimulated by some amino acids. Mainly valine, oxyproline, and tyrosine have the highest stimulating effect on biosynthesis of the antibiotic.

The yield of the antibiotic also depends on the age of the inoculum. Biosynthesis of chlortetracycline decreases significantly if the mycelium used for inoculation is older than 30 hours. The optimum age of the mycelium used for inoculation varies from 24 to 30 hours. The amount of the inoculum ensuring the effective biosynthesis of chlortetracycline is 5-6 % (v/v).

Phosphorus, which is important for the growth and development of the streptomycete, is also very important for the biosynthesis of chlortetracycline. If potassium phosphate K_2HPO_4 is added to the medium, the metabolism of the streptomycete is altered significantly to intensify assimilation of sugar and ammonium, to decrease pH of the substrate, and to promote accumulation of pyruvic acid in the medium. The growth of the mycelium is first accelerated but after the ammonium has all been consumed, its growth is slowed down. The biosynthesis of the antibiotic is inhibited, the degree of this inhibition being much higher than that of the mycelium growth.

Excess phosphorus in the medium produces a certain effect on the nucleic acid content of the mycelium and inhibits oxidation of pyruvic acid, which accumulates in the culture fluid.

A *S. aureofaciens* strain was selected [160] based on the ability to produce chlortetracycline in long-term cultures with phosphate concentrations of 70-110 mg/ml. The strain was morphologically and biochemicaly characterized. The production cultivation used a fermentor providing medium aeration, phosphate levels 100-110 mg/ml, and ammonia levels decreased by 35-50% of common concentrations used. The cultivation was run at pH 5.5-6.0 and 28-29 °C for 176-183 h.

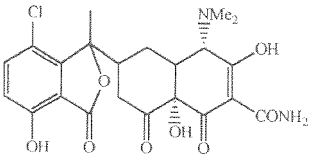
Cloning of the gene cluster of the chlortetracycline biosynthetic pathways of *S. aureofaciens* and its uses have also been described [161].

Chlorine is very important for the production of chlortetracycline. Mineral salts (NaCl, $MgCl_2$, and others) are normally used as sources of chlorine, but chlorine of organic compounds, can also be used by the microorganisms to synthesize chlortetracycline. Changes in the chlorine content of the medium have a significant effect on the biosynthesis of chlortetracycline. The increase of NaCl concentration from, *e.g.* 0 to 0.4 % intensifies the production of the antibiotic by the culture of *S. aureofaciens* LSB-2201 from 2800 to 6000 μg/ml.

Aeration of the culture is also very important for the biosynthesis of chlortetracycline. When the culture is grown in submerged conditions, continuous aeration is required to ensure the high yield of chlortetracycline (to 5000 μg/ml). Discontinuation of aeration for short periods decreases sharply the biosynthesis of the antibiotic. The culture is most sensitive to interruptions in aeration from the sixth to the twelfth hour of cultivation. A ten-minute interruption in aeration during this period of the streptomycete growth halves the yield of tetracycline (as compared with control). However, adding benzyl thiocyanate (2.5×10^{-5} M) removes this harmful effect on the biosynthesis of chlortetracycline. Impaired aeration of the culture and the increase in the concentration of the mineral source of phosphorus promote the production of isochlortetracycline (130) at the expense of chlortetracycline.

Chlortetracycline is isolated from the culture fluid (after its separation from the mycelium) by extraction, precipitation, or adsorption. Chlortetracycline is

poorly soluble in common organic solvents, but is soluble in water (0.55 mg/ml at 25 °C) arid insoluble in ether. The chemical structure of chlortetracycline was established in the fifties [162].



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Chemical and X-ray investigations had hitherto led to establishment of the relative configuration of naturally occurring tetracyclines [163]. it was found that formula [164] also expresses the absolute configuration of all these antibiotics. The crystal structure of chlortetracycline hydrochloride has also been achieved with the aid of X-ray data [164].

The antibiotic activity of chlortetracycline is the highest in an acid medium (pH 3.5-4). The antibiotic is rapidly destroyed in an alkaline medium and its antimicrobial properties are thus markedly decreased. At pH 14.0, chlortetracycline is inactivated by 50 % in 40 seconds, while at pH 7.6, in 12 hours. The stability of chlortetracycline depends on the salt in the form of which it is produced as a medicinal preparation. Chlortetracycline hydrochloride can be stored in sealed vials for 36 months (at 20-24 °C). if this salt is produced in the form of tablets, the stability increases to 48 months.

