Chapter Four

MACROLIDE AND POLYETHER POLYKETIDES
Biosynthesis and Molecular Diversity

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INTRODUCTION

Secondary Metabolites in Medicinal and Agricultural Chemistry

The healing and killing power of secondary metabolites has been explored by humans since prehistoric times. The very term "Pharmacology" was coined from the Greek word "Pharmakon" having the double meaning of both "Potion" and "Poison". Folklore references to such effects deal almost exclusively with plant extracts. Classic examples include the alkaloid metabolites atropine (**1**) and scopolamine (**2**) from *Atropa belladonna* (deadly nightshade), which are known to induce strong hallucinations, delirium, paralysis, and eventual death. Today, atropine is routinely used in ophthalmology for the dilation of the pupils during eye examinations, whereas scopolamine is used in the treatment of dizziness, gastrointestinal spasm, and motion sickness. Another striking example is that of tubocurarine (**3**), a metabolite of the Brazilian vine *Chondodendron tomentosum* and the key ingredient in *curare,* the extract used by South American Indians to poison their arrows. In modern medicine, tubocurarine is a useful clinical drug employed

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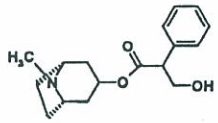
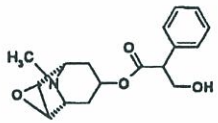
as a muscle relaxant during surgery.

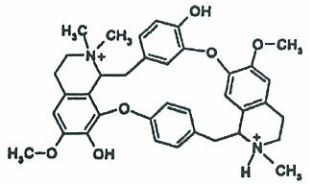
The existence of microorganisms was recognized only in the 17th century when the Dutch microscopist Anton van Leeuwenhoek turned a prototype microscope to the examination of water and decaying matter. However, the tremendous value of microbial metabolites was not realized until much after the



Atropine (1)

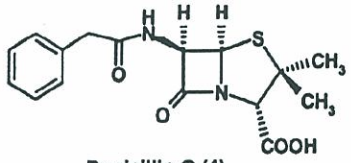


Scopolamine (2)



Tubocurarine (3)

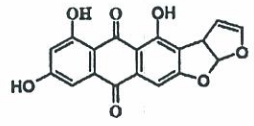
antagonistic effects of *Penicillium notatum* against *Staphylococcus aureus* had been recognized by Fleming and the isolation of penicillin G (**4**) had been achieved. This discovery launched a new era in medicine and the chemistry of natural products. In modern times, metabolites isolated from cultures of bacteria, fungi, or marine microorganisms play a pivotal role in the discovery of therapeutic agents. Recent reports indicate that secondary metabolites constitute approximately 60% of all the antitumor and antibiotic drugs on the market, as well as a significant portion of the new compounds undergoing clinical testing or development.[1]



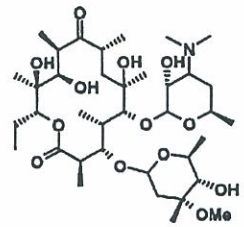
Penicillin G (4)

Polyketides constitute a special class of secondary metabolites, produced primarily by microorganisms and used in agriculture, food sciences, and especially as medicine for both humans and animals. Although these metabolites exhibit remarkable structural diversity, their biogenesis *in vivo* involves, primarily, the

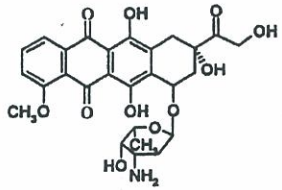
successive condensation of simple building blocks such as acetate, propionate, and butyrate. Examples of biologically important microbial poiyketides include the fungal aflatoxins, *e.g.* versicolorin A (**5**),[2] as well as numerous metabolites of filamentous bacteria, which include the antibiotics erythromycin A (**6**) and doxorubicin (**7**),[3,4] and the immunosuppressants FK506 (**8**),[5,6] and rapamycin (**9**).[7]



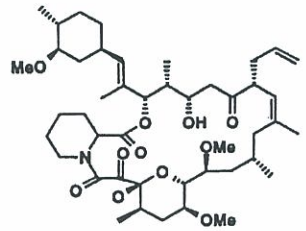
Versicolorin A (5)



