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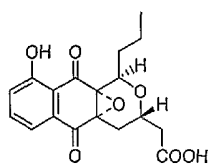
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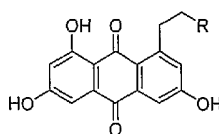
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(54) Title: HETEROLOGOUS GENES FOR ENCODING POLYKETIDES

Pharmaceutically Important Aromatic Polyketides

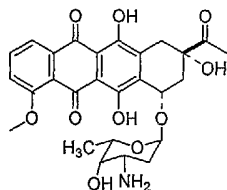


Frenolicin

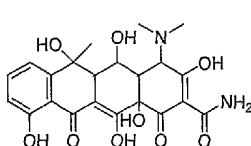


R1128

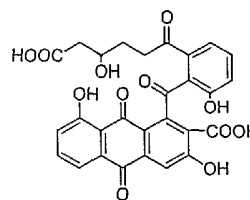
R=Me, Et, Pr, iPr



Daunorubicin



Oxytetracycline



Mumbaistatin

(57) Abstract: This invention relates to the engineered biosynthesis of new aromatic polyketides through novel starter unit incorporation. By coexpressing the proteins encoding for a PKS priming module, along with the components of a typical bacterial aromatic PKS in *Streptomyces coelicolor*, we are able to 1) biosynthesize aromatic polyketides with unique structures and cyclization patterns, and 2) insert uncommon functionalities into previously known aromatic polyketides. The invention can be applied to the biosynthesis of new, therapeutically useful polyketides.

HETEROLOGOUS GENES FOR ENCODING POLYKETIDES

Cross-Reference to Related Applications

[0001] This application is related to U.S. provisional application Serial No. 60/620,897, filed October 20, 2004, which is incorporated herein by reference in its entirety.

Field of the Invention

[0002] The invention relates to synthesizing novel polyketides by incorporating starter units that differ from the starter units used in the native production of polyketides. More specifically, the invention relates to a nucleic acid molecule that encodes such polyketides having a minimal aromatic polyketide synthases gene and heterologous gene encoding a priming module and the resultant polyketides.

Background

[0003] Polycyclic aromatic polyketides represent a large library of natural products that have important pharmacological properties, including antibacterial agents (tetracycline), anticancer agents (daunorubicin), anti-parasitic agents (frenolicin), estrogen receptor antagonists (R1128), and glucose-6-phosphate translocase inhibitors for the treatment of type II diabetes (mumbaistatin) (Figure 1). Most aromatic polyketides are found in bacteria from the actinomycetes group, such as *Streptomyces coelicolor* and *Streptomyces roseofulvus*.

[0004] Conventional or aromatic polyketides shown in Figure 1 are synthesized by their respective aromatic polyketide synthases (PKS), which consist of the “minimal PKS” and a collection of tailoring enzymes. Minimal PKS is a set of four enzymes that synthesizes nascent polyketide chains from malonyl-CoA units (Figure 3). The ketosynthase-chain length factor (KS-CLF) complex initiates chain synthesis through decarboxylation of one malonyl unit, and catalyzes the elongation of a polyketide chain until a fixed chain length is reached. An acyl-carrier protein (ACP) shuttles extender units to the active site of KS-CLF in the form of malonyl-ACP. The acyl transfer between malonyl-CoA and ACP is catalyzed by malonyl-CoA:ACP acyltransferase (MAT), which is shared between fatty acid synthases and PKSs. The starter unit incorporated by the minimal PKS is conventionally an acetate, which arises from the decarboxylation of malonyl-ACP. If the minimal PKS is co-expressed with tailoring

enzymes such as ketoreductases, aromatases, cyclases, oxidoreductases, methyltransferases and glycosyltransferases, more elaborate polyketides such as the compounds shown in Figure 1 are produced.

Summary of the Invention

[0005] Our understanding of the properties of ZhuG and ZhuH led us to the following: attaching the priming modules found in R1128 PKS to other minimal PKSs results in the incorporation of the same set of novel starter moieties into other aromatic polyketides. Similarly the priming modules found in frenolicin, Fren I and Fren J, attached to heterologous minimal PKS, also result in novel aromatic polyketides. Furthermore, by co-expressing the priming module with KS-CLFs of different chain length specificity, we have the potential to manipulate the final structures patterns of the new polyketide backbones. Finally, by co-expressing these bimodular PKSs with suitable tailoring enzymes, new analogs of polyketides such as those shown in Figure 1 can be produced.

