Review

Recent Developments in the Maytansinoid Antitumor Agents

John M. Cassady,[a] Kenneth K. Chan,[a] Heinz G. Floss,*[,b] and Eckhard Leistner[c]

[a] College of Pharmacy, The Ohio State University; 500 West 12th Avenue, Columbus, Ohio 43210, U.S.A.: [b] Department of Chemistry, Box 351700, University of Washington; Seattle, Washington 98195–1700, U.S.A.: and [c] Institut für Pharmazeutische Biologie, Rheinische Wilhelms-Universität; Nussallee 6, D-53115, Bonn, Germany.

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Maytansine and its congeners have been isolated from higher plants, mosses and from an Actinomycete, *Actinosynnema pretiosum*. Many of these compounds are antitumor agents of extraordinary potency, yet phase II clinical trials with maytansine proved disappointing. The chemistry and biology of maytansinoids has been reviewed repeatedly in the late 1970s and early 1980s; the present review covers new developments in this field during the last two decades. These include the use of maytansinoids as "warheads" in tumor-specific antibodies, preliminary metabolism studies, investigations of their biosynthesis at the biochemical and genetic level, and ecological issues related to the occurrence of such typical microbial metabolites in higher plants.

Key words maytansine; ansamitocin; *Actinosynnema pretiosum*; antitumor activity; biosynthesis; tumor-specific antibody

1. Introduction

The report by Kupchan and coworkers in 1972[1] on the bioassay-guided isolation of the potent cytotoxic agent, maytansine (**1**), from the Ethiopian shrub, *Maytenus serrata*, raised high hopes for its eventual use as a chemotherapeutic agent for the treatment of cancer. However, clinical trials with maytansine proved disappointing, showing no significant clinical benefits from its administration to human cancer patients.[2] Nevertheless, because of their extremely high potency, maytansine and its congeners continue to command interest.[3] Structurally, the maytansinoids are ansamycin antibiotics, which are unprecedented as constituents of higher plants but are typical microbial metabolites.[4] The subsequent isolation of maytansinoids, the ansamitocins, from an Actinomycete, *Actinosynnema pretiosum*,[5] is more in line with expectations based on their structures. This raised the question whether the maytansinoids isolated from higher plants are genuinely produced by the plant itself, suggesting a lateral gene transfer, or whether they are the result of an association or symbiosis between a microbial producer and the plant. Again, this is a topic of current interest. In the following article we will review the state of our knowledge on the maytansinoids, emphasizing particularly work carried out in the last twenty years, the time since several comprehensive reviews of the subject have appeared.[6—9] We will also address the prospects of deriving a clinically useful anticancer agent from this family of compounds.

2. Chemistry

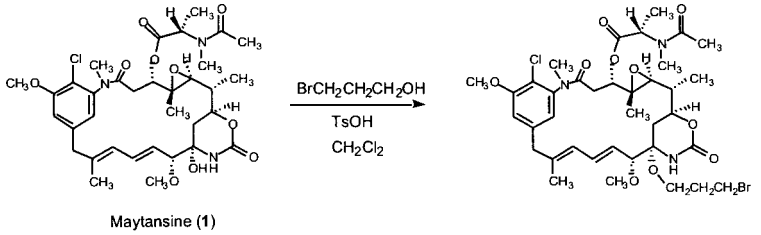
2.1. Maytansinoids from Higher Plants, Mosses and Microorganisms The maytansinoids are members of the ansamycin group of natural products[4] and are characterized by 19-member ansamacrolide structures attached to a chlorinated benzene ring chromophore.

Kupchan and co-workers isolated the unique parent compound maytansine (**1**) in 1972.[1] Its structure and stereochemistry were determined based on an X-ray crystallographic analysis of the 9-(3-bromopropyl) derivative (Fig. 1).

All of the natural maytansinoids of different origins are shown in Tables 1, 2 and 3 based on their sources. The majority of the forty-nine known naturally occurring members of this class were discovered and reported in the decade following the report on maytansine. Since 1984 when the last comprehensive reviews were written,[7—9] only five new members of this group have been reported and these are minor variants of the parent compounds.[10—13]

2.2. Total Synthesis of the Maytansinoids The novel and complex structures of the maytansinoids coupled with their potent cytotoxicity and potential for cancer treatment stimulated great interest in their total synthesis in the decade following their discovery. By 1984, total syntheses of naturally occurring maytansinoids had been reported by three different groups. These synthetic approaches are discussed in detail in reviews published in 1984.[8,9] Meyers and co-workers reported the total synthesis of racemic maytansinol in 1980.[14] The group used an anion intermediate to close the 19-member ring (Fig. 2).

Corey's group at Harvard reported the total synthesis of maytansine at almost the same time.[15] This group prepared an intermediate aldehyde that was similar to the Meyers intermediate. The aldehyde was converted to the desired precursor by a two-carbon chain extension followed by cyclization to the macrocyclic lactam precursor of maytansinol and in turn maytansine (Fig. 3).



Maytansine (1)

Fig. 1. Derivatization of Maytansine for X-Ray Crystallography

* To whom correspondence should be addressed. e-mail: floss@chem.washington.edu

Isobe and Goto's group reported a total synthesis of (±) maytansinol in 1982[16] and a stereocontrolled total synthesis of (−) maytansinol in 1984.[17] (−)-Maytansinol was synthesized stereoselectively from D-mannose. The key intermediate aminoaldehyde used by Corey and Meyer was prepared in optically active form and was converted to the natural product. Other approaches to the maytansinoids have been reported by Petrakis and Fried,[18] by Ho,[19,20] Hodgson and co-workers[21] and by Goodwin *et al*.[22,23]

Confalone and co-workers carried out model studies based on an intramolecular nitrile oxide–olefin [3+2] cycloaddition reaction to give the ansa-macrolactam skeleton[24—26] (Fig. 4).

Recently, a group led by Gao at the Shanghai Institute of Materia Medica reported the synthesis of maytansinol and maytansine from the fragment shown in Fig. 5.[27,28]

The chemistry of the maytansinoids and approaches to their synthesis have been the subject of studies carried out by several groups at the CNRS, Gif-sur-Yvette, France. The early studies by Barton and colleagues led to the synthesis of several potential intermediates to the maytansinoids.[29—31] These studies were continued by Bénéchie and Khuong-Huu and resulted in another total synthesis of (−)-maytansinol in

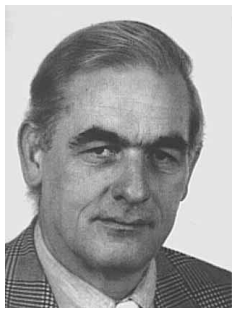
Dr. John M. Cassady is currently Professor of Medicinal Chemistry and Pharmacognosy at the Ohio State University College of Pharmacy, Columbus, Ohio. He received his Ph.D. in Organic Chemistry from Western Reserve University. He served as Dean of the College at Ohio State from 1988—2003. He held appointment as Glenn L. Jenkins Distinguished Professor at Purdue University, received the D. Sc. (Hon.) from DePauw University in 1990, and received the Research Achievement Award in Natural Products Chemistry from the American Pharmaceutical Association in 1990.

John M. Cassady

Heinz G.Floss

A native of Berlin and a naturalized US citizen, Prof. Floss received all his formal education in Germany, studying chemistry at the Technical Universities of Berlin and Munich. He was recruited to Purdue University's School of Pharmacy in 1966, then moved to Ohio State University's Department of Chemistry in 1982 and finally joined the University of Washington Department of Chemistry in 1988. He is now Professor Emeritus of Chemistry, but continues an active research program on the chemistry, biochemistry and molecular biology of natural products biosynthesis. He has published ~400 scientific papers and trained ~175 students, postdocs and scientific visitors.

Kenneth K. Chan



Eckhard Leistner

Dr. Kenneth K. Chan received his B.Sc. degree in Chemistry from California State University at San Jose in 1964, M.Sc. in Organic Chemistry from University of California at Davis in 1968, and Ph.D. in Pharmaceutical Chemistry from University of California at San Francisco in 1972. After a brief period of postdoctoral training at the University of Southern California, he joined the School of Pharmacy at USC as an Assistant Professor and rose to the rank of Professor in 1991. In 1992, he moved to the College of Pharmacy at the Ohio State University as Professor of Pharmaceutics and Internal Medicine. Dr. Chan also served as Director for Preclinical Drug Development and Co-Director of Developmental Therapeutics Program at the Comprehensive Cancer Center from 1992—1999. Dr. Chan was Science Advisor for Food and Drug Administration between 1983—1992 and received a presidential appointment from George Bush to serve as a member of National Cancer Advisory Board from 1991—1996. He is a fellow of American Association of Pharmaceutical Scientists. Dr. Chan's research interests include; pharmacokinetics, metabolite and clinical pharmacokinetics, clinical studies, drug interactions, drug metabolism, drug analytical method development, stable isotope labeling, mass spectrometry, application of stable isotope in pharmacokinetic and drug metabolism research, cancer chemotherapy, drug mechanism of action, and modulation, liposomal drug formulation and development. He published over 105 papers and book chapters and also serves as a journal reviewer and on the editorial board of a journal.

Dr. Eckhard Leistner is Head of the Institut fur Pharmazeutische Biologie of the Universitat Bonn, Germany. He received his Ph.D. under the supervision of Dr. Meinhart H. Zenk at the University of Munich in 1968. Subsequently Dr. Leistner spent two years as a post doctorate fellow with Dr. Ian D. Spenser at McMaster University in Hamilton, Ontario, Canada. He was appointed Associate Professor at the University of Münster in 1975 and eventually moved to Bonn in 1985. His current research is focussed on the biosynthesis of vitamin B6 and ginkgotoxin, the biosynthesis of ansa antibiotics (in collaboration with Dr. Heinz G. Floss) and on microbial endophytes of higher plants. Dr. Leistner is married to Doris Beyer since 1968. They have three sons at the age of 34, 32 and 26 years.

Table 1. Maytansinoids of Plant Origin

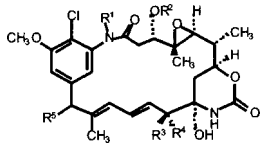
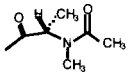
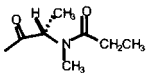
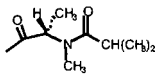
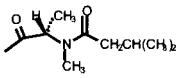
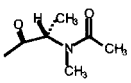
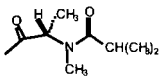
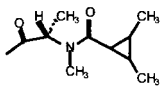
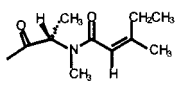
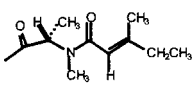
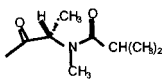
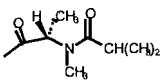
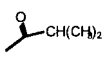
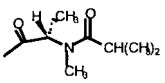
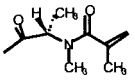
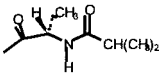
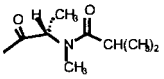
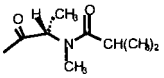
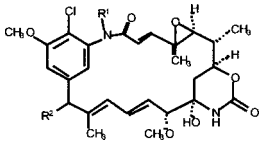
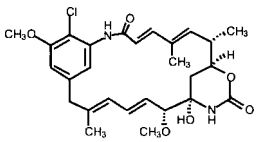
Name	R^1	R^2	R^3	R^4	R^5	Reference
						
Maytansine	CH_3		H	OCH_3	H	1, 7—9
Maytanprine	CH_3		H	OCH_3	H	1, 7—9
Maytanbutine	CH_3		H	OCH_3	H	1, 7—9
Maytanvaline	CH_3		H	OCH_3	H	1, 7—9
Maytanacine	CH_3		H	OCH_3	H	7—9
Maytansinol	CH_3		H	OCH_3	H	7—9
Normaytansine	H		H	OCH_3	H	7—9
2′-*N*-Demethylmaytanbutine	H		H	OCH_3	H	10
Normaytancyprine	H		H	OCH_3	H	7—9
Mallotusine	CH_3		H	OCH_3	H	11
Isomallotusine	CH_3		H	OCH_3	H	11
Colubrinol	CH_3		H	OCH_3	OH	7—9
Colubrinol acetate	CH_3		H	OCH_3		7—9
Maytanbutacine	CH_3		H	OCH_3		7—9
Trewiasine	CH_3		H	OCH_3	OCH_3	7—9

Table 1. (continued)

Name	R^1	R^2	R^3	R^4	R^5	Reference
Dehydrotrewiasine	CH_3		H	OCH_3	OCH_3	7—9
Demethyltrewiasine	CH_3		H	OCH_3	OCH_3	7—9
10-Epitrewiasine	CH_3		OCH_3	H	OCH_3	7—9
Nortrewiasine	H		H	OCH_3	OCH_3	7—9

Name	R^1	R^2	Reference
			
Maysine	CH_3	H	7—9
Trewsine	CH_3	OCH_3	7—9
Normaysine	H	H	7—9

Name	Reference
Maysenine 	7—9

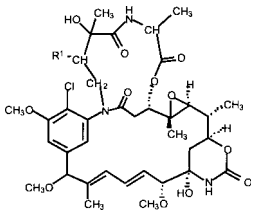
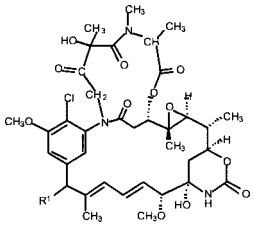
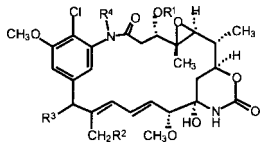
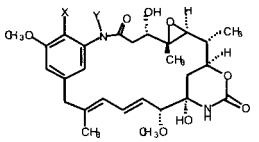
Name	R^1	Reference
		
Treflorine	H	7—9
Trenudine	OH	7—9
		
N-Methyltrenudone	OCH_3	7—9
Maytanbicyclinol	H	10

Table 2. Maytansinoids Isolated from Microorganisms (*Actinosynnema pretiosum*)



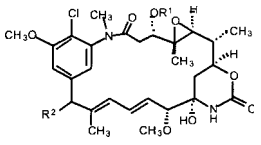
Name	R^1	R^2	R^3	R^4
Ansamitocin P-0 (maytansinol)*	H	H	H	CH_3
Ansamitocin P-1 (maytanacine)*	$COCH_3$	H	H	CH_3
Ansamitocin P-2	$COCH_2CH_3$	H	H	CH_3
Ansamitocin P-3	$COCH(CH_3)_2$	H	H	CH_3
Ansamitocin P-3′	$COCH_2CH_2CH_3$	H	H	CH_3
Ansamitocin P-4	$COCH_2CH(CH_3)_2$	H	H	CH_3
PHO-3	$COCH(CH_3)_2$	H	OH	H
epi-PHO-e	$COCH(CH_3)_2$	H	OH	H
PND-0	H	H	H	H
PND-1	$COCH_3$	H	H	H
PND-2	$COCH_2CH_3$	H	H	H
PND-3	$COCH(CH_3)_2$	H	H	H
PND-4	$COCH_2CH(CH_3)_2$	H	H	H
PHM-1	$COCH_3$	OH	H	CH_3
PHM-2	$COCH_2CH_3$	OH	H	CH_3
PHM-3	$COCH(CH_3)_2$	OH	H	CH_3
PHM-4	$COCH_2CH(CH_3)_2$	OH	H	CH_3
P4-βHY	$COCH_2C(OH)(CH_3)_2$	H	H	CH_3
P4-γHY	$COCH_2CH(CH_2OH)CH_3$	H	H	CH_3
PND-4-βHY	$COCH_2C(OH)(CH_3)_2$	H	H	H

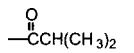
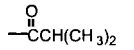
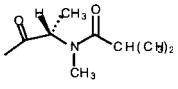
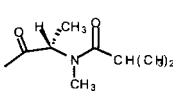


Name	X	Y
deCIQND-0	H	H
QND-0	CI	H
deCIQ-0	H	CH_3

From ref. 9. * Denotes compounds also isolated from plant sources.

