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The Use of Antibiotic-Producing Streptomycetes for Biotransformations

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1. Introduction

Streptomycetes are a genus of gram positive bacteria, well known for their ability to produce a vast range of structurally diverse natural products. At least some of these secondary metabolites are known to play a role in the physiology of the producing cells, while many others are commercially valuable products with antibiotic or related pharmacological activities (*e.g.* antifungal, antitumor, antiviral, antihelminthic, immunosuppressive). The starting materials for these impressive feats of synthesis are typically inexpensive fermentation ingredients such as soybean oil, soybean flour, and molasses, whereas the products often possess a high degree of structural and stereochemical complexity (see the *Formulae*). Over the past few years synergistic chemical and molecular genetic studies have led to a greatly increased knowledge of the enzymes active in antibiotic biosynthesis. This in turn has prompted interest in harnessing the synthetic potential of these enzymes for the rational design and production of novel or hybrid compounds through genetic manipulation of intact microorganisms, a part of what has been called 'metabolic engineering' [1].

Recent progress in this area is illustrated here by work on polyketide metabolites; molecules whose carbon backbones are built through the successive coupling of precursors derived from small fatty acids, most often acetic and propionic acids [2]. They vary greatly in size (*e.g.* 6-methylsalicylic acid and monazomycin), and are frequently subdivided into structural classes such as macrolides (*e.g.* erythromycin), polyethers (*e.g.* monensin), polyenes (*e.g.* amphotericin), and aromatic compounds (*e.g.* actinorhodin), to name but a few.

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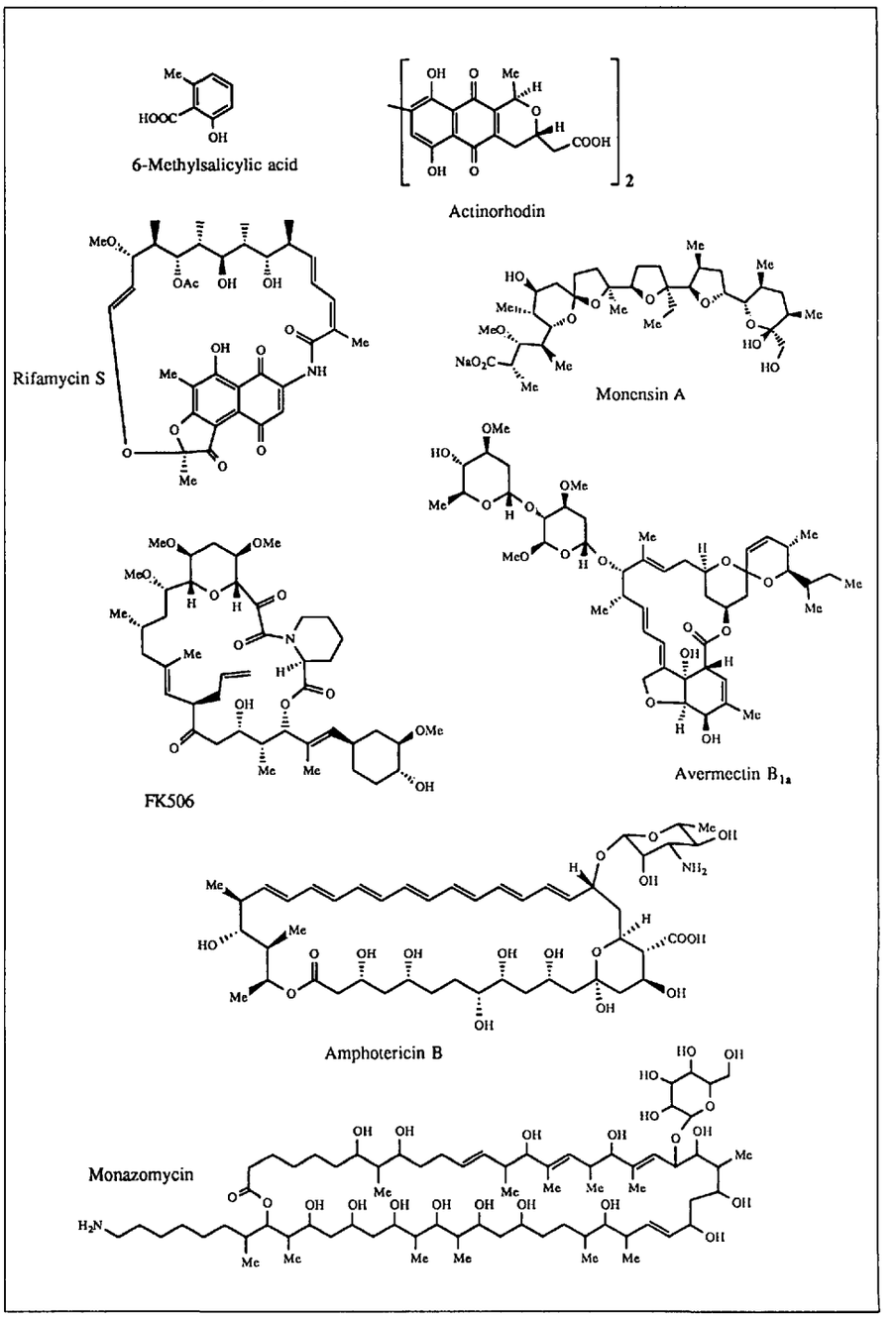
2. Fatty-Acid and Polyketide Biosynthesis

Birch and *Donovan* discussed in 1953 how a hypothetical poly(β-ketone), generated by the successive condensation of acetate units, might undergo well-precedented laboratory reactions, including aldol condensation, alkylation, reduction, dehydration, and oxidation, and thereby give rise to a large family of known polyketide metabolites [3][4]. Evidence

Polyketide Natural Products from Streptomycetes



accumulated since this time suggests that a close analogy exists between the mechanisms of long-chain fatty-acid synthesis, catalysed by fatty-acid synthases (FASs), and the assembly of polyketides, catalysed by polyketide synthases (PKSs), although very few PKSs have been characterized biochemically.

