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Secondary-Metabolite Biosynthesis and Metabolism

Edited by
Richard J. Petroski
and
Susan P. McCormick

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Richard J. Petroski and Susan P. McCormick

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PREFACE

This book was developed from the proceedings of the American Chemical Society, Division of Agricultural & Food Chemistry, subdivision of Natural Products Symposium "Biosynthesis and Metabolism of Secondary Natural Products" held in Atlanta, Georgia, April 1991. The objective of the conference was to bring together people from apparently diverse fields, ranging from biotechnology, metabolism, mechanistic organic chemistry, enzymology, fermentation, and biosynthesis, but who share a common interest in either the biosynthesis or the metabolism of natural products.

It is our intention to help bridge the gap between the fields of mechanistic bio-organic chemistry and biotechnology. Our thanks go to Dr. Henry Yokoyama, co-organizer of the symposium, the authors who so kindly contributed chapters, the conference participants, and to those who assisted in the peer review process. We also thank the financial supporters of the symposium: ACS/AGFD, NIH General Medical Sciences, and the agricultural, pharmaceutical, biotechnology, and chromatography companies. A full list of the supporting corporations and institutions is given on the following page. Pharma-Tech and P.C., Inc. are manufacturers of instrumentation for high-speed countercurrent chromatography.

We thank the Agricultural Research Service and the U.S. Department of Agriculture for granting me permission to co-organize the conference and for us to complete the book.

Richard J. Petroski
Susan P. McCormick

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Agricultural Utilization Research
Peoria, IL 61604

June 10, 1992

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ANTIBIOTICS

POLYKETIDE SYNTHASES: ENZYME COMPLEXES AND MULTIFUNCTIONAL PROTEINS DIRECTING THE BIOSYNTHESIS OF BACTERIAL METABOLITES FROM FATTY ACIDS

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INTRODUCTION

Microorganisms and plants produce from low-molecular weight fatty acids a collection of metabolites called polyketides that represent perhaps the largest group of secondary natural products[1]. These structurally diverse compounds typically contain oxygen atoms at alternate positions that are derived from the carbonyl groups of the fatty acid precursors by way of poly-β-ketoacylthioester intermediates. In fact, the name "polyketide" was coined about 100 years ago by Collie[2,3] as the signature of a concept in which he imagined that poly-β-ketone intermediates could account for the products produced upon treatment of polyacetyl compounds with weak alkali, and for the characteristic hydroxylation pattern of some aromatic metabolites whose structures were known at that time. Biochemical support of his idea was not provided until 1953 by the insightful studies of Birch and co-workers[4,5], who deduced from the isotopic labeling pattern of several fungal metabolites that they must have been made from acetic and malonic acids by a process like the biosynthesis of long-chain fatty acids. Polyketide chain growth must differ from fatty acid biosynthesis, however, because it lacks the faithful removal of each β-keto group, introduced by the condensation of acyl*S*R (R = protein) and malonyl*S*R intermediates, by an iterative reduction-dehydration-reduction process as in fatty acid biosynthesis. Further applications of the isotopic labeling method, augmented by the development of sophisticated nuclear magnetic resonance spectroscopic techniques[6,7], led by the end of the 1980's to a probable mechanism for the assembly and processing of poly-β-ketone intermediates in the early steps of polyketide biosynthesis. Synthesis of poly-β-ketones and -esters and studies of their behavior in solution when treated with acid or base, largely carried out by the Harris group[8], provided important insights about the chemical reactivity of such compounds in vitro and additionally resulted in the total synthesis of several important natural products[8,9].

