COMBINED MODULAR PKS WITH RETAINED SCAFFOLD

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ABSTRACT
Combinatorial libraries of polyketides can be obtained by suitable manipulation of a host modular polyketide synthase gene cluster such as that which encodes the PKS for erythromycin. The combinatorial library is useful as a source of pharmaceutically active compounds.
FIGURE 2
FIGURE 3
RECIPIENT: pCK5  
(\(A_{p^R}, Tc^R\))

DONOR: pCK6  
(\(Cm^R\), temperature-sensitive replicon)

\[ \text{FIGURE 4} \]

\(A_{p^R}, Cm^R \) @ 30°C

\(A_{p^R}, Cm^R \) @ 44°C

\(A_{p^R} \) @ 30°C

\(A_{p^R}, Cm^S, Tc^S \) @ 44°C
FIGURE 5
MODIFIED MODULAR PKS WITH RETAINED SCAFFOLD

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Ser. No. 08/486,645 filed Jun. 7, 1995 which is continuation-in-part of U.S. Ser. No. 08/238,811 filed May 6, 1994. The disclosures of these applications are incorporated herein by reference.

REFERENCE TO GOVERNMENT FUNDING

[0002] This work was supported in part by a grant from the National Institutes of Health, CA69736. The U.S. government has certain rights in this invention.

TECHNICAL FIELD

[0003] The invention relates to the field of combinatorial libraries. More particularly, it concerns construction of libraries of polyketide synthases by a multiplicity of polyketide synthases derived from a naturally occurring PKS, as illustrated by the erythromycin gene cluster.

BACKGROUND ART

[0004] Polyketides represent a large family of diverse compounds ultimately synthesized from 2-carbon units through a series of Claisen-type condensations and subsequent modifications. Members of this group include antibiotics such as tetracyclines, anticancer agents such as daunorubicin, and immunosuppressants such as FK506 and rapamycin. Polyketides occur in many types of organisms including fungi and mycelial bacteria, in particular, the actinomycetes.

[0005] The polyketides are synthesized by polyketide synthases (PKS). This group of enzymatically active proteins is considered in a different category from the fatty acid synthases which also catalyze condensation of 2-carbon units to result in, for example, fatty acids and prostaglandins. Two major types of PKS are known which are vastly different in their construction and mode of synthesis. These are commonly referred to as Type I or “modular” and Type II, “aromatic.”

[0006] The PKS scaffold that is the subject of the present invention is a member of the group designated Type I or “modular” PKS. In this type, a set of separate active sites exists for each step of carbon chain assembly and modification, but the individual proteins contain a multiplicity of such separate active sites. There may be only one multifunctional protein of this type, such as that required for the biosynthesis of 6-methyl salicylic acid (Bct., J. et al., *Eur J Biochem* (1990) 192:487-498; Davis, R. et al., *Abstracts of Genetics of Industrial Microorganisms Meeting*, Montreal, Abstract P288 (1994)). More 1 0 commonly, and in bacterial-derived Type I PKS assemblies, there are several such multifunctional proteins assembled to result in the end product polyketide. (Cortes, J. et al., *Nature* (1990) 348:176; Donadio, S. et al., *Science* (1991) 252:675; MacNeil, D. J. et al., *Gene* (1992) 115:119.)

[0007] The PKS for erythromycin, used as an illustrative system is a modular PKS. Erythromycin was originally isolated from *S. erythraeus* (since reclassified as *Saccharopolyspora erythraea*) which was found in a soil sample from the Philippine archipelago. Cloning the genes was described by Donadio, S. et al., *Science* (1991) 252:675. The particulars have been reviewed by Perun, T. J. in *Drug Action and Drug Resistance in Bacteria*, Vol. 1, S. Mitsuhashi (ed.) University Park Press, Baltimore, 1977. The antibiotic occurs in various glycosylated forms, designated A, B and C during various stages of fermentation. The entire erythromycin biosynthetic gene cluster from *S. erythraeus* has been mapped and sequenced by Donadio et al. in *Industrial Microorganisms: Basic and Applied Molecular Genetics* (1993) R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (eds.) (Amer Soc Microbiol) and the entire PKS is an assembly of three such multifunctional proteins usually designated DEBS-1, DEBS-2, and DEBS-3, encoded by three separate genes.

[0008] Type II PKS, in contrast, include several proteins, each of which is simpler than those found in Type I polyketide synthases. The active sites in these enzymes are used iteratively so that the proteins themselves are generally monofunctional or bifunctional.

[0009] For example, the aromatic PKS complexes derived from Streptomyces have so far been found to contain three proteins encoded in three open reading frames. One protein provides ketosynthase (KS) and acyltransferase (AT) activities, a second provides a chain length determining factor (CLDF) and a third is an acyl carrier protein (ACP).

[0010] The present invention is concerned with PKS systems derived from modular PKS gene clusters. The nature of these clusters and their manipulation are further described below.

DISCLOSURE OF THE INVENTION

[0011] The invention provides recombinant materials for the production of combinatorial libraries of polyketides wherein the polyketide members of the library are synthesized by various PKS systems derived from naturally occurring PKS systems by using these systems as scaffolds. Generally, many members of these libraries may themselves be novel compounds, and the invention further includes novel polyketide members of these libraries. The invention also includes methods to recover novel polyketides with desired binding activities by screening the libraries of the invention.

[0012] Thus, in one aspect, the invention is directed to a multiplicity of cell colonies comprising a library of colonies wherein each colony of the library contains an expression vector for the production of a different modular PKS, but derived from a naturally occurring PKS. In a preferred embodiment, the different PKS are derived from the erythromycin PKS. In any case, the library of different modular PKS is obtained by modifying one or more of the regions of a naturally occurring gene or gene cluster encoding an enzymatic activity so as to alter that activity, leaving intact the scaffold portions of the naturally occurring gene. In another aspect, the invention is directed to a multiplicity of cell colonies comprising a library of colonies wherein each colony of the library contains a different modular PKS derived from a naturally occurring PKS, preferably the erythromycin PKS. The invention is also directed to methods to produce libraries of PKS complexes and to produce libraries of polyketides by culturing these colonies, as well
as to the libraries so produced. In addition, the invention is directed to methods to screen the resulting polyketide libraries and to novel polyketides contained therein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 is a diagram of the erythromycin PKS complex from S. erythraea showing the function of each multifunctional protein.