[0006] This invention teaches how to make new polyfunctional aromatic polyketides by regioselectively replacing a naturally occurring methyl functional group in these natural products with diverse functional groups such as other alkanes, alkenes, acetylenes, cycloalkanes, aryl groups, haloalkanes and alcohols. The breadth of functional groups is limited by the availability of amino acids that can be catabolized by bacteria into corresponding acyl-CoA precursors, and by the substrate specificity of ZhuH. The former limitation can be further attenuated by metabolic engineering, whereas the latter limitation can be attenuated by structure-based protein engineering of ZhuH, whose X-ray crystal structure has recently been solved.

[0007] Thus, one embodiment is directed to a nucleic acid molecule comprising a minimal aromatic polyketide synthase (PKS) gene and a heterologous gene encoding a priming module. Preferably, the heterologous gene encodes a R1128 or frenolicin priming module. More preferably, the heterologous gene comprises a nucleic acid molecule encoding a first and a second polypeptide, wherein the first polypeptide has ketosynthase (KS) III analog activity and a second polypeptide has acyl carrier protein (ACP) activity. Most preferably, the heterologous gene comprises genes for encoding ZhuH and ZhuG, or Fren I and Fren J.

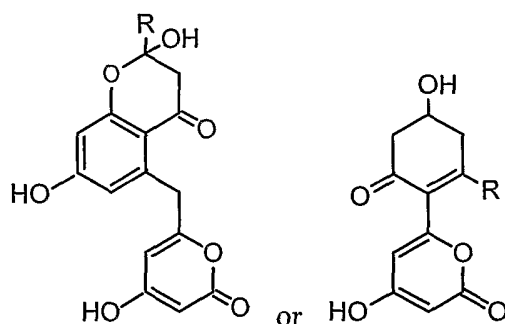
[0008] In another embodiment, the minimal polyketide synthase gene is a minimal actinomyces polyketide synthase gene. Preferably, the minimal actinomyces polyketide synthase gene is selected from the group of actinorhodin, tetracenomycin, frenolicin, R1128,

granaticin, and daunorubicin synthase genes. In a further embodiment, the minimal PKS gene comprises a gene selected from the group consisting of genes encoding FrenN, Gra-ACP, DpsG, ZhuN, Act and TcmKL.

[0009] The invention is also directed to a host cell comprising the nucleic acid molecule defined above, such as CH999. Another aspect is directed to a plasmid comprising the nucleic acid molecule described above, such as pYT46 or pYT82.

[0010] Also contemplated is a process for producing a polyketide comprising coexpressing proteins encoded by the nucleic acid molecule described above in the presence of starter units and extender units under conditions to produce an aromatic polyketide. Preferably, the starter units are non-acetate starter units, such as alkyl starter units. More preferably, the process provides an alkylacyl-ACP intermediate synthesized by the priming module which suppresses decarboxylative priming by the minimal PKS.

[0011] A further aspect is directed to a polyketide produced by such a method. Preferably, the polyketide is a compound having the formula:



or pharmaceutically acceptable salt or prodrug thereof, wherein R is a hydrocarbyl group greater than C2 optionally containing a heteroatom, such as O or halo. Preferably, R is selected from the group consisting of alkyl, alkene, acetylene, cycloalkyl, cycloalkenyl, aryl, haloalkyl, haloalkenyl, hydroxyalkyl, and hydroxyalkenyl. More preferably, the compound is selected from the group consisting of: YT82, YT82b, YT46, YT46b, YT46c, or pharmaceutically acceptable salt or prodrug thereof.

Brief Description of Figures

[0012] Figure 1 shows pharmaceutically important aromatic polyketides.

[0013] Figure 2 are aromatic polyketides. Actinorhodin, granaticin and tetracenomycin are primed by acetate primers. Frenolicin is primed by a butyryl group and R1128 is primed by a variety of acyl groups as shown.

[0014] Figure 3 shows a minimal PKS.

