Combinatorial biosynthesis of 'unnatural' natural products: the polyketide example

Multi-enzyme systems, such as those involved in the biosynthesis of polyketides, typically catalyze several distinct reactions that are combined in different ways to generate diverse natural products. The variability available in such systems has not been fully harnessed from nature. It may therefore be possible to create 'unnatural' natural products, which may be as structurally diverse and medicinally valuable as existing natural products, using combinatorial biosynthesis.

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Nature excels in the combinatorial biosynthesis of polyfunctional organic molecules. This was perhaps first recognized over a century ago, when Collie [1] suggested that many structurally diverse natural products might arise from multiple two-carbon condensation reactions. He called this class of natural products the 'polyketides' [2]. The discovery and analysis of the multi-enzyme systems that catalyze the polymerization, cyclization and tailoring of polyketides to a specific function has occupied many of the intervening decades.

Today, the understanding of polyketide biosynthesis has reached a point where we can now contemplate the idea of adapting the system to our own ends. The prospect of genetically manipulating polyketide biosynthesis to generate libraries of 'unnatural' natural products is no longer within the realm of fantasy. In this review, we evaluate recent progress in generating novel polyketide products, and the strategies suggested by these recent successes for the combinatorial generation of molecular diversity.

There are at least two good reasons for wishing to manipulate natural product synthesis in this way. First, such exercises can yield new insight into the function and mechanisms of enzymes involved in natural product biosynthesis. Second, libraries produced in this way would offer unique opportunities for drug discovery and development, since they combine the power of combinatorial chemistry with the proven track record of natural products as human therapeutic, veterinary and agrochemical agents.

Here we focus primarily on the class of polyketides for which most information is currently available, the bacterial aromatic polyketides. It is to be hoped that this type of analysis will soon also be possible for more complex polyketide pathways, such as those responsible for the synthesis of macrolides. Although the knowledge database for these pathways is relatively rudimentary, available insights from directed mutagenesis studies in these systems suggest that their potential for combinatorial biosynthesis could be substantially greater. We therefore provide a brief analysis of the overall functional plasticity in complex polyketide pathways. Even the complex polyketides are only the beginning, however; we speculate that there may be considerable combinatorial potential within other families of natural products.

Polyketide biosynthesis

The chemistry and biology of polyketide biosynthesis have been the subjects of several excellent reviews within the past three years [3–6]. Polyketides are a large family of structurally diverse natural products possessing a wide range of biological activities, including antibiotic and other pharmacological properties. They are synthesized by multifunctional polyketide synthase enzymes (PKSs). PKSs catalyze repeated condensation cycles between acyl thioesters (usually acetyl, propionyl, malonyl or methylmalonyl). Each cycle results in the formation of a β-keto group that may undergo all, part, or none of a series of reductive steps (Fig. 1). In the case of aromatic PKSs, the β-keto groups are left largely unmodified and the resulting highly reactive polyketide backbone undergoes a series of enzyme-catalyzed regiospecific cyclizations.

Types of PKSs

Over the past decade, cloning and sequence analysis of PKS genes have defined two types of PKSs (Fig.2). Complex or 'modular' PKSs, which catalyze the biosynthesis of macrolides such as erythromycin, are assemblies of large multifunctional proteins, with a different active site for each enzyme-catalyzed step in carbon-chain assembly and modification [7]. The active sites are clustered into modules, so that each module contains all the different sites required for one cycle in the iterative condensation process (Fig. 1). Once the product has gone through one cycle, for example the four-carbon to six-carbon condensation step for acetyl thioesters, it moves to the next enzyme module, which contains the active sites responsible for the six-carbon to eight-carbon condensation step. In contrast, the PKSs responsible for the

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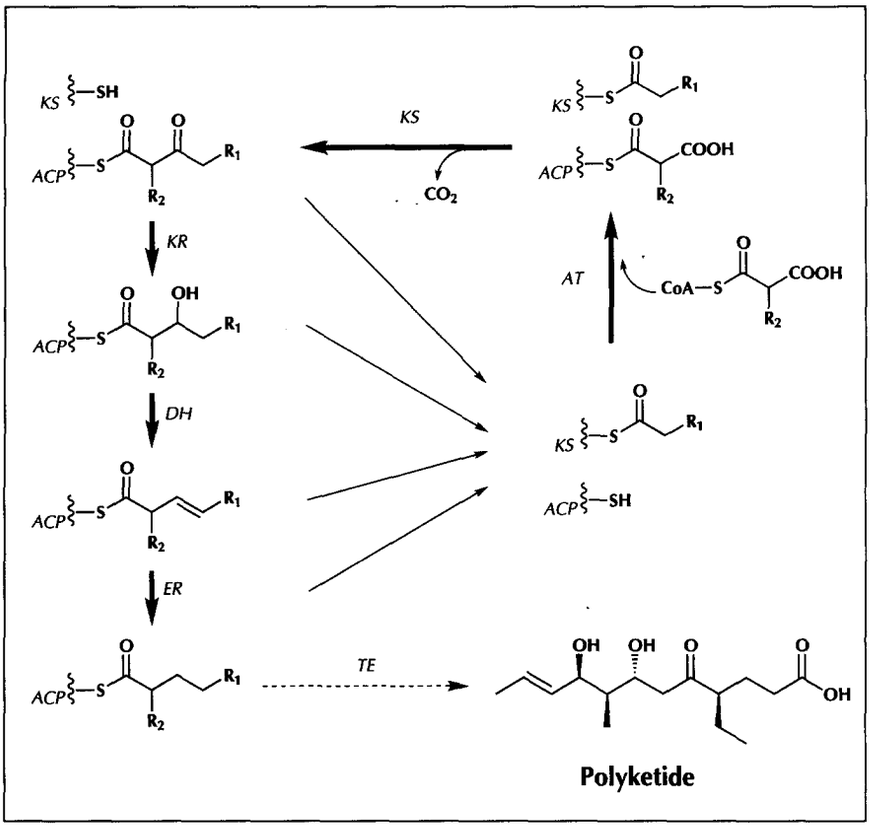