[0014] FIG. 2 is a diagram of DEBS-1 from S. erythraea showing the functional regions separated by linker regions.

[0015] FIG. 3 shows a diagram of a vector containing the entire erythromycin gene cluster.

[0016] FIG. 4 shows a method for the construction of the vector of FIG. 3.

[0017] FIG. 5 shows the structures of several polyketides produced by manipulating the erythromycin PKS gene cluster.

[0018] FIG. 6 shows the construction of derivative PKS gene clusters from the vector of FIG. 3.

MODES OF CARRYING OUT THE INVENTION

[0019] It may be helpful to review the nature of the erythromycin PKS complex and the gene cluster that encodes it as a model for modular PKS, in general.

[0020] FIG. 1 is a diagrammatic representation of the gene cluster encoding erythromycin. The erythromycin PKS protein assembly contains three high-molecular-weight proteins (>200 kD) designated DEBS-1, DEBS-2 and DEBS-3, each encoded by a separate gene (Caffrey et al., FEBS Lett. (1992) 304:225). The diagram in FIG. 1 shows that each of the three proteins contains two modules of the synthase—a module being that subset of reactivities required to provide an additional 2-carbon unit to the molecule. As shown in FIG. 1, modules 1 and 2 reside on DEBS-1; modules 3 and 4 on DEBS-2 and modules 5 and 6 on DEBS-3. The minimal module is typified in module 3 which contains a ketosynthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP). These three functions are sufficient to activate an extender unit and attach it to the remainder of the growing molecule. Additional activities that may be included in a module relate to 5 reactions other than the Claisen condensation, and include a dehydratase activity (DH), an enoylreductase activity (ER) and a keto-reductase activity (KR). The first module also contains repeats of the AT and ACP activities because it catalyzes the initial condensation, i.e. it begins with a “loading domain” represented by AT and ACP, which determine the nature of the starter unit. Although not shown, module 3 has a KR region which has been inactivated by mutation. The “finishing” of the molecule is regulated by the thioesterase activity (TE) in module 6. This thioesterase appears to catalyze cyclization of the macrocyclic ring thereby increasing the yield of the polyketide product.

[0021] FIG. 2 shows a detailed view of the regions in the first two modules which comprise the first open reading frame encoding DEBS-1. The regions that encode enzymatic activities are separated by linker or “scaffold”-encoding regions. These scaffold regions encode amino acid sequences that space the enzymatic activities at the appropriate distances and in the correct order. Thus, these linker regions collectively can be considered to encode a scaffold into which the various activities are placed in a particular order and spatial arrangement. This organization is similar in the remaining genes, as well as in other naturally occurring modular PKS gene clusters.

[0022] The three DEBS-1, 2 and 3 proteins are encoded by the genetic segments ery-AI, ery-AII and ery-AIII, respectively. These reading frames are located on the bacterial chromosome starting at about 10 kb distant from the erythromycin resistance gene (ermE or eryR).

[0023] The detailed description above referring to erythromycin is typical for modular PKS in general. Thus, rather than the illustrated erythromycin, the polyketide synthases making up the libraries of the invention can be derived from the synthases of other modular PKS, such as those which result in the production of rapamycin, avermectin, FK-506, FR-008, monensin, rifamycin, soraphen-A, spinocyn, squalstatin, or tylosin, and the like.

[0024] Regardless of the naturally occurring PKS gene used as a scaffold, the invention provides libraries, ultimately of polyketides, by generating a variety of modifications in the erythromycin PKS or other naturally occurring PKS gene cluster so that the protein complexes produced by the cluster have altered activities in one or more respects, and thus produce polyketides other than the natural product of the PKS. By providing a large number of different genes or gene clusters derived from a naturally occurring PKS gene cluster, each of which has been modified in a different way from the native cluster, an effectively combinatorial library of polyketides can be produced as a result of the multiple variations in these activities. All of the PKS encoding sequences used in the present invention represent modular polyketide synthases “derived from” a naturally occurring PKS, illustrated by the erythromycin PKS. As will be further described below, the mates and bounds of this derivation can be described on both the protein level and the encoding nucleotide sequence level.

[0025] By a modular PKS “derived from” the erythromycin or other naturally occurring PKS is meant a modular polyketide synthase (or its corresponding encoding gene(s)) that retains the scaffolding of all of the utilized portion of the naturally occurring gene. (Not all modules need be included in the constructs.) On the constant scaffold, at least one enzymatic activity is mutated, deleted or replaced, so as to alter the activity. Alteration results when these activities are deleted or are replaced by a different version of the activity, or simply mutated in such a way that a polyketide other than the natural product results from these collective activities. This occurs because there has been a resulting alteration of the starter unit and/or extender unit, and/or stereochemistry, and/or chain length or cyclization and/or reductive or dehydration cycle outcome at a corresponding position in the product polyketide. Where a deleted activity is replaced, the origin of the replacement activity may come from a corresponding activity in a different naturally occurring polyketide synthase or from a different region of the same PKS. In the case of erythromycin, for example, any or all of the DEBS-1, DEBS-2 and DEBS-3 proteins may be included in the derivative or portions of any of these may be included, but the scaffolding of an erythromycin PKS protein is retained in whatever derivative is considered. Similar comments pertain to the corresponding ery-AI, ery-AII and ery-AIII genes.
The derivative may contain preferably at least a thioesterase activity from the erythromycin or other naturally occurring PKS gene cluster.

In summary, a polyketide synthase "derived from" a naturally occurring PKS contains the scaffolding encoded by all or the portion employed of the naturally occurring synthase gene, contains at least two modules that are functional, and contains mutations, deletions, or replacements of one or more of the activities of these functional modules so that the nature of the resulting polyketide is altered. This definition applies both at the protein and genetic levels. Particular preferred embodiments include those wherein a KS, AT, KR, DH or ER has been deleted or replaced by a version of the activity from a different PKS or from another location within the same PKS. Also preferred are derivatives where at least one noncondensation cycle enzymatic activity (KR, DH or ER) has been deleted or wherein any of these activities has been mutated so as to change the ultimate polyketide synthesized.