Table 3. Maytansinoids Isolated from Mosses



Name	R^1	R^2	Reference
Ansamitocin P-3		H	12, 13
15-Methoxyansamitocin P-3		OCH_3	13
Maytanbutine		H	13
Trewiasine		OCH_3	13

1996.[32] The key steps in this synthesis are outlined in Fig. 6. All of these routes are convergent but still multi-step and do not provide a practical route to the maytansinoids as a source of analogs or clinical agents.

2.3. Routes to Maytansine Analogs by Chemical and Microbial Transformation of Natural Maytansinoids A limited amount of chemistry was carried out in the early studies of the maytansinoids as part of the efforts to establish their structures. These chemical studies were so limited because the compounds were isolated in minute quantities from the higher plant sources.

Some typical reactions that were carried out involved conversion of the C(9)-hydroxyl group (carbinolamide) to ethers. Attempts to hydrolyze the C-3 esters to the corresponding alcohol (*e.g.*, maytansine to maytansinol) led to the production of the corresponding Δ2,3 olefin, maysine. Alcohols corresponding to the 3-maytansinoid esters could be prepared by reaction with lithium aluminum hydride at low temperatures. So for example, maytansine could be converted to maytansinol. Reaction with $LiAlH_4$ at higher temperatures and longer reaction times gave the deschloro analogs. All of

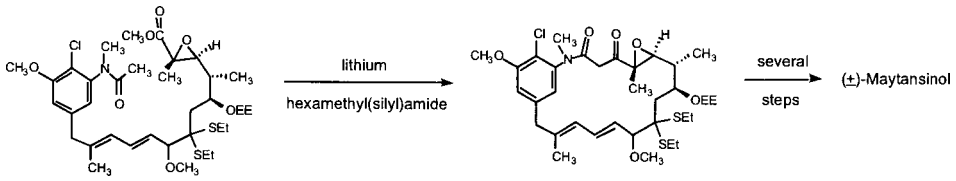


Fig. 2. Synthesis of Racemic Maytansinol by Meyers and Co-workers

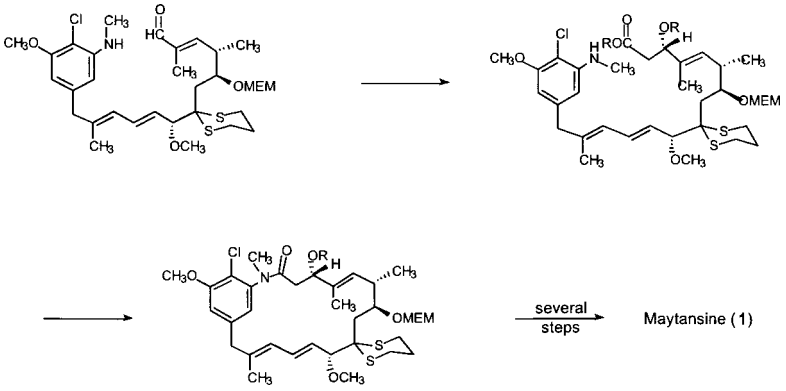


Fig. 3. Corey's Total Synthesis of Maytansine

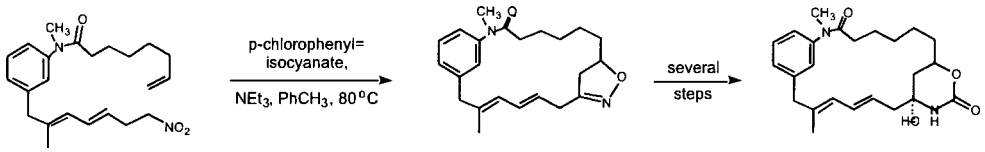


Fig. 4. Confalone's Nitrile Oxide-Olefin [3+2] Cycloaddition Approach to the Ansa-Macrolactam Framework

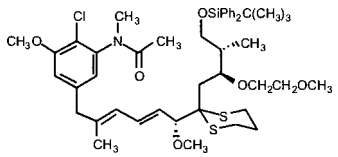


Fig. 5. Starting Material for Gao's Synthesis of Maytansine

this early chemistry was carried out from 1972 to 1984 and has been reviewed.[8,9)]

About five years after the first isolation of maytansinoids from higher plants, scientists at Takeda Chemical Industries in Osaka discovered maytansinoids, the ansamitocins, in a microorganism.[5)] This source produced the compounds in larger quantity and led groups at Takeda to carry out research on the production of maytansinoids[8,9)] and on chemical and microbial modifications to prepare analogs for testing. No attempt will be made to document all of this chemistry in detail as much of it is contained in the patent literature. A series of publications appeared in the literature from about 1980—1984 from the Takeda group.

Izawa and colleagues studied the microbial conversions of ansamitocins.[33—35)] These led to *N*-demethylation, deacylation, 15-hydroxylation and 20-*O*-demethylation. As mentioned previously, the maytansinoid esters can also be converted to maytansinol by reaction with $LiAlH_4$. The conversion products, especially the 20-OH and the 3-OH derivatives were converted into a variety of esters and ethers for testing (Fig. 7).[36—43)] The Takeda group also converted the maytansinoids into 9-thio derivatives by reaction with P_2S_5 (Fig. 8).[44)]

Maytansinol could be converted to the 3-epimaytansinoids by oxidation to 3-maytansinone and reduction with $NaBH_4$.[45)] The Takeda group also converted maytansinol to the 4,5-deoxymaytansinoids.[46)] The 3-epi analogs were inactive, however, the 4,5-deoxy compounds retained activity (Fig. 9).

Sneden has contributed significantly to our understanding of the structures and chemistry of the maytansinoids, first as a member of the Kupchan group, and later with his own group at Virginia Commonwealth. Chromatographic examination of outdated clinical samples supplied to the Sneden group by the NCI established that 40% of the maytansine had decomposed. The major decomposition product was shown to be maysine, which resulted from elimination of the C-3 ester side chain (Fig. 10).[47)] Other minor decomposition products included the C-15 hydroxy- and the 9- and 10-epimaytansines. Sneden also studied the reduction of maytansine using reagents that carried out chemistry at the 3-ester, the carbinolamide and the 11,13-diene functions (Fig. 11).[48—50)]

3. Biology

3.1. Antitumor Activity Since its concentration in the original source plant is very low (2×10^{-5}% of dry weight), maytansine would probably not have been discovered, were it not for its extremely potent cytotoxic activity against human KB cells *in vitro* (ED_{50} 10^{-4}—10^{-5} μg/ml) and strong inhibition of five standard animal tumor systems.[1)] Maytansine also

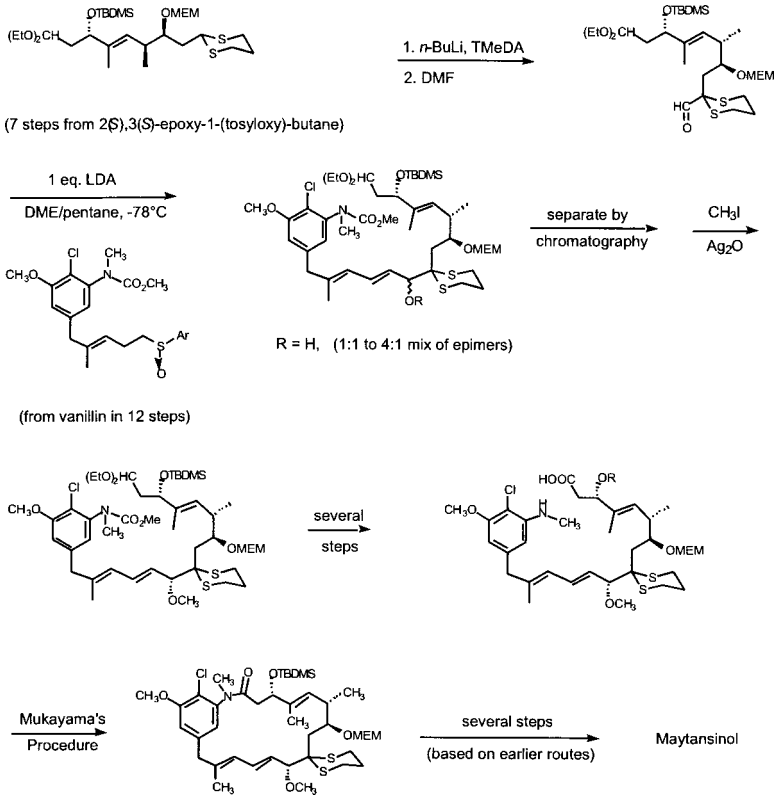


Fig. 6. Gif Route to (−)-Maytansinol

shows significant inhibitory activitiy against the Lewis lung carcinoma and B-16 melanocarcinoma solid murine tumor systems,[51] and antileukemic activity against P-388 lymphocytic leukemia over a 50—100 fold dosage range at a remarkable low dose per kg body weight.[52] More recently (1989), maytansine was tested in NCI's panel of 60 human tumor cell lines[53] and showed patterns of inhibition indicative of tubulin-interactive compounds (G. M. Cragg, personal communication). Closely related compounds, such as maytanprine and maytanbutine, but not maytansinol, showed a similar activity spectrum and potency as maytansine,[52] as did normaytansine.[54]

The 15-oxygenated maytansinoids isolated from *Colubrina texensis*, colubrinol and colubrinol acetate, exhibited activity against P388 lymphocytic leukemia at the microgram per kilogram level and cytotoxicity against KB cells at 10^{-4}—10^{-5} μg/ml.[55] Likewise, a series of maytansinoids isolated by Powell and his colleagues[56,57] from *Trewia nudiflora* L., including the novel compounds trenudine and treflorine, which contain two fused macrocyclic rings, fully retain activity against KB cells and P388 lymphocytic leukemia.[58] *Maytenus buchananii* also contains bicyclic maytansinoids, and maytanbicyclinol was shown to exhibit cytotoxic activity *in vitro* against KB cells and *in vivo* activity against P-388 lymphocytic leukemia.[10]

The bacterial maytansinoids, the ansamitocins, exhibit an activity spectrum and wide effective dose range similar to maytansine. They show antitumor activity against P388 leukemia at daily doses as low as 0.8 μg/kg, with a maximum at 25 μg. Ansamitocin P3 and ansamitocin P4 are also effective against B16 melanoma, sarcoma 180, Ehrlich carcinoma, and P815 mastocytoma but less active against leukemia L1210.[5]

The antitumor activity of the maytansinoids has been covered extensively in earlier review articles.[6—9]

3.2. Structure–Antitumor Activity Relationships Based on the X-ray crystallographic investigation[1] Kupchan reflects on the structural features of maytansine and how they may determine the biological activity[51]:

> "Structural elucidation of maytansine bromopropyl ether revealed that the two longer sides of the 19-membered ring are roughly parallel and separated by about 5.4 Å, so that there is a hole in the center of the ring. The two faces of the ring have a different character, the lower surface, opposite the ester residue, is predominantly hydrophobic, while the upper face is more hydrophilic. Furthermore, the ester residue is oriented in a manner which would hinder sterically the approach of reactants to the hydrophilic face. The ester function in the antileukemic maytansinoids may play a key role in the formation of highly selective molecular complexes with growth-regulatory biological macromolecules. Such molecular complex formation may be crucial for the subsequent selective alkylation of specific nucleophiles by the carbinol amide and epoxide function."

Investigations of the structure–activity relationships revealed that indeed the ester side chain plays an important role. Maytansinol esters, such as maytansine and ansamitocin P3 or P4, are highly active antileukemic agents, whereas compounds carrying a double bond (maysine, normaysine and maysenine) or hydroxy function (maytansinol) at C3 lack antileukemic activity and show only 1/10000 the cytotoxicity of maytansinol esters.[51,59] The importance of the ester group is also evident from the observation that a change from the α to the β configuration at C3 leads to loss of the inhibitory ac-

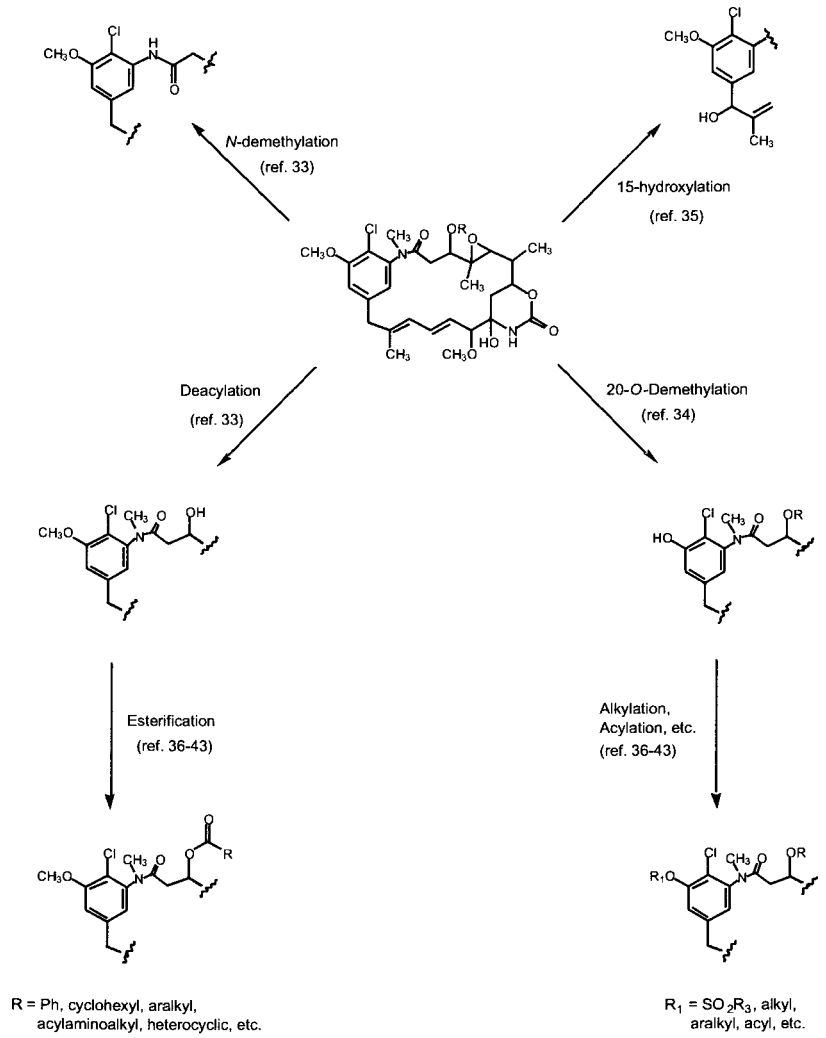


Fig. 7. Microbial Transformations of Ansamitocin and Follow-Up Chemistry Carried Out at Takeda Chemical Industries

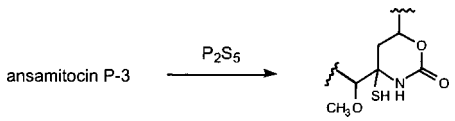


Fig. 8. Conversion of Ansamitocin P-3 into 9-Thio Derivatives

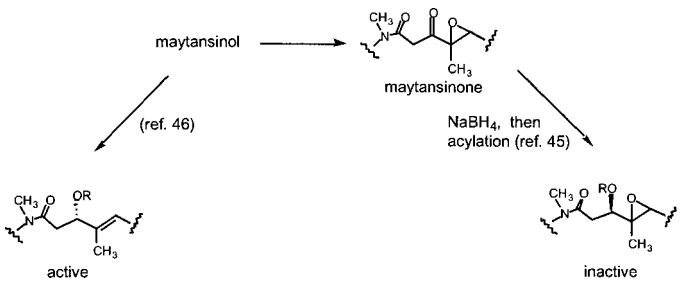


Fig. 9. Conversion of Maytansinol into 3-Epi- and 4,5-Deoxymaytansinoids

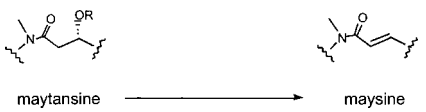


Fig. 10. Decomposition of Maytansine by Side-Chain Elimination to Maysine

tivities.[45] Twenty-nine semisynthetic maytansinoids bearing a variety of C3 acyloxy side chains were prepared synthetically from maytansinol. It was found that a change in the C3 ester group modulated the activity of the compound although almost all of them were active against B16 melanoma in mice.[36] The C3 hexadecanoyl ester and the C3 phenylglycinate both had a low activity in *in vitro* test systems but a dramatic *in vivo* antitumor activity. It was concluded that one cannot predict the degree of *in vivo* activity simply on the basis of the *in vitro* activity. Among the semi-synthetic compounds tested, the C3 phenylglycinate was most remarkable as it showed a two to four times better therapeutic ratio than maytansine itself.[36] These experiments reveal the key importance of the ester moiety for the biological activity of the maytansinoids.