Fig. 1. Catalytic cycle for polyketide backbone biosynthesis. Biosynthesis of the carbon-chain backbone of a polyketide involves the following enzyme-catalyzed reactions. At the start of any condensation cycle, the growing polyketide chain is attached via a thioester linkage to a cysteine residue in the active site of the ketosynthase (KS). An acyltransferase (AT) transfers the carboxylated extender unit from CoA to the 4' phosphopantetheine arm of the acyl carrier protein (ACP). The choice of the extender (R_2) is presumably dictated by the specificity of the AT. Following a condensation catalyzed by the KS, the β-carbonyl in the nascent chain can be subjected to all, part, or none of a series of steps catalyzed by a ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). After the chain has been through a fixed number of condensation cycles, it is released from the PKS via the action of a thioesterase (TE). Wherever applicable, the stereochemistry at any asymmetric center is uniquely controlled by the PKS.

biosynthesis of aromatic polyketides, such as 6-methylsalicylic acid and actinorhodin, generate the entire carbon-chain backbone using a single set of iterative active sites (Fig. 2). These active sites can perform the reactions required to add an extra acyl unit to a wide range of substrates of different chain length, and perform such additions repeatedly until the backbone reaches the length required.

Iterative PKSs can exhibit considerable architectural variety. For example, bacterial aromatic PKSs, such as the actinorhodin PKS, are made up of several distinct polypeptide subunits, each containing one or two active sites. In contrast, fungal PKSs such as the 6-methylsalicylic acid PKS consist of a single multi-domain polypeptide which includes all the active sites required for the biosynthesis of 6-methylsalicylic acid. In some cases, the nascent polyketide chain undergoes subsequent cyclizations and other regiospecific modifications. Some of these steps may also be catalyzed by the PKSs themselves. Although the combinatorial biosynthesis potential in modular PKSs may be greater, as the iterative PKSs are smaller they have been more convenient targets for manipulation thus far.

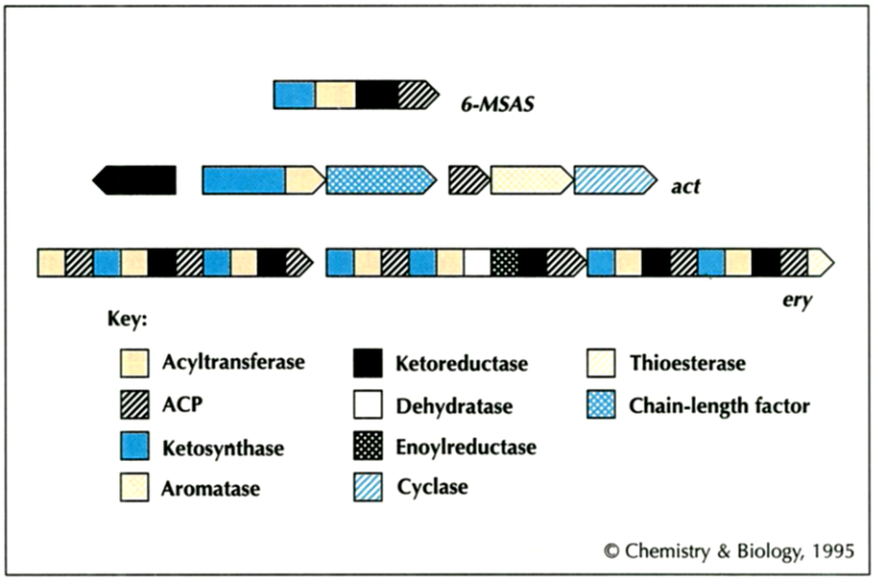

Fig. 2. Polyketide synthase gene clusters. The gene clusters encoding three representative gene clusters are schematically shown. The *6-MSAS* gene cluster from *Penicillium patulum* encodes a multifunctional protein responsible for the synthesis of 6-methylsalicylic acid. The *act* gene cluster from *Streptomyces coelicolor* encodes a multi-subunit PKS responsible for the biosynthesis of the bicyclic precursor of actinorhodin (Fig. 4). The *ery* gene cluster from *Saccharopolyspora erythraea* encodes three large multifunctional proteins that catalyze the biosynthesis of 6-deoxyerythronolide B, a precursor of erythromycin (see Fig. 6). Each shaded region is indicative of a putative active site domain identified either via mutagenesis or through sequence comparison with enzymes having known functions. For further details on the functions of these active sites, see text or Figure 1 legend.

Genetic construction of combinatorial PKS libraries

To explore the combinatorial potential in PKSs, a multi-disciplinary strategy for the engineered biosynthesis of novel natural products was recently developed [8]. The strategy involves a host-vector system capable of high-level expression of subsets of biosynthetic genes. The heterologous host, *Streptomyces coelicolor* CH999, contains a chromosomal deletion of the entire *act* gene cluster, which encodes the biosynthesis of the aromatic polyketide actinorhodin. Shuttle plasmids are used to express recombinant PKSs in CH999. Such plasmids typically include a colEI replicon, an appropriately truncated SCP2* *Streptomyces* replicon, two *act*-promoters to allow for bidirectional cloning, the gene encoding the *act*II-ORF4 activator which induces transcription from *act* promoters during the transition from growth phase to stationary phase, and appropriate marker genes. Restriction endonuclease cleavage sites have been engineered into these vectors to facilitate the combinatorial construction of PKS gene clusters starting from cassettes encoding individual subunits (or domains) of naturally occurring PKSs.