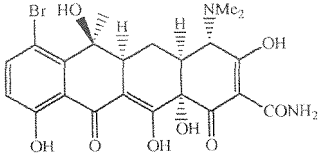
Chlortetracycline is effective in most cases as a bacteriostatic preparation. It follows therefore that if the antibiotic is present for a long time in the medium together with sensitive microbes the main mass of the bacteria begins to increase again after the preparation has been decomposed or after the microbial cells have been separated from the antibiotic. The antibiotic is bacteriostatic only at certain concentrations. When the concentration of chlortetracycline increases, it can produce a bactericidal effect.

Microorganisms sensitive to chlortetracycline develop resistance to it, but this resistance develops slowly, much slower than, for example, to streptomycin. The excessive use of chlortetracycline in medicine increases the formation of microbes ı-existant to it. Chlortetracycline should preferably be used to treat diseases whose causative agents are stable against penicillin or streptomycin. Chlortetracycline is successfully used to treat bacterial pneumonia, brucellosis, tularemia, pertussis, scarlet fever, anthrax, and other bacterial diseases. Moreover, chlortetracycline can be used to treat various forms of louse-borne typhus, various rickettsioses, and also some viral diseases (trachoma, lymphogranuloma).

Chlortetracycline is given *per* or in equal doses 3 or 4 times a day. Each dose of the antibiotic should not exceed 500 mg. *i.e.* the daily dose should he 1.5-2 g. No serious complications or side reactions due to toxicity of chlortetracycline occur. Some patients, however. complain of intestinal pain. diarrhea, and vomiting.

Severe affections of liver after reception of large doses of chlortetracycline or other antibiotics of this group were recently reported. Chlortetracycline is sometimes used in agriculture. Small portions of this antibiotic added to fodder for poultry, calves, lambs, pigs. *etc.* intensify their growth. Tetracyclines arc also the antibiotic of choice to prevent or to treat bacterial infections in USA fish farms. The quantities consumed are stunning - a "light user" of tetracyclines will apply 8 kg in a single dose, whereas a "heavy user" will typically apply 168 kg of antibiotic in a single prophylactic dosage. In Germany, oxytetracycline and chlortetracycline are the only antibiotics licensed for use in aquaculture.

The discovery of a new antibiotic of the tetracycline nature, bromotetracycline **(131)**, was reported in 1955. The bromotetracycline was produced [165] by culturing a bromotetracycline strain of S. *aureofaciens* RO 1441. The aqueous medium contained from 3 to 10 % of a carbohydrate, 0.1 to 1.5 % $(NH_4)_2SO_4$, 0.2 to 0.9 % $CaCO_3$, 0.02 to 0.5 % of an alkaline metal or alkaline earth metal bromide and 1 to 3 % of a special malt extract as sole organic nitrogen source. The pH was 6.5 initially: the temperature 27-31 °C; the duration of cultivation was up to 160 hours and was preferably under submerged aerobic conditions.



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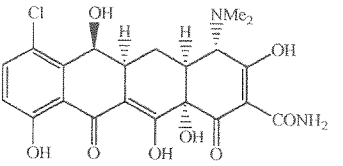
The study of the antibacterial propel-tics of bromotetracycline showed that *in vitro* the effect of the new antibiotic was very similar to that produced by the other tetracyclines. Bromotetracycline differs from chlortetracycline by higher activity against some microorganisms, *e.g.* against *Sarcina lutea* (twice as active), *Proteus vulgaris* (2.5 times), and *Bacillus cereus* (6 times more active than chlortetracycline).

Demethylchlorotetracycline **(132)** was isolated and identified [166] in 1957. This antibiotic is produced by the mutant strain of *S. aureofaciens* with upset mechanism of methyl group transfer. However. demethyl derivatives of chlortetracycline can also be produced by the common strain of *S. aureofaciens*.

'Po that end antimetabolites of methionine, the main donor of methyl groups, should be added to the medium for cultivation of the streptomycete. Ethionine, D-norleucine, and D-methionine may be used for the purpose. The demethyl derivatives differ from chlortetracycline or bromotetracycline by tire absence of the methyl group in the sixth position of the main tetracycline molecule. Demethyltetracyclines arc more stable to acids and alkali than their methyl homologues. The antimicrobial activity *in vitro* of the tetracycline antibiotics used in medicine can be shown by relating the activity of some of them against *Staphylococcus aureus*.