Functional groups at the hydrophobic ("lower") side of the molecule are also important structural features that contribute to the biological activity: The C9 carbinolamide was proposed to function as an alkylating agent intimately involved in antitumor activity. Interestingly, blockage of the carbinol amide by etherification of the C9 alcohol results in a marked decrease in antitumor activity and cytotoxicity against KB cells when compared to maytansine and maytanbutine.[59] Epimerisation at C10, *e.g.*, in trewiasine to give 10-

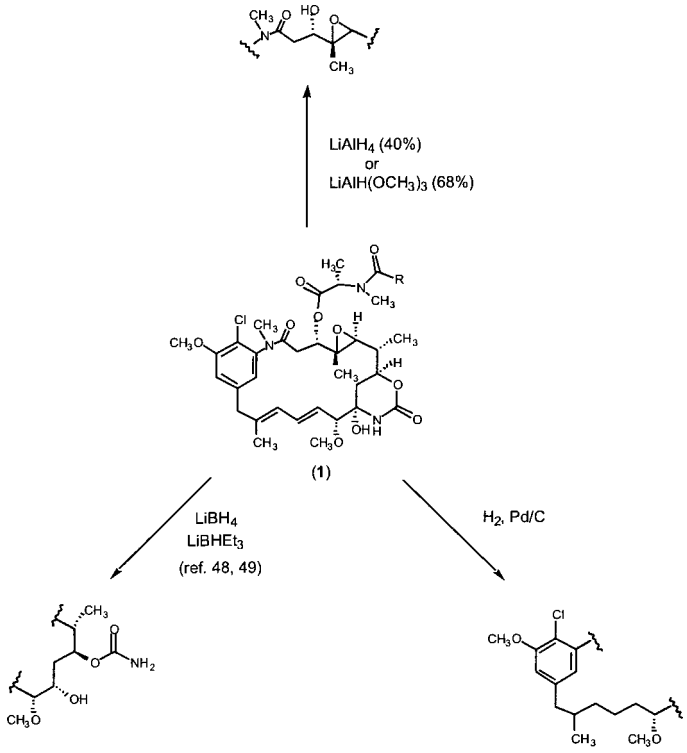


Fig. 11. Reductive Transformations of Maytansine Reported by Sneden and Co-workers

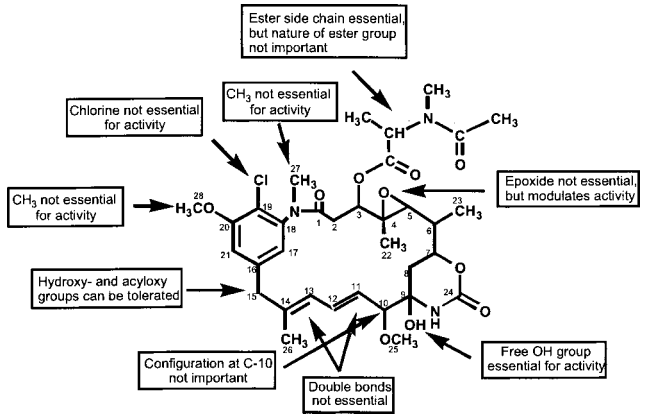


Fig. 12. Structure–Antitumor Activity Relationship of Maytansine

epitrewiasine, has little influence on the biological activity[58] and a hydroxy group at C15, as in colubrinol, is compatible with potent antitumor activity.[35,55]

Neither the amide *N*-methyl group[60] nor the 20-*O*-methyl group[34] seem to be necessary for biological activity; in fact, 20-*O*-demethylansamitocin P3 is more active against P-388 and L-1210 leukemia *in vivo* than AP-3.[35] Surprisingly, the epoxide function also is not absolutely essential for antitumor activity, although it seems to modulate the activity.[45] The structure–activity relationship for the maytansinoid antitumor agents is summarized in Fig. 12.

Rode and co-workers have reported a QESAR study comparing the structures and antitumor activities of the maytansinoids to the quassinoids and cucurbitacins using semiempirical SCF-MO calculations.[61]

3.3. Mode of Antitumor Action Early work established that maytansine[62—64] and the ansamitocins[65] have antimitotic activity due to their binding to tubulin which results in inhibition of microtubule assembly.[62,63] The reaction of maytansinoids with tubulin results in cytological changes in Ascites P388 cells in which chromosomes are scattered at random in the arrested cells in metaphase. A number of cells were enlarged and were observed to be multinucleate. The cytological effects resembled those of vincristine. Almost the same metaphase arrest and alteration of morphology were observed in SN36 cells after a single treatment with ansamitocin P3. Ansamitocins have properties that prevent the polymerisation of tubulin and depolymerize the polymeric tubulin. Lysis of tumor cells was also observed.[65]

Maytansine competitively inhibits vincristine binding with a K_i value of 0.4×10^{-6} M. Binding of maytansine was also competitively inhibited by vincristine, but not by colchicine, with a K_i for vincristine of 1×10^{-5} M.[66] The maytansinoids thus bind to tubulin at a site overlapping the Vinca alkaloid binding site, different from the colchicine binding site. The Vinca alkaloid binding site has been mapped to the tubulin β-subunit,[67] and the maytansinoids also bind to this subunit of tubulin.[68] The structure–activity relationship of maytansinoids for tubulin binding correlates well with their activity against P388 mouse leukemia[69,70]; this and the absence of other primary effects[7,62] suggests that mitotic inhibition represents the main mechanism of antitumor action of the maytansinoids. However, activity against many more recently discovered targets has never been evaluated.

The maytansine binding site on tubulin is also the target of the macrolide rhizoxin isolated from the fungus *Rhizopus chinensis*.[71,72] β-Tubulins from *Aspergillus nidulans* and several yeasts which are sensitive to rhizoxin contain an asparagine residue in position 100, whereas those from resistant strains carry an Asn100Ile or –Val mutation. Site directed mutagenesis of the resistant β-tubulin gene at Ile100 to Asn100 in *Schizosaccharomyces pombé* resulted in an increased sensitivity to ansamitocin P-3.[73,74]

It has been suggested that maytansine acts by binding to key sulfhydryl groups of tubulin.[63] Certain sulfhydryl groups in β-tubulin are highly reactive and their reactivity is sensitive to the presence of tubulin binding ligands. Reaction with *N*,*N*′-ethylenebis-(iodoacetamide) results in two intrachain cross-links; formation of one of these is blocked by colchicine and podophyllotoxin, whereas formation of the other is blocked completely by maytansine and partly by Vinca alkaloids.[75] Maytansinoids are useful tools for the study of tubulin structure; fluorescent probes and photoaffinity labeling reagents incorporating the ansamitocin P-3 structure have been described.[76]

3.4. Other Biological Activities Maytansinoids not only exhibit antitumor activity, but are also effective against eukaryotic systems such as protozoa, yeasts, fungi in general, insects and plants. They do not affect growth of bacteria or the plaque forming ability of different coliphages.[77]

Ansamitocins P3 and P4 inhibit the growth of fungi such as *Hamigera avellanea* (IFO 7721, also called *Penicillium avellaneum*), *Tricophyton mentagrophytes* (IFO 7522) and *Cryptococcus neoformans* (IFO 0410) but not *Candida albicans* (IFO 0583) or *Saccharomyces cerevisiae* (IFO 0209).[77] *Filobasidium uniguttulatum* (IFO 0699), selected out of 396 yeasts, was the most suitable test organism for the bioassay

of maytansinoids, giving a 20 mm inhibition zone with 45 ng of ansamitocin P4,[78] whereas with *Penicillium avellaneum* 240 ng of maytansine were required to give an inhibition zone of the same size.[79]

While these test systems make use of the antibiotic activity of maytansinoids, protozoa can be used to test for their antitubulinic activity.[80—82] Protozoa may be equipped with axopods (cilia) containing bundles of microtubules. Recovery of the motility of deciliated *Tetrahymena pyriformis* cells can be used to test for the presence of maytansinoids.[80,81] Ansamitocin P-3 completely inhibits cilia regeneration at a concentration of 1 μM, about 20-fold lower than that of vinblastine. A control group of *Tetrahymena* recovers from removal of cilia within 60 min. In this assay system, the ansamitocins had slightly stronger inhibitory activity than maytansine. Ansamitocins also synchronized cell division in *Tetrahymena pyriformis*.[83]

A similar test system was employed using *Actinophris sol* Ehrenberg and *Actinosphaerium eichhorni* Ehrenberg. Retraction of axopods occurs at a concentration of 1 μM maytansine.[82] The system can be used for a semiquantitative determination of maytansinoids. In a control experiment vinblastine, vincristine and vindesine were found also to be effective. This bioassay can be used to distinguish between microtubule stabilizing and destabilizing agents. While maytansinoids, vinblastine, vincristine and vindesine cause retraction of axopods with degradation of microtubules, axopods form bundles under the influence of taxol. This can be observed using a light microscope.[82]

Maytansine at a concentration of 6×10^{-8} M inhibits cell division of clam eggs and of two different species of sea urchin eggs.[63]

Extracts of *Trewia nudiflora* seeds were also shown to be active against a number of insects including species belonging to the *Lepidoptera* and *Coleoptera*, pests of fruits and vegetables. A dose–mortality relationship for maytansinoids isolated from *Trewia nudiflora* was presented.[84] Maytansinoids proved also 100% effective against the chicken body louse.

Since maytansine has strong cytotoxic and antitumor activity, it was anticipated that it would also inhibit plant cell division and consequently reduce the height of the whole plant by retarding meristematic activity. Indeed, there is a striking inhibitory activity of maytansine on cell division of tobacco callus tissue. Similarly, growth of rice seedlings is inhibited. In contrast and unexpectedly, maytansine has a promoting activity on the growth of *Avena coleoptiles* at 100 ppm.[85] During these experiments it was also demonstrated that maytansine is taken up by roots of rice seedlings and *Avena* plantlets. This is a very interesting observation in view of a possible ecological interaction between root-associated bacteria producing a precursor molecule and maytansinoid accumulating plants (see Chapter 6.3).[82]

4. Clinical Studies

4.1. Pre-clinical Pharmacology, Toxicology and Metabolism Maytansine (**1**) was developed for clinical trials under the auspices of the National Cancer Institute's Drug Discovery Program. After the report of the discovery of this compound in 1972 by Kupchan's group,[1] extensive studies were carried out in order to enter the compound into human clinical trials. The pre-clinical results with maytansine have been reviewed by Issell and Crooke.[6]

Presumably due to a shortage of material and the lack of sufficiently sensitive analytical methods at the time, no metabolism studies were apparently carried out in conjunction with the initial pre-clinical and clinical studies on maytansine. To remedy this lack of information and to generate data which might be of use in designing maytansinoid analogs with improved clinical potential, studies on the metabolism of ansamitocin P-3 and maytansine were recently initiated.[86]

The approach of these studies was to first examine the mass spectral fragmentation characteristics of ansamitocin P-3, using electrospray (ESI) LC/MS, with multi-stage mass fragmentation (MS^n) capability on an ion-trap instrument (LCQ, Finnigan, San Jose, CA, U.S.A.). Then metabolism studies of ansamitocin P-3 were carried out *in vitro* with cryopreserved human liver microsomes, cryopreserved rat liver microsomes, rat liver homogenate, and finally *in vivo* in the rat. Following extraction with ethyl acetate, the metabolites were characterized and tentative structures assigned based on the MS^n properties in comparison with those of the parent compound. The existence of the chlorine atom was used as a guide to rapidly locate the possible metabolic products by virtue of the isotope ratio ($^{35}Cl/^{37}Cl$, 3 : 1) from the biological matrices. Thus, any dechlorinated metabolites were not considered at this time.