The primary advantages of the above strategy are that (i) all relevant biosynthetic genes are plasmid-borne and therefore are amenable to facile manipulation and mutagenesis in *Escherichia coli*; (ii) the entire library of PKS gene clusters is expressed in the same bacterial host which is genetically and physiologically well characterized and presumably contains most, if not all, ancillary activities required for *in vivo* production of polyketides; (iii) polyketides are produced only after log-phase growth, alleviating the toxic effects of synthesizing potentially bioactive compounds *in vivo*; and (iv) molecules produced in this manner undergo fewer side reactions than if the same pathways were expressed in wild-type organisms or in mutants in which the naturally occurring pathway is blocked at a particular step. This system has been successfully used to express functional, recombinant aromatic PKSs and produce several novel aromatic polyketides [8–17]. To extrapolate this technology to larger gene clusters (such as the modular PKSs), an *in vivo* recombination strategy has been developed for the design and construction of plasmids containing more than 20 kb of PKS gene sequence [18].

Aromatic polyketide biosynthetic pathways

As a prerequisite for combinatorial biosynthesis, it is necessary to elucidate the functions and specificities of various enzymes in naturally occurring aromatic polyketide pathways. A typical pathway for polyketide biosynthesis is schematically outlined in Figure 3. Figure 4 shows the steps involved in the biosynthesis of a representative aromatic polyketide, actinorhodin. Polyketide biosynthesis begins with the loading of a primer unit into the active site of the condensing enzyme, β-keto acyl synthase (KS; Fig. 2). An extender unit (usually malonate) is then transferred to the pantetheinyl arm of the acyl carrier protein (ACP; Fig. 2), and the KS catalyzes the condensation between the ACP-bound malonate and the starter unit. Additional extender units are added sequentially until the nascent polyketide chain has grown to a desired chain length determined by the protein chain length factor (CLF; Fig. 2), perhaps together with the KS. Thus, the KS, CLF and the ACP form a minimal set of proteins necessary to generate a polyketide backbone, and are together called the 'minimal PKS' (Fig. 4). The nascent polyketide chain is then subjected to regiospecific ketoreduction by a ketoreductase (KR; Fig. 4) if it exists. Cyclases (CYC; Fig. 4) and aromatases (ARO; Fig. 4) later catalyze regiospecific ring formation events through intramolecular aldol condensations. The cyclized intermediate may then undergo additional regiospecific and/or stereospecific modifications (e.g. O-methylation, hydroxylation, glycosylation) controlled by downstream tailoring enzymes (Fig. 3). The absence of an enzyme activity beyond the minimal PKS typically results in the formation of a shunt product.

Combinatorial potential in aromatic polyketide biosynthesis

I) From monomers to partially cyclized polyketide backbones

The initial steps in polyketide biosynthesis provide several possible ways to modify the synthetic pathway that could be exploited to generate structural diversity.

a. Established degrees of freedom

Expression and analysis of numerous recombinant bacterial PKSs has led to significant advances in our understanding of aromatic PKS function and specificity. In turn, these insights have led to the proposal of a set of design rules for rational or stochastic manipulation of

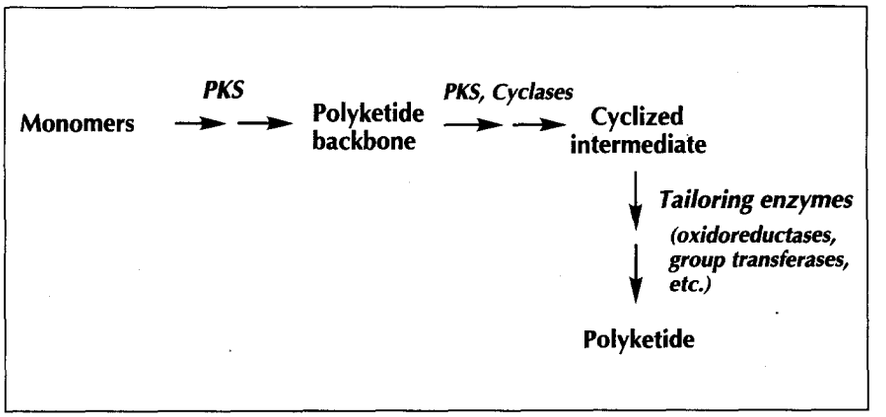

Fig. 3. Overall biosynthetic pathway for a typical polyketide natural product. A typical polyketide pathway can be thought of as occurring in three stages. In the first stage, catalyzed by the PKS, a nascent polyketide backbone is generated from monomeric CoA thioesters. In the second stage, this backbone is regiospecifically cyclized. While some cyclization reactions are controlled by the PKS itself, others are performed by downstream enzymes. In the final stage, the cyclized intermediate is modified further by the action of mechanistically diverse 'tailoring enzymes', giving rise to the natural product.