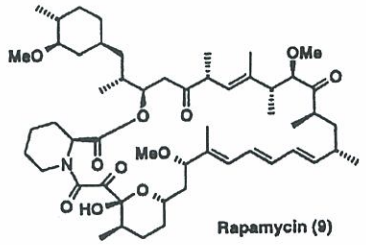
Erythromycin A (6)



Doxorubicin (7)



FK506 (8)



Rapamycin (9)

As with the plant alkaloids **1-3**, toxicity is also common among most clinically useful polyketides, thus compromising their therapeutic value. For example, the third most widely used class of antibiotics worldwide, the

erythromycins (*e.g.* metabolite **6**) are often associated with serious gastrointestinal disorders. Similarly, the clinical usefulness of doxorubicin (**7**) is limited by its powerful cardiotoxic, myelosuppresive, and DNA damaging effects.

In this millennium, the key challenges for natural product chemists will be: (a) to fully understand Nature's catalytic processes that are involved in the biogenesis of complex structures, such as those shown above (metabolites **5-9**), and (b) to selectively redesign or reprogram the biosynthetic pathways allowing for the production of new "unnatural" natural products with exquisite efficiency and selectivity.

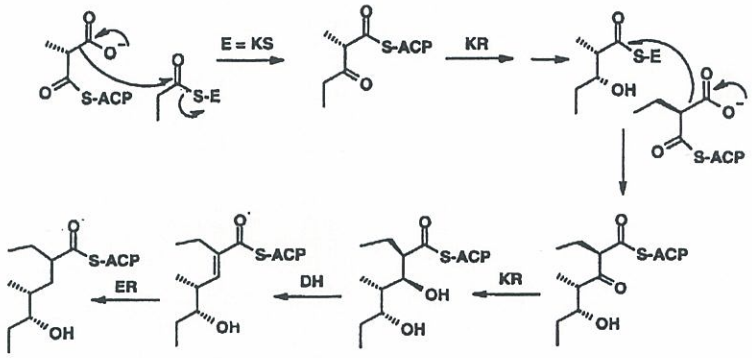
It is evident that by increasing structural diversity we will improve the chances of discovering new compounds with beneficial biological properties and without undesirable side effects. In the last few years, a number of achievements in this area have provided strong evidence that this goal is realistic and have sparked intense enthusiasm among scientists in the field of natural product biosynthesis and genetics. The main purpose of this chapter is to highlight some examples from this hybrid field of chemistry and biology that are relevant to the author's own research interests. However, it is not intended to be a thorough review of the field; a number of excellent reviews on this topic were published in 1997 as a collective volume in *Chemical Reviews* (volume 97, issue number 7).

BIOSYNTHESIS OF MICROBIAL MACROLIDE AND POLYETHER POLYKETIDES

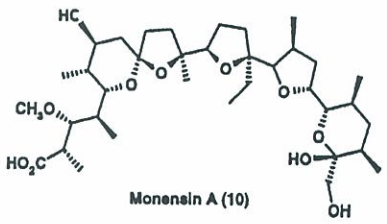
Biosynthesis, Enzymology, and Genetics

Over the last ten years, extensive investigations into the biosynthesis of microbial polyketides have demonstrated that their formation is catalyzed by a class of enzymes collectively known as the polyketide synthases (PKSs). These enzymes share many mechanistic similarities with those associated with the carbon backbone assembly of fatty acids. Biosynthetic studies and molecular genetics have demonstrated that PKSs catalyze the synthesis of oligoketides from primary precursors *via* repeated decarboxylative Claisen condesnation, analogous to the mechanism of fatty acid synthases (FASs) (Scheme 1).[8,9] However, several catalytic variables are unique to PKS enzymes, such as the choice of starter unit, extender units at each step, control of reductive steps on the ß-keto group of the growing carbon chain, as well as control of stereochemistry of the alkyl substituents (Scheme 1).

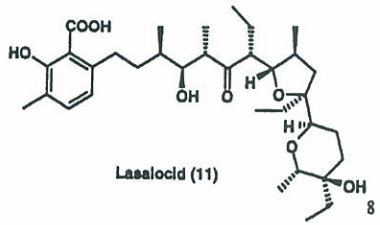
Scheme 1: Key catalytic steps in carbon chain elongation of macrolide and polyether polyketides.