[0015] Figures 4A and 4B show steps in the synthesis of polyketides by minimal PKSs and proposed mechanism of chain initiation in *fren* and R1128 aromatic PKSs. In Figure 4A, minimal PKSs consist of *act* or *tcm* KS-CLF are able to synthesize C16 or C20 polyketides from eight or ten malonyl-CoA, respectively. The minimal PKS initiates polyketide synthesis through decarboxylation of one malonyl-ACP. Additional malonyl-ACP are recruited by and condensed with KS-CLF to elongate the polyketide chain until the desired chain length is reached. Without additional tailoring enzymes, SEK4/4B and SEK15/15B are formed by *act* and *tcm* minimal PKS, respectively. In Figure 4B, from *fren* and R1128 PKSs, a loading module is proposed to synthesize the starter unit. The loading module consists of a KSIII homolog, an additional ACP and MAT. KSIII catalyzes the condensation between ACP and acyl-CoA to form the β -ketoacyl-ACP. The β -ketoacyl-ACP is then reduced to an acyl-ACP by most likely homologs of FAS KR, DH and ER. The minimal PKS is primed acyl-ACP, followed by full length polyketide synthesis as in part A.

[0016] Figure 5 shows the putative primary module for R1128 PKS.

[0017] Figure 6 illustrates SDS-PAGE of purified proteins assayed. Lane 1: *act* KS-CLF; 2: *tcm* KS-CLF; 3: ZhuH; 4: FrenI; 5: FrenN; 6: ZhuN; 7: Gra; 8: DpsG; 9: ZhuG., and 10: FrenJ.

[0018] Figure 7 shows MAT catalyzed labeling of Holo-ACP by malonyl-CoA. The extent of ACP labeled by [2-¹⁴C]malonyl-CoA at three time points (30, 60 and 120 seconds) are visualized by SDS-PAGE and autoradiography. In each reaction, 1 nM MAT, 100 μ M holo-ACP and 200 μ M malonyl-CoA are present. All ACPs are labeled by MAT at comparable rates. The k_{cat} for ZhuG, ZhuN, FrenJ, FrenN, DpsG and Gra ACP are 8330, 3617, 6518, 5016, 5839, and 3340 min⁻¹, respectively.

[0019] Figure 8 shows *in vitro* titration of KS:ACP interactions.

[0020] Figure 9 illustrates that KS/CLF prefers minimal PKS ACPs.

[0021] Figure 10 illustrates that KSIII prefers priming Module ACP_{ps}.

[0022] Figure 11 shows representative kinetics of PKS activity assay. The kinetics of SEK4/SEK4B synthesis starting from malonyl-CoA (1.5 mM), catalyzed by *act* KS-CLF (0.7 μ M), MAT (100 nM) and different ACPs are shown. Minimal PKS ACPs FrenN (◆), ZhuN (□), DpsG (◇) and Gra ACP (▲) all supported polyketide formation at comparable rates.

Priming ACPs ZhuG (■) were not active in the assay. Not shown: FrenJ. Details are shown in Table 2 below.

[0023] Figures 12A and 12B are KSIII acyl-transfer assays. Figure 12A shows ZhuH (0.05 μ M)-catalyzed transfer of [14 C]propionyl-CoA (100 μ M) to malonyl-ACP; Figure 12B shows FrenI (0.05 μ M)-catalyzed transfer of [14 C]acetyl-CoA (100 μ M) to malonyl-ACP. Malonyl-ACP was generated *in situ* from MAT (100 nM), malonyl-CoA (300 μ M) and holo-ACP (50 μ M). Priming ACPs ZhuG (■) and FrenJ (Δ) were competent acceptors of acyl groups in each assay. Minimal PKS ACPs FrenN ($\blacklozenge$), ZhuN ($\square$) and DpsG ($\blacklozenge$) were not labeled by either acyl-CoA. Gra ACP ($\blacktriangle$) showed low levels of labeling in both assays.

[0024] Figure 13 shows multiple-sequence alignment of ACPs from different PKSs and FASs. The conserved catalytic serine residue is marked by *. The residue identified as important in KSIII:ACP interactions is highlighted with a box. The PKS ACPs referred to in the figure are as follows: MtmS (mithramycin), Otc_ACP (oxytetracycline), Act_ACP (actinorhodin), Gris_ACP (griseusin), SnoA3 (noglamicin), AknD (alkavinone), TcmM (tetracenomycin) and WhiE_ACP (*Streptomyces avermitilis* spore-pigment). All these PKSs are primed by acetate primers. Ecoli_ACPp and Scoe_ACPp are the FAS ACPp from *E. coli* and *S. coelicolor*, respectively.