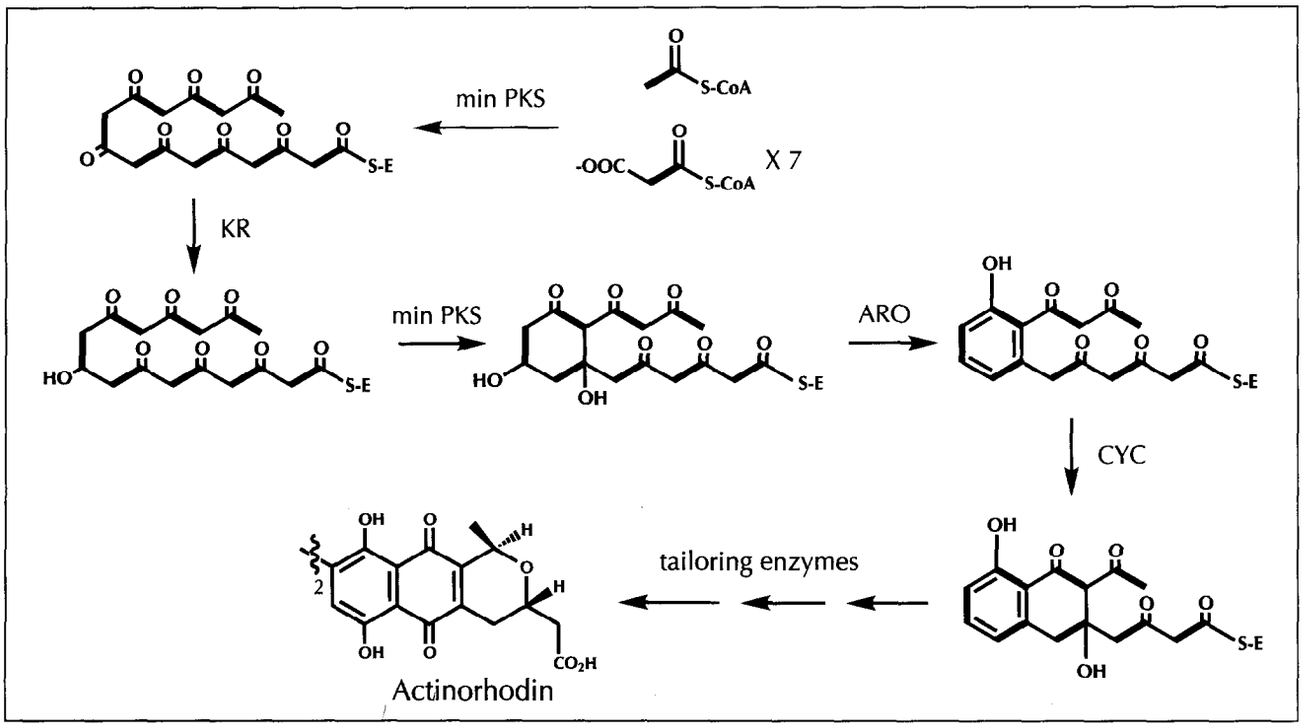

Fig. 4. Biosynthetic pathway for the aromatic polyketide, actinorhodin. The 'minimal PKS' (min PKS), composed of a ketosynthase, a chain-length factor, and an acyl carrier protein, synthesizes an unreduced octaketide backbone, which is then reduced at the C-9 carbonyl by a ketoreductase (KR). The reduced backbone is then cyclized into a bicyclic intermediate through the action of the minimal PKS, an aromatase (ARO), and a cyclase (CYC). This intermediate is finally converted into actinorhodin through the action of several tailoring enzymes. None of the above intermediates have been directly isolated; the pathway has instead been deduced through an analysis of shunt products isolated from a series of deletion mutants in the PKS gene cluster.

early biosynthetic steps in aromatic polyketide pathways, including chain synthesis, C-9 ketoreduction, and the formation of the first two aromatic rings [17]. If each biosynthetic degree of freedom was independent of all others, then it should be possible to design a single combinatorial library of $N_1 \times N_2 \times N_3 \times \ldots$ clones, where N_i is the number of ways in which the i^{th} degree of freedom can be exploited, each of which should generate a different polyketide product. In practice, however, not all enzymatic degrees of freedom are independent [17]. Therefore, to minimize redundancy, it is preferable to design several sub-libraries of aromatic polyketide producing clones. For example, for reduced polyketides, the relevant degrees of freedom include the chain length (which can be manipulated in seven ways using naturally occurring minimal PKSs with different chain-length specificities), the first ring aromatization (which gives two possible outcomes, aromatized or not), and the second ring cyclization (which occurs only after the first ring aromatization, so that there are three possible outcomes in total). For unreduced polyketides, the regiospecificity of the first cyclization can be manipulated (two possibilities) in addition to chain length (seven possibilities). Thus, the known combinatorial potential is $7 \times 3 = 21$ for reduced chains and $7 \times 2 = 14$ for unreduced chains. These numbers do not include additional minor products, of the order of 5 to 10 per major product, that are produced in the recombinant strains through non-enzymatic or non-specific enzyme catalyzed steps. Thus, the number of polyketides that can be generated from combinatorial manipulation of only the first few steps in aromatic polyketide biosynthesis is of the order of a few hundred. It is important to note that, based on limited structural analysis of the ~25 compounds produced thus far using this method, most of the compounds in such libraries are likely to be novel chemical entities [8–17]. Thus, genetically engineered biosynthesis appears to represent an untapped source of chemical diversity for drug discovery.

b. Starter unit

There are other steps in aromatic polyketide biosynthesis that are attractive targets for expanding aromatic polyketide libraries even further via combinatorial manipulation. For example, although acetyl CoA is the usual starter unit for most aromatic polyketides, malonamyl CoA [19] and propionyl CoA [20] are primers for many members of the tetracycline and anthracycline classes of polyketides, respectively (Fig. 5). Isotope labeling studies have shown that the complete PKS for the anthracycline daunorubicin can accept acetate, butyrate, and isobutyrate as starter units as well [21,22]. More recently, the observation that acetate can also be used as a primer by the daunorubicin minimal PKS [23] and by the oxytetracycline PKS [14] has provided further evidence that some PKSs possess relaxed specificity for the primer unit. Although the enzymatic basis for non-acetate primer incorporation remains obscure, recent studies by Hutchinson and coworkers suggest that this