Successful cloning and sequencing of the genes for a variety of PKSs has established the primary protein structures of synthase enzymes from a number of different sources. For example, PKSs have been found that resemble the classical FASs type I, characteristic of fungi and vertebrates, in which all of the catalytic sites, acyl transferase (AT), ketosynthase (KS), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) are present as domains along the length of a multifunctional protein. PKS type II enzymes, characteristic of bacterial and plant metabolites, have also been identified. The latter systems are analogous to the type II FASs in which each catalytic site is carried on a separate protein subunit. A number of gene clusters associated with PKS type II enzymes have already been studied. These catalyze primarily the biosynthesis of aromatic polyketides, such as doxorubicin (**7**),[3,4] tetracenomycin C,[10,11] actinorhodin,[12] and frenolicin.[13] However, detailed discussion of the biosynthesis and enzymology of aromatic polyketides is beyond the scope of this article.



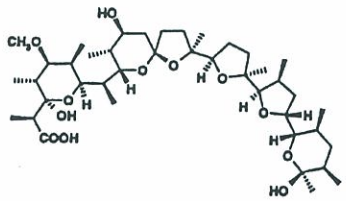
Monensin A (10)



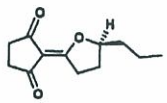
Lasalocid (11)

In this chapter, we focus on the PKS type I enzymes that are associated with the biosynthesis of macrolides, such as erythromycin A (**6**),[14-16] niddamycin,[17] FR-008,[18] FK506 (**8**),[19,20] rapamycin (**9**),[21] polyether polyketides, such as monensin A (**10**), lasalocid (**11**), mutalomycin (**12**), oudenone (**13a**, **13b**), and the family of marine toxins typified by brevetoxin A (**14**).

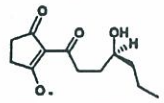
Erythromycin A (**6**), a metabolite of the mycelia-forming bacterium



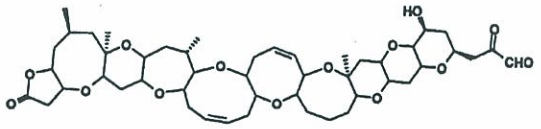
Mutalomycin (12)



Oudenone (13a)

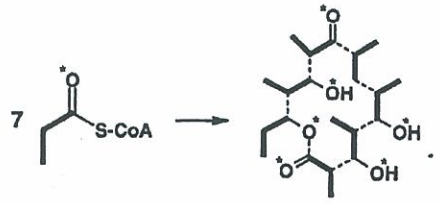


Oudenone (13b)



Brevetoxin A (14)

Scheme 2: Incorporation of ^{13}C, ^{18}O-labeled propionate into 6-dEB (**15**).

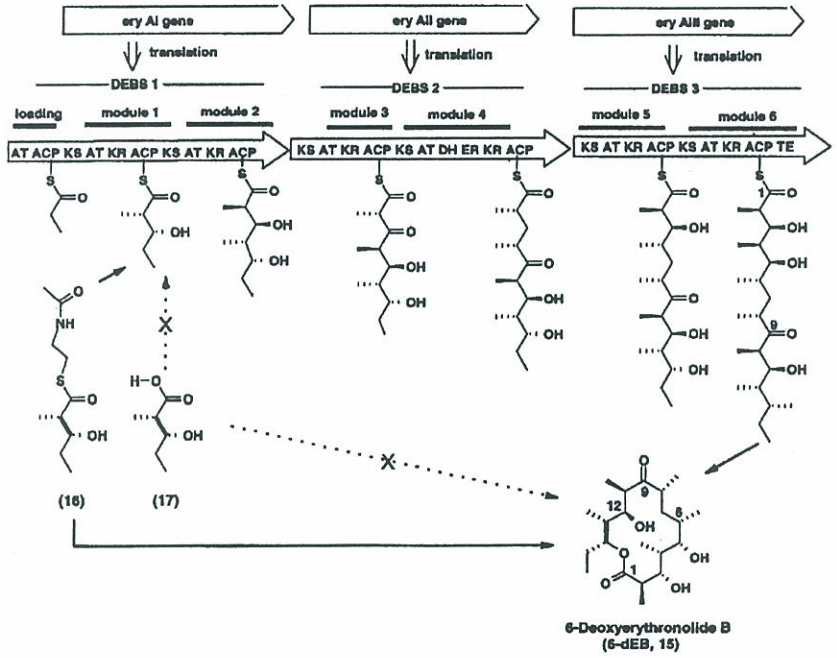


6-dEB (15)