During these thirty or so years, knowledge of the enzymology of the polyketide synthases (PKS) languished due to the apparent intractability of their in vitro assay and purification. Only three PKS's were purified by 1985: 6-methylsalicylic acid synthase (6-MSAS) from the fungus *Penicillium patulum*[10,11], naringenin chalcone synthase (CHS) from the parsley plant *Petroselinum hortense*[12,13], and resveratrol (stilbene) synthase from the peanut plant *Arachis hypogaea*[14]. Studies of these three enzymes established the basic characteristics of a PKS, yet their distinctly different properties (6-MSAS is an ≈800 kDa, probably tetrameric multifunctional protein[14a] to which all the substrates are covalently attached, whereas CHS is a homodimer composed of 42 kDa subunits[15] that acts on the coenzyme A (CoA) esters of the substrates and lacks a functionality equivalent to an acyl carrier protein (ACP)) did not lead to a secure prediction of how a bacterial PKS would be organized. Would it consist of individual enzymes as in the plant or large, multifunctional enzymes as in the fungi and yeast? Further uncertainty arose from the fact that while bacterial fatty acid synthases (FAS) typically are enzyme complexes composed of at least seven different proteins (type

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II FAS)[16], examples of ones that are multifunctional enzymes (type I FAS) are also known[16a,17]. These issues and other aspects of bacterial PKS's have been clarified during the past few years through investigations of the genetics of antibiotic production by *Streptomyces* and related bacteria[18].

GENETICS OF ANTIBIOTIC PRODUCTION

Antibiotic Production is a Dispensible Characteristic Definable by Specific Mutations

Secondary metabolism in microorganisms and plants is often thought of as a collection of specialized biosynthetic pathways unnecessary for growth but useful for an organism's survival in competition with the other inhabitants of its ecological niche. Antibiotics, as one kind of secondary metabolite, are apparently not required for the growth and development of bacteria (and plants) and thus non-producing mutants can be isolated that exhibit wild-type growth and morphological characteristics. (Nonetheless, there is considerable evidence that morphogenesis and antibiotic production are temporally intertwined processes in microorganisms.) By studying the metabolic properties of such mutants, the intermediates of the biosynthetic pathway and the sequence of events in it can usually be defined[19]. Our study of the biosynthesis of the anthracycline antibiotic tetracenomycin C (Tcm C) provides an illustrative example. Since Tcm C inhibits the growth of many other actinomycetes, mutagenized cells of *Streptomyces glaucescens* GLA.0 could be screened for the lack of a zone of growth inhibition around colonies growing on a solid medium seeded with the Tcm C-sensitive *Streptomyces coelicolor* M111 strain (its *strA1* mutation provided the needed resistance to the hydroxystreptomycin also produced by the GLA.0 strain) to identify Tcm C non-producing mutants, phenotypically designated by Tcm C$^-$. Thirty-four Tcm C$^-$ mutants were isolated and by testing them for their ability to make Tcm C when grown pairwise in liquid culture or as overlapping cross-streaks on solid culture, we could order the *tcm* mutations according to the probable sequence of steps in Tcm C