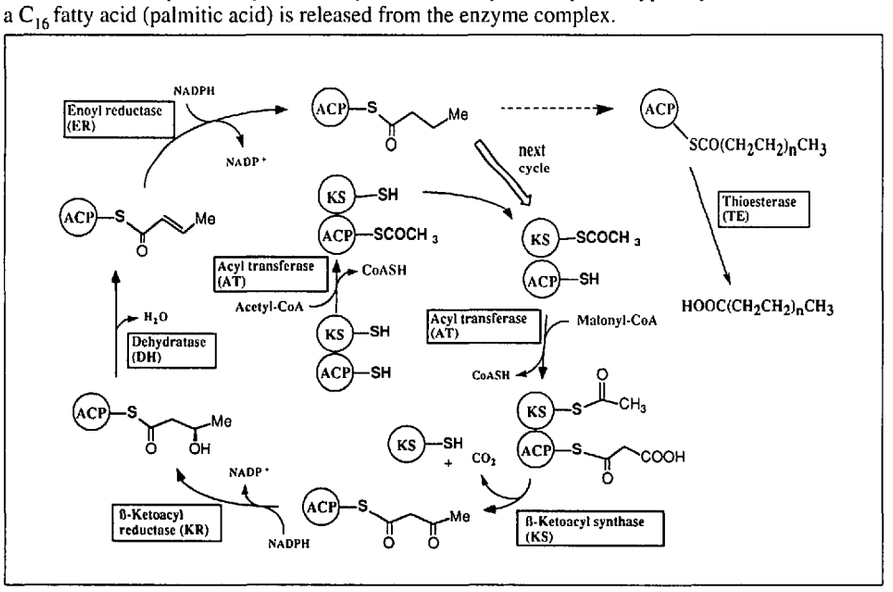
Although the same basic chemistry (*Scheme 1*) is followed during fatty-acid biosynthesis in all organisms, the FASs fall into two broad structural classes [5]. The type-I enzymes, typically found in higher organisms, are large multifunctional polypeptides that fold into catalytic domains, where each domain catalyses one or several consecutive steps in the FAS cycle. The type-II systems, characteristic of plants and bacteria, consist of several, relatively small, monofunctional proteins, each responsible for catalysing a single step in the pathway. In most cases, acetyl-CoA (sometimes other linear or branched chain acyl-CoA derivatives) is the starter unit, and malonyl-CoA is the extender unit. During the keto-reduction, dehydration, and enone-reduction, the growing fatty-acid chain remains attached through a thioester to the acyl carrier protein (ACP), a small protein of *ca.* 80 amino acids in the type-II systems, or a homologous ACP-domain in the type-I systems.

Whereas PKSs and FASs are believed to catalyse analogous reactions, by similar or identical mechanisms, the way these reactions are concerted, or 'programmed', by PKSs is different to that seen in fatty acid biosynthesis [6]. As depicted in *Scheme 2*: *1*) a variety of different starter units (linear or branched, aliphatic or aromatic carboxylic acids) may be used for polyketides; *2*) methylmalonyl-CoA and ethylmalonyl-CoA, as well as malonyl-CoA, are frequently used as extender units, and new chiral centres generated after the decarboxylative condensation may be either *R* or *S*, perhaps dependent upon whether (*S*)- or (*R*)-methyl(ethyl)malonyl-CoA is used as substrate; *3*) the extent of 'processing' (*i.e.* β-keto-thioester → β-hydroxy-thioester → α,β-unsaturated thioester → reduced acylthioester) may not be identical at each chain-elongation step, unlike fatty-acid synthesis where the same cycle of reactions is repeated several times [7]. In polyketide biosynthesis, new cycles may initiate with an acyl chain containing a β-keto- (*R*)- or (*S*)-β-hydroxy, (*E*)- or (*Z*)-ene, or fully reduced acyl chain; *4*) termination of the assembly process may involve thioester hydrolysis, or for example, macrolide ring formation, or amide-bond formation, all of which are believed to occur, while the acyl chain is still attached to the PKS. For those PKSs involved in aromatic polyketide biosynthesis, the cyclase, dehydratase, and aromatase activities presumably needed to catalyse aromatic-ring formation, from a polyketide precursor, may also constitute part of the PKS.

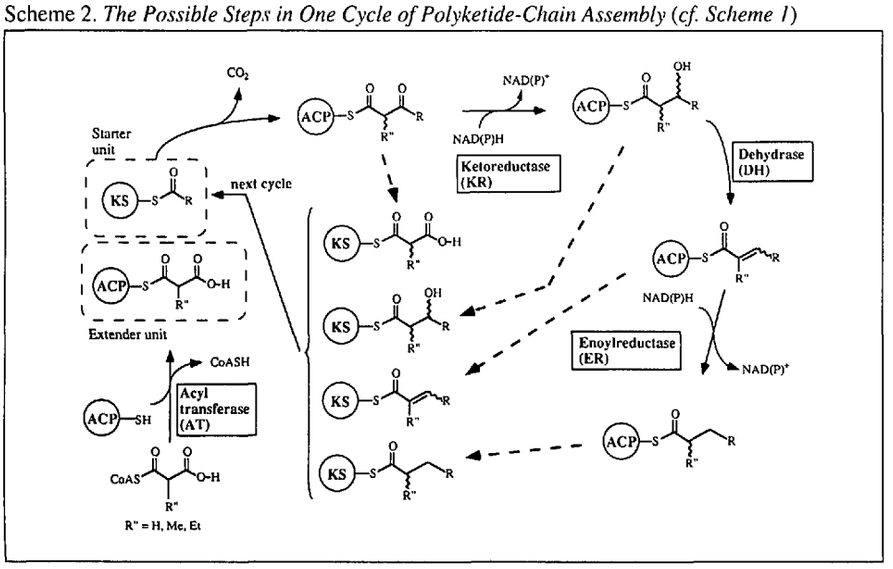
Thus, by using an appropriate combination of *1*) starter units, *2*) extender units, *3*) chain elongation processes, and *4*) termination strategies, an enormous variety of different polyketides can, in principle, be made. It seems clear from the enormous range of polyketide metabolites already discovered that Nature has indeed taken advantage of this potential for generating structural diversity. Important questions then arise: how are individual PKSs programmed to catalyse the synthesis of a unique polyketide chain? And how can this information be used rationally to allow the production of new metabolites or analogues of known products? Lastly, it should be remembered that biosynthetic steps that typically follow polyketide assembly (*e.g.* oxidation or glycosylation) amplify yet further the scope for generating novel structures.