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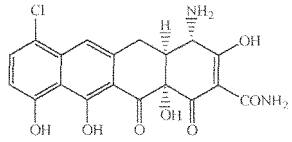
In a further study [169] production of high levels of tetracycline derivatives was observed in genetically altered *Streptomyces* strains. in particular, the production of demethyltetracycline and/or demethylchlortetracycline by strains of *S. aureofaciens* in which the CTC *03*. CTC 09, and/or CTC 11 genes are altered was described. CTC *03* encodes an enzyme responsible for the chlorination of the C-7 position of tetracycline, CTC 09 encodes an enzyme responsible for the methylation of the C-6 position, and CTC 11 encodes a putative transcription activator. The recombinant *S. aureofaciens* cells comprise at least one CTC 11 gene, and optionally a CTC 09 gene, a CTC *03* gene or a combination thereof. Vectors for allelic replacement in a S. *aureofaciens* host cell were also described comprising: a functional Escherichia coli origin of replication, a functional *Streptomyces* origin of replication, a functional gene that imparts a positive selectable phenotype on the host cell and a ribosomal *S12* gene which is expressed in *Streptomyces* such that it imparts sensitivity to streptomycin to the host cell.

Strains that produce demethylchlortetracycline also produce a melanin-like pigment [170]. The correlation between melanin-like pigment production and demethylchlortetracycline biosynthesis was investigated by using *S. aureofaciens* NRRL 3203. Ten chlortetracycline-producing recombinants were derived from demethylchlortetracycline producing mutant NRRL 3203 by gene replacement. Further examination showed that melanin-like pigment might be synthesized *via* the demethylchlortetracycline biosynthetic pathway; mutants defective in demethylchlortetracycline biosynthesis were derived from a demethylchlortetracycline producer. Production of demethylchlortetracycline

and production of melanin-like pigment were restored simultaneously by integrative transformation with the corresponding demethylchlortetracycline biosynthetic genes, indicating that some of demethylchlortetracycline biosynthetic enzymes are indispensable for melanin-like pigment production

One of the inventions [171] in this field relates to the production of high levels of tetracycline derivatives such as, for example, demethyltetracycline and demethylchlortetracycline by genetically altered *Streptomyces* strains such as *S aureofaciens*

Anhydrodemethylchlortetracycline (133) was successfully isolated [167] from mutant *S aureofaciens* II 13, a derivative of a demethylchlortetracycline-producing parental strain Mutant II 61 I? normally produces an about 10-25 μg/ml of demethylchlortetracycline In that part of the biosynthetic pathway to the tetracyclines, which involves tetracyclic intermediates, blocked mutants have been reported for each step



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In the study [168] of the biosynthesis tetracycline antibiotics, it was observed that the hydrotetracyclines are biologically reconverted to the tetracyclines from which they were derived The conversions were accomplished by the action of the tetracycline-producing species of the genus *Streptomyces* This observation was interpreted to mean that the anhydrotetracyclines were normal biosynthetic intermediates to the tetracyclines, and this concept successfully pointed the way to the identification of yet earlier stages in the biosynthetic pathway

Table 10. Activities of common antibiotics of tetracycline group

Antibiotic	Activity %
Chlorotetracycline	100
Demethylchlorotetracycline	75
Tetracycline	25
Oxytetracycline	24

The tetracyclines have long been used in practical medicine, and microorganisms resistant to them have therefore developed. This has stimulated the investigators to work out synthetic derivatives of natural tetracyclines with improved antimicrobial properties. With the aim of obtaining compounds with

new properties many tetracycline biosynthetic or semisynthetic compounds were prepared, which also provided numerous data on structure function relationships of tetracyclines.

These relationships can be summarized as follows:

1. The antibacterial activity is not substantially influenced by substitution in positions 5,6,7 and 9. Thus for instance hydrogen in position 5 can be substituted by hydroxyl group (oxytetracycline) without any substantial effect on its antimicrobial activity. similarly, chlorine or bromine can he placed in position 7 (chlortetracycline, bromtetracycline). Semisynthetic 9-halogen derivatives arc also highly efficient.
2. Substitutions in positions 2,11a and 12a yield biologically active compounds only if the resulting products can be hydrolyzed to the original compounds inside the or-ganisn-i.
3. Substitution of the dimethylamino group in position 4 by hydrogen, its quarternization or- epimerization resulted in a substantially decreased or fully suppressed biological activity. Epitetracyclines arc formed in aqueous solutions at elevated temperatures and pH 2-6.
4. 5-Epimer, 5a,11a-dehydro- and 5a,6-dehydroanalogs are also biologically inactive. In an acidic environment (pH < 2) tetracyclines with hydroxyl group in position 6 arc transformed; water molecule is cleaved off and the C ring is aromatized resulting in anhydrotetracyclines. Hydroxyl group in position 12a is essential for this conversion.