Thus, there are five degrees of freedom for constructing a polyketide synthase in terms of the polyketide that will be produced. First, the polyketide chain length will be determined by the number of modules in the PKS. Second, the nature of the carbon skeleton of the PKS will be determined by the specificities of the acyl transferases which determine the nature of the extender units at each position—e.g., malonyl, methyl malonyl, or ethyl malonyl, etc. Third, the loading domain specificity will also have an effect on the resulting carbon skeleton of the polyketide. Thus, the loading domain may use a different starter unit, such as acetyl, propionyl, and the like. Fourth, the oxidation state at various positions of the polyketide will be determined by the dehydratase and reductase portions of the modules. This will determine the presence and location of ketone, alcohol, alkene substituents or whether a single -OH bond will result at particular locations in the polyketide. Finally, the stereochemistry of the resulting polyketide is a function of three aspects of the synthase. The first aspect is related to the AT/KS specificity associated with substituted malonlys as extender units, which affects stereochemistry only when the reductive cycle is missing or when it contains only a ketoreductase since the dehydratase would abolish chirality. Second, the specificity of the ketoreductase will determine the chirality of any P-OH. Finally, the enoyl reductase specificity for substituted malonlys as extender units will influence the result when there is a complete KR/DH/ER available.

In the working examples below, all of the foregoing variables other than the loading domain specificity which controls the starter unit have been varied.

Thus, the modular PKS systems, and in particular, the erythromycin PKS system, permit a wide range of polyketides to be synthesized. As compared to the aromatic PKS systems, a wider range of starter units including aliphatic monomers (acetyl, propionyl, butyryl, isovaleryl, etc.), aromatics (aminohydroxybenzoyl), aticyclydes (cyclohexanoyl), and heterocycles (thiazoyl) are found in various macrocyclic polyketides. Recent studies have shown that modular PKSs have relaxed specificity for their starter units (Kao et al. Science (1994), supra). Modular PKSs also exhibit considerable variety with regard to the choice of extender units in each condensation cycle. The degree of β-ketoreduction following a condensation reaction has also been shown to be altered by genetic manipulation (Donadio et al. Science (1991), supra; Donadio, S. et al. Proc Natl Acad Sci USA (1993) 90:7119-7123). Likewise, the size of the polyketide product can be varied by designing mutants with the appropriate number of modules (Kao, C. M. et al. J Am Chem Soc (1994) 116:11612-11613). Lastly, these enzymes are particularly well-known for e.g. generating an impressive range of asymmetric centers in their products in a highly controlled manner. Thus, the combinatorial potential within modular PKS pathways based on any naturally occurring modular, such as the erythromycin PKS scaffold, is virtually unlimited.

Methods to Construct Multiple Modular PKS Derived from a Naturally Occurring PKS

The derivatives of the a naturally occurring PKS can be prepared by manipulation of the relevant genes. A large number of modular PKS gene clusters have been mapped and/or sequenced, including erythromycin and rapamycin, which have been completely mapped and sequenced, and soraphen A, FK506 and oleandomycin which have been partially sequenced, and coccidin, avermectin, and nemadectin which have been mapped and partially sequenced. Additional modular PKS gene clusters are expected to be available as time progresses. These genes can be manipulated using standard techniques to delete or inactive activity encoding regions, insert regions of genes encoding corresponding activities form the same or different PKS system, or otherwise mutated using standard procedures for obtaining genetic alterations. Of course, portions of, or all of, the desired derivative coding sequences can be synthesized using standard solid phase synthesis methods such as those described by Jaye et al., J Biol Chem (1984) 259:6361 and which are available commercially from, for example, Applied Biosystems, Inc.

In order to obtain nucleotide sequences encoding a variety of derivatives of the naturally occurring PKS, and thus a variety of polyketides for construction of a library, a desired number of constructs can be obtained by “mixing and matching’ enzymatic activity-encoding portions, and mutations can be introduced into the native host PKS gene cluster or portions thereof.

Mutations can be made to the native sequences using conventional techniques. The substrates for mutation can be an entire cluster of genes or only one or two of them; the substrate for mutation may also be portions of one or more of these genes. Techniques for mutation include preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a PKS subunit using restriction endonuclease digestion. (See, e.g., Kunkel, T. A. Proc Natl Acad Sci USA (1985) 82:448; Geisselsoder et al. BioTechniques (1987) 5:786.) Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length) which hybridizes to the native nucleotide sequence (generally cDNA corresponding to the RNA sequence), at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located. Zoller and Smith, Methods Enzymol (1983) 100:468. Primer extension is effected using DNA poly-
merase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Selection can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. See, e.g., Dlabie-McFarland et al. Proc Natl Acad Sci USA (1982) 79:6409. PCR mutagenesis will also find use for effecting the desired mutations.

[0035] Random mutagenesis of selected portions of the nucleotide sequences encoding enzymatic activities can be accomplished by several different techniques known in the art, e.g., by inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during in vitro DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants or by damaging plasmid DNA in vitro with chemicals. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as nitrosoquinuclidine, 5-bromouracil, 2-aminopurine, or acridine intercalating agents such as proflavine, acriflavin, quinacrine, and the like. Generally, plasmid DNA or DNA fragments are treated with chemicals, transformed into E. coli and propagated as a pool or library of mutant plasmids.

[0036] In addition to providing mutated forms of regions encoding enzymatic activity, regions encoding corresponding activities from different PKS synthases or from different locations in the same PKS synthase can be recovered, for example, using PCR techniques with appropriate primers. By “corresponding” activity encoding regions is meant those regions encoding the same general type of activity—e.g., a ketoreductase activity in one location of a gene cluster would “correspond” to a ketoreductase-encoding activity in another location in the gene cluster or in a different gene cluster; similarly, a complete reductase cycle could be considered corresponding—e.g., KR/DH/ER would correspond to KR alone.

[0037] If replacement of a particular target region in a host polyketide synthase is to be made, this replacement can be conducted in vitro using suitable restriction enzymes or can be effected in vivo using recombinant techniques involving homologous sequences framing the replacement gene in a donor plasmid and a receptor region in a recipient plasmid.

[0038] Such systems, advantageously involving plasmids of differing temperature sensitivities are described, for example, in PCT application WO 96/40968.