The key to understanding and manipulating 'polyketide programming' lies in learning more about the structure and catalytic functions of individual PKS complexes. Unfortunately, biochemical studies with PKSs have been fraught with difficulties, principally because they are difficult to assay for catalytic activity, and are probably not very stable in crude cell extracts. Although 6-methylsalicylic acid synthase from *Penicillium patulum* [8][9], and a few PKSs of plant origin have been characterised [10], until very recently none had been isolated from polyketide producing streptomycetes. This bleak picture has changed dramatically, however, with the emergence of molecular-genetic techniques, which have come to play an impor-

Scheme 1. *The Steps in Fatty-Acid Biosynthesis.* The cycle is repeated typically seven times before a C_{16} fatty acid (palmitic acid) is released from the enzyme complex.



Scheme 2. *The Possible Steps in One Cycle of Polyketide-Chain Assembly* (*cf. Scheme 1*)



tant role in the cloning of genes encoding PKS complexes [6]. One feature to have emerged from such studies is that the biosynthetic genes for a given pathway tend to be clustered in one relatively small region of the genome of the producing organism. Once a method has been found to isolate one gene in a pathway, the others can usually be found easily in the adjacent DNA.

3. Re-programming Aromatic Polyketide Biosynthesis

The genetically best studied streptomycete is S. *coelicolor* A3(2), which produces the pigmented isochromanequinone antibiotic actinorhodin. A biosynthetic pathway to actinorhodin has been proposed, based upon results from labelling studies [11], the isolation of intermediates from mutants blocked at specific stages in the pathway [12], and more recently the analysis of the cloned biosynthetic genes [6][13] (*Scheme 3*). The biosynthetic genes (*act* cluster) span *ca.* 22 Kb of the *S. coelicolor* chromosome, and include *ca.* 20 open reading frames (ORFs = protein coding regions). The *act*I and *act*III genes (4 ORFs) encode the enzymes needed to couple eight acetate-derived precursors, and include a heterodimeric (ORF1 + ORF2) condensing enzyme (KS), an ACP (ORF3), and a dehydrogenase (*act*III) for reduction at the C(9) position [14]. The *act*VII gene product is a putative bifunctional cyclase/dehydratase [15], most likely catalysing aromatic-ring formation. When this gene is inactivated by mutation (*act*VII mutants), the compound mutactin is produced [16], apparently by aberrant (spontaneous?) cyclisation/dehydration. The next step, C(5)-C(14) dehydration is determined by *act*IV, followed by reduction at C(3) with pyran-ring formation, determined by the *act*VI genes. The *act*VI mutants produce the known natural product aloesaponarin II. The *act*VA genes (6 ORFs) determine oxidation at C(6) and C(8), and the *act*VB gene is responsible for dimerization to afford actinorhodin. The *act*VB mutants produce the known natural product kalafungin.

A number of closely related isochromanequinone antibiotics are produced by other streptomycetes, including medermycin and dihydrogranaticin (*Table*). When clones containing *act*VA were introduced into the medermycin-producer, a new pigmented antibiotic was produced, called mederrhodin A, most likely since the *act*VA encoded C(8)-hydroxylase from the actinorhodin pathway also used medermycin as substrate [17]. In a second experiment, the entire *act* cluster was introduced into the dihydrogranaticin-producer, which led to the production of the hybrid compound called dihydrogranatirhodin. This molecule has the dihydrogranaticin configuration at C(15) and the actinorhodin configuration at C(3), most likely as a result of the exchange of *act* and *gra*-encoded C(3) reductase/dehydratases. These pioneering experiments provided an important precedence for the transfer of biosynthetic genes between different antibiotic-producing organisms, with the resulting formation of novel hybrid compounds.

The primary sequences of PKSs active in actinorhodin (*act*) [14], tetracenomycin (*tcm*) [18], and granaticin (*gra*) [19] biosynthesis (amongst others) have been deduced from the gene sequences, and they form a highly homologous family of pro-

Scheme 3. *The Biosynthetic Pathway to Actinorhodin and the Actinorhodin Gene Cluster* (*act*). Several known or presumptive intermediates are shown together with the genes responsible for different steps of the pathway, *e.g. act*I and *act*III genes for the early steps, *act*VA and *act*VB genes for the late steps. At the top, the large arrows depict the protein-coding regions (ORFs) identified in the DNA sequence, which encode the biosynthetic enzymes. The arrows indicate the sizes of the genes (and gene products) and their relative direction of transcription/translation in the genomic DNA. The *act*II region includes ORFs which have a regulatory function.