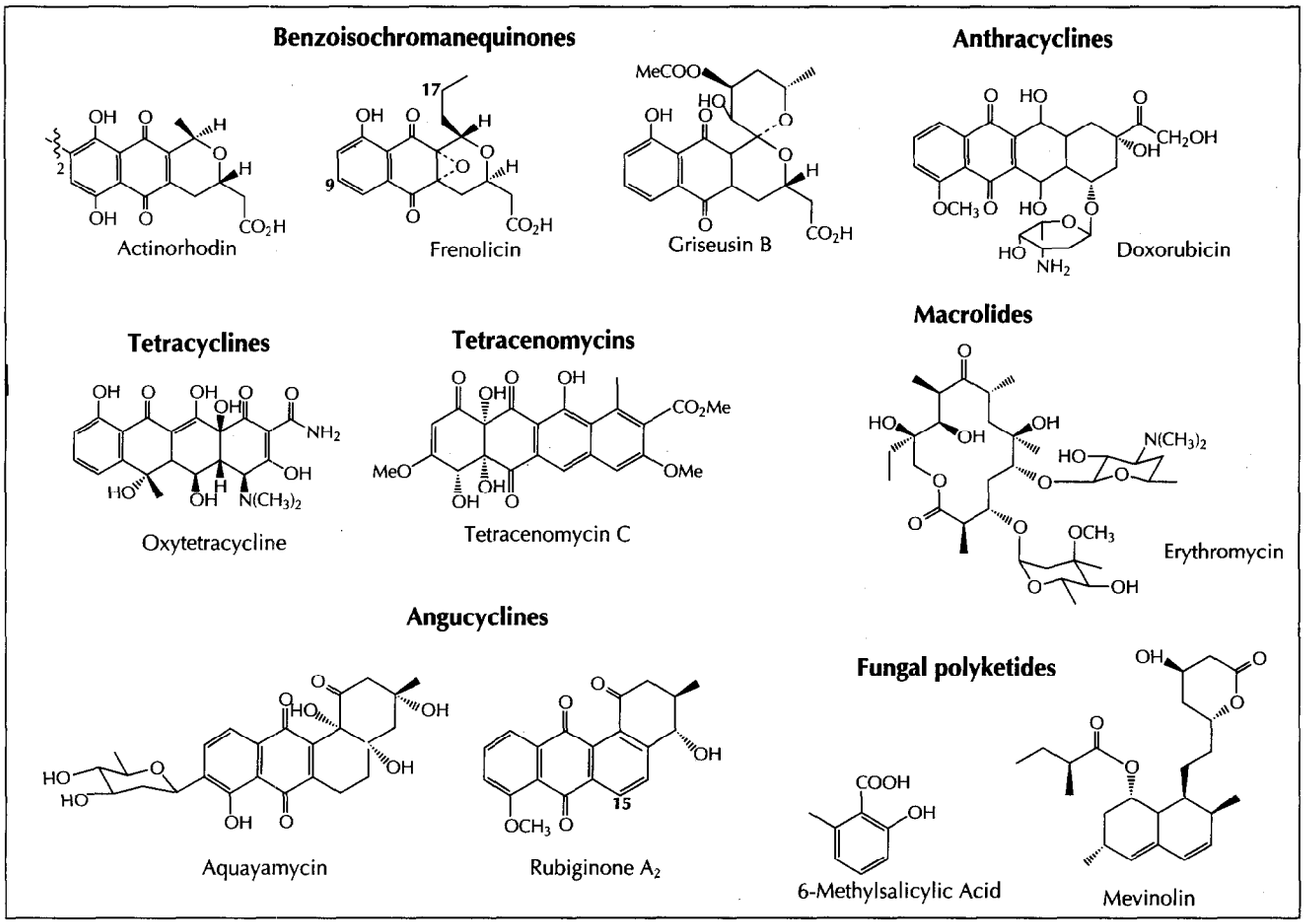

Fig. 5. Structures of several polyketide natural products referred to in the text.

property may be due to the presence of a specific acyltransferase and/or an additional KS-like protein [24]. If these subunits can productively interact with minimal PKSs that have other chain length specificities, they would be attractive targets for incorporation into the above combinatorial format.

c. Ketoreduction

The *act* KR can productively interact with all minimal PKSs studied thus far and is both necessary and sufficient to catalyze a C-9 ketoreduction. Although homologous KRs have been found in other PKS clusters, they catalyze ketoreduction with the same regiospecificity and therefore do not provide an additional source of diversity. However, the structures of frenolicin, griseusin and daunorubicin suggest that an additional C-17 ketoreduction occurs in these biosynthetic pathways (Fig. 5). Several angucyclines also undergo an additional ketoreduction at C-15, before the nascent polyketide chain is cyclized [25]. The ketoreductases responsible for C-15 or C-17 reductions have not yet been identified; however, two homologous KR sequences have been found in the daunorubicin PKS cluster [26,27]. It is probable that they catalyze the C-9 and C-17 reductions. Thus, KRs responsible for regiospecific reduction of the carbon-chain backbone at positions other than C-9 may also be useful in the construction of combinatorial libraries.

II) From partially cyclized polyketide backbones to fully cyclized intermediates

As discussed above, the formation of the first two six-membered rings in the biosynthesis of most naturally occurring bacterial aromatic polyketides is controlled by PKS subunits. Further ring closures are controlled by additional cyclases and modifying enzymes. The structural diversity introduced through these reactions appears to be greater than that for the first two cyclizations. Certain preferred patterns are observed, however, which suggests that at least some of these downstream cyclases may be combinatorially useful. For example, the pyran ring in isochromanequinones (Fig. 5) invariably forms via cyclization between C-3 and C-15, and two stereochemically distinct classes of products are observed (see, for example, the structures of actinorhodin and frenolicin in Fig. 5). In anthracyclines and tetracyclines a third aldol condensation usually occurs between C-3 and C-16, whereas in unreduced tetracenomycins and related compounds it occurs between C-5 and C-18, and in angucyclines it occurs between C-4 and C-17. Representative genes encoding a few of these enzymes have already been cloned [28,29]. It is possible that at least some cyclases might recognize chains of altered lengths and/or degrees of reduction, thus increasing the diversity of aromatic polyketide combinatorial libraries.