It follows from the above-mentioned data that the basic skeleton of tetracycline molecules and chromophore keto-enol systems in the rings A and BCD and basic function in the ring A are required for their high biological activity. Configuration on asymmetric centers C-4, C-4a and C-12a is essential, whereas that on C-5, C-5a anti C-6 can vary without a substantial effect on tlic biological activity. Hydrogen of the amide group can be replaced with a methyl group; longer residues have ɜ negative effect with exception of those that arc cleaved off in water. The dimethylamino group can be substituted by a primary amino group.

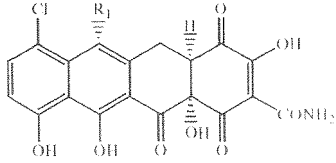
The tetracyclines can be accumulated selectively in tumor tissue. When tumors containing tetracyclines are irradiated. they begin fluoresce. Demethylchlorotetracycline proved to be most effective when given *per os*. while the highest effect in intravenous injection is attained with oxytetracycline.

Tetracyclines belong to the most commonly used antibiotics. In addition to their extensive application in human and veterinary medicine. they are still used. as medicated food additives for domestic animals since the addition of small amounts of tetracyclines to the fodder highly accelerates growth and increases the weight of the animals.

Table 11. The names of some important tetracycline metabolites.

No	Name
134	4-oxoanhydrochlortetracycline
135	methylchlorpretetramid
136	methylhydroxychlorpretetramid
137	chloraureovocidin
138	methylchlortetramid-blue
139	6-demethyl-6-deoxy-7-chloraureovocidin
140	chlorpretetramid
141	chloro-A-C-diquinone
142	chlortetramid-blue
143	chlortetramid-green
144	4-oxoanhydrodemethylchlortetracycline
145	6-demethyl-6-deoxy-7-chlortetramid-green
146	4-oxoanhydrodemethylchlortetracycline
147	4-aminoanhydrodemethylchlor tetracycline
148	4-aminoanhydrochlortetracycline
149	anhydrochlortetracycline
150	dehydrodemethylchlortetracycline
151	dehydrochlortetracycline
152	4-oxodemethylchlortetracycline
153	4-oxochlortetracycline
154	4-oxodehydrochlortetracycline

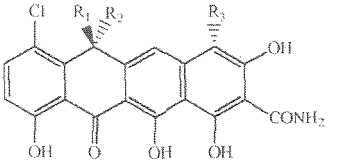
The formulae depicted bellow are only some important intermediates that are produced during biosynthesis ot end products from tetracycline family



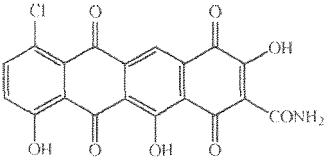
134 R_1 = Me

135 R_1 = H

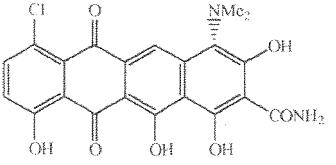
More recently, dactylocyclines A **(155)**, B **(156)**, D **(157)** and I- **(158)** have been isolated from a *Dactylosporangium* strain ATCC 53693 [172-176]; these have the opposite stereochemistry at C-6. Dactylocyclinones *i.e.* 8-methoxy-chlortetracycline (Sch 36969) **(159)**, 2′-*N*-Methyl-8-methoxychlortetracycline



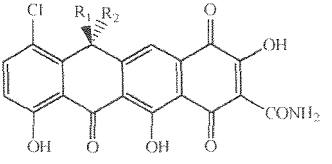
136 $R_1 = H$, $R_2 = Me$, $R_3 = H$
137 $R_1 = OH$, $R_2 = Me$, $R_3 = H$
138 $R_1 = OH$, $R_2 = Me$, $R_3 = OH$
139 $R_1 = OH$, $R_2 = Me$, $R_3 = NMe_2$
140 $R_1 = H$, $R_2 = H$, $R_3 = OH$
141 $R_1 = R_2 = R_3 = H$