Saccharopolyspora erythraea, is one of the most widely used clinical antibiotics against Gram-positive bacteria. It is also used for many pulmonary infections such as Legionnaire's disease and as an alternative treatment for patients allergic to b-lactam antibiotics (*e.g.* penicillins). Over the last two decades, the biosynthetic origin of this structurally complex molecule has been the subject of numerous studies.[22-24] The 14-membered macrolactone core of erythromycin, 6-deoxyerythronolide B (6-dEB, **15**), is derived from one propionyl CoA starter unit and six methylmalonyl CoA extender units (Scheme 2). Furthermore, all of the oxygen atoms of 6-dEB originate from propionate and not molecular oxygen or water.[25]

The polymerization mechanism leading to the formation of the carbon backbone involves a condensation step of each propionate unit (activated as a methylmalonate unit), which is followed by a reduction to give the required oxidation state and absolute stereochemistry, before the addition of the next propionate unit (Scheme 3). Strong evidence in support of the mechanism outlined in Scheme 3 was first provided by Cane's group in a pioneering experiment showing that an *N*-acetylcysteamine (NAC) thioester derivative of the advanced intermediate diketide **16** could be taken-up by the enzymatic machinery and incorporated *intact* into the final metabolite erythromycin *via* the key intermediate 6-dEB (Scheme 3).[26,27] It is worth noting that the incorporation of the intact carboxylic acid **17** into erythromycin could not be achieved, due to the efficient *in vivo* degradation of fatty acids by β-oxidation. However, NAC thioester derivatives are known to be far better substrates for PKS enzymes than their corresponding carboxylic acids, most likely because they exhibit a high degree of structural homology with the 4'-phosphopantetheine sidearm of the active acyl carrier protein (ACP) of the PKSs, as well as that of coenzyme A.[28,29]

Scheme 3: Model for the biosynthesis of 6-dEB (**15**) catalyzed by the complete 6-deoxyerytheronolide B synthase (three multifuntion proteints: DEB1, 2, and 3)



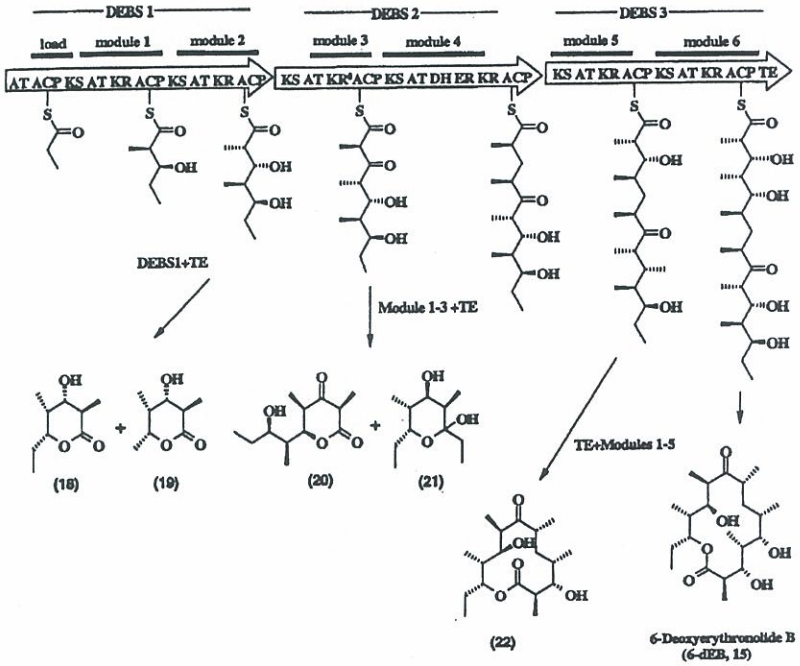
In spite of numerous earlier attempts to isolate the fully active forms of the PKS enzymes involved in the construction of the 6-dEB macrolide (**15**) from *S. erythraea,* it was only in 1995 that Khosla and co-workers reported the first method for the preparation of a recombinant 6-deoxyerythronolide B synthase (DEBS 1, 2, and 3).[30] This enzyme preparation exhibited complete modular function, possessing at least 28 distinct active sites, and was subsequently used in the first cell-free enzymatic synthesis of 6-dEB (**15**).[30]