[0025] Figure 14 shows engineered biosynthesis of novel aromatic polyketides.

[0026] Figure 15 illustrates aromatic polyketides with new structures.

[0027] Figure 16 shows engineered biosynthesis of existing aromatic polyketides containing novel starter units.

Detailed Description of Invention

[0028] Recently, the gene cluster encoding the R1128 PKS was sequenced and a putative priming module was identified (Figure 5). The priming module consists of a ketosynthase III analog (ZhuH) and an additional ACP (ZhuG). As a result of the relaxed primer unit specificity of ZhuH, the priming module synthesizes a variety of alkylacyl-ACP moieties, which are in turn elongated by the R1128 minimal PKS (the elongation module) to synthesize full-length polyketide backbones. Thus the alkylacyl-ACP intermediates are able to suppress decarboxylative priming by the minimal PKS, resulting in the replacement of the acetate primer with alkyl chains of various lengths (see structure of R1128) (Figure 3). Such suppression also occurs with frenolicin (*fren*).

[0029] Biochemical studies indicate the ketosynthases in the priming and minimal PKS modules have orthogonal ACP specificities. *In vitro* assay with four minimal PKS ACPs, two priming ACPs (including ZhuG), two KSIIIs (including ZhuH) and two KS-CLFs revealed the following: KS-CLFs displayed a clear preference towards minimal ACPs over priming ACPs. The k_{cat}/K_m values of ZhuG were attenuated by at least 50 fold in the minimal PKS assay. In the KSIII assay, the opposite specificity patterns were observed: while ZhuG was rapidly labeled by ZhuH, none of the minimal PKS ACPs were recognized by ZhuH. Thus we confirmed that ZhuH and ZhuG are indispensable elements of the priming module.

[0030] Conventional Type II polyketide synthases (PKS) (Hopwood, D. A. *et al.*, *Annu. Rev. Genet.* (199) 24:37-66) are responsible for the biosynthesis of aromatic polyketides, many of which are pharmacologically valuable compounds (Figure 2). The organization of genes within PKSs and functions of encoded enzymes parallel closely to that of type II fatty-acid synthases (FAS) (Hopwood, D. A. *Chem. Rev.* (1997) 97:2465-2498). Heterologous expression of proteins from different type II PKS clusters in engineered hosts (McDaniel, R. *et al.*, *Science* (1993) 262:1546-50) has allowed the elucidation of individual protein functions and has facilitated the rational reassembly of enzymes towards generation of novel polyketides (McDaniel, R. *et al.*, *Nature* (1995) 375:549-54; Shen, Y. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1999) 96:3622-7). The work by our laboratory (McDaniel, R. *et al.*, *J. Am. Chem. Soc.* (1993) 115:11671-11675; Fu, H. *et al.*, *Chem. Biol.* (1994) 1:205-10; Fu, H. *et al.*, *Biochemistry* (1994) 33:9321-6; Fu, H. *et al.*, *Biochemistry* (1996) 35:6527-32; McDaniel, R. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1994) 91:11542-6; Carreras, C. W. *et al.*, *Biochemistry* (1998) 37:2084-8; Dreier, J. *et al.*, *J. Biol. Chem.* (1999) 274:25108-12) and others (Yu, T. W. *et al.*, *Microbiology* (1995) 141(11):2779-91; Shen, B. *et al.*, *Science* (1993) 262:1535-40) has revealed a "minimal PKS" that is essential for the construction of a polyketide chain (Figure 4B). The minimal PKS is a set of four monofunctional enzymes that catalyze the initiation and elongation of a polyketide chain from malonyl-CoA. The ketosynthase (KS) and the chain length factor (CLF) form a heterodimer (KS-CLF) that catalyzes condensation reactions between successive malonyl units. The KS-CLF also controls the overall chain length (McDaniel, R. *et al.*, *Science* (1993) 262:1546-50) and influences the regiospecificity (McDaniel, R. *et al.*, *J. Am. Chem. Soc.* (1993) 115:11671-11675) of some of the cyclization events that occur on the full-length chain. An acyl-carrier protein (ACP) shuttles malonyl units to the active site of KS-CLF in the form of a malonyl-ACP. Acyl transfer between malonyl-

CoA and ACP is catalyzed by the malonyl-CoA:ACP transacylase (MAT), which is shared between FAS and PKSs (Summers, R. G. *et al.*, *Biochemistry* (1995) 34:9389-402).