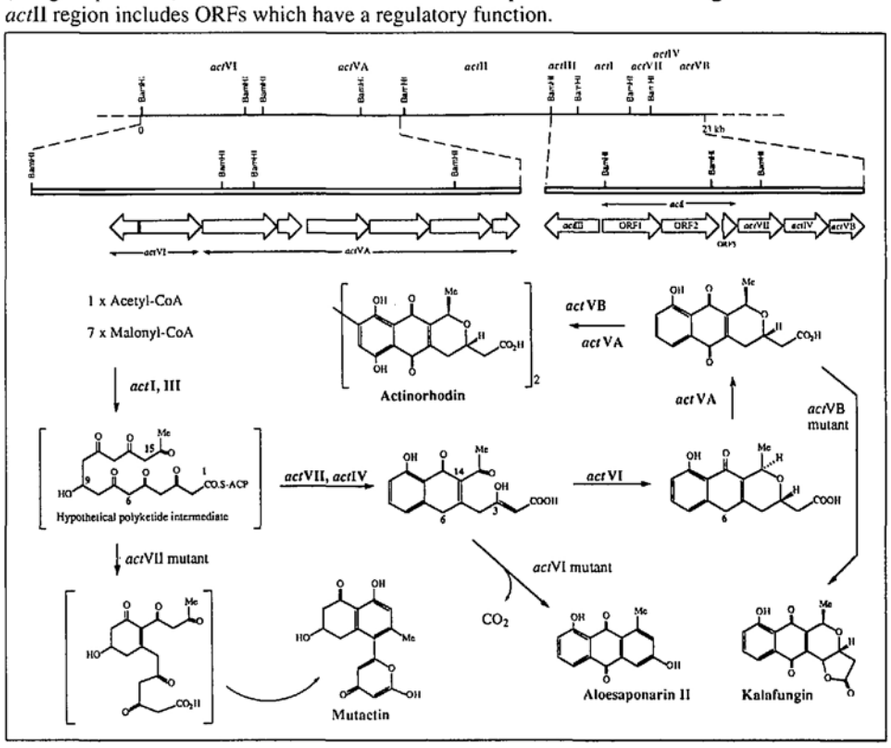
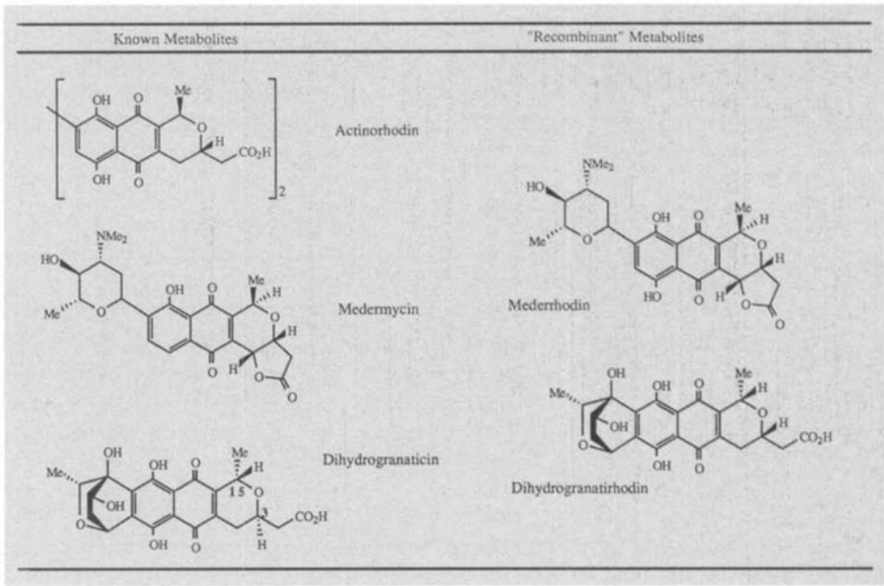


Table. *New Products Derived from Medermycin- and Dihydrogranaticin-Producers Containing Some or All of the Act Genes*



teins (*Fig.*) [6]. How the pattern of polyketide-chain folding is determined is not yet clear. In each case, the PKSs resemble the type-II bacterial and plant FASs, since each consists of relatively small discrete enzymes; a heterodimeric ketoacyl synthase (KS) encoded by ORFI and ORF2; an acyl carrier protein (ACP, ORF3); a ketoacylreductase (KR, ORF5) (absent in the *tcm* PKS), and a bifunctional cyclase/dehydratase (ORF4; cyclase/*O*-methyltransferase in the case of *tcm*). One way to explore the possible functions of such proteins is to substitute one for another, and monitor the effects upon metabolite production. This can be achieved either by examining the effects of introducing the new gene (on a plasmid) into a mutant strain where the counterpart has been inactivated, or by replacing the host gene directly with the new gene from a different pathway.

One interesting example involves various *S. galilaeus* strains that produce anthracyclinones (*Scheme 4*). The mutant strain 31671 produces 2-hydroxyaklavinone, rather than aclacinomycin A. Introduction of a plasmid carrying the KR gene from the actinorhodin pathway (*act*III) resulted in restoration of aklavinone production [20]. This indicates that the 2-hydroxy form results from a failure to reduce the polyketide chain at the ninth C-atom from the carboxyl terminus during synthesis, and that the KR involved in actinorhodin production can associate with the aklavinone PKS to reduce the chain precisely at this point. Also the aklavinone PKS is clearly able to assemble a complete polyketide despite the absence of a functional KR.

As indicated earlier (*Scheme 3*), the *act*I, *act*III, *act*VII, and *act*IV genes in *S. coelicolor* are sufficient for the production of aloesaponarin II. Introduction of a DNA fragment containing *act*I ORFs 1 and 2 (the KS) into wild type *S. galilaeus* resulted in aloesaponarin II production [20] (*Scheme 5*). The same fragment in mutant 31671 led to the production of the hydroxylated counterpart of aloesaponarin II, desoxyerythrolaccin. This indicates that the ACP, KR, cyclase, and dehydratase involved in aklavinone biosynthesis can interact with the ORF 1 and 2 proteins of the actinorhodin PKS, which alone control the length of the polyketide chain produced, and lead to aloesaponarin production. When the KR function is lost, the corresponding hydroxylated product is seen.

4. Re-programming Complex Polyketide Biosynthesis

The so-called complex polyketides are those whose C skeletons have undergone reductive and dehydrative processes during assembly (*Scheme 2*) so poly(β-ketone) derivatives are not formed, and the C backbone typically contains ketone, alcohol, and alkene functionality. The stereochemistry and appropriate degree of processing of the β-carbonyl groups in the growing chain are adjusted prior to subsequent elongation cycles. Included amongst these are the macrolide antibiotics (erythromycin A and tylosin) and the polyethers (monensin and nonactin). Evidence that the processive strategy of polyketide assembly occurs during macrolide biosynthesis has accrued from two directions. Firstly, putative chain elongation intermediates, activated as *N*-acylcysteamine thioesters, have been incorporated specifically into erythromycin [21] and tylosin [22]. Secondly, a series of complex fatty acids have been identified in fermentation broths of mycinamycin producers, which match the stereochemistry and functional groups expected in chain elongation intermediates attached to the mycinamycin PKS [23][24]. These metabolites apparently arise by premature release from the PKS, before chain assembly is complete.