The availability of this cell-free system allowed the *in vitro* synthesis of 6-dEB (**15**), as well as the synthesis of several smaller lactones (compounds **18-22**) from truncated DEBS mutants (Scheme 4).[31] In addition to altering the macrolide ring size, modifications of its functional groups became possible through

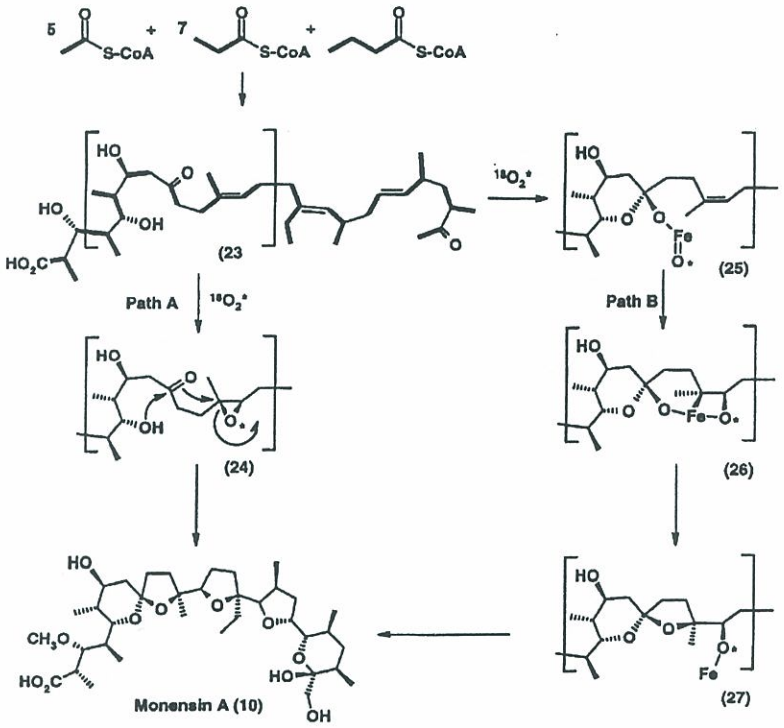
reprogramming of the polyketide biosynthetic pathway. Multiple genetic modifications of this system, in a highly selective fashion, have led to the design of mutants that produce novel "unnatural" natural products. These studies have also expanded our knowledge of microbial genetics and enriched the structural diversity of metabolites with potential pharmaceutical or agrochemical value.[32,33]

Although it is generally believed that the biosynthetic steps associated with the skeletal construction of polyether antibiotics are also catalyzed by modular, multifunctional PKS type I enzymes (analogous to those described in the cases of 6-dEB, Scheme 3), little is known about the specific enzymes and genetics associated with this family of natural products. An idea that was initially proposed by Westley for the biogenesis of lasalocid (**11**),[34] and later modified by Cane *et al.* as a unified hypothesis for the biosynthesis of all polyether polyketides [*e.g.*, monesin A (**10**)], is

Scheme 4: Synthesis of novel "unnatural" natural lactones from DEBS mutants.



Scheme 5: Models for the biosynthesis of tetrahydrofuranyl and tetrahydropyranyl moieties in monesin A (**10**).