[0031] In well-characterized PKSs such as the actinorhodin (**1**, *act* in Figure 4A) (Dreier, J. *et al.*, *J. Biol. Chem.* (1999) 274:25108-12) and tetracenomycin (**3**, *tcm* in Figure 4A) (Bao, W. *et al.*, *Biochemistry* (1998) 37:8132-8), the KS-CLF is primed by an acetate unit through decarboxylation of a malonyl-ACP. This acetyl group is then transferred to the active site of KS, followed by dissociation of ACP and association of a second equivalent of malonyl-ACP. Residues that are critical for the decarboxylation reaction have been located on both the KS (Dreier, J. *et al.*, *Biochemistry* (2000) 39:2088-95) and CLF (Bisang, C. *et al.*, *Nature* (1999) 401:502-5) subunits, although the exact priming mechanism remains elusive. Some aromatic PKSs use non-acetate primers (Moore, B. S. *et al.*, *Nat. Prod. Rep.* (2002) 19:70-99). For example, frenolicin (Bibb, M. J. *et al.*, *Gene* (1994) 142:31-9), an antimalarial agent produced by *S. roseofulvus*, is primed by a butyryl unit (although nanaomycin (see Tsuzuki, K. *et al.*, *J. Antibiot (Tokyo)* (1986) 39:1343-5), the acetate-primed analog of frenolicin, is also produced by the same PKS in significantly higher abundance). R1128, an estrogen receptor antagonist (see Hon, Y. *et al.*, *J. Antibiot.* (1993) 46:1069-1075; Hon, Y. *et al.*, *J. Antibiot.* (1993) 46:1055-1062; Hon, Y. *et al.*, *J. Antibiot.* (1993) 46:1063-1068), is primed by a variety of primer units excluding acetate. Daunorubicin, a widely-used antitumor drug, is primed by a propionyl unit (see Otten, S. L. *et al.*, *J. Bacteriol.* (1990) 172:3427-34; Bao, W. *et al.*, *Biochemistry* (1990) 38:9752-7; Bao, W. *et al.*, *J. Bacteriol.* (1999) 181:4690-5). Two unique proteins have been located in the gene clusters of these PKSs, and are believed to be responsible for chain initiation (Marti, T. *et al.*, *J. Biol. Chem.* (2000) 275, 33443-48): (1) A ketosynthase (FrenI in *fren* PKS, ZhuH in R1128 PKS and DpsC in *dnr* PKS) homologous to the FAS ketoacyl synthase III (KSIII); and (2) A second ACP (FrenJ in *fren* PKS and ZhuG in R1128 PKS) in addition to the putative minimal PKS ACP (FrenN and ZhuN). DpsG is the only ACP found in the *dnr* PKS (Otten, S. L. *et al.*, *J. Bacteriol.* (1990) 172:3427-34). The KSIII from a bacterial FAS is responsible for chain initiation, and catalyzes condensation between malonyl-ACP and a short-chain acyl-CoA to yield a β -ketoacyl-ACP (Davies, C. *et al.*, *Structure Fold. Des.* (2000) 8:185-95; Tsay, J. T. *et al.*, *J. Biol Chem.* (1992) 267:6807-14). The β -carbonyl of this intermediate is then reduced before the nascent fatty acid chain is transferred to another KS that catalyzes further chain elongation. By analogy, ZhuH and FrenI play a similar role in the R1128 and frenolicin pathways, respectively (Figure 4B) (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). The recently elucidated X-ray

crystal structure of ZhuH has provides a useful starting point for mechanistic dissection of chain initiation (Pan, H. *et al.*, *Structure* (2002) 10:1559-68).