[0039] The vectors used to perform the various operations to replace the enzymatic activity in the host PKS genes or to support mutations in these regions of the host PKS genes may be chosen to contain control sequences operably linked to the resulting coding sequences in a manner that expression of the coding sequences may be effected in an appropriate host. However, simple cloning vectors may be used as well.

[0040] If the cloning vectors employed to obtain PKS genes encoding derived PKS lack control sequences for expression operably linked to the encoding nucleotide sequences, the nucleotide sequences are inserted into appropriate expression vectors. This need not be done individually, but a pool of isolated encoding nucleotide sequences can be inserted into host vectors, the resulting vectors transformed or transfected into host cells and the resulting cells plated out into individual colonies.

[0041] Suitable control sequences include those which function in eucaryotic and prokaryotic host cells. Preferred host include fungal systems such as yeast and prokaryotic hosts, but single cell cultures of, for example, mammalian cells could also be used. There is no particular advantage, however, in using such systems. Particularly preferred are yeast and prokaryotic hosts which use control sequences compatible with Streptomyces spp. Suitable controls sequences for single cell cultures of various types of organisms are well known in the art. Control systems for expression in yeast, including controls which effect secretion are widely available are routinely used. Control elements include promoters, optionally containing operator sequences, and other elements depending on the nature of the host, such as ribosome binding sites. Particularly useful promoters for prokaryotic hosts include those from PKS gene clusters which result in the production of polyketides as secondary metabolites, including those from aromatic (Type II) PKS gene clusters. Examples are act promoters, tcm promoters, spiramycin promoters, and the like.

[0042] However, other bacterial promoters, such as those derived from sugar metabolizing enzymes, such as galactose, lactose (lac) and maltose, are also useful. Additional examples include promoters derived from biosynthetic enzymes such as tryptophan (trp), the β-lactamase (bla), bacteriophage lambda PL, and T5. In addition, synthetic promoters, such as the tac promoter (U.S. Pat. No. 4,551,433), can be used.

[0043] Other regulatory sequences may also be desirable which allow for regulation of expression of the PKS replacement sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

[0044] Selectable markers can also be included in the recombinant expression vectors. A variety of markers are known which are useful in selecting for transformed cell lines and generally comprise a gene whose expression confers a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium. Such markers include, for example, genes which confer antibiotic resistance or sensitivity to the plasmid. Alternatively, several polyketides are naturally colored and this characteristic provides a built-in marker for screening cells successfully transformed by the present constructs.

[0045] The various PKS nucleotide sequences, or a cocktail of such sequences, can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, e.g., a single promoter. The PKS subunits or cocktail components can include flanking restriction sites to allow for the easy deletion and insertion of other PKS subunits or cocktail components so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.
As described above, particularly useful control sequences are those which themselves, or using suitable regulatory systems, activate expression during transition from growth to stationary phase in the vegetative mycelium. The system contained in the illustrated plasmid pCK7, i.e., the act/actIII promoter pair and the actII-ORF4, an activator gene, is particularly preferred. Particularly preferred hosts are those which lack their own means for producing polyketides so that a cleaner result is obtained. Illustrative host cells of this type include the modified S. coelicolor CH999 culture described in PCT application WO 96/40968 and similar strains of S. lividans.

[0047] The expression vectors containing nucleotide sequences encoding a variety of PKS systems for the production of different polyketides are then transformed into the appropriate host cells to construct the library. In one straightforward approach, a mixture of such vectors is transformed into the selected host cells and the resulting cells plated into individual colonies and selected for successful transformants. Each individual colony will then represent a colony with the ability to produce a particular PKS synthase and ultimately a particular polyketide. Typically, there will be duplications in some of the colonies; the subset of the transformed colonies that contains a different PKS in each member colony can be considered the library. Alternatively, the expression vectors can be used individually to transform hosts, which transformed hosts are then assembled into a library. A variety of strategies might be devised to obtain a multiplicity of colonies each containing a PKS gene cluster derived from the naturally occurring host gene cluster so that each colony in the library produces a different PKS and ultimately a different polyketide. The number of different polyketides that are produced by the library is typically at least four, more typically at least ten, and preferably at least 20, more preferably at least 50, reflecting similar numbers of different altered PKS gene clusters and PKS gene products. The number of members in the library is arbitrarily chosen; however, the degrees of freedom outlined above with respect to the variation of starter, extender units, stereochemistry, oxidation state, and chain length is quite large.

[0048] Methods for introducing the recombinant vectors of the present invention into suitable hosts are known to those of skill in the art and typically include the use of CaCl2 or other agents, such as divalent cations, lipofection, DMSO, protoplast transformation and electroporation.

[0049] The polyketide producing colonies can be identified and isolated using known techniques and the produced polyketides further characterized. The polyketides produced by these colonies can be used collectively in a panel to represent a library or may be assessed individually for activity.

[0050] The libraries can thus be considered at three levels: (1) a multiplicity of colonies each with a different PKS encoding sequence encoding a different PKS cluster but all derived from a naturally occurring PKS cluster; (2) colonies which contain the proteins that are members of the PKS produced by the coding sequences; and (3) the polyketides produced. Of course, combination libraries can also be constructed wherein members of a library derived, for example, from the erythromycin PKS can be considered as a part of the same library as those derived from, for example, the rapamycin PKS cluster.

[0051] Colonies in the library are induced to produce the relevant synthases and thus to produce the relevant polyketides to obtain a library of candidate polyketides. The polyketides secreted into the media can be screened for binding to desired targets, such as receptors, signaling proteins, and the like. The supernatants per se can be used for screening, or partial or complete purification of the polyketides can first be effected. Typically, such screening methods involve detecting the binding of each member of the library to receptor or other target ligand. Binding can be detected either directly or through a competition assay. Means to screen such libraries for binding are well known in the art.

[0052] Alternatively, individual polyketide members of the library can be tested against a desired target. In this event, screens wherein the biological response of the target is measured can more readily be included.