In the absence of downstream cyclases, polyketide chains undergo non-enzymatic reactions. Recently, some degree of predictability has emerged within this repertoire of possibilities as well [17]. For instance, hemiketals and benzene rings are two common moieties seen on the methyl end. Hemiketal formation typically involves an enol and is then followed by dehydration. Benzene rings are formed if the uncyclized methyl terminus of a chain consists of more than three ketide units. On the carboxyl terminus, a γ-pyrone ring formed by three ketide units is frequently observed. Spontaneous decarboxylations occur on free carboxyl ends activated by the existence of a β-carbonyl.

III) From cyclized intermediates to natural products

A cyclized intermediate can undergo various types of modifications to generate the final natural product. The recurrence of certain structural motifs among naturally occurring aromatic polyketides suggests that some tailoring enzymes, particularly group transferases, may be combinatorially useful. Two examples are discussed below.

a. O-methyl transfer

O-methylation is a common downstream modification. Although several S-adenosylmethionine-dependent O-methyltransferase genes have been found in PKS gene clusters [30], their specificities have not yet been systematically studied. Perhaps some of them could be useful for combinatorial biosynthesis. For instance, O-11 methylation occurs in several members of the anthracycline and angucycline classes of aromatic polyketides (Fig. 5). Likewise, the 2-hydroxyl-5-methylbenzoic acid moiety of tetracenomycin C is often observed in natural and engineered products; the tetracenomycin O-methyltransferase should therefore be a good candidate for modification of these products.

b. Glycosyltransfer

Both aromatic and complex polyketides are often glycosylated. In many cases (e.g. doxorubicin and erythromycin) absence of the sugar group(s) results in considerably weaker bioactivity. There is tremendous diversity in both the types and numbers of sugar units attached to naturally occurring polyketide aglycones, although deoxy- and aminosugars are commonly found. Regiochemical preferences can be detected in many glycosylated natural products. Among anthracyclines, O-17 is frequently glycosylated, whereas among angucyclines, C-10 is usually glycosylated. Elegant studies by Katz and coworkers [7] suggest that the glycosyltransferases involved in erythromycin biosynthesis have relaxed specificities for the aglycone moiety. More unusual but significant is the recent report by Decker *et al.* [31] that an elloramycin glycosyltransferase may be able to recognize an unnatural NDP-sugar unit and attach it regiospecifically to an aromatic polyketide aglycone. These early results suggest that glycosyltransferases derived from secondary metabolic pathways have unique properties and may be useful in the generation of combinatorial libraries. The potential for manipulating glycoside biosynthesis is discussed below.

Combinatorial biosynthesis: beyond bacterial aromatic polyketides

I) Modular polyketide synthases

Although modular PKSs have not been extensively analyzed, the one-to-one correspondence between active sites and product structure (Fig. 6), together with the incredible chemical diversity observed among naturally occurring 'complex' polyketides, suggests that the combinatorial potential within these multi-enzyme systems could be considerably greater than that for aromatic PKSs. For example, a wider range of primer units including aliphatic monomers (acetate, propionate, butyrate, isovalerate, etc.), aromatics (aminohydroxybenzoic acid),

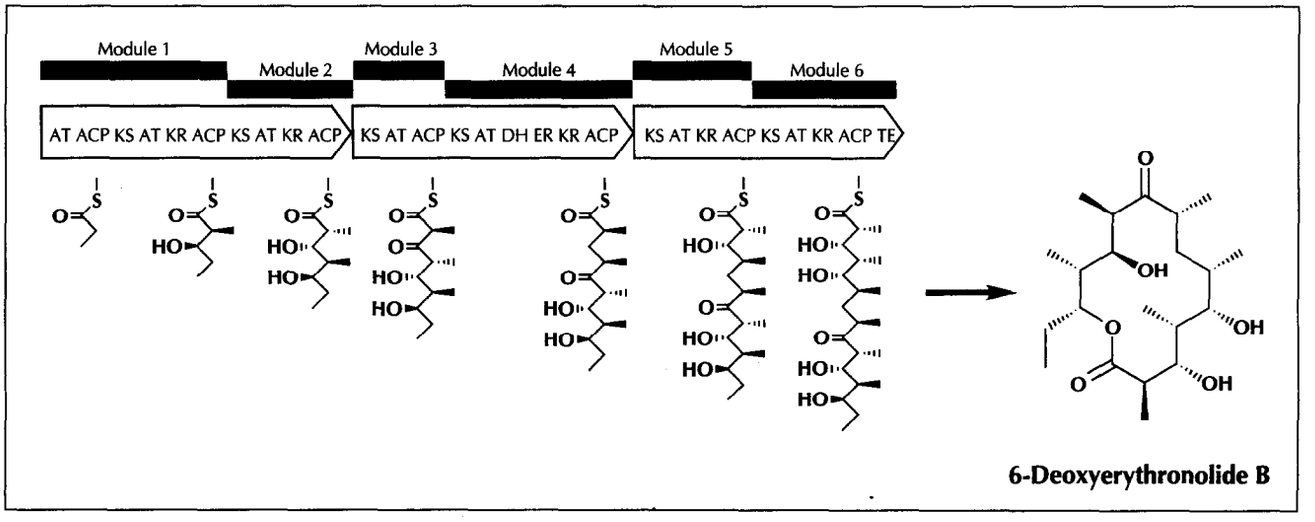

Fig. 6. Biosynthetic pathway for 6-deoxyerythronolide B synthase (DEBS). 6-Deoxyerythronolide B is the macrocyclic precursor of erythromycin. DEBS is made up of three large multifunctional proteins, each composed of a number of active site domains (abbreviated as in Fig. 1). These domains are organized into modules; each module includes all the active sites required for catalyzing a condensation reaction, together with associated reductive modifications. Following synthesis of an appropriately reduced heptaketide precursor, the chain is regiospecifically cyclized into a 14-membered macrolactone, presumably through the action of the TE. The above pathway has been deduced through sequence comparisons with known enzymes and through analysis of a few site-directed mutants.