[0032] The role of the additional ACP found in the *fren* and *R1128* clusters is not understood. We have previously shown that both apo-ZhuG and apo-ZhuN inhibited the ZhuH-catalyzed condensation between propionyl-CoA and malonyl-ZhuG, suggesting equivalent roles for both proteins in the priming mechanism (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). Furthermore, only one ACP is found in the PKS cluster of daunorubicin, thus raising the possibility of ACP gene redundancy in *fren* and *R1128*. Deciphering the precise choices of ACPs by the loading and elongation modules is critical to uncover the unusual chain initiation mechanism of non-acetate priming PKSs. Towards this end, we expressed and purified two KS-CLFs, two KSIII proteins and six ACPs from a variety of PKSs. Two *in vitro* assays were used to directly probe the abilities of the ACPs to support either polyketide synthesis catalyzed by KS-CLF, or chain initiation catalyzed by KSIII. From kinetic analysis of KS-CLF:ACP and KSIII:ACP interactions, we show that FrenJ and ZhuG are indispensable in the priming of *fren* and *R1128* PKSs, respectively, whereas FrenN and ZhuN are the preferred components of the elongation module.

[0033] Frenolicin and *R1128* PKSs are the only two aromatic PKSs sequenced to date that contain both a KSIII homolog and an additional ACP. Our results in the Examples have provided new insight into the roles of these additional ACPs. Specifically, we have shown that 1) secondary ACPs are indispensable for the activities of priming modules; but 2) they are not appropriate substrates for KS-CLF heterodimers. Similarly, while ACPs from minimal PKSs are fully interchangeable among each other, they are unable to serve as substrates for either ZhuH or FrenI (with the exception of Gra ACP, which supports low levels of chain initiation). Thus, it appears that the initiation and elongation modules of PKSs such as the frenolicin and *R1128* PKSs have orthogonal ACP specificity. It is noteworthy that DpsG from the daunorubicin pathway is a good substrate for KS-CLF proteins, but not for KSIII proteins. Since the daunorubicin gene cluster encodes only a single ACP, it may be that DpsC (the KSIII homolog from that pathway) has ACP recognition features that resemble those of KS-CLF heterodimers.

[0034] Our previous work with ZhuG, ZhuN and ZhuH led to the suggestion that ZhuG and ZhuN are interchangeable substrates of ZhuH (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). The proposal was based on the observation that apo-ZhuN, when added to a reaction containing ZhuH, malonyl-ZhuG and propionyl-CoA, was able to competitively

inhibit chain initiation. We have directly evaluated the ability of ZhuH to interact with malonyl-ZhuN, and shown thereby that malonyl-ZhuN is not a substrate of ZhuH.

[0035] The concomitant production of nanaomycin and frenolicin by the *fren* PKS in *S. roseofulvus* presumably arises as a result of competition between decarboxylative priming of the KS-CLF by malonyl-FrenN and chain transfer from butyryl-FrenJ to the KS-CLF. In contrast, the R1128 PKS does not produce polyketides derived via decarboxylative priming. Thus, the R1128 KS-CLF must accept acyl chains from acyl-ZhuG in preference to catalyzing decarboxylation of malonyl-ZhuG or malonyl-ZhuN. Reconstitution of the R1128 KS-CLF with both the loading module and minimal PKS components will provide insight into how decarboxylative priming is suppressed in R1128 PKS. In turn, this insight leads to the engineering of novel starter units into aromatic polyketides.

[0036] The resulting polyketide compound is active *in vitro* and *in vivo* for activity against a panel of representative microorganisms. The compounds of the invention thus exhibit a sufficient diversity in specificity to cover the spectrum of antibiotic activities desired.

[0037] For use in treating infectious disease, the compounds of the invention are formulated into suitable compositions which will include typical excipients, pharmaceutically acceptable counterions if the compound is a salt, further additives as desired, such as antioxidants, buffers, and the like, and administered to animals or humans. The types of formulations that are appropriate for these compounds are similar to those for the macrolide antibiotics in general. Formulations may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., latest edition. The compounds can be administered by any desired route, including injection, oral administration, transdermal administration, transmucosal administration, or any combination. The compounds of the invention can also be administered with additional active ingredients if desired.

[0038] The compounds of the invention include any stereoisomeric forms of these compounds as shown. By modifying the expression system for the PKS, or by altering the chirality of the diketide, or by synthetic chemical conversion, other stereoisomers may also be prepared. Additional chiral centers may be present in R. The stereoisomers may be administered as mixtures, or individual stereoisomers may be separated and utilized as is known in the art.

[0039] A compound of the invention contains an R group which preferably is other than methyl and preferably has more than two carbons in a hydrocarbyl residue which may contain at least one heteroatom.