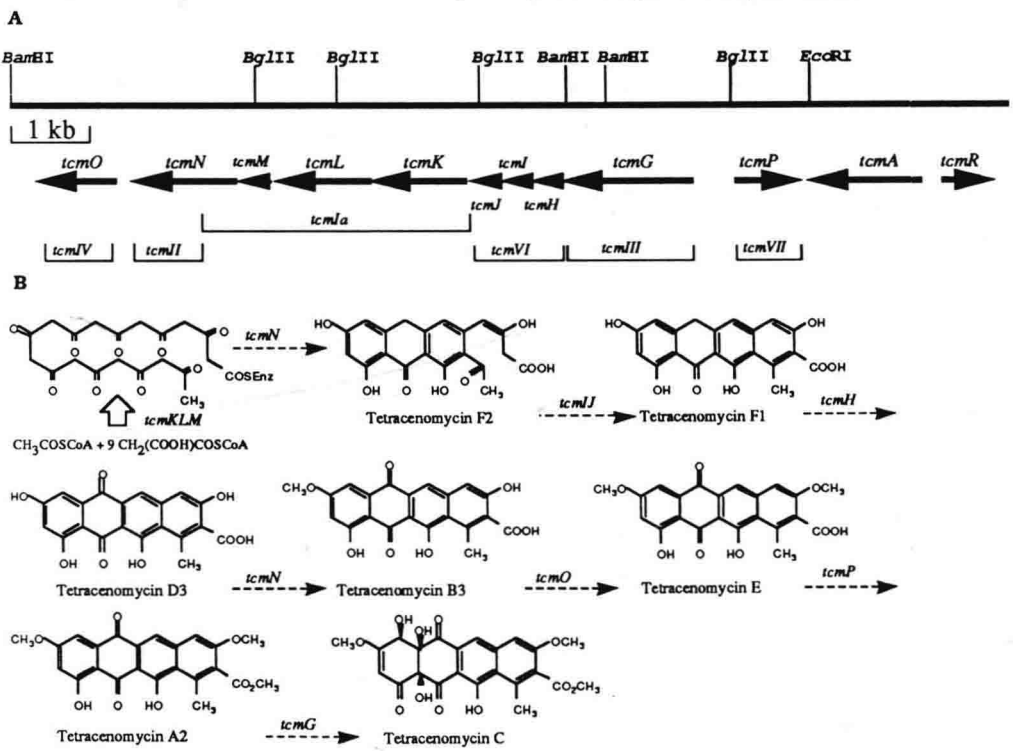

Fig. 1. (**A**) A physical map of the Tcm C gene cluster showing the location of some restriction sites and all of the known *tcm* genes, whose size and direction of transcription are indicated by the thick arrows. The bracketed regions below the *tcm* genes indicate the locations of six different classes of Tcm C$^-$ mutants, which accumulate the following compounds enclosed in parentheses: *tcmII* (Tcm D3), *tcmIII* (Tcm A2), *tcmIV* (Tcm B3), *tcmVI* (Tcm F2) and *tcmVII* (Tcm E). *tcmA* and *tcmR* are, respectively, the Tcm C resistance gene and a repressor gene that regulates expression of *tcmA*. (**B**) The Tcm C biosynthetic pathway showing the principal intermediates and the steps governed by the *tcm* genes.

biosynthesis[20]. We then were able to determine the nature of the pathway intermediates and the steps in the pathway (Fig. 1B) by structural characterization of the metabolites accumulated by each class of mutant[21].

Mutations in polyketide synthase genes typically exhibit a characteristic phenotype

The 13 members of the *tcmIa* class of *S. glaucescens* Tcm C$^-$ mutants do not accumulate or secrete detectable anthracycline metabolites but are able to convert the compounds secreted by the other classes of mutants to Tcm C. Since the *tcmIa* mutation affects one of the earliest steps of Tcm C biosynthesis, we assumed that these mutants lacked the ability to construct the decaketide skeleton of Tcm F2, the earliest intermediate of the pathway that we have been able to isolate (Fig. 1B). Blocked mutants (*actI*) with an analogous behavior had been isolated earlier in the laboratory of David Hopwood during the initial stage of his genetic study of the production of actinorhodin[22], an isobenzochromane quinone antibiotic made by *S. coelicolor* A3(2) from an octaketide intermediate[23]. Similarly, the characterization of mutations blocking macrolide biosynthesis by *Streptomyces fradiae*[24] (designated *tylG*) and *Saccharopolyspora erythraea*[25] (designated *eryA*) showed that the most frequently isolated class of mutants did not secrete pathway intermediates and were unable to carry out the assembly of the polyketide-derived portion of tylosin and erythromycin, the principal antibiotics made by these two bacteria, respectively. One would expect that poly-β-ketone biosynthetic intermediates should be difficult to isolate since their solution chemistry[8] shows that they are notoriously reactive compounds, and by analogy to fatty acid acid biosynthesis, they should remain covalently attached to the PKS until the final carbon chain has been assembled. Hence, the effect of mutations in PKS genes should result in a phenotype precisely like that of the *tcmIa*, *actI*, *tylG* and *eryA* mutants. Such mutants have played key roles in the identification of PKS genes.