The ESI mass spectrum of ansamitocin P-3 as obtained under infusion in 50.0% acetonitrile/0.9% formic acid showed a major doublet at *m*/*z* 635/637 in a ratio of 3 : 1, corresponding to the $[M+H]^+$ of ansamitocin P-3 with one chlorine atom. The collision induced dissociation (CID) mass spectrum of the parent ion at *m*/*z* 635 at 23 eV. showed one major fragment ion at *m*/*z* 547 (MH^+-88 Da), probably formed by elimination of isobutyric acid from the parent ion. To obtain further information on the major fragment at *m*/*z* 547, the MS^3 mass spectrum at *m*/*z* 635 was acquired. There was one major fragment ion at *m*/*z* 435, several fragment ions of significant intensity at *m*/*z* 529, 515, 486, 485, 468, 453, 407 and 355. Most of these fragment ions arise by straight-foreward sequential losses of water, methanol, the carbamate group or CO_2, as shown in Fig. 13. The base fragment ion at *m*/*z* 435 is probably not formed in one straightforward step, but may possibly arise by several elimination and rearrangement processes from the ion at *m*/*z* 547. Conceivably, dehydration of the ion at *m*/*z* 453 could give rise to this fragment ion, but the dehydration may involve a rather complex process through epoxide ring opening assisted by the enamine to form an amino alcohol. Subsequently, elimination of water and rearrangement form a pyridine ring. The MS^4 mass spectrum of the transition of ions at *m*/*z* 635→547→435 was acquired and there were three major fragment ions at *m*/*z* 407, 399 and 355. The fragment ion at *m*/*z* 407 is presumably formed through ring contraction by elimination of CO. The fragment ion at *m*/*z* 355 is proposed to be formed by elimination of 1,3-butyne from the fragment ion at *m*/*z* 407. The reason for the facile formation of the ion at *m*/*z* 435 remains obscure. Figure 13 depicts the proposed major fragmentation pathways of ansamitocin P-3 based on the ESI MS^n experiments, bearing in mind that these pathways have not been verified by accurate mass measurements or metastable scans. Other explanations are therefore possi-

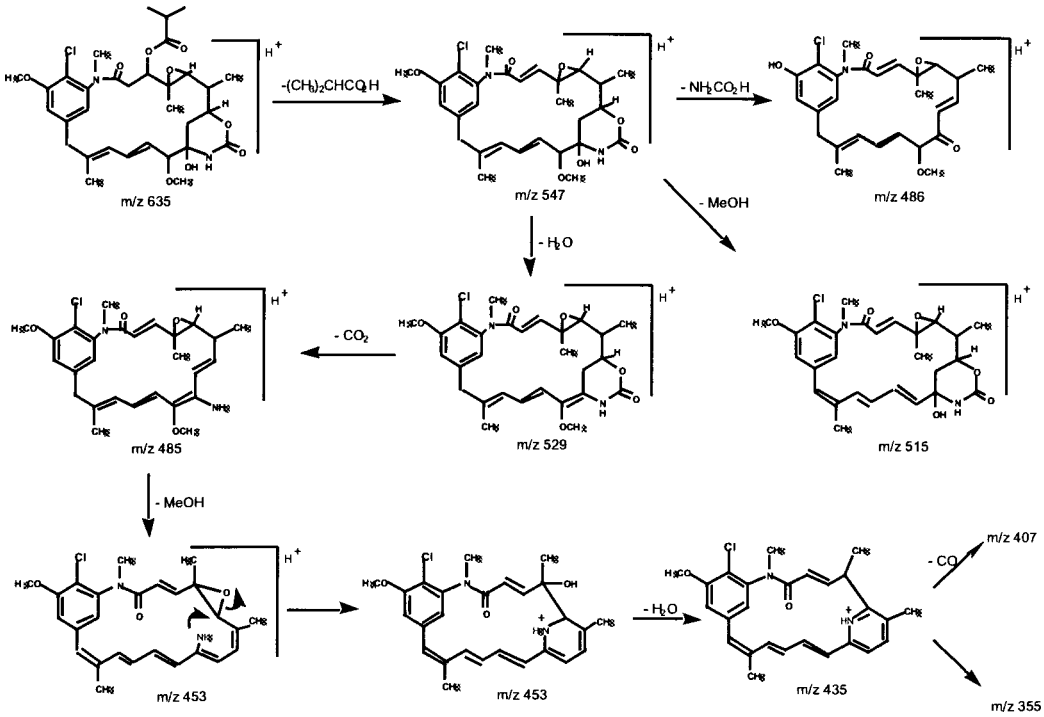


Fig. 13. Collision-Induced Mass Spectral Fragmentation Pattern of Ansamitocin P-3

ble. Nevertheless, the present study represents the first detailed ESI-LC-MS/MS investigation of a maytansinoid. The results are consistent with the limited mass spectral data on maytansinoids found in the literature,[12,35,47,87,88] most of which are derived from EI-MS and provide little or no fragmentation information. These mass spectral fragmentation patterns of ansamitocin P-3 have provided guidance in the subsequent structure elucidation of ansamitocin P-3 metabolites.

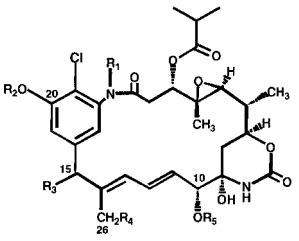
The metabolism of ansamitocin P-3 was then investigated first with human liver microsomes, using standard metabolism protocols and parallel controls of blank microsomes and drug alone. The ethyl acetate extracts were analyzed by LC-ESI-MS/MS and ten metabolites were characterized as summarized in Table 4. The possible structures for these metabolites are also shown in Table 4. The major metabolic pathway of ansamitocin P-3 in human liver microsomes appears to be 10-*O*-demethylation (Structure I). Oxidation and sequential oxidation/demethylation also occurred, although to a lesser extent. There were trace contaminants of some of these compounds in the initial ansamitocin P-3 sample, but they were discerned when quantitative metabolism was considered (see below). These structural assignments may need confirmation by more rigorous structural analysis using complementary techniques such as NMR and use of stable isotope labeling to confirm mass fragmentations. No ansamitocin P-3 or metabolites were found in the aqueous layer of the incubation mixture, although no attempt to concentrate this fraction was made.

The metabolism of ansamitocin P-3 in rat liver microsomes was investigated similarly. Again, the 10-*O*-demethylated ansamitocin P-3 at *m/z* 621 (M-14) (Structure I, Table 4) was found to be the major metabolite. Several minor metabolites such as demethylated ansamitocin P-3 (Structure II or III), didemethyl ansamitocin P-3 (Structures XII and XIII), and oxidized ansamitocin P-3 (Structure IV or V) were also detected. Assuming that the mass response of ansamitocin P-3 and its metabolites are the same, based on the area of peaks corresponding to ansamitocin P-3 and its metabolites, it was estimated that about 70% of ansamitocin P-3 was converted to its metabolites in human liver microsomes; however, only 20% of ansamitocin P-3 was converted to metabolites in rat liver microsomes under the same condi-

Table 4. Summary of Metabolites of Ansamitocin P-3 Detected and Characterized in Human Liver Microsomes

Number	ID	Ansamitocin P-3±nDa	T_{ret} [a] (min)	*m/z*	Proposed structure(s)
0	Ansamitocin P-3	−0	24.1	635/637	MH^+
1	–10O–Me	−14	22.4	621/623	I
2	–20O or N–Me	−14	21.3	621/623	II—III
3	+C26–OH	+16	20.5	651/653	V
4	+C15–OH	+16	21.9	651/653	IV
5	–Me+OH	+2	19.5	637/639	VI—XI
6	–Me+OH	+2	20.6	637/639	VI—XI
7	No assignment	+2	19.8	637/639	?
8	–(N–Me & 10O–Me)	−28	19.7	607/609	XIII
9	–(20O & 10O–Me)	−28	20.9	607/609	XII
10	–2Me+OH	−12	18.7	623/625	XIV—XVII

a) Relative retention time.



I: R_1, R_2 = CH_3; R_3, R_4, R_5 = H
II: R_1,R_5 = CH_3; R_2, R_3, R_4 = H
III: R_1, R_3, R_4 = H; R_2,R_5 = CH_3
IV: R_1, R_2, R_5 = CH_3; R_3 = OH; R_4 = H
V: R_1, R_2, R_5 = CH_3; R_3 = H; R_4 = OH
VI: R_1, R_2 = CH_3; R_3 = OH; R_4, R_5 = H
VII: R_1, R_2 = CH_3; R_3, R_5 = H; R_4 = OH
VIII: R_1, R_5 = CH_3; R_2, R_4 = H; R_3 = OH
IX: R_1, R_5 = CH_3; R_2, R_3 = H; R_4 = OH
X: R_1, R_4 = H; R_2, R_5 = CH_3; R_3 = OH
XI: R_1, R_3 = H; R_2, R_5 = CH_3; R_4 = OH
XII: R_1= CH_3; R_2, R_3, R_4, R_5 = H
XIII: R_1, R_3, R_4, R_5 = H; R_2= CH_3
XIV: R_1= CH_3; R_2, R_4, R_5 = H; R_3 = OH
XV: R_1= CH_3; R_2, R_3, R_5 = H; R_4 = OH
XVI: R_1, R_4, R_5 = H; R_2= CH_3; R_3 = OH
XVII: R_1, R_3, R_5 = H; R_2= CH_3; R_4 = OH

tions.

Metabolism of ansamitocin P-3 in rat liver homogenate was also examined. Since ansamitocin P-3 was found to be contaminated with traces of compounds identical to some of the metabolic products, this caused complications in the detection of ansamitocin P-3 metabolites, as was also the case in the *in vivo* experiment. However, by comparison of the ratios of the peak areas of the potential metabolites to that of the parent compound in the metabolite extracts with those in the starting material the origin of the potential metabolites could be discerned. Analysis of mass spectra and tandem mass spectra demonstrated that there was a new component with an ion doublet at *m/z* 565/567 (−70 Da from ansamitocin P-3). This species was interpreted as an ester hydrolysis metabolite of ansamitocin P-3. The tandem mass spectrum of *m/z* 565 showed a base peak at 453 (+18 Da from the equivalent fragment ion at *m/z* 435 in the MS^3 mass spectrum of ansamitocin P-3). Therefore, this major metabolite was assigned to be the alcohol maytansinol formed by ester hydrolysis of ansamitocin P-3. To examine whether there were other metabolites previously detected in human microsomes generated in the incubation of ansamitocin P-3 with rat liver homogenate, the total ion current (XIC) of each potential metabolite was scanned and the corresponding peak was integrated. Table 5 shows the area ratios of ion peaks to that of ansamitocin P-3 and the corresponding values in the original formulation. As shown, no significant increase in the ratio was seen in the five potential metabolites monitored. Therefore, no significant metabolism other than ester hydrolysis was detected in rat liver homogenate incubated with ansamitocin P-3.

In an effort to detect these metabolites *in vivo*, a Sprague–Dawley rat, weighing about 300 g, was dosed with ansamitocin P-3 at 3 mg/kg, and the 24 h cumulative urine was collected. The ethyl acetate extracts of the predose urine sample and the 24 h urine sample were examined for evidence of metabolism using similar methodologies as described above. Based on the peak area intensity, about 4% of the unchanged dose was recovered from urine. Mass spectral analysis of the ansamitocin P-3 formulation showed that there was a small amount of demethylated ansamitocin P-3 (Structure I, Table 4) and oxidized ansamitocin P-3s (Structures IV and V, Table 4) present. Therefore, the ratios of the intensities of each of these ions to that of the parent from the 24 h urine extract were compared to those in the dose. As shown in Table 6, the ratio of the extracted ion at *m/z* 621, the 10-*O*-demethylansamitocin P-3, to that of ansamitocin P-3 gave a value of about 3%, about 3-fold higher than that in the formulation (1%). Therefore, it is likely that 10-*O*-demethylansamitocin P-3 is an *in vivo* metabolite of ansamitocin P-3. On the other hand, the ratio of the ion at *m/z* 651 (oxidized ansamitocin P-3) to ansamitocin P-3 in urine was 1%, as compared to 2% in the formulation. Therefore, the oxidized ansamitocin P-3 observed is entirely accounted for by the contaminant in the formulation.

According to the results, there appears to be some species difference in ansamitocin P-3 metabolism. Although the same major metabolite, 10-*O*-demethylansamitocin P-3, and four minor metabolites (the others were not detectable based on current sensitivity) were detected in rat and human liver microsomes, it appears that rat liver microsomes metabolize

Table 5. The Comparison of Area Ratios of Potential Metabolite Peaks to that of Ansamitocin P-3 in the Formulation and in Rat Liver Homogenate

Source of ansamitoc in P-3	Area ratio of MH^+ of components to that of ansamitocin P-3 (rrt[a] of HPLC peaks)				
	651/635	621/635 (20.79)	621/635 (21.94)	621/635 (22.77)	565/635 (21.48)
In dose	0.06	0.005	0.02	0.01	0.001
In RLH[b]	0.01	0.005	0.01	0.007	0.025

a) Relative retention time. *b*) Rat liver homogenate.

Table 6. The Area Intensity of Peaks at *m/z* 635, 621 and 651 in the Extracted Ion Chromatogram of Ansamitocin P-3 and in the Urine of Rat Dosed with Ansamitocin P-3 at 3 mg/kg

Source of ansamitocin P-3	635 (Area)	621 (Area)	651 (Area)	Ratio of 621 to 635	Ratio of 651 to 635
In dose	3.2e8	3.40e6	9.03e6	1 : 100	3 : 100
In urine	1.5e8	5.2e6	3.43e6	3 : 100	2 : 100

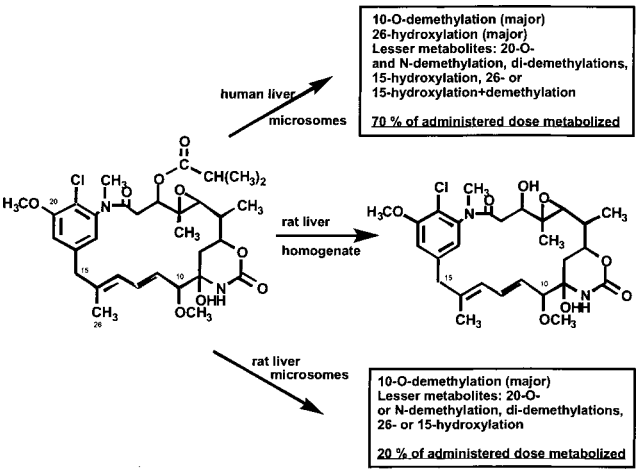


Fig. 14. Summary of Ansamitocin P-3 Metabolism

ansamitocin P-3 substantially less efficiently than human liver microsomes. The comparison with rat liver homogenate *in vitro* and with the whole rat *in vivo* raises the question whether the liver microsome system truely reflects drug metabolism *in vivo*. In rat liver homogenate, ansamitocin P-3 was only metabolized by hydrolysis of the ester side chain. The rat liver homogenate was prepared freshly and the detection sensitivity for these systems is believed to be similar. Therefore, it is possible that the liver microsome system may be somewhat artificial. Differences were also seen *in vivo*, where only the demethylated ansamitocin P-3 but none of the other metabolites was detected. The metabolism of ansamitocin P-3 is summarized in Fig. 14.

4.2. Clinical Trials Early clinical trials were initiated by the NCI in 1975 and the pre-clinical and phase I results prior to 1980 have been summarized by Komoda and Kishi.[7] These trials were carried out in patients with advanced disease refractory to conventional therapy. Dose limiting toxicity was established to be in the range of 1—2 mg/m^2 and side effects included neurotoxicity, gastrointestinal toxicity, weak-

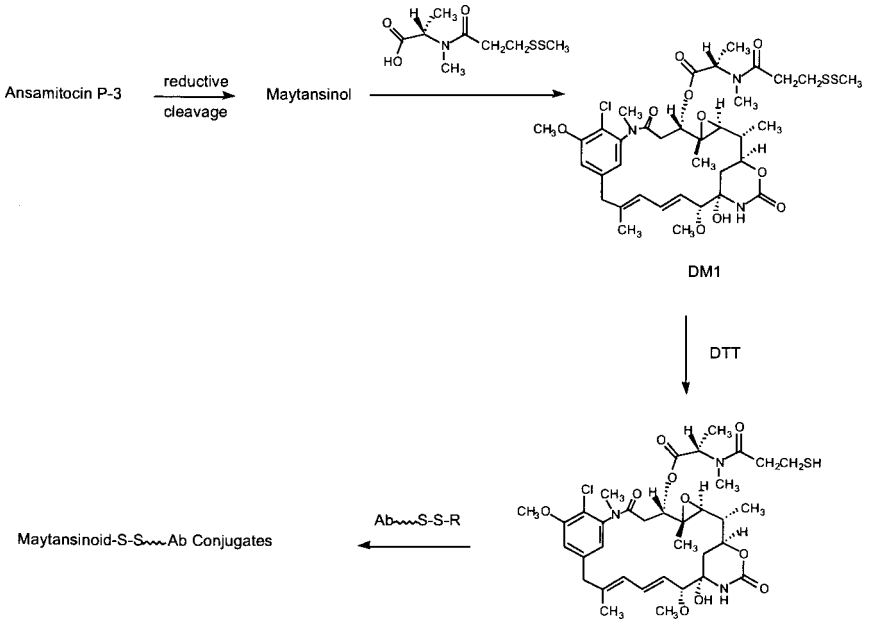


Fig. 15. Synthesis of the Compound DM1 Used to Conjugate Maytansinoids to Tumor-Specific Antibodies

ness, nausea, vomiting and diarrhea. A few patients responded positively, so the compound was entered into phase II clinical trials. Phase II clinical trials involving maytansine alone and in combination with other agents were carried out from about 1977 to 1984. A summary of the results of all of the pre-clinical toxicology and clinical trials results is given in a report from the NCI to the FDA.[89] The compound was evaluated as a single agent in over 35 tumor types in more than 800 patients. Only one patient with islet cell carcinoma showed a complete response. About 20 patients showed partial responses.[90—105] Based on these results, NCI eventually closed the investigational new drug application (IND) for maytansine.