**EXAMPLES**

**Materials and Methods**

**General Techniques**

**Bacterial Strains, Plasmids, and Culture Conditions**

**S. coelicolor CH999** described in WO 95/05848, published Mar. 30, 1995 was used as an expression host.

**DNA manipulations were performed in Escherichia coli MC1061. Plasmids were passaged through E. coli ET12567 (dam dcm bsd S Cm) (MacNeil, D. J. J. Bacteriol (1988) 170:5607) to generate unmethylated DNA prior to transformation of S. coelicolor. coli strains were grown under standard conditions. S. coelicolor strains were grown on R2YE agar plates (Hopwood, D. A. et al. Genetic manipulation of Streptomyces. A laboratory manual. The John Innes Foundation: Norwich, 1985). prRM5, also described in WO 95/08548, includes a colEI replicon, an appropriately truncated SCP2* Streptomyces replicon, two act-promoters to allow for bidirectional cloning, the gene encoding the actII-ORF4 activator which induces transcription from act promoters during the transition from growth phase to stationary phase, and appropriate marker genes. Engineered restriction sites facilitate the combinatorial construction of PKS gene clusters starting from cassettes encoding individual domains of naturally occurring PKSs.

**When prRM5 is used for expression of PKS, (i) all relevant biosynthetic genes are plasmid borne and therefore amenable to facile manipulation and mutagenesis in E. coli, (ii) the entire library of PKS gene clusters can be 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 in a secondary metabolite-like manner, thereby alleviating the toxic effects of synthesizing potentially bioactive compounds in vivo, and (iv) molecules thus produced undergo fewer side reactions than if the same pathways were expressed in wild-type organisms or blocked mutants.
Polymerase chain reaction (PCR) was performed using Taq polymerase (Perkin Elmer Cetus) under conditions recommended by the enzyme manufacturer. Standard in vitro techniques were used for DNA manipulations (Sambrook, et al. Molecular Cloning: A Laboratory Manual, Current Edition). E. coli was transformed with a Bio-Rad E. Coli Pulsing apparatus using protocols provided by Bio-Rad. S. coelicolor was transformed by standard procedures (Hopwood, D. A. et al. Genetic manipulation of Streptomyces. A laboratory manual. The John Innes Foundation: Norwich, 1985) and transformants were selected using 2 ml of a 500 mg/μl thiostrepton overlay.

Production and Purification of Polyketides.

For initial screening, all strains were grown at 30°C as confluent lawns on 150 mm Petri plates containing 50 ml of R2YE agar supplemented with 50 mg/ml thiostrepton poured over a 125 mm disc of Whatman 52 filter paper. After 2-3 days of growth, the agar disc was lifted from the dish and placed atop a layer of 6 mm glass beads mixed with 60 ml of liquid R2YE medium and 3 g of Amberlite XAD-16 absorption resin in a 150 mm Petri dish. Growth was continued for an additional 6 days at 30°C. The agar disc was removed, and the XAD-16 resin was collected by vacuum filtration. After washing with water, the resin was shaken with 15 ml of ethanol for 30 min. The ethanol extract was decanted from the resin, and the extraction was repeated twice more. The combined ethanol extracts were then evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate, washed once with saturated aqueous NaHCO₃, then analyzed by HPLC (water-acetonitrile-acetic acid gradient, C18-reversed phase) with mass spectrometric detection. For purification, extracts were separated on silica gel columns of silica gel preparative thin-layer chromatography using ethyl acetate-hexane mixtures as eluents.

Preparation A

Construction of the Complete Erthromycin PKS Gene Cluster

Recovery of the Erthromycin PKS Genes

Although various portions of the erthromycin PKS gene cluster can be manipulated separately at any stage of the process of preparing libraries, it may be desirable to have a convenient source of the entire gene cluster in one place. Thus, the entire erthromycin PKS gene cluster can be recovered on a single plasmid if desired. This is illustrated below utilizing derivatives of the plasmid pMAK705 (Hamilton et al. J Bacteriol (1989) 171:4617) to permit in vivo recombination between a temperature-sensitive donor plasmid, which is capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature, and recipient plasmid. The eryA genes thus cloned gave pCK7, a derivative of pRM5 (McDaniel et al. Science (1993) 262:1546). A control plasmid, pCK7f, was constructed to carry a frameshift mutation in eryAl. pCK7 and pCK7f possess a ColEI replicon for genetic manipulation in E. coli as well as a truncated SCP2* (low copy number) Streptomyces replicon.

These plasmids also contain the divergent actI/actIII promoter pair and actII-ORF4, an activator gene, which is required for transcription from these promoters and activates expression during the transition from growth to stationary phase in the vegetative mycelium. High-level expression of PKS genes occurs at the onset of the stationary phase of mycelial growth. The recombinant strains therefore produce the encoded polyketides as secondary metabolites.

In more detail, pCK7 (FIG. 4), a shuttle plasmid containing the complete eryA genes, which were originally cloned from pSI1 (Tuan et al. Gene (1990) 90:21), was constructed as follows. The modular DEBS PKS genes were transferred incrementally from a temperature-sensitive “donor” plasmid, i.e., a plasmid capable of replication at a first, permissive temperature and incapable of replication at a second, non-permissive temperature, to a “recipient” shuttle vector via a double recombination event, as depicted in FIG. 5. A 25.6 kb Sphi fragment from pSI1 was inserted into the Sphi site of pMAK705 (Hamilton et al. J Bacteriol (1989) 171:4617) to give pCK6 (Cm8), a donor plasmid containing eryAI, eryAll, and the 3’ end of eryAI. Replication of this temperature-sensitive pSC101 derivative occurs at 30°C but is arrested at 44°C. The recipient plasmid, pCK5 (Ap8, Tc5), includes a 12.2 kb eryA fragment from the eryAI start codon (Caffrey et al. FEBs Lett (1992) 304:225) to the XcmI site near the beginning of eryAI, a 1.4 kb EcoRI-BsmI pBR322 fragment encoding the tetracycline resistance gene (Tc), and a 4.0 kb NotI-EcoRI fragment from the end of eryAI. PacI, NdeI, and ribosome binding sites were engineered at the eryAI start codon in pCK5. pCK5 is a derivative of pRM5 (described above). The 5’ and 3’ regions of homology are 4.1 kb and 4.0 kb, respectively. pCK5 was transformed with pCK5 and pCK6 and subjected to carbenicillin and chloramphenicol selection at 30°C. Colonies harboring both plasmids (Ap8, Cm8) were then restreaked at 44°C on carbenicillin and chloramphenicol plates. Only co-integrates formed by a single recombination event between the two plasmids were viable. Surviving colonies were propagated at 30°C. Under carbenicillin selection, for the resolution of the co-integrates via a second recombination event. To enrich for pCK7 recombinants, colonies were restreaked again on carbenicillin plates at 44°C. Approximately 20% of the resulting colonies displayed the desired phenotype (APR, TcS, Cms). The final pCK7 candidates were thoroughly checked via restriction mapping. A control plasmid, pCK7F, which contains a frameshift error in eryAI, was constructed in a similar manner. pCK7 and pCK7F were transformed into E. coli ET1525/7 (MacNeil J Bacteriol (1988) 170:5607) to generate unmethylated plasmid DNA and subsequently moved into Streptomyces coelicolor CH999.