It is not always possible to identify mutations in PKS genes unambiguously, however, since they can be null mutations that do not interrupt a clear precursor-product relationship in a biosynthetic pathway, unlike the *tcmIII* mutation, for example, which causes the accumulation of Tcm A2 by blocking its oxidation to Tcm C (Fig. 1). Nearly all of the non-producing mutants of bacteria that make polyether antibiotics, such as lasalocid A from *Streptomyces lasaliensis*[26] and monensin from *Streptomyces cinnamonensis*[27], fail to accumulate or secrete metabolites that are likely intermediates of polyether biosynthesis and do not exhibit antibiotic cosynthesis when grown in pairwise combinations. This behavior suggests that polyether biosynthesis is characterized by enzyme-bound intermediates that cannot be detected extracellularly; yet other explanations, like the frequent isolation of pleiotropic mutations preventing antibiotic production through the disruption of regulatory circuits, rather than the function of the structural genes encoding the pathway enzymes, are equally plausible. This pitfall has so-far prevented the identification of the PKS genes for polyether antibiotics.

BACTERIAL POLYKETIDE SYNTHASE GENES

Cloning of PKS Genes

The assumption was made that the *S. glaucescens tcmIa* and *S. coelicolor actI* mutations[22] lay in the PKS genes. Cloning of these genes was accomplished by introducing, through transformation, segments of DNA from the wild-type organism into representative blocked mutants and looking for complementation. Since the *tcm*[28] and *act*[29] gene clusters had been identified by shotgun cloning before the complementation experiments were actually done (this was not a prerequisite for their success, however), fragments of DNA from each cluster could then be subcloned and tested for the desired property. DNA hybridization experiments showed that the sequences of the cloned *tcmIa* (renamed *tcmKLM*) and *actI* genes were very similar, and furthermore, that the genomic DNA of many *Streptomyces* known to produce polyketide metabolites contain DNA that hybridizes to the *actI* gene[30]. This result has simplified cloning additional PKS genes from other bacteria that produce aromatic polyketide metabolites by eliminating the need to isolate strains with mutations in these genes first.

The PKS genes involved in the biosynthesis of macrolide, polyether and polyene antibiotics, whose largely aliphatic structures indicate a more frequent occurrence of biochemical reductions during the assembly of their polyketide-derived portions compared with the formation of aromatic metabolites like Tcm C, usually do not exhibit significant hybridization to the *actI* or *tcmKLM* genes. Consequently, the first representative of this type of PKS gene (from the erythromycin gene cluster) was also cloned by complementation of an *eryA* mutation[31], thus providing a DNA probe that should be useful for the isolation of further members of this class of PKS gene.

PKS Gene Products Have Highly Conserved Sequences

Sequence analysis of the *S. coelicolor actI*[32], *actIII*[33], and *actVII*[32], *S. glaucescens tcmKLMN*[34,35], *Streptomyces violaceoruber gra*[36] (for granaticin, the principal polyketide metabolite of this organism) and *S. erythraea eryA*[37,38] genes has revealed the characteristics of the PKS's in these organisms, thereby establishing a paradigm for each of two classes of PKS genes. Following the FAS nomenclature, type I PKS genes are represented by *eryA1* to *-3* (plus the 6-MSAS gene[14]), and type II by *actI/III/VII*, *tcmKLMN*, and *gra* because the sequence data suggest that the *eryA123* genes encode three large, multifunctional enzymes and the *actI/III/VII*, *tcmKLMN* and *gra* PKS genes encode at least four separate enzymes. [The CHS[15] and resveratrol synthase[15a] genes could be classified as type III PKS genes since their products are relatively small proteins that function as dissociable homodimers but do not contain an acyl carrier prosthetic group[12-14].] The event governed by *actIII* (see below) may not be essential for formation of the polyketide intermediate, but this enzyme is assumed nonetheless to be part of the PKS. The homologous functional relationships among the deduced products of these bacterial genes are illustrated in Fig. 2.