Important insights into how the macrolide PKSs determine the processing steps in polyketide assembly have come recently from analyses of the deduced primary sequence of the 6-deoxyerythronolide PKS [25–28]. 6-Deoxyerythronolide B, the first isolable intermediate in erythromycin biosynthesis, is assembled from a propionyl-CoA starter unit and six methylmalonyl-CoA extender units. A cluster of genes for erythromycin biosynthesis, including a resistance gene, have been isolated from *S. erythraea*. The PKS genes extend over 30 kb of DNA, within which three large ORFs were identified, that each encode a protein of *ca.* 300 kDa. Biochemical studies have shown recently that three such large proteins are indeed produced in *S. erythraea* [29]. Based upon sequence comparisons to known PKS and FAS enzymes, each is predicted to be a multifunctional polypeptide with an array of putative FAS-like activities (KS, ACP, KR, ER, DH; *cf. Scheme 1*) distributed along the length of the peptide chain (*Scheme 6*). In this respect the 6-deoxyerythronolide PKS resembles more closely the type-I multi-

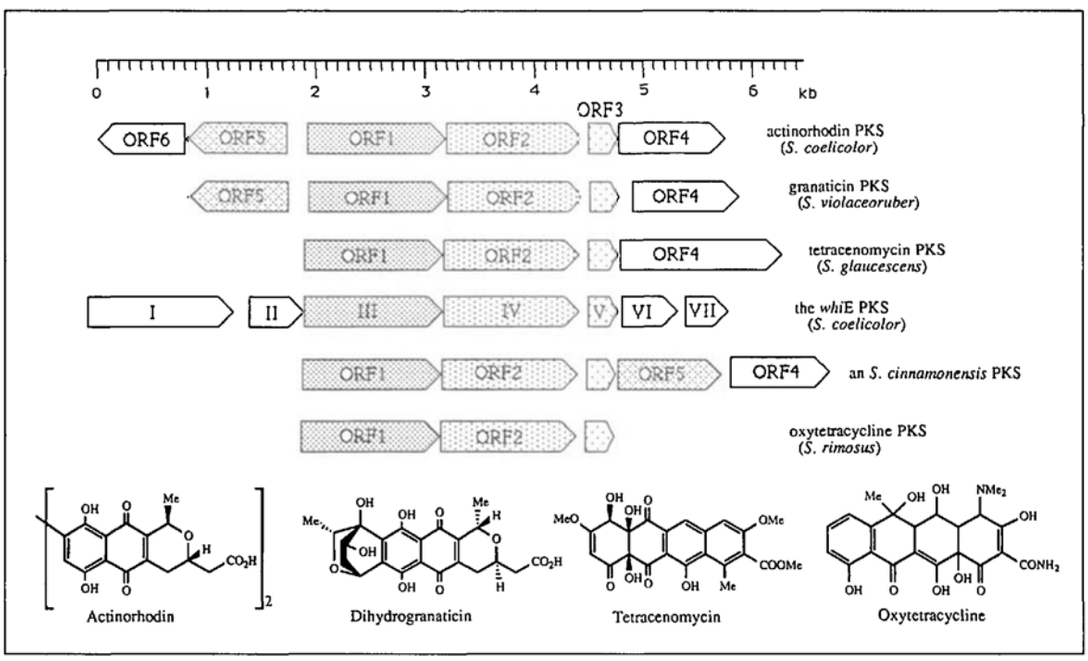


Figure. *The organization of ORFs in the actinorhodin* (*act*), *dihydrogranaticin* (*gra*), *tetracenomycin* (*tcm*), *whi*E [38],S. cinnamonensis [35] *and oxytetracycline* (*otc*) [6] *PKS gene clusters.* The deduced functions for these ORFs are: ORFI + ORF2 (ORFIII + ORFIV) = β-ketoacyl synthase (KS); ORF3 (ORFV) = acyl carrier protein (ACP); ORF4 = cyclase/dehydratase (*act*, *gra*, and *S. cinnamonensis*), or cyclase/*O*-methyltransferase (*tcm*); ORFS = ketoreductase (KR); ORF6 = ketoreductase.