Upon growth of CH999/pCK7 on R2YE medium, the organism produced abundant quantities of two polyketides. The addition of propionic (300 mg/L) to the growth medium resulted in approximately a two-fold increase in yield of polyketide product. Proton and 13C NMR spectroscopy, in conjunction with propionic-13C acid feeding experiments, confirmed the major product as 6DEB (>40 mg/L). The minor product was identified as 8,8a-deoxyxylendiol (10 mg/L, which apparently originates from an acetate starter unit instead of propionate in the 6 DEB biosynthetic pathway. 13C2 sodium acetate feeding experiments confirmed the incorporation of acetate into the minor product. Three high molecular weight proteins (>200 kDa), presumably DEBS1, DEBS2, and DEBS3 (Caffrey et al. FEBs Lett (1992) 304:225), were also observed in crude
extracts of CH999/pCK7 via SDS-polyacrylamide gel electrophoresis. No polyketide products were observed from CH999/pCK7. The inventors hereby acknowledge support provided by the American Cancer Society (IRG-32-34).

Example 1

Preparation of Cassettes from the Rapamycin PKS

A cosmid library of genomic DNA from Streptomyces hygroscopicus ATCC 29253 was used to prepare DNA cassettes prepared from the rapamycin PKS gene cluster to be used as replacements in the enzymatic activity regions of the erythromycin gene cluster. Cassettes were prepared by PCR amplification from appropriate cosmids or subclones using the primer pairs listed in Table 1.

(The rapDH/ER/KR1 cassette sequence was amplified in two halves, then joined at the engineered XhoI site.)

Example 2

Replacement of DEBS Modules by Rapamycin PKS Cassettes

a) Replacement of DEBS DH/ER/KR4. A portion of the erythromycin gene of module 4 (eryDH/ER/KR4) was replaced either with the corresponding rapamycin activities of the first rapamycin module (rapDH/ER/KR1) or of module 4 of rapamycin (rapDH/ER/KR4). The replacement utilized the technique of Kao et al. Science (1994) 265:509-512. A donor plasmid was prepared by first amplifying 1 kb regions flanking the DEBS DH/ER/KR4 of DEBS to contain a PsI site at the 3' end of the left flank and an XbaI site at the 5' end of the right flank. The fragments were ligated into a temperature-sensitive donor plasmid, in a manner analogous to that set forth for KR6 in paragraph b) of this example and the rapamycin cassettes prepared as described in Example 1 were inserted into the PsI/XbaI sites. The recipient plasmid was pCK7 described in Preparation A. The in vivo recombination technique resulted in the expression plasmid pKOS011-19 (eryDH/ER/KR4→rapDH/ER/KR1) and pKOS011-21 (eryDH/ER/KR4→rapDH/ER/KR4). The junctions at which the PsI and XbaI sites were introduced into DEBS in both vectors are as follows:

[0069] GAGCCGACGGTTCTGCTGCAG rap cassette TCTAGAGCCGGTCGAGCGCCGGCCCG

[0070] The resulting expression vectors were transformed into S. coelicolor CH999 and successful transformants grown as described above. The transformant containing the rapDH/ER/KR1 cassette produced the polyketide shown in FIG. 6 as 11-19a; the transformant containing the plasmid with rapDH/ER/KR4 cassette produced the polyketide shown in FIG. 6 as 11-21 a. As shown, these polyketides differ from 6-deoxyerythronolide B by virtue of a 6,7-alkene in the case of 11-21 a and by the C6-methyl stereochemistry in the case of 11-19a.

b) Replacement of DEBS KR6. In a manner analogous to that set forth in paragraph a), plasmid pKOS01 1-25, wherein eryKR6 was replaced by rapDH/KR4, was prepared by substituting regions flanking the KR6 domain of DEBS in construction of the donor plasmid.

Approximately 1 kb regions flanking the eryKR6 domain were PCR amplified with the following primers:

Example 3

Left forward 5'-TTTGGATCCGTTTTCGTCTTCCCAGGTCAG 5'-TTTCTGGAGCCAGTACCGCTGGGGCTCGAA 5'-TTTTCTAGAGCGGTGCAGGCGGCCCGGGCCCG 5'-AAAATGCATCTATGAATTCCCTCCGCCCA

Example 4

These fragments were then cloned into a pMAK705 derivative in which the multiple cloning site region was modified to accommodate the restriction sites of the fragments (i.e., BamHI/PsiI for the left flank and XbaI/NsiI for the right flank). Cassettes were then inserted into the PsI/XbaI sites of the above plasmid to generate donor plasmids for the in vivo recombination protocol. The resulting PsI and XbaI junctions engineered into DEBS are as follows:

[0073] GAACACCCAGGCGTCTTCTGGCTGCAG rap cassette TCTAGAGCCGGTCGAGCGCCGGCCCG

[0075] Transformants of S. coelicolor CH999 resulted in the production of the polyketide shown in FIG. 6 as 11-25 a,b. Regions flanking the KR6 domain of DEBS were used to construct the donor plasmids.

[0076] Replacement of DEBS KR2. The eryKR2 enzymatic activity was replaced in a series of vectors using in vitro insertion into the PsI/XbaI sites of pKAO206. pKAO206 is a derivative of pCK13 described in Kao, C. M. J Am Chem Soc (1996) 118:9184-9185. It was prepared by
introducing the PstI and XbaI restriction sites positioned identically to those in the analogous 2-module DEBS system described by Bedford, D. et al. *Chem. ant Biol.* (1996) 3:827-831. Three expression plasmids were prepared: pKO2009-7 (eryKR2→rapDH/HRK); pKOAO392 (eryKR2→rapKR2); and pKOAO410 (eryKR2→rapDH/ER/ KR1). These plasmids, when transformed into *S. coelicolor* CH999, resulted in the production of polyketides with the structures 9-7 a,b; 392 a,b and 410 a,b,c in Fig. 6, respectively. An additional vector, pKOAO400 (eryKR2→rapKR4) produced the same results as pKOAO392.