4.3. Conjugates of Maytansinoids as Target-Specific Cancer Chemotherapeutic Agents The use of conjugates between cytotoxic agents and monoclonal antibodies (MAbs) or receptor agonists that bind selectively to cancer cells has met with limited success. One of the problems is the inability to deliver a high enough concentration of the cytotoxic agent to the tumor cell to kill it. One approach to overcoming this limitation is to use highly cytotoxic compounds such as the maytansinoids.

Two companies, Takeda Chemical Industries and ImmunoGen, Inc., filed patents covering delivery systems of antibody conjugates of maytansinoids in the early 1990s.[106,107] The Japanese group reported on the construction of a bispecific monoclonal antibody reactive to ansamitocins and human transferrin receptor (TfR). The conjugate was more effective than ansamitocin P-3 alone in suppressing mouse tumor xenografts.[108]

The ImmunoGen group took a different approach which has led to the development of a number of conjugates that are now entering early clinical trials for the treatment of cancer.[109] The approach taken by Chari and co-workers involved the synthesis of thiol-containing maytansinoids from the natural esters of either plant (maytansine) or microbial (ansamitocin P-3) origin. The general approach to the intermediate referred to in the literature as DM1 is shown in Figure 15. After reductive cleavage of DM1 with dithiothreitol (DTT), the resulting thiol could be coupled with a variety of MABs or receptor agonists to generate conjugates of interest for clinical trials. The detailed processes for preparation and purification of thiol-containing maytansinoid conjugates are reported in two patents.[110,111] Chari and co-workers at ImmunoGen have reported on the preparation of conjugates of DM1 with the TA.1, A7 and 5E9 antibodies[112] and the monoclonal antibody C242.[3] C242 recognizes a mucin-type glycoprotein expressed by human colorectal cancers. The C242-DM1 conjugate cured mice bearing sub-cutaneous COLO 205 human colon tumor xenografts. The same group reported a new folate-maytansinoid in 1997, which was designed to target selectively various carcinomas over-expressing the folate receptor.[113]

ImmunoGen has entered into several joint ventures with major pharmaceutical companies to use their DM1 Tumor Activated Prodrug (TAP) in conjunction with tumor-specific MABs. For example, Boehringer-Ingelheim has coupled DM1 to their anti-CD44V6 antibody to give a conjugate, bivatuzumab mertansine, which is entering clinical trials. Other conjugates entering clinical trials include huN901-DM1[114] licensed to Glaxo Smith Kline and BB-10901 licensed to British Biotech.[115] Other products in development include trastuzumab-DM1 by Genentech[116] and MLN591 DM1 by Millennium.[117] The Millennium compound is in phase I clinical trials for patients with metastatic androgen-independent prostate cancer. A recent paper reported the preparation of conjugates of DM1 with monoclonal anti-PSCA antibodies as targeting agents for prostate cancer.[118] It will be of great interest to see if the maytansinoid conjugates produce better clinical results than the parent drugs.

5. Biosynthesis

Since the maytansinoids structurally belong to the family of ansamycin antibiotics, their biosynthesis can be predicted

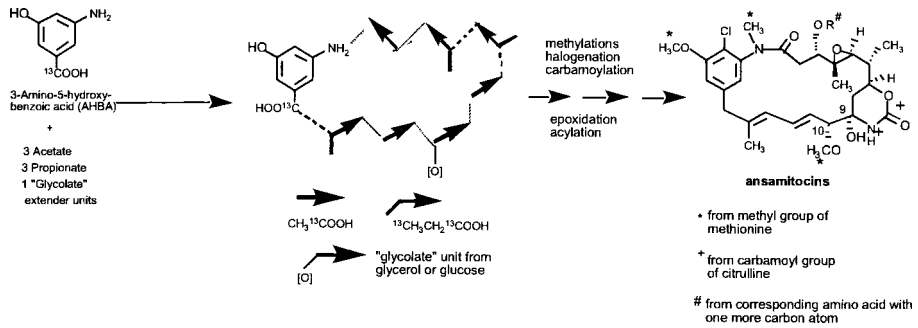


Fig. 16. Biosynthetic Building Blocks of the Ansamitocins as Deduced from Feeding Experiments with Isotopically Labeled Precursors

based on what is known about the formation of other ansamycins, particularly geldanamycin,[119] rifamycin[120] and ansatrienin/mycotrienine.[121] They can thus be envisioned to be assembled on a type I modular polyketide synthase (PKS) from an aromatic starter unit by a series of chain extension steps which give rise to a 19-membered macrocyclic lactam. This initial "proansamitocin" is then further elaborated into the bioactive final products by a series of post-PKS modification reactions that introduce several methyl groups, a carbamoyl group, an epoxide and an ester side chain (Fig. 16). The work that will be summarized in this section has confirmed the validity of this general prediction, it has revealed many details of the biosynthetic process and it has provided the tools for the diversion of the natural biosynthetic machinery into generating structural analogs.

5.1. Building Blocks All the biosynthetic studies on the maytansinoids were carried out with the ansamitocin producer, *Actinosynnema pretiosum* ATCC 31565. The basic building blocks were identified by *in vivo* feeding experiments with isotope-labeled precursors. As in other ansamycin antibiotics, 3-amino-5-hydroxybenzoic acid (AHBA) was identified as the specific precursor of the aromatic polyketide starter unit. Feeding of [7-^{13}C]-AHBA resulted in ansamitocin P-3 (AP-3) specifically enriched at C-15 (almost 100% specific incorporation).[122] Feeding of unlabeled AHBA to the fermentation gave a 30% stimulation of ansamitocin production, whereas several analogs of AHBA (3-amino-, 3-hydroxy-, 3,5-diamino-, and 3,5-dihydroxybenzoic acid) were all strongly (71—87%) inhibitory. The remainder of the macrocyclic lactam backbone is built up by seven chain extension reactions which incorporate three acetate and three propionate units. This was demonstrated by enrichment of the expected positions in AP-3 upon feeding [2-^{13}C]acetate (C-2, C-8, C-12), [1-^{13}C]propionate (C-3, C-5, C-13) and [3-^{13}C]propionate (C-22, C-23, C-26).[125] The third chain extension step, giving rise to C-9 and C-10, incorporates a unique oxygenated 2-carbon unit (often called a "glycolate" unit) which is not derived from acetate, but originates somehow from glucose metabolism.[123] Such "glycolate" extender units are also present in some other antibiotics, all presumably assembled on type I PKSs, such as geldanamycin,[124] leucomycin,[125] soraphen A,[126] FK520 and FK506,[127] concanamycin A,[128] aflastatin[129] and zwittermicin A.[130] Despite numerous feeding experiments their biochemical origin has, until recently, remained obscure. Feeding of D-[6-^{13}C]glucose labeled C-10 of AP-3, the oxygenated carbon of the "glycolate" unit, whereas [2-^{13}C]glycine enriched only the three *O*- and *N*-methyl groups, ruling this compound out as a more proximate intermediate.[123] Later feeding experiments showed that the two carbon atoms of this unusual extender unit in ansamitocin were derived intact from [U-$^{13}C_3$]glycerol, but that neither ^{13}C-labeled L-serine nor D,L-glycerate were incorporated.[131]

In the further elaboration of AP-3 from the initial cyclic polyketide, both the two *O*-methyl and the *N*-methyl group are labeled by L-[*methyl*-^{13}C]methionine, indicating that they are introduced by transmethylation reactions from S-adenosylmethionine (AdoMet). The enrichment of the same three positions by [2-^{13}C]glycine is presumably due to formation of methyl-labeled methionine *via* the methylenetetrahydrofolate pathway. The carbamoyl group of AP-3 is derived specifically from the carbamoyl group of L-citrulline, not the guanido group of L-arginine. This was shown by ^{14}C-labeling and degradation, which recovered 86% of the incorporated label in the CO_2 obtained by hydrolysis of the AP-3.[123] Figure 16 summarizes these labeling patterns.

The acyl moieties attached to 3-*O* of the ansamitocin backbone are derived from the corresponding acids, which presumably undergo activation. They can also arise from the corresponding amino acids with one more carbon atom (through transamination and oxidative decarboxylation) or the corresponding alcohols or aldehydes (through oxidation).[132] The pattern of ansamitocins accumulated in a fermentation is highly responsive to the addition of particular side chain precursors. Whereas without precursor addition the organism produces ansamitocins P-2, P-3 and P-4, carrying propionyl, isobutyryl and isovaleryl side chains, respectively, in a ratio of 15 : 55 : 30, addition of valine, isobutyrate, isobutyraldehyde or isobutanol results in accumulation of over 90% of the total ansamitocins as a P-3. This allows the production of specific ansamitocins with desired side chains and greatly simplifies the work-up of fermentations.[132]

5.2. Biosynthesis of the AHBA Starter Unit Our knowledge of the biosynthesis of AHBA is mostly derived from studies on the biosynthesis of other ansamycins, particularly rifamycin B, but the presence of the same essential genes in the ansamitocin biosynthetic gene cluster indicates that the same general pathway is operative here. Early work on the origin of the AHBA-derived mC_7N unit revealed labeling patterns matching those of shikimic acid, but feeding experiments gave no incorporation of labeled shikimate or earlier intermediates in the shikimate pathway (*cf.* refs. 133, 134). Hornemann[135] first postulated a 4-amino analog of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) as

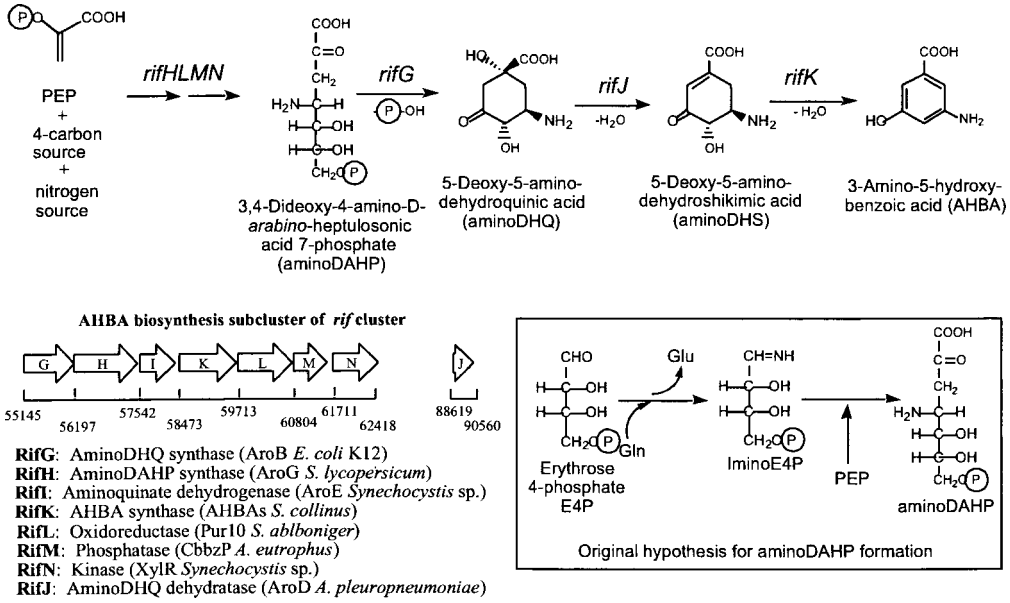


Fig. 17. Original Hypothesis for the Formation of AminoDAHP and AHBA, and the AHBA Biosynthesis Genes from the Rifamycin Biosynthetic Gene Cluster

an early intermediate, which he envisioned arising from DAHP. Based on a report that L-glutamine is the best nitrogen source for the mC_7N unit of rifamycin[136] and on the analogy to enzymes such as anthranilate synthase and *p*-aminobenzoate synthase, we postulated an alternative mode of 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP) formation by direct condensation of phosphoenolpyruvate (PEP) with the imine of erythrose 4-phosphate (E4P).[137,138] This imine we envisioned arising through the reaction of a bifunctional glutamine amidohydrolase/DAHP synthase, which would hydrolyze glutamine to generate ammonia in the active site of the enzyme, condense it with the aldehyde function of E4P and then catalyze the condensation with PEP. The postulated intermediates in this hypothetical pathway (Fig. 17) were synthesized[139,140] and shown to be converted into AHBA in cell-free extracts of the rifamycin and the ansatrienin producers, whereas the normal shikimate pathway intermediate DAHP was not.[140] The conversions of aminoDAHP, 5-deoxy-5-amino-3-dehydroquinic acid (aminoDHQ) and 5-deoxy-5-amino-3-dehydroshikimic acid (aminoDHS) into AHBA were very efficient (41—95%), confirming the pathway from aminoDAHP to AHBA. However, only small amounts of AHBA (about 6% conversion) were formed from PEP and E4P as substrates. Also, the amide nitrogen of [*amide*-^{15}N]glutamine was very inefficiently incorporated into newly synthesized AHBA. Formation of [^{14}C]aminoDAHP from [1-^{14}C]PEP was demonstrated, albeit in low and variable yield.[140]

The terminal enzyme in this new pathway was then purified to homogeneity from the rifamycin B producer, *Amycolatopsis mediterranei*, and the encoding gene was cloned by reverse genetics and overexpressed in *E. coli*.[141] The enzyme contains pyridoxal phosphate as a cofactor, which led to a proposed reaction mechanism for the dehydration/aromatization of aminoDHS to AHBA.[141] Support for this mechanism comes from a crystal structure of the recombinant enzyme[142] and from the preparation and analysis of a number of site specific AHBA synthase mutants.[143] The native, functional enzyme is a homodimer with two shared active sites, as demonstrated by the generation of chimeric dimers with partial activity from inactive mutants carrying substitutions of essential amino acids contributed to the active site from different subunits.[143] The requirement for AHBA synthase in rifamycin biosynthesis was confirmed by inactivation of the encoding gene in the producing organism. Surprisingly, this mutation did not result in the accumulation of aminoDHS or its breakdown product, protocatechuic acid.[141]