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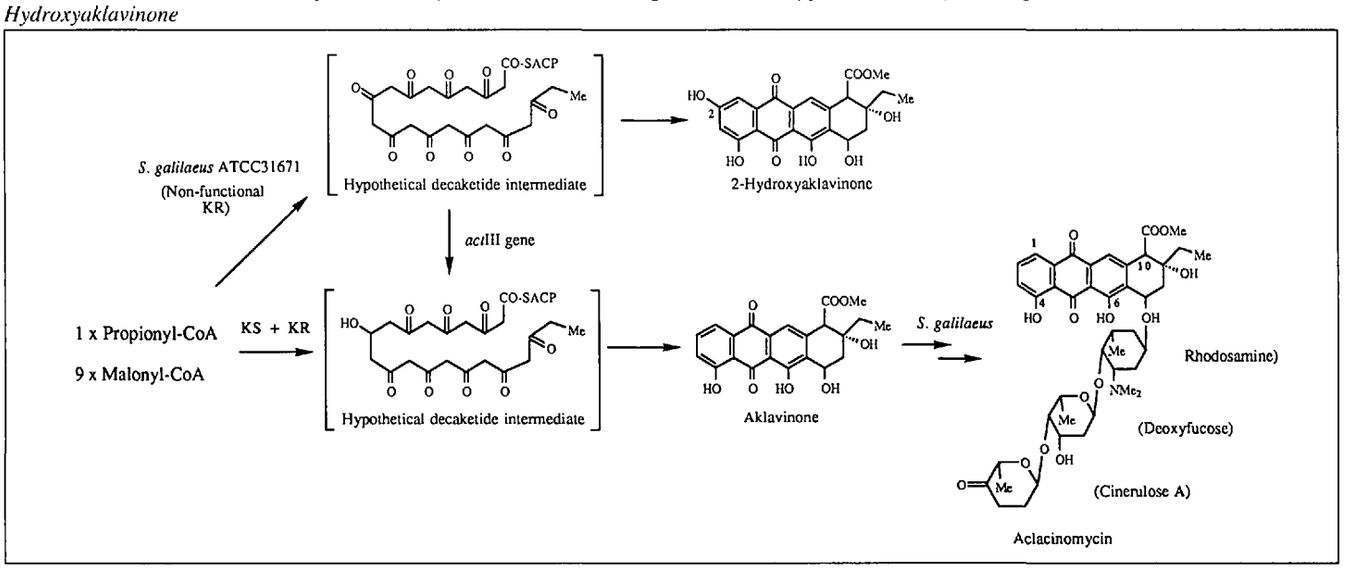
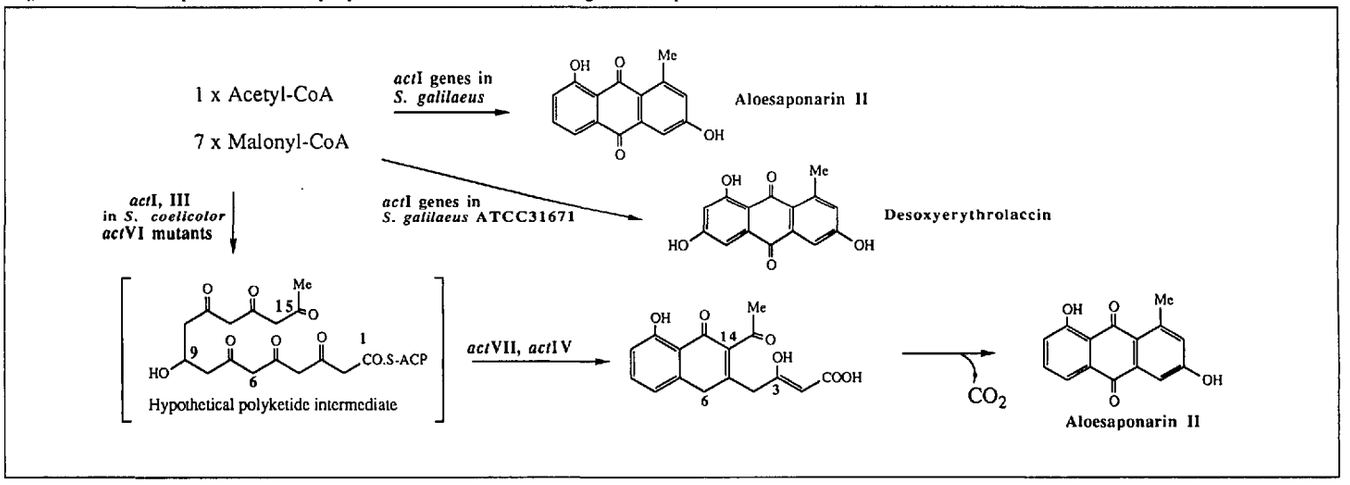
Scheme 4. *Metabolites Generated from Anthracyclinone Producers,* S. galilaeus (wild-type), *Aclacinomycin;* S. galilaeus (ATCC31671 mutant), *2-Hydroxyaklavinone*



Scheme 5. *Aloesaponarin is Produced by an* S. coelicolor act*VI Mutant* (see *Scheme 3*), *and by* S. galilaeus (wild-type) *Containing the* act*I Genes.* The *S. galilaeus* mutant produces desoxyerythrolaccin, when the *act*I genes are present.

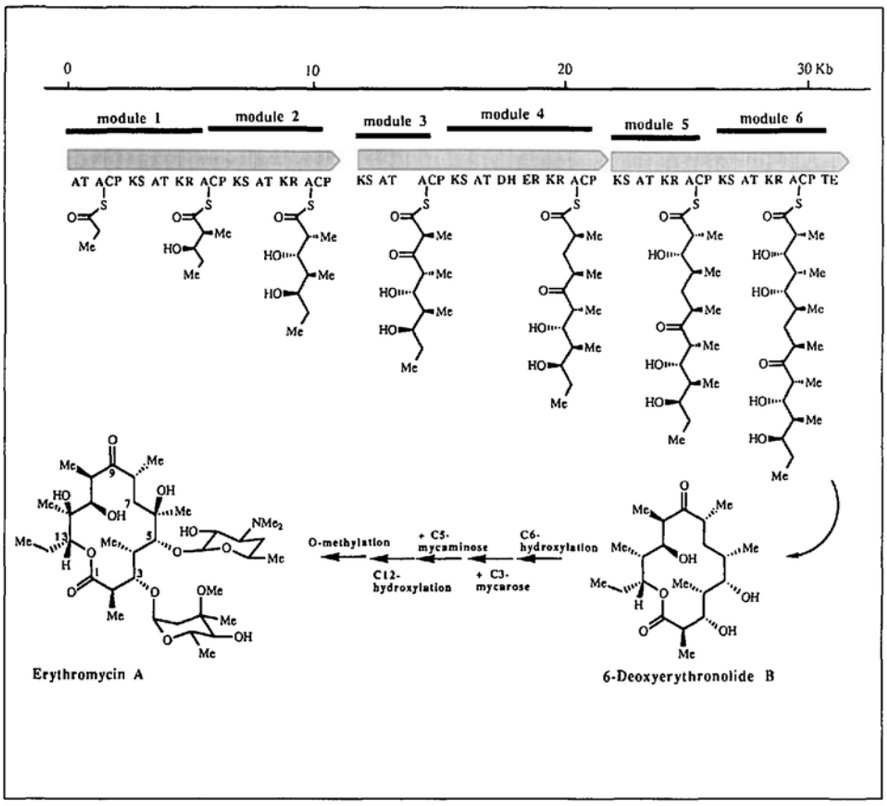


functional FASs, than the smaller type-II aromatic PKSs discussed above.