[0040] R may be an alkyl, alkenyl or alkynyl group. "Alkyl" refers to a saturated straight-chain, branched chain or cyclic hydrocarbyl (or cycloalkyl) moiety containing a specified number of carbons and that may contain one or more suitable heteroatoms; similarly, alkenyl and alkynyl (or acetylene) refer to straight or branched chain or cyclic (*i.e.*, cycloalkenyl or cycloalkynyl) hydrocarbon substituents containing one or more double bonds or one or more triple bonds, respectively and that may contain one or more suitable heteroatoms. Preferred alkyl groups with respect to the R groups are propyl, butyl, dimethylpropyl, and dimethylbutyl. In one preferred embodiment, these preferred groups are substituted with halo or hydroxy. Preferably, the R group contains 3-20 carbon atoms, more preferably 3-6 carbon atoms, and may contain a heteroatom between the carbon atoms and/or may be substituted.

[0041] R may also be "haloalkyl," "haloalkenyl," or "haloalkynyl", which refers to substituents where a halo group is linked to the substituted moiety through an alkyl, alkenyl, or alkynyl linkage.

[0042] Similarly, R may be "hydroxyalkyl," "hydroxyalkenyl," and "hydroxyalkynyl", which refers to substituents where a hydroxy group is linked to the substituted moiety through an alkyl, alkenyl, or alkynyl linkage, respectively.

[0043] "Halogen" or "halo" includes fluoro, chloro, bromo and iodo.

[0044] R may be "aryl", which refers to an aromatic substituent that may contain one or more suitable heteroatoms such as phenyl. Preferably, the aryl group contains 5-20, or more preferably 5-10, carbon or backbone atoms.

[0045] R may also be "arylalkyl," "arylalkenyl," or "arylalkynyl", which refers to substituents wherein an aryl group is linked to the substituted moiety through an alkyl, alkenyl or alkynyl linkage, respectively.

[0046] Thus, included among the defined substituents herein are "heteroalkyl," "heteroalkenyl," "heteroalkynyl," "heteroaryl," "heteroarylalkyl," and the like. Suitable heteroatoms include halo, N, O, and S.

[0047] Preferably, the R group has three or more carbon atoms.

[0048] All of the foregoing substituents may be unsubstituted or may be further substituted. Typical substituents include R, -OR, -SR, -NR₂, -COR, -COOR, -CONR₂, -OOCR, -NRCOR, -OCONR₂, -CN, -CF₃, -NO₂, -SOR, -SO₂R, halogen wherein each R is independently H or is alkyl, alkenyl, alkynyl, aryl, arylalkyl, or the hetero forms of these as defined above. In addition, alkyl, alkenyl and alkynyl may be substituted by aryl or heteroaryl, which may, themselves, be further substituted.

Examples

[0049] *Materials.* [1-¹⁴C]Propionyl-CoA (55 mCi/mmol) and [1-¹⁴C]acetyl-CoA (50 mCi/mmol) were purchased from Moravsek Biochemicals; [2-¹⁴C]-Malonyl-CoA (55 mCi/mmol) was from American Radiolabeled Chemicals. Flag peptide, Anti-FLAG® M2 monoclonal antibody, Anti-FLAG® M1 agarose affinity gel and all other biochemicals, including unlabeled CoA derivatives, were purchased from Sigma. Phenyl-sepharose resin and Hitrap Q anion-exchange column were purchased from Amersham Biosciences.

Example 1

DNA Manipulation and Mutagenesis

[0050] All cloning steps were performed in *E. coli* strain XL-1 Blue. Site directed mutagenesis was performed using the Quickchange kit from Stratagene. Primers CTCGCTCGCGCTGTACGAGACCGCCGCC and CGACTCGCTCGCCGTCCTGGAGGTCGTCAC and their complementary oligonucleotides were used to introduce the M42Y in ZhuN and Y45L in ZhuG mutations, respectively.