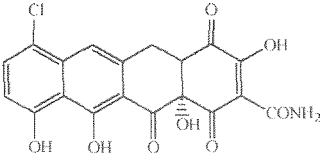
142

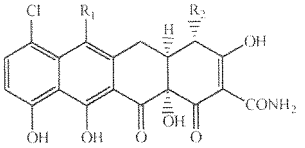


143

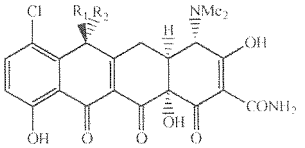


144 $R_1 = OH$, $R_2 = Me$
145 $R_1 = H$, $R_2 = H$

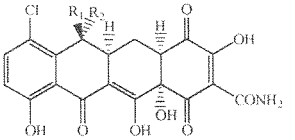




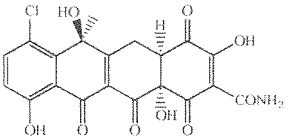
147 R_1 = H, R_2 = NH_2
148 R_1 = Me, R_2 = NH_2
149 R_1 = Me, R_2 = NMe_2



150 R_1 OH R_2 H
151 R_1 OH R hle



152 R_1 OH R_2 H
153 R_1 *Oil* R_2 Me

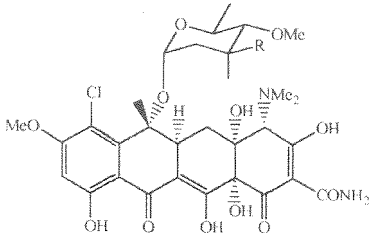


154

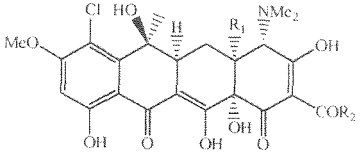
(Sch 33256) **(160)** and 4a-hydroxy-8-methoxychlor tetracycline (Sch 34164) **(161)** are the parent members of the family that are the 6-epimers of X-methoxychlortetracycline i~om *Ictinomadura brunnea* [177 i78] ihe dactylocyclinones contain unusual sugar moieties, with dactylocycline A being the biologically most active member A group of tetracyclines including SF 2575 (162) has been isolated from *Streptomyces* species that have a C-glycosidic linkage at C -9 and a salicyloyl ester at C-4

12. Miscellaneous compounds

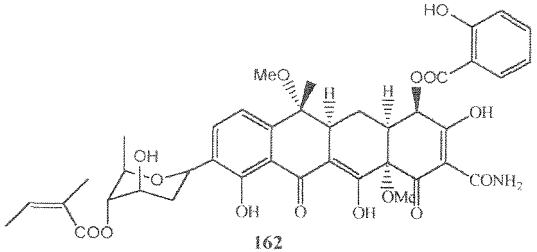
A new crystalline antitumor agent, compound 593A **(163)**, has been discovered [179] in fermentation broths of S. *griseoluteus*. The strain was isolated from soil collected in the area of Richmond, South Africa. Its presence



155 R NHOH
156 R YO,
157 R NHOAc
158 R OH

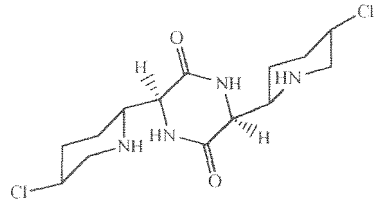


159 R_1 = H, R_2 = NH_2
160 R_1 = H, R_2 = NHMe
161 R_1 = OH, R_2 = NH_2



162

in the fermented broth was detected by the human tumor-egg host system, which was used also to help guide fermentation and isolation studies. KB cell and bacterial test systems provided supplementary guidance for these studies. The compound has been shown to inhibit growth of the 1,1210 lymphocytic leukemia Krebs 2 murine tumor systems, the rat Walker 256 carcinosarcoma, and several neoplastic cell lines. Recent clinical trials have revealed that 593A is effective against certain solid tumors and leukemia. On a weight basis, antitumor compound 593A is very potent against the human adenocarcinoma in the embryonated egg. It also inhibits, in the egg, growth of the human sarcoma and metastasis but not primary growth of the human epidermoid carcinoma.