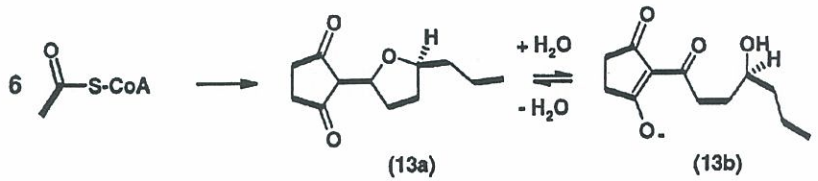


the "polyepoxide cascade" mechanism (Scheme 5, Path A).[35] In this three-step process, the open-chain oligoketide precursor **23** is formed from five acetate, seven propionate, and one butyrate unit *via* repeated decarboxylative Claisen condensations and subsequent modifications of the β-keto group. All of these steps are believed to be catalyzed by the multifunctional PKS type I enzyme(s). The second step is proposed to be an enzymatic polyepoxidation reaction of the acyclic hydroxypolyene precursor **23** to give intermediate **24**, followed by a cascade of intramolecular nucleophilic attacks on the keto and epoxide moieties of metabolite **24**, eventually leading to the formation of the tetrahydrofuran and tetrahydropyran rings of the final product **10** (Scheme 5, Path A).

More recently, the alternative mechanism of "*syn*-oxidative polycyclization" of the hydroxypolyene precursor **23** was proposed by Townsend *et al.* (Scheme 5, Path B).[36-38] In this model, an alkoxy-bound oxo metal derivative **25** is proposed to undergo a series of consecutive intramolecular [2+2] cycloadditions with the double bonds to give the corresponding metallaoxetane intermediate **26**, followed by reductive elimination of the metal and closure of the tetrahydrofuran or tetrahydropyran rings (Scheme 5, Path B).

Each of these models may be relevant in the biogenesis of polyether polyketides from different sources; nonetheless, both proposals remain unproven.

Scheme 6. Incorporation of ^{13}C-labeled acetate into oudenone (**13a** and **13b**)



This is mainly due to the chemical complexity and instability of the precursors and intermediates associated with the biosynthesis of such structurally complex molecules as monensin A (**10**) or brevetoxin A (**14**). Recently, we reported our on-going investigations into the biosynthesis and enzymology of the fungal metabolite oudenone (**13a** and **13b**),[39] a structurally unique hexaketide (Scheme 6) characterized by a tetrahydrofuran and a 1,3-cyclopentadione moiety (**13a**). The structure of oudenone in anhydrous organic solvents is that of **13a**. However, in aqueous solvents, **13a** is in dynamic equilibrium with the β-trione anion **13b** *via* the simple addition of water (pHa'=4.1).

We speculated that due to the small molecular structure of oudenone this metabolite could serve as a model for exploration into the enzymology and genetics associated with the biogenesis of tetrahydrofuranyl and tetrahydropyranyl moieties of polyether-type polyketides. We began our investigation by showing that the N-acetylcysteamine (NAC) thioester derivative of (5S)-5-hydroxyoctanoic acid (Scheme 7, NAC-derivative of intermediate **28**) can serve as a substrate of the PKS enzyme(s) catalyzing the biosynthesis of **13** in cultures of *Oudemansiella radicata*. Furthermore, the enzymatic incorporation of the deuterium-labeled NAC-derivative

of **28** into the tetrahydrofuran moiety of oudenone (**13a**) was achieved without any change in the absolute stereochemistry of the C9 chiral center. Subsequent studies allowed structure determination of the complete pre-cyclization polyketide precursor of oudenone. Based on our previous experimental data, two plausible hexaketide open-chain precursors were proposed, **29** and **31**, which could be supported by either the "polyepoxide cascade" or the "oxidative polycyclization" models, respectively (Scheme 7).

Recently, we reported the synthesis and successful *in vivo* transformation of the deuterium labeled, NAC-derivative of β-diketone **30** into oudenone (**13a**).[40] This result strongly suggests a biosynthetic mechanism analogous to the "polyepoxide cascade" model (Scheme 7, Path A) and confirms that the linear hexaketide α-diketone **30** is the open-chain precursor of oudenone (**13**). Thus, it is reasonable to assume that the cyclization of **30** proceeds *via* an intramolecular β-addition, followed by a Claisen-type intramolecular condensation and dehydration to give **13** (Scheme 6, Path A).