Example 5

**Manipulation of Macrolide Ring Size by Directed Mutagenesis of DEBS**

Using the expression system of Kao, C. M. et al. *Science* (1994) 265:509-512, the 5 expression of DEBS1 alone (1+2), in the absence of DEBS2 and DEBS3 (in plasmid pCK9), resulted in the production of (2R,3S,4S,5R)-2,4-dimethyl-3,5-dihydroxy-n-heptanoic acid L-lactone ("the heptanoic acid L-lactone" (PK3) (see Fig. 7)) (1-3 mg/L), the expected triketide product of the first two modules (Kao, C. M. et al. *J Am Chem Soc* (1994) 116:11611-11613). Thus, a thioesterase is not essential for release of a triketide from the enzyme complex.

**Example 2**

**TABLE 2**

<table>
<thead>
<tr>
<th>plasmid</th>
<th>modules</th>
<th>genotype</th>
<th>products</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKOS008-5</td>
<td>3</td>
<td>eryKR2→ARdx</td>
<td>5-4a,b</td>
</tr>
<tr>
<td>pKOS008-51</td>
<td>2</td>
<td>eryAT2→rapAT2</td>
<td>5-1a,b</td>
</tr>
<tr>
<td>pKOS009-7</td>
<td>3</td>
<td>eryKR2→mpDH/HRK4</td>
<td>5-7b,a,c</td>
</tr>
<tr>
<td>pKOS011-13</td>
<td>6</td>
<td>eryKR6→ARdx</td>
<td>31-1a,3b,c</td>
</tr>
<tr>
<td>pKOS011-19</td>
<td>6</td>
<td>eryDH/ER/HRK4→rapDH/ER/HRK1</td>
<td>31-1a,9b,c</td>
</tr>
<tr>
<td>pKOS011-21</td>
<td>6</td>
<td>eryDH/ER/HRK4→rapDH/ER/HRK4</td>
<td>31-1a,21</td>
</tr>
<tr>
<td>pKOS011-22</td>
<td>6</td>
<td>eryDH/ER/HRK4→ΔARdx</td>
<td>31-22b</td>
</tr>
<tr>
<td>pKOS011-25</td>
<td>6</td>
<td>eryKR6→mpDH/HRK4</td>
<td>31-25a,b,c</td>
</tr>
<tr>
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<td>6</td>
<td>eryAT1→rapAT2</td>
<td>31-28a,b,c</td>
</tr>
<tr>
<td>pKOS014-9</td>
<td>2</td>
<td>eryAT2→rapAT4</td>
<td>CK 12a,b,c</td>
</tr>
<tr>
<td>pKOAO392</td>
<td>3</td>
<td>eryKR2→mpKR2</td>
<td>392b,a,c</td>
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<td>pKOAO410</td>
<td>3</td>
<td>eryKR2→mpDH/ER/HRK1</td>
<td>410b,a,c</td>
</tr>
</tbody>
</table>

**Example 4**

**Summary of DEBS Constructs**

Using the foregoing techniques, the DEBS constructs shown in Table 2 were constructed.
supra) and was constructed using the in vivo recombination strategy described earlier (Kao et al. Science (1994), supra). pCK15 is identical to pCK7 with the exception of a deletion between KR-5 and ACP-6, which occurs between residues GI 372 and A2802 of DEBS3, and the insertion of a blunt Sall fragment containing a kanamycin resistance gene (Ok A. et al. J Mol Biol (1981) 147:217) into the blunt HindIII site of pCK7. An arginine residue is present between GI372 and A2802 so that the DNA sequence at the fusion is GGGCGGGCC.

[0089] Plasmids pCK12 and pCK15 were introduced into S. coelicolor CH999 and polyketide products were purified from the transformed strains according to methods previously described (Kao et al. Science (1994), supra).

[0090] The products obtained from various transformants: CH999/pCK12 and CH999/pCK15 as well as CH999/pCK9 described above, are shown in FIG. 7. CH999/pCK12 produced the heptanoic acid L-lactone (PK3) (20 mg/L) as determined by $^1$H and $^{13}$C NMR spectroscopy. This triketide product is identical to that produced by CH999/pCK9, which expresses the unmodified DEBS1 protein alone described above. However, CH999/pCK12 produced PK3 in significantly greater quantities than did CH999/pCK9 (>10 mg/L vs. 1 mg/L), indicating the ability of the TE to catalyze thiolysis of a triketide chain attached to the ACP domain of module 2. CH999/pCK12 also produced significant quantities of PK4, a novel analog of PK3, (10 mg/L), that resulted from the incorporation of an acetate start unit instead of propionate. This is reminiscent of the ability of CH999/ pCK7, which expresses the intact PKS, to produce 8,8-deoxyoleandolide (PK1) in addition to 6DEB (PK2) described above.

[0091] Since PK4 was not detected in CH999/pCK9, its facile isolation from CH999/pCK12 provides additional evidence for the increased turnover rate of DEBS1 due to the presence of the TE. In other words, the TE can effectively recognize an intermediate bound to a “foreign” module that is four acyl units shorter than its natural substrate, 6DEB (PK2). However, since the triketide products can probably cyclize spontaneously into PK3 and PK4 under typical fermentation conditions (pH 7), it is not possible to discriminate between a biosynthetic model involving enzyme-catalyzed lactonization and one involving enzyme-catalyzed hydrolysis followed by spontaneous 5 lactonization. Thus, the ability of the 1+2+TE PKS to recognize the C-5 hydroxyl of a triketide as an incoming nucleophile is unclear.