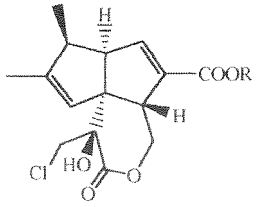


163

An X-ray crystallographic analysis [180] of 593A determined the appropriate structure including its absolute configuration. The compound is a piperazinedione composed of two identical and hitherto unknown amino acids characterized by a unique 3-chloropiperidine ring.

The isolation of a new antitumor antibiotic DC89-A1, which has the molecular formula $C_{26}H_{26}N_3O_8Cl$ from a culture broth of a streptomycete, was described [181]. The producing organism was isolated from a soil collected at Mt. Rokko in Flyogo, Japan, and was taxonomically classified as *Streptomyces sp.* (FERM BP 988) based on both whole cell wall analysis and morphology. The antibacterial activity was detected in both mycelium and extracellular medium. DC89-A1 showed antimicrobial activity. mainly against Gram-positive bacteria. Its structure is still unknown.

As a result of screening cultures of actinomycetes for metabolites having antibacterial properties against Gram-positive and Gram-negative bacteria, including acid-fast bacteria, the acidic lipophilic antibiotics pentalenolactones **(164, 165)** from the fermentation broth of *Streptomyces sp.* strain AA-57 were isolated [182].



164 R = H
165 R = Me

A new antibiotic, ferramido chloromycin (FACM), has been identified [183] in the fermentation broth of *Streptomyces* AS 13 isolated from the soil of Egypt. The antibiotic is a polypeptide containing sulfur, chlorine, and iron. FACM is mainly active against Gram-positive bacteria and fungi. The producing

organism, culture conditions, isolation of the antibiotic as well as its chemical, physical and biological properties have been described but its structure has not yet been elucidated.

13. Halogenating enzymes

In this subchapter, the catalysis of halogenation of organic substrates in streptomycetes will be briefly discussed. Two outstanding chapters on this topic have already been published [2, 184]. *Streptomyces phaeochromogenes*, a chloramphenicol producer, produces a halogen peroxidase, an enzyme that was first discovered in bacteria in 1985. This enzyme catalyzes bromination of organic compounds in the presence of bromine ions and hydrogen peroxide. Subsequently, a high number of bromoperoxidases were described in different *Streptomyces* species. A bromoperoxidase was isolated from S. *phaeochromogenes* catalyzing bromination of pyrrolnitrin in position 2 of the pyrrole ring. *S. aureofaciens*, a tetracycline producer, and *S. griseus*, contain at least two and four bromoperoxidases, respectively. S. *venezuelae* producing chloramphenicol contains a bromoperoxidase, however, this enzyme was found to catalyze chlorination of an acetic acid derivative in chloramphenicol biosynthesis. It follows from the above data that a chlorination enzyme has not yet been described in bacteria but that it is substituted for by bromoperoxidases. Haloperoxidases can be divided into two basic groups according to the presence or absence of prosthetic groups containing metal ions. Heme (protoporphyrin IX) containing enzymes react with hydrogen peroxide giving rise to a hydroperoxide of the enzyme (E) which reacts with a halogen anion (X) yielding the compound EOX. Unfortunately, the mechanism of this reaction has not yet been clarified. The data on substrate and stereospecific and steric specificity have so far been unsatisfactory and no differences between the chemical and enzymatic halogenation could he detected. Non-heme chloroperoxidases have not yet been discovered in bacteria. On the contrary. non-heme haloperoxidases were discovered in *S. lividans*, a bacterium that does not produce any known chlorinated metabolite. On the basis of the hitherto data it can he concluded that no halogenation enzymes could be detected and that thus the whole biosynthesis (introduction of halogen to a molecule of an organic compound) has not been conclusively clarified.

14. Conclusion

It can be concluded that the research of halogenated metabolites can proceed in two directions. The first includes the exploitation of sites from which new streptomycetes can be isolated, *e.g.* marine environment, or locations with an extremely high content of salts (surroundings of industrial plants, salt lakes).

The second direction utilizes methods of molecular biology and genetics for the production of new halogenated metabolites The creation of novel polyketides has been achieved through genetic manipulation of polyketide synthases The modular nature of the Type 1 polyketide synthases allows for domain exchange between different polyketide synthase genes resulting in hybrid genes, which produce polyketide synthases with altered properties that result, in turn in modified macrolide structures Thus, it is possible to control chain length, choice of chain extender unit, degree of 3-carbon oxidation level, and some degree stereochemistry [1Xi]

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