The AHBA synthase gene probe then allowed the cloning and sequence analysis of an *ca.* 95 kb gene cluster from *Am. mediterranei* encoding the biosynthesis of rifamycin B.[120] Within this cluster we identified a subset of eight genes, most of them in a single operon, which are involved in AHBA formation (Fig. 17). Gene inactivation experiments and heterologous expression of a cassette of all these genes, resulting in AHBA formation in a transformant of *Streptomyces coelicolor*, and deletion of individual genes from this cassette defined the positions of these genes in the pathway.[144] The involvement of *rifH*, *G* and *J* was expected based on their sequence homologies, and their mutations could be partially complemented by the corresponding normal shikimate pathway enzymes. *RifI* as expected was not required for AHBAsynthesis, but three additional genes, *rifL*, *M* and *N* were each absolutely essential for aminoDAHP formation. No gene with homology to aminohydrolases or any other plausible candidate gene for the introduction of the nitrogen was identified in the *rif* gene cluster [an aminotransferase, *rif orf 6* was shown by inactivation not to be involved in AHBA formation[144]]. The accumulated evidence cast doubt on the original hypothesis for aminoDAHP formation (Fig. 17) and suggested a more complex pathway to this committed intermediate. It also suggested the possibility that the AHBA synthase, RifK, could have a second function earlier in the pathway as the enzyme introducing the nitrogen. Notably, the closest homology of *rifK* is to *stsC*, encoding a glutamine-dependent *scyllo*-inosose aminotransferase involved in streptomycin biosynthesis.[145] Also, the linked arrangement of

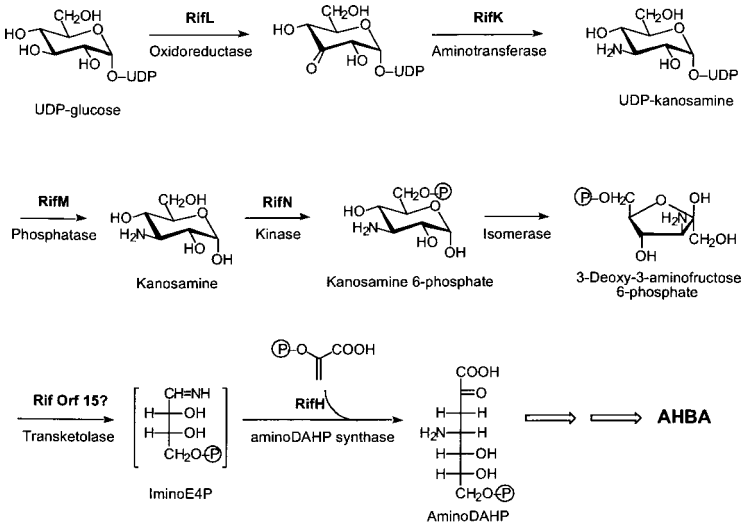


Fig. 18. New Hypothesis for the Formation of the AHBA Precursor, AminoDAHP, *via* Kanosamine

rifK, L and M in one operon is highly conserved in all the AHBA synthesis clusters analyzed.

Recently, Guo and Frost[146] showed that aminoDAHP, together with large amounts of DAHP, can be produced by the action of *E. coli* transketolase on 3-amino-3-deoxyfructose 6-phosphate (aminoF6P) in the presence of PEP, an acceptor for the transketolase reaction, such as ribose 5-phosphate, and RifH or cell-free extract of *A. mediterranei*, but not of *E. coli* DAHP synthase. Presumably, the action of transketolase on aminoF6P gives rise to iminoE4P which is then condensed with PEP to aminoDAHP (Fig. 18). The authors propose this reaction sequence as the normal pathway for aminoDAHP formation and suggest as the source of the aminoF6P the known amino sugar, kanosamine (3-amino-3-deoxyglucose). Support for this suggestion comes from the finding that the kinase encoded by *rifN* is a highly specific ATP-dependent kanosamine 6-kinase.[147] Its product, kanosamine 6-phosphate, in turn was shown by Guo and Frost to substitute for aminoF6P when yeast phosphoglucose isomerase was included in the incubation.[148] The biosynthesis of kanosamine has been studied in a *Bacillus* sp. by Umezawa and colleagues and found to involve dehydrogenation and transamination of UDP-glucose followed by hydrolysis of the presumed intermediate, UDP-kanosamine.[149—151] Preliminary evidence suggests a similar pathway in *A. mediterranei*. Guo and Frost demonstrated formation of UDP-kanosamine from UDP-glucose by *Am. mediterranei* cell-free extract in the presence of NAD and glutamine.[148] Preliminary data[147] suggest that the oxidoreductase RifL forms a complex with RifK which can catalyze pyridine nucleotide-dependent dehydrogenation and glutamine-dependent transamination of UDP-glucose but not dTDP-glucose. This leads to a new hypothetical pathway of AHBA formation as shown in Fig. 18, with RifK in complex with RifL acting as the transaminase which introduces the nitrogen, and the phophatase RifM serving to release kanosamine from its UDP derivative. Notably, the *rif* cluster does also contain a transketolase gene (*rif orf 15*), and it is conceivable that the product of this gene cooperates specifically with RifH to channel iminoE4P into aminoDAHP formation. Work is in progress to test this new hypothesis of aminoDAHP formation.

5.3. Cloning and Analysis of the Ansamitocin (*asm*) Biosynthetic Gene Cluster Using a PCR approach with conserved sequences of the AHBA synthase gene, rifK, two different AHBA synthase genes (*asm24* and *asm43*) were isolated from *A. pretiosum* DNA. Both were shown to be competent, *asm43* by expression in *E. coli* and demonstration of AHBA synthase activity, *asm24* by complementation of a *rifK* mutant of *Am. mediterranei* to restore rifamycin production. Each of these genes was isolated on a large cosmid and the surrounding DNA was analyzed for the presence of other putative ansamitocin biosynthesis genes. The cosmid containing *asm43* did not carry any PKS genes, but did contain three other AHBA biosynthesis genes, *asm44*, *45* and *47*, homologous to *rifL*, *rifM* and *rifG*. In contrast, *asm24* on its cosmid was located adjacent to a modular type I PKS gene (*asmA*) with a loading domain closely resembling in its recognition sequence that of the *rif* PKS. Chromosome walking then allowed the sequencing of a 96 kb region of DNA containing most of the genes expected to be required for ansamitocin biosynthesis (Fig. 19, Table 7).[152] A deletion of the *asmB* gene was constructed and this mutant no longer produced ansamitocin, demonstrating that the cloned type I PKS was indeed the *asm* PKS and that cluster I is essential for ansamitocin biosynthesis. However, cluster II surrounding *asm43* must also be essential, since cluster I lacks some genes known to be indispensable for AHBA formation and these, *asm44*, *45* and *47*, are present in cluster II. This was confirmed by a deletion from the wild-type DNA of the 35.3 kb of DNA corresponding to cosmid pDHc1 (Fig. 19) carrying the entire cluster II region. The mutant no longer produced ansamitocin, but production was restored by the addition of AHBA to the fermentation, demonstrating that cluster II only contains genes required for AHBA formation. Extensive mapping showed that the two AHBA synthase genes, *asm24* and *asm43*, were located 68 kb apart from each other on the genome, and that the ends of the two clusters were separated by 30 kb of DNA. A mutant was then constructed in which this 30 kb of DNA, including parts of *asm37* and *38* was deleted. This mutant showed no phenotypic change in either ansamitocin production or growth and

Table 7. Deduced Functions of the Open Reading Frames in the *asm* Biosynthetic Gene Cluster

Polypeptide	Amino acids	Sequence similarity	Proposed function [Function of homologue]	Polypeptide	Amino acids	Sequence similarity	Proposed function [Function of homologue]
AsmA	4684		Polyketide synthase	Asm19	378	MdmB *S. mycarofaciens*	3-*O*-acyltransferase
Loading domain		RifA *A. mediterranei* S699	ADE ACP	Asm20	115	ORFX *S. meliloti*	Unknown
Module 1		RapA *S. hygroscopicus* NRRL 5491	KS AT DH ER KR ACP	Asm21	668	NolnO *Rhizobium* sp. NGR234	*O*-carbamoyltransferase
Module 2		PimS1 *S. natalensis*	KS AT DH KR ACP	Asm22	251	MitS *S. lavendulae*	Kinase
AsmB	3073			Asm23	144	RifJ *A. mediterranei* S699	aDHQ dehydratase
Module 3		RifB *A. mediterranei* S699	KS AT DH KR ACP	Asm24	388	RifK *A. mediterranei* S699	AHBA synthase
Module 4		PimS1 *S. natalensis*	KS AT ACP	Asm25	402	OleG2 *S. antibioticus*	Glycosyltransferase
AsmC	1589			Asm26	80	NcnC *S. arenae*	[Acyl carrier protein]
Module 5		RifA *A. mediterranei* S699	KS AT KR ACP	Asm27	341	YxnA *B. subtilis*	[Glucose 1-dehydrogenase]
AsmD	3324			Asm28	154		Unknown
Module 6		RifB *A. mediterranei* S699	KS AT DH KR ACP	Asm29	193	BetI *S. meliloti* 102F34	[Transcriptional regulator]
Module 7		PimS1 *S. natalensis*	KS AT KR ACP	Asm30	1005	YrhJ *B. subtilis*	Cytochrome P450
Asm01	241	ORF4 *S. coelicolor* A3(2)	[Quinone oxidoreductase]	Asm31	348	RpoT *M. leprae*	[Sigma factor]
Asm02	214	NonG *S. griseus* subsp. griseus	[Transcriptional repressor]	Asm32	67		Unknown
Asm03	88		Unknown	Asm33	190	EC 1.5.1.3 *T. maritima*	[Dihydrofolate reductase]
Asm04	748	Slr2019 *Synechocystis* sp.	[ABC transporter]	Asm34	204	SCD95A.38c *S. coelicolor* A3(2)	[Transcriptional regulator]
Asm05	416	PatA *Synechocystis* sp.	[Na/H antiporter]	Asm35	392	RP698 *R. prowazekii*	[Bicyclomycin resistance]
Asm06	97		Unknown	Asm36	105		Unknown
Asm07	348	EC 2.1.1.38 *S. anulatus*	Methyltransferase	Asm37	558partial		Unknown
Asm08	1117		Transcriptional regulator	Asm38	426	LysA *M. tuberculosis*	[DAP-decarboxylase]
Asm09	259	RifF *A. mediterranei* S699	Amide synthase	Asm39	144	AbaA-ORFA *S. coelicolor*	[Regulator]
Asm10	294	TcmP *S. glaucescens*	Methyltransferase	Asm40	128	SCH5. 12c *S. coelicolor* A3(2)	[Sigma factor antagonist]
Asm11	480	MtmOII *S. argillaceus*	Oxygenase	Asm41	190	SCJ1. 02c *S. coelicolor* A3(2)	Glycosyl hydrolase
Asm12	441	PltA *P. fluorescens*	Halogenase	Asm42	336	EpiH *S. epidermidis*	[Transmembrane protein]
Asm13	341	EC 1.1.1.157 *E. coli*	[3-Hydroxyacyl-CoA dehydrogenase]	Asm43	388	RifK *A. mediterranei* S699	Transaminase [AHBA synthase]
Asm14	90	Dltc *S. mutans*	ACP [D-alanyl carrier protein]	Asm44	387	RifL *A. mediterranei* S699	Oxidoreductase
Asm15	357	I30A.22c *S. coelicolor* A3(2)	[Acyl-CoA dehydrogenase]	Asm45	212	RifM *A. mediterranei* S699	Phosphatase
Asm16	437	EC 1.2.99.2 *C. thermoaceticum*	Unknown [CO dehydrogenase]	Asm46	266		Unknown
Asm17	167	MdmC *S. mycarofaciens*	*O*-Methyltransferase	Asm47	342	RifG *A. mediterranei* S699	aDHQ synthase
Asm18	913	SnoA *S. nogalater*	[Transcriptional activator]	Asm48	518 partial	MalT *E. coli*	[Transcriptional activator]

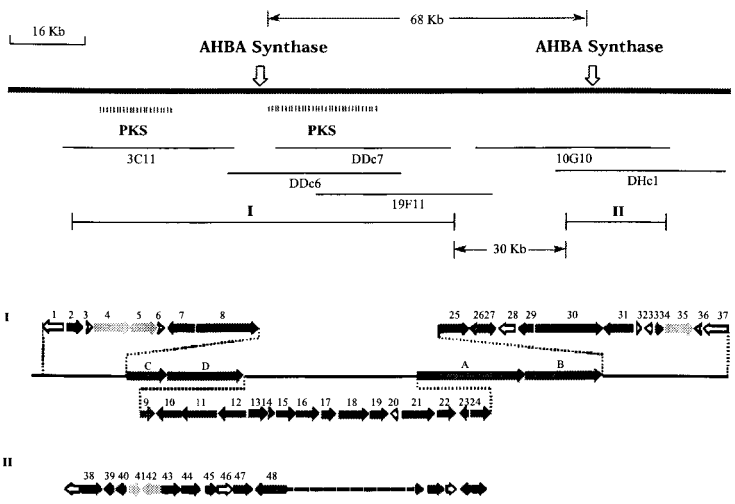


Fig. 19. Organization of the Ansamitocin (*asm*) Biosynthetic Genes in *Actinosynnema pretiosum*

Shown are six overlapping cosmids covering the region, and the arrangement of the genes in the two non-connected clusters I and II (see Table 7).

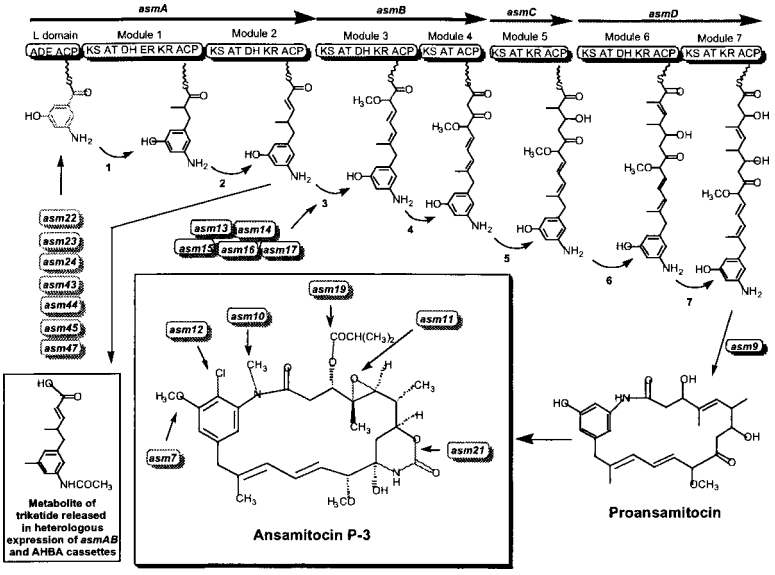


Fig. 20. Summary of the Biosynthesis of Ansamitocin P-3 by the *asm* Biosynthetic Genes

Shown are the genes for the formation of the AHBA starter and the methoxymalonate chain extension units, the assembly of the polyketide on the *asm* PKS, its release and cyclization to proansamitocin by *asm9*, and the post-PKS processing of proansamitocin to ansamitocin P-3. The structure of a triketide metabolite resulting from expression of a truncated *asm* PKS in *S. coelicolor* is also shown.

morphology. The 30 kb region between the two clusters is, thus, not essential for primary or secondary metabolism of the organism. The *asm* biosynthetic gene cluster represents one of the first examples defying the general paradigm that in the genomes of Actinomycetes the genes encoding the biosynthesis of a given antibiotic are always clustered. However, the separation of the *asm* genes into two clusters seems to be merely an inconsequential accident resulting from an evolutionary gene rearrangement which inserted 30 kb of non-essential DNA into the otherwise contiguous cluster.[152]

5.3.1. The AHBA Biosynthesis Genes: The combined cluster contains seven genes related to the formation of the AHBA starter unit, including two copies of the AHBA synthase gene. This is in accordance with the emerging notion that the AHBA synthase protein may have two functions in AHBA formation, the aromatization of aminoDHS to AHBA and the earlier introduction of the nitrogen into a precursor by transamination. Although both *asm24* and *asm43* were shown to be competent to catalyze AHBA formation from aminoDHS, it seems likely that one enzyme, probably Asm24, has been optimized to function as the AHBA synthase and the other, Asm43, as the transaminase. The assembly of *asm43*, *44* and *45* may serve as a subcluster responsible for the synthesis of the AHBA precursor, kanosamine. Absent relative to the *rif* AHBA subcluster (Fig. 17) are a shikimate dehydrogenase homolog (*rifI*) which is not essential for AHBA formation in *Am. mediterranei*, and, more notably, a homolog of DAHP synthase. In rifamycin biosynthesis the function of the aminoDAHP synthase (RifH) can be complemented, albeit poorly, by the organism's normal DAHP synthases functioning in aromatic amino acid biosynthesis. Such an enzyme must also be responsible for aminoDAHP formation in ansamitocin biosynthesis. It has been demonstrated that the *A. pretiosum* genome contains a single plant type DAHP synthase gene, which is not located in the 250 kb mapped region surrounding clusters I and II. Unlike in rifamycin biosynthesis, the supply of AHBA seems to be rate-limiting in ansamycin formation, and this may be related to the absence of a dedicated aminoDAHP synthase gene. Also absent from the cluster is a transketolase gene corresponding to *rif orf 15*.