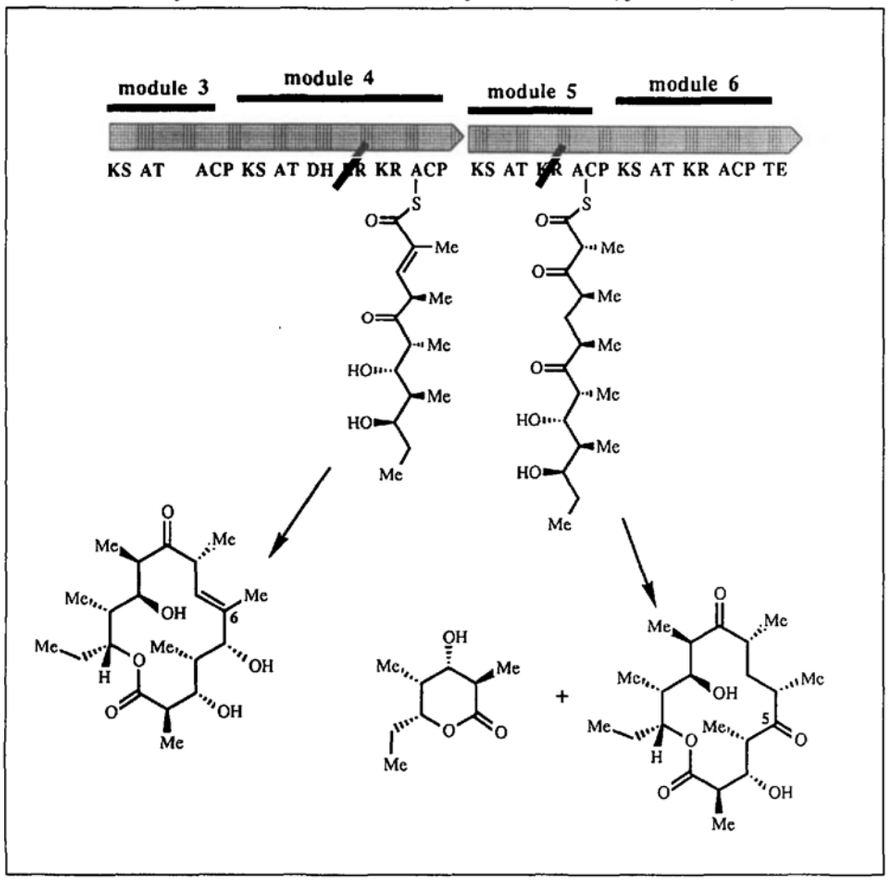
The most intriguing aspect, however, is that almost a linear correlation exists between the activities identified in the PKS and those needed for the stepwise assembly of the macrolide, where each active domain is used for just one step in the assembly process. As illustrated in *Scheme 6*, the deduced activities encoded in each ORF can be grouped into six modules, where each module contains the enzymes needed for one round of chain elongation and processing. Module 4, *e.g.*, contains, in addition to the condensing enzyme (KS) and keto reductase (KR), also the dehydratase (DH) and enoyl reductase (ER) necessary for forming the C(7) methylene. Modules 1, 2, 5, and 6 encode KS and KR domains, consistent with the formation of an alcohol at C(3), C(5), C(11), and C(13), and module 3 encodes only a functional KS, consistent with the retention of a CO group at C(9). At the end of module 6 is a sequence showing high similarity to known thioesterases, which may catalyse macrolide-ring formation, thereby releasing the substrate from the PKS. It seems that the extent of processing in each elongation cycle may simply be programmed by the presence or absence of the requisite catalytic activities, the choice of extender units incorporated may rest on the specificities of the acyl transferase domains in each module, and the absolute configuration of chiral centres may depend upon the stereospecificities of the individual KS, KR, and ER domains. More detailed biochemical studies are needed to elucidate the mechanism and specificity of chain transfer from CoASH to ACP to KS, as well as between the three PKS polypeptides, catalysed presumably by the various acyl transferases (AT).

This blueprint for polyketide assembly inferred from these sequence comparisons immediately suggests a strategy for altering the specificity of the PKS. If each enzymic activity is involved in a single biochemical step in the pathway, inactivation of any one should affect only a single step in the pathway [27]. Confirmation that this is at least possible came by inactivating the KR domain of module 5 by deleting a section of the gene encoding this portion of the PKS [26][30]. This KR domain was proposed to reduce the β-keto group of a C(13)-intermediate, with the resulting OH group appearing at C(5) in the macrolide ring (*Scheme 7*). The strain carrying this deletion now produced as the main products the 5-oxo derivative of 6-deoxyerythronolide B and 3-α-mycarosyl-5,6-dideoxy-5-oxoerythronolide B, where the 5-oxo group has been retained through the rest of the synthesis. A minor component of the fermentation was also identified as 3,5-dihydroxy-2,4-dimethylheptanoic-acid-δ-lactone [27], which is pre-

Scheme 6. *The Three Shaded Arrows Represent Three Large ORFs Identified Amongst the Erythromycin Biosynthetic Genes (ery), and Encode the* ery *PKS.* Within each sequence, various catalytic domains have been identified where; KS = β-ketoacyl synthase; KR = β-keto reductase; ACP = acyl-carrier domain; AT = acyl transferase; DH = dehydratase; ER = enoyl reductase; TE = thioesterase [26].



Scheme 7. *Mutations Introduced Specifically into the ER Domain of Module 4, Or the KR Domain of Module 5, in the* ery *PKS, Leads to the Formation of New Products (cf. Scheme 6)*



dicted to arise from cleavage of a C(7) acyl chain from module-2 of the PKS.