alicyclics (cyclohexanoic acid), and heterocyclics (pipecolic acid) are found in various macrocyclic polyketides. Recent studies have shown that modular PKSs have relaxed specificity for their starter units [18]. The degree of β-ketoreduction following a condensation reaction can be altered by genetic manipulation [7,32], and the size of the polyketide product can also be varied by designing mutants with the appropriate number of modules [33]. Modular PKSs also exhibit considerable variety with regard to the choice of extender units in each condensation cycle, although it remains to be seen to what extent this property can be manipulated. These enzymes are also particularly well known for generating an impressive range of asymmetric centers in their products in a highly controlled manner. It is therefore tempting to speculate that the combinatorial potential within modular PKS pathways could be virtually unlimited.

II) Fungal PKSs

Like the actinomycetes, filamentous fungi are a rich source of polyketide natural products. The fact that fungal PKSs, such as the 6-methylsalicylic acid synthase (6-MSAS) and the mevinolin synthase, are encoded by single proteins having multiple domains ([34] and Davis, R. *et al.*, abstract P228 Genetics of Industrial Microorganisms Meeting, Montreal, 1994) suggests that they may also be well suited for combinatorial mutagenesis. Moreover, functional fungal PKSs can be expressed in *S. coelicolor* CH999 using the genetic strategy outlined above [35]. As yet, no evidence exists for the functional plasticity of domains within fungal PKSs; based on a comparative analysis of fungal polyketides, however, one can speculate on the potential for designing combinatorial libraries using fungal PKSs. For example, chain lengths not observed in bacterial aromatic polyketides (e.g. tetraketides, pentaketides and hexaketides) can be found among fungal aromatic polyketides [3]. The cyclization patterns of fungal aromatic polyketides are also quite different from those observed in bacterial aromatic polyketides [3]. In contrast with modular PKSs from bacteria, branched methyl groups are introduced into fungal polyketide backbones by S-adenosylmethionine-dependent methyltransferases; in the case of the mevinolin PKS, this activity is encoded as one domain within a monocistronic PKS (Davis, R. *et al.*, as above). It is now possible to experimentally evaluate whether these and other sources of chemical diversity in fungal polyketides are indeed amenable to combinatorial manipulation.

III) Other families of natural products

PKSs are probably not unique among natural product biosynthetic enzymes in terms of their potential for combinatorial biosynthesis. The biosynthesis of many other natural products involves controlled assembly of discrete 'building block units' followed by subsequent cyclization and modification reactions. For example, non-ribosomal peptides such as gramicidin, enniatin, and cyclosporin [36] are also synthesized by multifunctional enzymes consisting of homologous domains with similar functions but different specificities. Similarly, the enzymes involved in the biosynthesis of diverse glycosides, such as streptomycin and the lipopolysaccharide O-chains in *Salmonellae* are also homologous [37]. Given currently available tools for facile genetic manipulation, it may soon be possible to evaluate whether these pathways are also amenable to the 'mix-and-match' approach. After all, nature's strategy for the evolution of its vast ensemble of structurally intricate and pharmaceutically useful organic molecules has been precisely that.

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References

1. Collie, J.N. (1893). The production of naphthalene derivatives from dehydracetic acid. *J. Chem. Soc.* **63**, 329–337.
2. Collie, J.N. (1907). Derivatives of the multiple ketene group. *Proc. Chem. Soc.* **23**, 230–231.
3. O'Hagan, D. (1991). *The Polyketide Metabolites.* Ellis Horwood, Chichester, U.K.
4. Katz, L. & Donadio, S. (1993). Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* **47**, 875–912.
5. O'Hagan, D. (1995). Biosynthesis of fatty acid and polyketide metabolites. *Nat. Prod. Rep.* **12**, 1–32.
6. Hutchinson, C.R. & Fujii, I. (1995). Polyketide synthase gene manipulation: a structure–function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* **49**, 201–238.
7. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J. & Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. *Science* **252**, 675–679.
8. McDaniel, R., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1993). Engineered biosynthesis of novel polyketides. *Science* **262**, 1546–1550.
9. McDaniel, R., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1993). Engineered biosynthesis of novel polyketides: manipulation and analysis of an aromatic polyketide synthase with unproven catalytic specificities. *J. Am. Chem. Soc.* **115**, 11671–11675.
10. McDaniel, R., Ebert-Khosla, S., Fu, H., Hopwood, D.A. & Khosla, C. (1994). Engineered biosynthesis of novel polyketides: influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase. *Proc. Natl. Acad. Sci. USA* **91**, 11542–11546.
11. McDaniel, R., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1994). Engineered biosynthesis of novel polyketides: *actVII* and *actIV* genes encode aromatase and cyclase enzymes, respectively. *J. Am. Chem. Soc.* **116**, 10855–10859.
12. Fu, H., McDaniel, R., Hopwood, D.A. & Khosla, C. (1994). Engineered biosynthesis of novel polyketides: stereochemical course of two reactions catalyzed by a polyketide synthase. *Biochemistry* **33**, 9321–9326.
13. Fu, H., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1994). Engineered biosynthesis of novel polyketides: dissection of the catalytic specificity of the Act ketoreductase. *J. Am. Chem. Soc.* **116**, 4166–4170.
14. Fu, H., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1994). Relaxed specificity of the oxytetracycline polyketide synthase for an acetate primer in the absence of a malonamyl primer. *J. Am. Chem. Soc.* **116**, 6443–6444.
15. Fu, H., Hopwood, D. A. & Khosla, C. (1994). Engineered biosynthesis of novel polyketides: evidence for temporal, but not regiospecific, control of cyclization of an aromatic polyketide precursor. *Chemistry & Biology* **1**, 205–210.
16. McDaniel, R., Hutchinson, C.R. & Khosla, C. (1995). Engineered biosynthesis of novel polyketides: analysis of TcmN function in tetracenomycin biosynthesis. *J. Am. Chem. Soc.*, in press.
17. McDaniel, R., Ebert-Khosla, S., Hopwood, D.A. & Khosla, C. (1995). Rational design of aromatic polyketide natural products by modular assembly of enzymatic subunits. *Nature*, in press.
18. Kao, C.M., Katz, L. & Khosla, C. (1994). Engineered biosynthesis of a complete macrolactone in a heterologous host. *Science* **265**, 509–512.
19. Gatenbeck, S. (1961). The biosynthesis of oxytetracycline. *Biochem.*