5.3.2. The *asm*PKS: The loading domain and seven chain extension modules of the asmPKS are organized into four separate proteins. Each module contains the modification domains (ketoreductase, KR; dehydratase, DH; and enoylreductase, ER) expected from the product structure. The loading domain, as in the *rif* PKS,[153] represents a nonribosomal peptide synthase (NRPS) adenylation (AD)-acyl carrier protein (ACP) didomain, which activates AHBA to the adenylate and transfers it to the cognate ACP. Although the four PKS genes are not arranged in the order in which they operate in the biosynthesis, within each gene the modules are indeed co-linear with their function, allowing for the synthesis of a set of PKS proteins that can be assembled into a complex in which all modules are arranged co-linear with their function (Fig. 20). The last PKS module is followed by an amide synthase gene (*asm9*) which must be responsible for release of the fully assembled polyketide from the PKS and its cyclization to a proansamitocin, as has been demonstrated for the homologous *rifF* in rifamycin biosynthesis.[154,155]

The ansamitocin structure reveals two notable features. One is the incorporation of the unusual "glycolate" extender unit in the third chain elongation step. The acyltransferase (AT) domain of module 3 of the PKS, which must recognize the substrate for this glycolate extender unit, does not show any unusual sequence signature which would allow its distinction from malonyl-CoA- or methylmalonyl-CoA-specific AT domains.[152] A second unusual feature of the ansamitocin structure is the position of the conjugated double bonds at Δ11,12 and Δ13,14, different from the Δ10,11 and Δ12,13 positions into which normal polyketide assembly would place them. The timing of this double bond shift, either during polyketide assembly or immediately after release of the

initial PKS product, is unclear. The fact that a mutant blocked in the first identified post-PKS modification reaction, the halogenation step, accumulates a proansamitocin with already shifted double bonds[156] favors double bond migration during polyketide assembly on the *asm* PKS. However, no obvious candidate domain or separate gene for this double bond rearrangement has been identified, and the sequence of the DH of module 3 is not obviously different from normal DH domains. The possibility that the migration of the double bonds is related to the presence of the oxygen function at C-10 was ruled out by the formation, in a mutant unable to synthesize the unique substrate for the third chain extension step, of 10-demethyoxy-AP-3 with the double bonds in the Δ11,12 and Δ13,14 positions.[157]

To further probe the function of the *asm*PKS, an expression vector was constructed carrying on one side of the bi-directional *actI*/*actIII* promoter the *rif* AHBA synthesis cassette[144] and on the other side various parts of the *asm*PKS. Expression of the AHBA cassette plus *asmA* yielded no new products other than AHBA, but expression of the AHBA cassette with *asmA+B* gave a new compound which was identified as the *N*-acetyl metabolite of the triketide predicted to be synthesized on the *asmA* protein alone.[152] The requirement for the presence of the *asmB* protein can be relinquished by fusing the thioesterase (TE) domain of the 6-deoxyerythronilide B synthase (DEBS) to the terminus of the AsmA protein.[158] These results indicate that in the absence of a TE the triketide cannot be released from the PKS when it is attached to its cognate ACP. However, if it can be transferred to the next KS domain but then not processed further, due to lack of the substrate for the next chain extension step, it can now be released from the enzyme by an unknown mechanism.

5.3.3. The Unusual "Glycolate" Chain Extension Unit: In analogy to the substrates for the incorporation of acetate and propionate units, malonyl-CoA and methylmalonyl-CoA, one would predict the substrate for the incorporation of a "glycolate" extender unit to be 2-hydroxymalonyl-CoA or 2-methoxymalonyl-CoA. The latter alternative takes account of the fact that in most natural products containing such "glycolate" extender units the extra oxygen is methylated.[124—126,128,129,159] This prediction was tested by synthesizing [1-^{13}C]hydroxymalonyl-*N*-acetyl-cysteamine (SNAC) thioester and [1-^{13}C]methoxymalonyl-SNAC and feeding them to cultures of *A. pretiosum*. Surprisingly, no incorporation of ^{13}C into ansamitocin was observed. Likewise, neither compound complemented a mutant blocked in the endogenous synthesis of the substrate for the third chain extension step to restore ansamitocin P3 formation.[157] The SNAC esters are recognized as competent cell-permeable analogs of the corresponding CoA esters and their acceptance by type I PKSs is well documented.[160—162] This finding, therefore, argues against 2-hydroxy- or 2-methoxymalonyl-CoA as substrate for incorporation of the "glycolate" unit, although it does not rule out different thioesters of hydroxy- or methoxymalonate.

The *asm* cluster contains a set of five genes forming an operon, with homology to dehydrogenases (*asm13* and *15*), an ACP (*asm14*), an *O*-methyltransferase (*asm17*) and an unknown protein (*asm16*) (Fig. 19). A similar subcluster was found in the biosynthetic gene cluster for FK520, an antibiotic also containing "glycolate" units.[127] It was therefore hypothesized that this cluster might be responsible for synthesizing the substrate for the incorporation of the "glycolate" extender unit as a thioester on the ACP encoded by *asm14*. This notion was confirmed by inactivating each of these genes individually.[157,163] None of the five mutants produced any AP-3, and the mutant with inactive *asm14* produced no other ansamitocin-related compounds. In contrast, the mutants with inactive *asm13*, *asm15*, *asm16* or *asm17* produced small amounts of a new metabolite which was identified as 10-demethoxy-AP-3.[157] Notably, the *Δasm17* mutant did not produce any 10-demethyl-AP3.[163] These experiments demonstrated that each of the five genes was necessary for formation of the substrate for the oxygenated chain extension unit. In a second experiment it was shown that the five genes are also sufficient for the formation of this chain extension substrate by constructing a *Streptomyces* vector for the expression of a cassette of these five genes under the control of the *actI*/*actIII* promoter.[164] In collaboration with Dr. Peter Revill at Kosan Biosciences, this cassette was co-expressed with a vector expressing *eryABC*, encoding the entire DEBS PKS, in which the methylmalonate-specific AT6 domain had been replaced by the presumably hydroxymalonate-specifying AT8 domain from the *fkb* PKS.[165] Expression of the latter vector alone resulted in the production of only 6-deoxyerythronolide B (6-DEB) and 2-desmethyl-6-DEB, whereas its co-expression with the methoxymalonate cassette abolished the formation of these metabolites and resulted in accumulation of a single new compound identified as 2-desmethyl-2-methoxy-6-DEB (Fig. 21).[164] This compound evidently results from the incorporation of 2-methoxymalonate in the last chain elongation step on the modified DEBS. No 2-desmethyl-2-hydroxy-6-DEB was detected in the fermentation. In a second experiment the methoxymalonate cassette was modified to delete most of the coding sequence for the *O*-methyltransferase, Asm17. The co-transformant of this vector with the modified DEBS gave the same product as the transformant expressing only the modified DEBS; no 2-desmethyl-2-hydroxy-6-DEB was detectable. Therefore, the five genes *asm13—17* are not only necessary but also sufficient to provide the substrate for the incorporation of the "glycolate" chain extension unit. The substrate for this chain extension step must be 2-methoxymalonyl-ACP rather than 2-hydroxymalonyl-ACP, and the cognate AT domain of the PKS can accept this substrate only when delivered as the ACP thioester, not the CoA thioester.[164] The ACP as delivery vehicle is evidently also necessary for the aberrant incorporation of a malonate unit in the mutants with inactivated *asm13*, *15*, *16* or *17*, since the *asm14* mutant, lacking this ACP, does not produce 10-demethoxy-AP-3.[163]

Based on a comparison of the *asm*[152] and *fkb*[127] methoxymalonate subclusters with the functionally corresponding

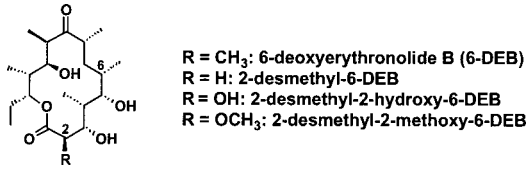


Fig. 21. Structures of 6-Deoxyerythronolide B (6-DEB) and Analogs

genes in the soraphen (*sor*)[166] gene cluster from the myxobacterium, *Sorangium cellulosum*, it has been suggested[164] that the unknown protein Asm16 must catalyze the activation of a carboxylic acid precursor of methoxymalonate which then is transferred to the ACP encoded by *asm14* and methylated by the Asm17 protein. The structure of this precursor carboxylic acid is not known. Its labeling by glucose and glycerol but not by a variety of other precursors suggests that it may be a phosphorylated compound, perhaps phosphoglycerate. Two dehydrogenation steps would then complete the formation of methoxymalonyl-ACP (Fig. 22).

5.3.4. Post-PKS Modification Reactions: The *asm* biosynthetic gene cluster I also contains a number of genes which, based on their sequence homologies, are candidates to encode all the post-PKS processing enzymes required for the conversion of proansamitocin into AP-3. These are two additional methyltransferases (*asm7* and *10*), a halogenase (*asm12*), a carbamoyl transferase (*asm21*), an acyltransferase (*asm19*) and an epoxidase (*asm11* or *30*).[152] Their functions and the order of their operation in the biosynthetic pathway were probed by individually inactivating each of these genes and observing the phenotypic changes. Some genes were also expressed in *E. coli* and the resulting proteins enzymatically characterized. From each mutant which no longer produced AP-3, the main new compound accumulated was isolated and its structure fully characterized by MS, MS/MS, 1D- and 2D-NMR; the structures of the additional compounds were inferred from MS and MS/MS data.[156,167] Overall, the results suggest that most of the downstream processing enzymes have relaxed substrate specificities, *i.e.*, many can work not only on their natural substrate but also, albeit less efficiently, on an analog in which one or more prior modifications have not taken place. Thus, most mutants accumulate a major compound, but in addition often one or more additional products resulting from aberrant processing of the initially accumulated compound. Conversion experiments with the accumulated compounds indicate that this represents a metabolic grid with several parallel routes to the end product. It is clear however, that there is a predominant pathway from proansamycin to ansamitocin P3, as shown in Fig. 23.

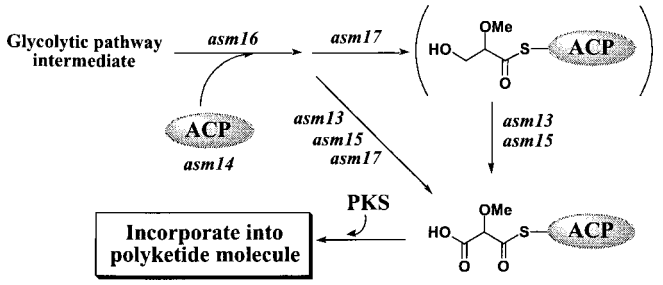


Fig. 22. Proposed Biosynthesis of Methoxymalonyl-ACP, the Precursor of the "Glycolate" Chain Extension Unit, Catalyzed by the *asm13—17* Gene Products

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The first step in the post-PKS pathway is the halogenation catalyzed by Asm12. The *Δasm12* mutant accumulates as its least substituted product proansamitocin. Its structure confirms that the *O*-methylation catalyzed by Asm17 must indeed occur during polyketide assembly, and also suggests that the same holds true for the migration of the double bonds. The spectrum of additional compounds detected shows that every one of the subsequent modifying reactions can take place in the absence of the chlorine. Similarly, the mutant blocked in the next enzyme, the carbamoyl transferase Asm21, produces 19-chloro-proansamitocin as the least substituted compound, but also accumulates compounds with additional modifications. Surprisingly, transfer of the ester side chain, catalyzed by Asm19, is not the last reaction in the biosynthesis, despite the fact that the alcohol maytansinol is a natural product both in plants and in *A. pretiosum*. This follows from the accumulation of *N*-desmethyl-desepoxymaytansinol by the *Δasm19* mutant and from bioconversion experiments with the *ΔasmB* mutant which cannot synthesize proansamitocin but contains all the post-PKS processing machinery. *N*-Desmethyl-desepoxymaytansinol, but not maytansinol itself, was efficiently converted into AP-3.[167] The presumed broad substrate range of the enzyme for

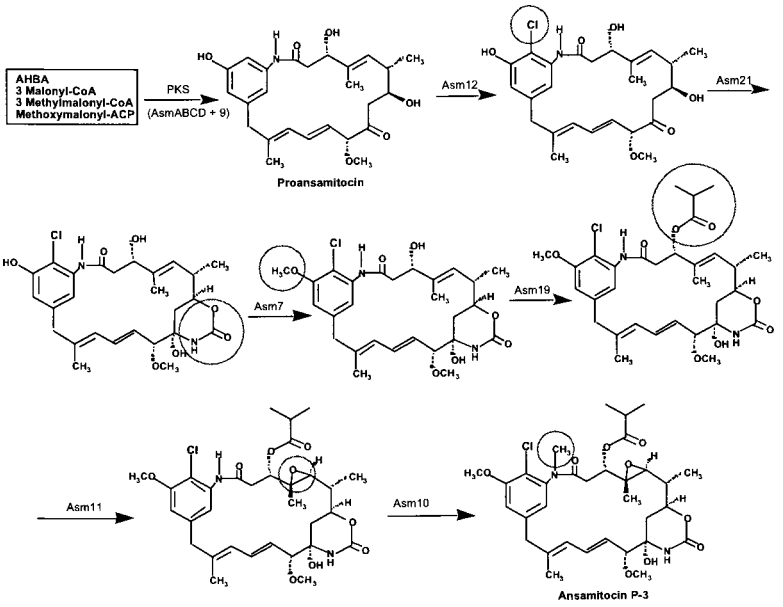

Fig. 23. Pathway of Post-PKS Processing of Proansamitocin to Ansamitocin P-3

its acyl substrate, inferred from the work of the Takeda group,[132] was confirmed by expressing the Asm19 protein in *E. coli*. The recombinant protein converted *N*-desmethyl-4,5-desepoxy-AP-3 with acetyl-CoA, propionyl-CoA, isobutyryl-CoA, *N*-butyryl-CoA or isovaleryl-CoA, respectively, or an equimolar mixture of all five, as cosubstrate into the respective 3-*O*-acylated ansamitocins. Both alone and in competition with the other four substrates, isovaleryl-CoA proved to be the best substrate, despite the fact that in the normal fermentation the isobutyryl compound AP-3 is a more prominent product than the isovaleryl compound AP-4. The product distribution therefore must be determined not only by the specificity of the acyltransferase but also by the supply of precursor.[168]

Inactivation of *asm10* resulted in the exclusive accumulation of *N*-desmethyl-AP3, indicating that this gene encodes the *N*-methyltransferase, which catalyzes the last step in the reaction sequence. In contrast, inactivation of *asm7* resulted in accumulation of both *O*-desmethyl-AP-3 and *N*,*O*-didesmethyl-AP3. Therefore, *asm7* encodes the 20-*O*-methyltransferase which must operate prior to the *N*-methyltransferase. Two genes were candidates to encode the epoxidizing enzyme, *asm30* which is homologous to fusion proteins between a cytochrome P450 and its associated electron carrier protein, and *asm11*, which is homologous to flavin-dependent oxygenases. Surprisingly, the deletion of a major part of *asm30* had no significant effect on AP-3 production, whereas inactivation of *asm11* resulted in the accumulation of *N*-desmethyl-desepoxy-AP-3. This has been confirmed by expression of *asm11* in *E. coli* and demonstration of epoxidation of *N*-desmethyl-desepoxy-AP-3 by the recombinant protein. Thus, *asm11* catalyzes the double bond epoxidation and this reaction takes place before the final *N*-methylation.[156] This work has thus identified all the genes involved in the post-PKS modifications leading to AP-3, setting the stage for their heterologous expression and full characterization of the resulting recombinant proteins. It has also generated a number of ansamitocin analogs carrying peripheral structural modifications.