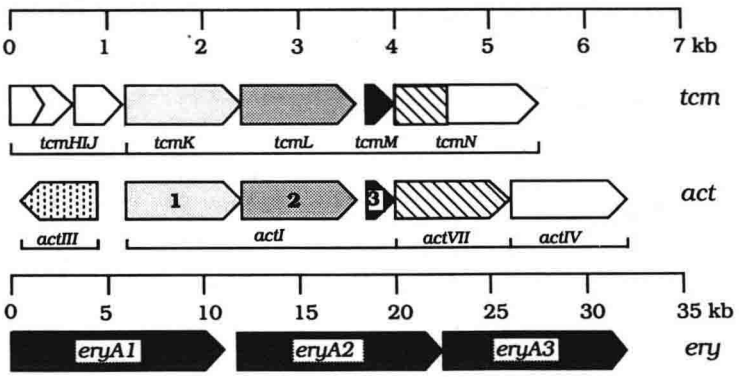

Fig. 2. Three sets of PKS genes. The *eryA123* genes represent type I and the *tcm* and *act* genes, type II. The rulers at the top and middle of the figure indicate the size of the genes in kilobase pairs of DNA. Identically shaded genes or regions within an open reading frame (orf) are predicted to have an equivalent function. The unshaded genes are included to show the relationship between the location of the PKS and adjacent *tcm* and *act* genes.

Type II PKS genes encode proteins with the following identifiable active site motifs (Table 1): condensing enzyme (β-ketoacyl:ACP synthase), acyltransferase, and ACP. A β-ketoreductase function has been assigned to the ActIII protein and its homologs due to their strong resemblance to ribitol dehydrogenase and other *bona fide* dehydrogenases that catalyze the oxidation and reduction of hydroxyl and ketone groups[33,36]. Furthermore, introduction of the *actIII* gene into other *Streptomyces* sp. can result in the reduction of ketone groups in putative polyketide intermediates[39]. The amino acid sequence of the product of the gene just downstream of the condensing enzyme orf (*actI orf1* and *tcmK* in Fig. 2) is very similar to the condensing enzyme[32,34,36] but lacks the two active site motifs that characterize the latter enzyme (Table 1). This distinct difference suggests that these genes (*actI orf2* and *tcmL*) could govern some property of the PKS complex not carried out by the condensing enzyme or ACP; e.g., regulating the number of times the malonate-derived unit reacts with the acylSR intermediate, which fixes the length of the polyketide chain. From its extensive resemblance to known ACP's and ability to be acylated by malonylCoA *in vitro*[40], the role of the ACP as the carrier of the intermediates of polyketide assembly is certain. In contrast, the function of the fourth type II PKS gene, *actVII* and *tcmN*, must be inferred from the phenotype of *actVII* mutants and of strains that carry the *tcmKLMN* genes on a plasmid vector. Since *actVII* mutants accumulate mutactin, the product of the imperfectly cyclized and dehydrated octaketide intermediate of actinorhodin biosynthesis[39,40], and *S. lividans(tcmKLMN)* or *S. glaucescens tcmIc(tcmKLMN)* but not -(*tcmKLM*) transformants produce Tcm F2 in quantity[35], it seems that the *actVII* and *tcmN* genes encode a type of polyketide cyclase/dehydrase[42], whose role is to fix the correct conformation of the respective enzyme-bound, octa- or decaketide substrate so that the enzyme can catalyze the equivalent of a Dieckmann or intramolecular Claisen

condensation. This results in the formation of two or three aromatic rings and release of a product (Tcm F2 in Fig. 1B) having a free carboxylic acid at the position where the terminus of the polyketide intermediate had been attached to the enzyme. These "cyclase" enzymes consequently appear to have at least two activities: bond formation and dehydration, with thioester hydrolysis a likely third unless the latter property eventually is assigned to one of the other, non-ACP components of the type II PKS. Interestingly, the C-terminal portion of the TcmN protein must have *O*-methyltransferase activity since this domain directs the C-3 *O*-methylation of Tcm D3 to Tcm B3[35,42] (Fig. 1B).