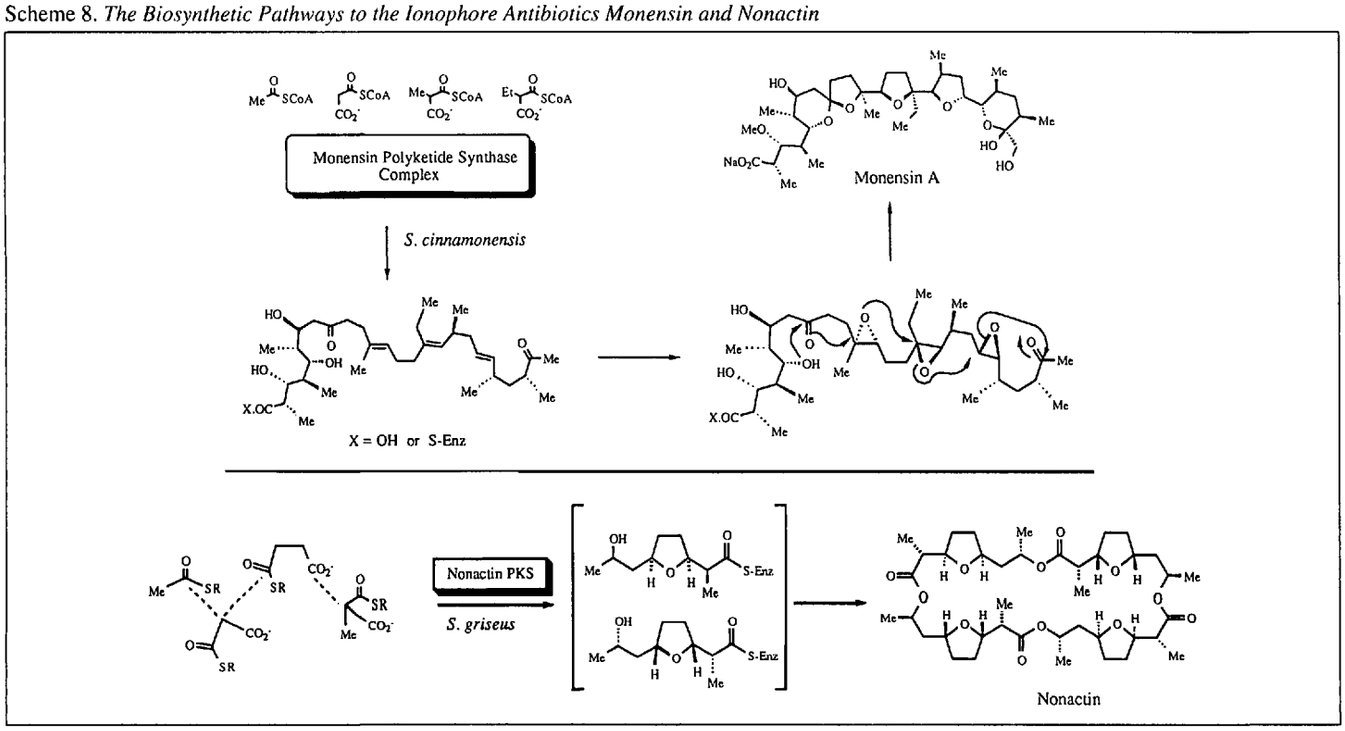
A second change involved module 4, where the ER domain, responsible for reduction of an enoyl-SACP intermediate to the fully saturated form, was inactivated by altering residues essential for enoyl reductase activity [31][32]. As anticipated, the strain carrying this mutation accumulated 6,7-anhydroerythromycin C (*Scheme 7*). These studies indicate that the polyketide chain can be passed between different domains without great regard for the structure of the acyl chain, and that the enzymes functioning later in the synthesis can transform acyl chains bearing new arrays of functional groups. Many more similar experiments are needed to define where the limits lie to this type of PKS engineering [32].

5. Modifying Steps after Polyketide Assembly

As discussed earlier, the products of polyketide assembly are often modified by other enzymes, such as oxidases, methyltransferases, aminotransferases, and glycosidases. Inactivation of these enzymes in blocked mutants generated randomly can be exploited for the production of new metabolites (*cf. Scheme 3*). However, targeted genetic manipulation offers a potentially more powerful approach for obtaining novel structures, not only by deleting activities, but also by adding new activities to an antibiotic-producing organism.

For example, a gene from *S. thermotolerans* encoding an acyl transferase responsible for adding an isovaleryl or butyryl group to the 4"-hydroxyl of the mycarose moiety of carbomycin was introduced into the spiramycin producer *S. ambofaciens* [33]. The resulting strain produced a novel 4"-isovalerylspiramycin, indicating that the acyl transferase recognised the spiramycin analogue as a substrate. As a final example, consider again the pathway to erythromycin A (*Scheme 6*). In the wild type *S. erythraea,* addition of the sugar mycarose to the macrolide ring at C(3) occurs after C(6)-hydroxylation, catalysed by a specific cytochrome P450 monooxygenase. A recombinant mutant in which this monooxygenase had been inactivated by gene disruption now produced 6-deoxyerythromycin A, an antibiotic with improved acid stability, that could not easily have been produced by chemical synthesis [34]. Clearly, the enzymes working after this hydroxylase can function on 6-deoxyerythronolide B to form a new biologically active erythromycin derivative lacking the C(6)-OH group.

Scheme 8. *The Biosynthetic Pathways to the Ionophore Antibiotics Monensin and Nonactin*



6. Outlook

Much more remains to be learnt about the structure, function, and specificity of enzymes in polyketide antibiotic biosynthesis. For example, the features governing chain length and chain folding in aromatic PKSs, the extent to which other macrolide and polyether PKSs conform to the module model seen with the erythromycin PKS, and the specificity of post-polyketide assembly enzymes. In Zurich, the enzymes involved in monensin [35] and nonactin [36] biosynthesis are presently under investigation (*Scheme 8*). Of special interest here are the PKSs and the enzymes catalysing ether-ring formation [37]. Although it is not always straightforward to clone and manipulate the large gene clusters encoding these pathways, an alliance of chemical and molecular-genetic techniques provides a powerful armoury for the characterization and future exploitation of these secondary metabolic pathways. As knowledge advances in these and related areas, it will be interesting to see whether, from a library of biosynthetic genes, completely new pathways to novel structures may be designed *denovo*.

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