5.4. The Prospects for Creating Targeted Modifications by Genetic Engineering The work described above has provided the genetic information and tools to manipulate the biosynthetic machinery in order to produce modified ansamitocin structures. Of particular interest, since they are difficult to achieve by chemical synthesis, are backbone modifications of the ansa ring system by targeted mutations of the *asm* PKS. Two principal approaches are being explored. One is the expression of all the *asm* genes, assembled into several cassettes, in a heterologous host, such as *S. coelicolor* or *S. lividans*. This approach is the most promising in terms of elucidating the detailed biosynthetic process, but it has some major practical drawbacks, not the least of which is that, even if it is successful, the titers of product are likely to be very low. The alternative approach is to engineer mutations in the *asm* PKS in the parent producer organism. This has the advantage that all the regulatory elements and resistance mechanisms required for ansamitocin formation are present and functional. Obviously, one cannot predict with certainty whether the downstream modules of the PKS or the post-PKS modifying enzymes can handle structural changes in the polyketide being assembled. However, the demonstrated relaxed substrate specificity of many of the post-PKS enzymes and the fact that the *asm13* and *asm15—17* mutants produced 10-demethoxy-AP-3 encourage hope for the success of this approach. Since the metabolite flux through an engineered type I PKS is evidently highly dependent on maintaining the precise protein structure and domain interactions,[168,169] wherever feasible, mutations in the PKS should be introduced by site-specific mutagenesis, using the riskier domain-swap approach only where necessary.

6. Evolutionary and Ecological Aspects

6.1. Role of Plants: Lateral Gene Transfer or Plant-Microbe Interaction Among higher plants the orders *Celastrales*, *Euphorbiales* and *Rhamnales* represent a developmental line within the class of *Rosopsida*. The three orders are not only characterized by morphological but also chemotaxonomic features: Benzylisoquinoline alkaloids and alkaloids with a polypeptide skeleton are present and support the view of a close taxonomic link between these orders.[170] This view may be strengthened by the observation that members of the same orders are the only known higher plant taxa to contain representatives of the maytansinoids. One particular maytansinoid, *i.e.*, maytansine itself, occurs in *Celastrales* only and thus would even be a suitable character to distinguish *Celastrales* from *Euphorbiales* and *Rhamnales*. It may be questionable, however, to use the occurrence of maytansinoids within the *Rosopsida* as chemotaxonomic marker because a prerequisite for the use of any kind of taxonomic character is that it should be confined to certain taxa. Maytansinoids, however, are not only constituents of the three orders mentioned above[8,9] but occur also in taxonomically unrelated taxa such as a gram positive bacterium (*Actinosynnema pretiosum*)[5] and even mosses (*Claopodium crispifolium* (Hook) Ren. & Card, *Anomodon attenuatus* (Hedw.) Hueb,[12] *Isothecium subdiversiforme* Broth. and *Thamnobrium sandei* (Besch.) Iwatsuki[13]).

The occurrence of maytansinoids in unrelated taxa could be explained by the assumption that the biosynthesis of these remarkable natural products has been repeatedly invented during evolution. However, the basic ansa skeleton including the configuration of four to eight stereo centers of the maytansinoids is identical for all known maytansinoids (except for 10-epitrewiasine).[9] This and the fact that approximately 48 genes are involved in the bacterial biosynthesis of maytansinoids[152] makes it highly unlikely that maytansinoids have been repeatedly invented during evolution.

One could assume, however, that genes for maytansinoid biosynthesis were introduced into mosses and higher plants during a horizontal (lateral) gene transfer with a bacterium being the donor and an ancestral plant functioning as an acceptor. An alternative assumption would be that mosses and higher plants acquired the ability to harbour symbiotic microorganisms capable of producing maytansinoids. The latter assumption would imply that the orders *Celastrales*, *Euphorbiales* and *Rhamnales* as well as some mosses are not characterized by their ability to synthesize maytansinoids but rather by their ability to harbour a symbiotic maytansinoid-producing microorganism.

Ansa macrolactams in general have been frequently isolated from bacteria.[4] Hence, a maytansinoid-producing bacterium might live in close association with a plant. Challenge

of such a symbiotic community by a pathogenic fungus could result in the formation of maytansinoids which exhibit a cytostatic activity against eucaryotes and thus would be able to ward off pathogenic fungi. Such a model would not be without precedent as it is known that *Streptomyces hygroscopicus* var. *geldanus*, a bacterium that produces the ansamycin antibiotic geldanamycin is able to protect a pea plant (*Pisum sativum* L.) from fungal attack by *Rhizoctonia solani*.[171] A symbiotic association between a bacterium and mosses or higher plants raises the question as to the identity of such a microorganism and invokes a possible microorganism/plant coevolution.

6.2. Evidence against Production of Maytansinoids by Plants The plausibility of the assumption that a microorganism is responsible for the accumulation of maytansinoids in higher plants should not lead to the conclusion that the host plants are unable to synthesize maytansinoids. Taxol (paclitaxel) a natural product used in cancer therapy is an example to the contrary. This important chemotherapeutic compound was first isolated from the bark of *Taxus brevifolia* NUTT.[172] It was later concluded that an endophytic fungus named *Taxomyces andreanae* isolated from the bark of *Taxus brevifolia* is capable of taxol production.[173] Similar observations were reported later: *Taxus wallichiana* ZUCC. seems to harbour three endophytic taxol-producing fungi named *Sporormia minima*, *Trichothecium* sp. and an unidentified fungal isolate.[174] Also, from *Taxus mairei* (LEMEE & LEVEILLE) S. YHU a *Tubercularia* strain was isolated which is a taxol producer[175] and *Taxus baccata* L. and *Taxus brevifolia* are the source of 150 fungal isolates, 10% of which produce taxanes.[176] In addition, a taxol-producing fungus, *Pestalotia heterocormis*, was even isolated from the soil of a *Taxus* forest.[177] In every case the taxol production was low, ranging from ng to μg per liter of culture.

There is convincing evidence, however, that taxol is also a product of *Taxus* plants. Thus, cell suspension cultures raised from *Taxus* plants were shown to produce taxol.[178—181] This observation is significant because cell suspension cultures are believed to be sterile and formation of taxol should therefore be a genuine feature of *Taxus* cells. This conclusion is supported by the work of Croteau and his associates who isolated genes responsible for taxol biosynthesis from a *Taxus* plant. Interestingly, the taxadiene synthase gene has a long N-terminal targeting sequence for localization to and processing in the plastids indicating that this gene is plant-derived rather than a fungal product.[182]

All evidence seems to point to the fact that the situation with maytansinoids is different while the hypothesis that maytansinoids are produced by associated microorganisms is plausible. No evidence has been found that cultured plant cells themselves produce maytansinoids: Maytansine was neither found in cell suspension cultures grown in various media from a *Maytenus buchananii* (LOES.) R. WILCZEK plant,[183] nor in a callus culture raised from *Maytenus wallichiana* R. et B.[184] nor in a callus culture derived from *Putterlickia verrucosa*.[82] This is in line with the result of an extensive search for the AHBA synthase gene in a *Putterlickia verrucosa* (E. MEYER ex SONDER) SZYSZYL. cell culture.[82] This unique gene is involved in ansamycin, including maytansinoid, biosynthesis (see sections 5.2 and 5.3.1). An extensive PCR based homology screen using oligonucleotides adjusted to the *Putterlickia verrucosa* codon usage gave negative results only.

There is a third observation which points to the conclusion that plants do not produce maytansinoids *ab initio*. Chemical analysis of individual *Putterlickia verrucosa*, *Putterlickia retrospinosa* van WYK and MOSTERT and *Putterlickia pyracantha* (L.) SZYSZYL. plants collected from their natural habitats in South Africa showed that some individual plants contained maytansinoids whereas in others maytansinoids did not seem to be present.[82] This was also experienced by the late Dr. Morris Kupchan and his associates.[82] It is also in line with results reported on the occurrence of maytansinoids in the moss *Claopodium crispifolium*.[185] A large variation in antitumor activity of plant extracts against a lymphocytic leukemia and a KB cell culture was assumed to indicate that not the plant but rather some kind of "infective organism" was responsible for the presence of maytansinoids in mosses and that the presence of maytansinoids may not be the result of the biosynthetic capacity of the moss itself.

6.3. Search for a Plant-Associated Microbial Producer of Maytansinoids Conclusive evidence for the presence in higher plants of a maytansinoid-producing microorganism can only be obtained by tracing the organism (or organisms) responsible for maytansinoid accumulation in plants. At first, fungal endophytes were isolated from individual plants known to contain maytansine. The host plants belonged to *Putterlickia verrucosa*, *Putterlickia pyracantha* and *Putterlickia retrospinosa*. The fungi were identified and the isolates assigned to the following genera (given in parenthesis are the number of identified species): *Alternaria* [2], *Aspergilllus* [1], *Aureobasidium* [2], *Cladosporium* [1], *Coleophoma* [1], *Colletotrichum* [1], *Macrophoma* [1], *Melanconium* [1], *Penicillium* [2], *Pestalotiopsis* [3], *Phialophora* [1] *Phoma* [2], *Phomopsis* [15] *Rhizoctonia* [1] and *Xylaria* [1]. Thus, a total of 35 fungal species was isolated and checked for the presence of maytansinoid compounds using a maytansine-sensitive strain of *Penicillium avellaneum*. No indication for the presence of maytansine was obtained, however.[186]

Subsequently, the bacterial community of the *Putterlickia verrucosa* and *Putterlickia retrospinosa* plants was investigated. Bacterial isolates obtained from the wood, leaves and the rhizosphere were checked by PCR for the presence of an AHBAsynthase gene. One isolate (F18—98) from the rhizosphere was found to carry this gene. Overexpression and biochemical analysis showed that it encodes a functionally active AHBAsynthase (*asm24*, *43* homologue). This gene was associated with genes showing high homology to those required for ansa macrolactam biosynthesis: Kinase (*asm22*), aminoDHQ dehydratase (*asm23*), oxidoreductase (*asm44*), phosphatase (*asm45*). Indications for the presence of a polyketide synthase (*asmA—D*) 3-*O*-acyltransferase (*asm19*), aminoDHQ synthase (*asm47*) and aminoDAHP-synthase) were also obtained by Southern blot analysis.[187]

The strain F18—98 was properly described, found to belong to the genus *Kitasatospora* and named *Kitasatospora putterlickiae*.[188] Incubation of this strain with a sample of [carboxy-^{14}C]-AHBA gave three labeled compounds, one of which showed antibiotic activity against *Penicillium avellaneum*. None of these three compounds, however, is identical with maytansine. Thus, the enigma of how maytansine accu-

mulates in *Putterlickia* plants is still unresolved at present. Progress in this field has to await the structure elucidation and investigation of the metabolism of these compounds in *Putterlickia* plants.

6.4. Plant Contribution to the Biosynthesis Although the available evidence favors production of the core structure of the plant maytansinoids by an associated microorganism, an active role of the plant in the overall biosynthesis cannot be excluded and, in fact, seems likely. This is suggested by the finding that each maytansinoid-carrying plant family produces unique structures. *Colubrina texensis* (*Rhamnaceae*) produces 15-oxygenated maytansinoids, which are absent from the plants in the other two families, and *Trewia nudiflora* (*Euphorbiaceae*) produces compounds with unique bicyclic ester moieties, which are rarely[10] seen in the other two families. Furthermore, maytansine and similar compounds with a more complex side chain are not produced by *A. pretiosum* and, unlike the simple ester moieties of the ansamitocins, the ester side chain of maytansine cannot be attached to the ansamycin backbone by the acyltransferase (Asm19) of *A. pretiosum*.[189] It seems likely, therefore, that the plant itself participates in the biosynthesis of maytansine and other plant-derived maytansinoids by converting a bacterially synthesized precursor into the final biologically active compound. In this process, the plants in the three families exhibit their different unique chemical capabilities.

Secondly, it is possible that maytansine is only produced as a consequence of a pathogen attack on the plant. The plants may contain a biologically inactive bacterially produced precursor, which is only converted into the biologically active final product in response to a signal resulting from the pathogen attack. Alternatively, and more plausibly, the bacterial production of the maytansinoid precursor could be triggered by a plant signal in response to the pathogen attack. This would explain why certain individual *Putterlickia* plants do contain maytansinoids whereas others do not seem to contain bioactive compounds.

7. Conclusion

Can the Clinical Potential of the Maytansinoid Antitumor Agents be Resurrected? The fact that despite high *in vitro* and animal *in vivo* potency, maytansine proved to be ineffective as an antitumor agent in phase II clinical trials in humans, is rather surprising. One may assume that the clinical trials with maytansine failed due to dose-limiting toxicity. Since the compound was effective in animals, the apparently higher toxicity in humans may be due to differences in metabolism. Comparison of the metabolism of maytansine in animals *vs*. man may thus reveal sites in the molecule which may be modified in order to reduce human toxicity. A first step in this direction has been taken with the preliminary metabolism studies reported in section 4.1. In addition, it may be possible to design molecules which combine the pharmacophoric features of maytansine with those of the clinically interesting geldanamycin-derivatives, 17-AAG and 17-DMAG, potent inhibitors of heat shock protein 90 (HSP-90),[190—194] to give antitumor agents with a dual mode of action. Target compounds may be prepared by semi-synthesis from available natural products or by genetically engineered biosynthesis, based on the knowledge and tools made available by the biosynthetic studies reported in section 5. Importantly, the therapeutic potential of newly generated compounds has to be evaluated early by not only determining their potency in representative antitumor assays, but also their metabolic, pharmacokinetic and toxicological profiles in a battery of *in vitro* tests. Maytansine analogs with reduced toxicity in humans but high antineoplastic activity should be promising clinical anticancer drug candidates indeed.

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