Table 1. Sequences of conserved amino acids in the active sites of the products of bacterial PKS genes, deduced from comparisons with the corresponding enzymes or domains of bacterial and eukaryotic FAS.

Gene	Amino acid sequence
tcmK	GPVTVVSTG**C**TSGLDAV[a] . . . SMVGH**S**LGAIGS[b]
actI orf1	GPVTVVSTG**C**TSGLDAV . . . SMIGH**S**LGAIGS
eryA1	GPAISVDTA**C**SSSLVAV . . . AVIGH**S**QGEIAA
	GPALTVDTA**C**SSSLVAL . . . AVIGH**S**QGEIAA
	AVIGH**S**QGEIAA
tcmM	QDLGYD**S**IALLE[c]
actI orf3	EDIGYD**S**LALME
eryA1	RELGLD**S**VLAAQ
	AELGVD**S**LSALE
	KELGFD**S**LAAVR
tcmN	IA**D**L**GGG**DGW**F**LAQILR[d]

[a]The condensing enzyme active site; **C** indicates the cysteine residue essential for substrate attachment.
[b]The acyltransferase active site; **S** indicates the serine residue essential for substrate attachment.
[c]The ACP active site; **S** indicates the essential serine residue to which 4'-phosphopantetheine is attached.
[d]The nucleotide binding site in the C-terminal region responsible for the *O*-methyltransferase function; the most important conserved residues are shown in bold face.

The products of bacterial type I PKS genes, which currently are represented only by the *eryA123* genes, appear to be vastly larger than those of the type II PKS and consist of many domains, whose order and function mirror the architecture of the multifunctional enzymes of a eukaryotic FAS[37,38]. EryA1, for instance, is predicted to be a 365 kDa protein with two modular regions, each consisting of several distinct domains that collectively catalyze the assembly of the first nine carbon intermediate in the biosynthesis of the 6-deoxyerythronolide B (6dEB) macrolide[38]. Module 1 contains the following active site motifs: two ACP, two acyltransferase, one condensing enzyme and one ketoreductase. These are believed to provide the activities needed for attachment of the propionate starter unit to the cysteine-SH of the condensing site by transacylation from propionylCoA (or from propionyl*S*ACP), its reaction with a 2-methylmalonyl extender unit attached to the ACP site (obtained from 2-methylmalonylCoA by transacylation), followed by reduction of the β-keto group of the (2*S*)-2-methyl-3-ketopentanoyl*S*R intermediate formed in the condensation reaction. The activities present in module 2 (one each of the ACP, acyltransferase, condensing enzyme, and ketoreductase motifs) would extend the resulting (3*R*)-3-hydroxyacylthioester by three more carbons, provided by 2-methylmalonylCoA, and reduce the ensuing β-keto group to give a (2*R*, 3*S*, 4*S*, 5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoate thioester. This intermediate would be passed on to the EryA2 protein and then to EryA3 for further extension and modification *in sequo*, followed by eventual lactonization to release the 6dEB product[38].

Expression of PKS Genes in *Escherichia coli* and *Streptomyces* Provides Large Quantities of PKS Enzymes

Investigations of the mechanistic and physical properties of either type of PKS will be facilitated by overexpression of the relevant genes in *E. coli* or *Streptomyces*, since it is typically difficult to even assay these enzymes in the wild-type strain, let alone attempt their isolation. Each of the *tcmKLM* genes has been expressed in *E. coli* using the T7-based, two plasmid system of Tabor and Richardson[43]. This expression vector capitalizes on the fact that phage T7 RNA polymerase provided by one of the plasmids will only transcribe genes placed under the control of a T7 phage promoter on the other plasmid; the cloned gene is transcribed robustly without involvement of the *E. coli* genes, which simplifies the task of enzyme