[0092] CH999/pCK15, produced abundant quantities of (S,R,S)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethonolide (the 10-deoxymethonolide (PK5) (10 mg/L), demonstrating that the pentamodular PKS is active. PK5 was characterized using $^1$H and $^{13}$C NMR spectroscopy of natural abundance and $^{13}$C-enriched material, homonuclear correlation spectroscopy (COSY), heteronuclear correlation spectroscopy (HETCOR), mass spectrometry, and molecular modeling. PK5 is an analog of 10-deoxymethonolide (Lambalot, R. H. et al. J Antiinfectives (1992) 45:1981-1982), the aglycone of the macrolide antibiotic methymycin. The production of PK5 by a pentamodular enzyme demonstrates that active site domains in modules 5 and 6 in DEBS can be joined without loss of activity. Thus, it appears that individual modules as well as active sites are independent entities which do not depend on association with neighbor-ing modules to be functional. The 12-membered lactone ring, formed by esterification of the terminal carboxyl with the C-11 hydroxyl of the hexaketide product, indicated the ability of the 1+2+3+4+5+6+7+TE PKS, and possibly the TE itself, to catalyze lactonization of a polyketide chain one acyl unit shorter than the natural product of DEBS, 6DEB. Indeed, the formation of the PK5 may mimic the biosynthesis of the closely related 12-membered hexaketide macrolide, methymycin, which frequently occurs with the homologous 14-membered heptaketide macrolides, picromycin and/or narbomycin (Cane, D. E. et al. J Am Chem Soc (1993) 115:522-566). The erythromycin PKS scaffold can thus be used to generate a wide range of macrolactones with shorter as well as longer chain lengths.

[0093] The construction of the 1+2+3+4+5+6+7+TE PKS resulted in the biosynthesis of a previously uncharacterized 12-membered macrolactone that closely resembles, but is distinct from, the glycone of a biologically active macrolide. The apparent structural and functional independence of active site domains and modules as well as relaxed lactonization specificity suggest the existence of many degrees of freedom for manipulating these enzymes to produce new modular PKSs.

1. A method to prepare a nucleotide sequence encoding a modified PKS from a nucleotide sequence encoding a naturally occurring modular PKS wherein said naturally occurring modular PKS contains first regions which encode enzymatic activities and second regions which encode scaffolding amino acid sequences, which method comprises modifying at least one said first region.

2. The method of claim 1 wherein said modifying comprises deleting or inactivating at least one said first region.

3. The method of claim 1 wherein said modifying comprises replacing at least one said first region with a region encoding the corresponding enzymatic activity from a different naturally occurring PKS gene or from a different region of the same naturally occurring PKS gene.

4. The method of claim 1 wherein said nucleotide sequence encodes at least three PKS modules.

5. The method of claim 1 wherein said modifying results in utilization of a different extender unit.

6. The method of claim 1 wherein said modifying results in utilization of a different starter unit.

7. The method of claim 1 wherein said modification results in a polyketide of a different chain length.

8. A method to construct a library of colonies containing expression vectors for a multiplicity of different polyketide synthases which method comprises transforming recombiant host cells with a mixture of expression vectors containing the nucleotide sequences obtained by the method of claim 1; and

separating the transformed cells into individual colonies, and culturing the colonies.

9. A method to prepare a polyketide combinatorial library which method comprises culturing the library of colonies obtained by the method of claim 8 under conditions wherein said polyketides are produced.

10. A multiplicity of cell colonies comprising a library of colonies wherein each colony of the library contains an expression vector comprising a nucleotide sequence encoding a modular PKS derived from a naturally occurring PKS gene cluster wherein at least one enzymatic activity has been deleted and/or replaced by a different version of said activity
or is mutated so as to result in a polyketide other than that produced by said naturally occurring PKS and

wherein the nucleotide sequence contained in each colony in the library encodes a different PKS.

11. The multiplicity of cell colonies of claim 10 wherein said naturally occurring PKS gene cluster is the erythromycin gene cluster.

12. The multiplicity of cell colonies of claim 10 wherein, in at least one colony of said library, said different version is the corresponding enzymatic activity from a different modular PKS or from another location in the same PKS gene cluster.

13. The multiplicity of cell colonies of claim 10 wherein the number of PKS modules contained in the expression vector is different in at least two colonies of the library.

14. The multiplicity of cell colonies of claim 10 wherein the extender unit utilized by the encoded PKS is different in at least two colonies of said library.

15. A method to produce a library of modular PKS proteins which method comprises culturing the multiplicity of cell colonies or the library of colonies of claim 10 under conditions wherein said expression vectors effect production of said modular PKS proteins.


17. A multiplicity of cell colonies comprising a library of colonies wherein each colony of the library contains a modular PKS derived from a naturally occurring PKS wherein at least one enzymatic activity has been deleted or replaced by a different version of said activity or is produced from a mutated form of said gene so as to result in a polyketide other than that produced by said naturally occurring PKS, and

each colony in the library contains a different PKS.

18. The multiplicity of cell colonies of claim 17 wherein said naturally occurring PKS is the erythromycin PKS.

19. The multiplicity of claim 17 wherein the number of modules of PKS is different in at least two colonies of the library.

20. The multiplicity of claim 17 wherein the extender unit utilized by the PKS is different in at least two colonies of the library.

21. The multiplicity of claim 17 wherein the reduction cycle specificities are different in at least two colonies of said library.

22. A method to produce a combinatorial library of polyketides which method comprises culturing the cell colonies or library of colonies of claim 17 under conditions wherein polyketides whose synthesis is effected by said different PKS proteins are produced.

23. A combinatorial library of polyketides prepared by the method of claim 22.

24. A multiplicity of polyketides which comprises a combinatorial library of polyketides which results from culturing colonies containing polyketide synthases derived from a naturally occurring PKS wherein at least one enzymatic activity has been deleted and/or replaced by a different version of said activity or is mutated so as to result in a polyketide other than that produced by said naturally occurring PKS, wherein each PKS in said library produces a different polyketide.

25. The library of claim 24 wherein the chain length is different in at least two polyketides.

26. The library of claim 24 which contains at least two polyketides formed from different extender units.

27. The library of claim 24 which contains at least two polyketides of different oxidation states.

28. The library of claim 24 which contains at least two polyketides of differing stereochemistry.

29. The library of claim 24 which contains at least two polyketides formed from different starter units.

30. A method to identify a successful candidate polyketide which binds to or reacts with a target moiety, which method comprises screening the library of claim 24 by contacting each polyketide in said library with the target moiety under conditions wherein a successful candidate would form a complex with said target moiety, and
detecting any complex formed, thus identifying a polyketide of the library as the successful candidate.

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