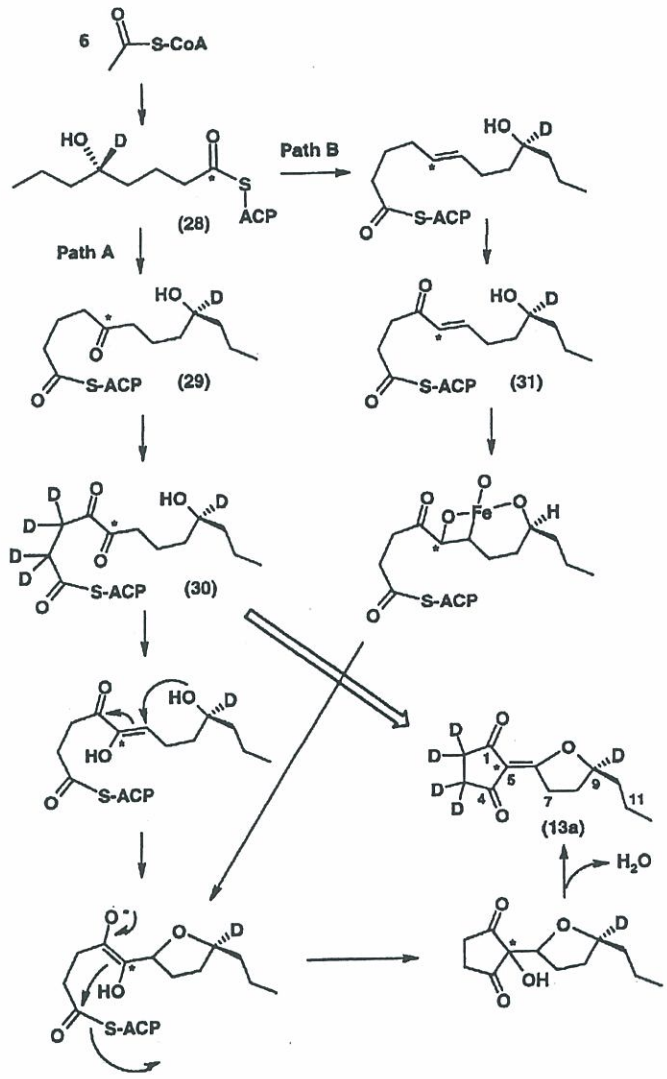
It should be noted that although the proposed mechanism for the biogenesis of oudenone (**13**) bears many similarities to the "polyepoxide cascade" model (Scheme 7), it is also reminiscent of the catalytic pathway leading to the formation of the 2-amino-3-hydroxycyclopent-2-enone, a unique structural moiety found in several antibiotics, including reductiomycin[41] and asukamycin.[42] A pyridoxal phosphate-dependent cyclization mechanism leading to the formation of this moiety was proposed by Floss in the biosynthesis of reductiomycin.[27]

Further studies on the genetics associated with the biosynthesis of metabolite **13** are currently in progress in our laboratory and hopefully will shed more light on the details of the enzymes and the catalytic mechanisms involved in the formation of oudenone (**13**).

SUMMARY

The biosynthesis of macrolides and polyether polyketides represent only one of the many remarkable examples of Nature's ability to synthesize complex compounds with exquisite efficiency and selectivity. It also presents natural product chemists and genetic engineers with a unique opportunity to harness the catalytic diversity and power of PKS enzymes in mutant microorganisms that can produce novel molecular structures of pharmaceutical or agricultural value. Furthermore, of the estimated 3 million species of bacteria and 1.5 million species of fungi believed to occupy our planet, only 0.1% of the bacteria and 5% of the fungi have been described. An important obstacle in identifying many of the yet unknown microorganisms is our inability to grow these species in the laboratory. However,

Scheme 7: Plausible hexaketide precursors and their cyclization to oudenone (**13a**)



it should be possible to isolate the chromosomal DNA of microorganisms from small samples (some researchers refer to this DNA as "environmental DNA"), and by using sequence homology of known PKSs we should be able to discover gene clusters encoding for new PKS enzymes. Expressing these genes in a heterologous host may subsequently lead to the discovery of new natural products. There is little doubt that a proliferation of studies on the rational manipulation of biosynthetic genes will occur over the next few years. The key results will be the rapid progress in our understanding of complex biosynthetic pathways and a complementary explosion in the molecular diversity of secondary metabolites, or "unnatural" natural products, that will be discovered.

ACKNOWLEDGMENTS

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