Biophys. Res. Commun. **6**, 422–426.
20. Paulick, R.C., Casey, M.L. & Whitlock, H.W. (1976). A ^{13}C-nuclear magnetic resonance study of the biosynthesis of daunomycin from $^{13}CH_3{}^{13}CO_2Na$. *J. Am. Chem. Soc.* **98**, 3370–3371.
21. Oki, T., Matsuzawa, Y., Kiyoshima, K. & Yoshimoto, A. (1981). New anthracyclines, feudomycins, produced by the mutants from *Streptomyces coeruleorubidus* ME130-A4. *J. Antibiot. (Tokyo)* **34**, 783–790.
22. Yoshimoto, A., Johdo, O., Nishida, H., Okamoto, R. & Takeuchi, T. (1993). Anthracycline metabolites from baumycin-producing *Streptomyces sp.* D788: III. New anthracycline metabolites produced by blocked mutants 4L-660 and YDK-18. *J. Antiobiot. (Tokyo)* **46**, 1758–1761.
23. Meurer, G. & Hutchinson, C.R. (1995). The daunorubicin type II polyketide synthase enzymes DpsA and DpsB neither determine the choice of starter unit nor the folding pattern of aromatic polyketides. *J. Am. Chem. Soc.*, in press.
24. Shen, B. & Hutchinson, C.R. (1993). Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A. *Science* **262**, 1535–1540.
25. Gould, S.J., Cheng, X-C & Halley, K.A. (1992). Biosynthesis of dehydrorabelomycin and pd 116740 prearomatic deoxygenation as evidence for different polyketide synthases in the formation of benz-a-anthraquinones. *J. Am. Chem. Soc.* **114**, 10066–10068.
26. Grimm, A., Madduri, K., Ali, A. & Hutchinson, C.R. (1994). Characterization of the *Streptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. *Gene* **151**, 1–10.
27. Ye, J., Dickens, M.L., Plater, R., Li, Y., Lawrence, J. & Strohl, W.R. (1994). Isolation and sequence analysis of polyketide synthase genes from the daunomycin-producing *Streptomyces sp.* strain C5. *J. Bacteriol.* **176**, 6270–6280.
28. Fernández-Moreno, M.A., Martínez, E., Caballero, J.L., Ichinose, K., Hopwood, D.A. & Malpartida, F. (1994). DNA sequence and functions of the *actVI* region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). *J. Biol. Chem.* **269**, 24854–24863.
29. Shen, B. & Hutchinson, C.R. (1993). Tetracenomycin F2 cyclase: intramolecular aldol condensation in the biosynthesis of tetracenomycin C in *Streptomyces glaucescens*. *Biochemistry* **32**, 11149–11154.
30. Decker, H., Motamedi, H. & Hutchinson, C.R. (1993). Nucleotide sequences and heterologous expression of *tcmG* and *tcmP* biosynthetic genes for tetracenomycin C in *Streptomyces glaucescens*. *J. Bacteriol.* **175**, 3876–3886.
31. Decker, H., Haag, S., Udvarnoki, G. & Rohr, J. (1995). Novel genetically engineered tetracenomycins. *Angew. Chem. Int Ed. Engl.*, in press.
32. Donadio, S., McAlpine, J.B., Sheldon, P.J., Jackson, M. & Katz, L. (1993). An erythromycin analog produced by reprogramming of polyketide synthesis. *Proc. Natl. Acad. Sci. USA* **90**, 7119–7123.
33. Kao, C.M., Luo, G., Katz, L., Cane, D.E. & Khosla, C. (1994). Engineered biosynthesis of a triketide lactone from an incomplete modular polyketide synthase. *J. Am. Chem. Soc.* **116**, 11612–11613.
34. Beck, J., Ripka, S., Siegner, A., Schiltz, E. & Schweizer, E. (1990). The multifunctional 6 methylsalicylic acid synthase gene of *Penicillium patulum*: its gene structure relative to that of other polyketide synthases. *Eur. J. Biochem.* **192**, 487–498.
35. Bedford, D.J., Schweizer, E., Hopwood, D.A. & Khosla, C. (1995). Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). *J. Bacteriol.*, in press.
36. Kleinkauf, H. & Von Doehren, H. (1990). Nonribosomal biosynthesis of peptide antibiotics. *Eur. J. Biochem.* **192**, 1–15.
37. Piepersberg, W. (1994). Pathway engineering in secondary metabolite-producing actinomycetes. *Crit. Rev. Biotechnol.* **14**, 251–285.

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