Example 2

Protein Expression and Purification

[0051] (1) *act* and *tcm* KS-CLF. *S. coelicolor* strains CH999/pSEK38 (Dreier, J. *et al.*, *Biochemistry* (2000) 39:2088-95) and CH999/pSEK33 (McDaniel, R. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1994) 91:11542-6) were used to obtain *act* KS-CLF and *tcm* KS-CLF, respectively. pSEK38 encodes the *act* CLF with a N-terminal FLAG-tag. Spores suspensions of each strain were used to inoculate 3x1L of SMM media containing 50 mg/L thiostrepton. Mycelia from the stationary phase cultures (3 days) were collected by centrifugation and resuspended in 40 mL of disruption buffer. Mycelia were disrupted via French Press and insoluble cellular debris was removed by centrifugation (24000 g, 1 hr). DNA was precipitated by adding 0.2% polyethyleneimine (PEI) and was removed by centrifugation (24000 g, 1 hr). KS-CLF proteins were precipitated between 30% and 50% (NH₄)₂SO₄. Precipitated proteins were redissolved in Buffer A (100 mM NaH₂PO₄, 2 mM DTT, 2 mM EDTA, pH 7.4) and loaded onto a phenyl-sepharose column preequilibrated with Buffer B (buffer A plus 1.5 M (NH₄)₂SO₄). The following gradient was applied to the column: 0-30 minutes, 100% B; 30-60 minutes, 40% B; 60-200 minutes, 10% B; 200-240 minutes, 0 % B. Both *act* and *tcm* KS-CLF

complexes eluted near the end of the gradient. Fractions containing *act* KS-CLF were pooled and buffer exchanged into 50 mL of TBS buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, 10 mM CaCl₂) and loaded onto a column packed with Anti-FLAG® M1 agarose affinity gel (5 mL). The column was washed with 30 mL of TBS and KS-CLF was eluted with 3 x 5 mL of TBS containing 100 µg/mL FLAG peptide. The eluent was concentrated, buffered exchanged into Buffer A containing 20% glycerol, aliquoted, flash frozen with liquid nitrogen and stored at -80°C.

[0052] Fractions containing *tcm* KS-CLF were pooled and buffer exchanged into Buffer A and loaded onto a Hitrap Q column preequilibrated with Buffer A. The following gradient was applied to the column: 0-20 min, 0% Buffer C (Buffer A + 400 mM NaCl); 20-30 min, 35% C; 30-40 min, 35% C; 40-70 min, 60% C; 70-100 min, 100% C. The target protein eluted at 50% C. The eluent was concentrated, aliquoted and frozen as described above.

Example 3

KSIII Purification

[0053] The gene encoding FrenI was amplified from plasmid pIJ5214 (Bibb, M. J. *et al.*, *Gene* (1994) 142:31-9) and cloned into pET28a to yield pYT30. *E. coli* strains BL21(DE3)/pESM8 and BL21(DE3)/pYT30 were used to obtain ZhuH and FrenI, respectively. Purification procedures for ZhuH were described in detail previously (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). FrenI was purified using a similar protocol.

Example 4

ACP and MAT Purification

[0054] *S. coelicolor* MAT was expressed in *E. coli* and purified as described before (Summers, R. G. *et al.*, *Biochemistry* (1995) 34:9389-402). *E. coli* expression strains BAP1/pFRN, BAP1/pSK73, BAP1/pANS401, BAP1/pESM10, BAP1/pESM11 and BAP1/pYT21 were used to obtain holo versions of FrenN, Gra ACP (Sherman, D. H. *et al.*, *EMBO J.* (1989) 8:2717-25), DpsG, ZhuG, ZhuN and FrenJ, respectively. Protein expression was induced at OD₆₀₀ = 0.5 with 100 µM IPTG and allowed to proceed at 30°C for 10 hours. The cells were then harvested and lysed with sonication. ZhuG, ZhuN and FrenJ contained N-terminal His₆ Tags and were purified using Ni-NTA resin under native conditions (Qiagen),

followed by anion-exchange chromatography on a HiTrap Q column (0-100 % NaCl in 60 min, ACPs elute between 300 mM and 400 mM NaCl). FrenN, Gra ACP and DpsG were first partially purified by anion-exchange chromatography, and then purified to homogeneity using a phenyl-sepharose chromatography step (1.5 M – 0 M (NH₄)₂SO₄ in 100 min, ACPs elute between 300 mM and 100 mM (NH₄)₂SO₄). The relative amounts of holo- and apo-form of each ACP were quantified by MALDI-TOF mass spectrometry. FrenN, Gra ACP, DpsG and ZhuN were all found to be nearly 100% in the holo-protein form. ZhuG and FrenJ were found to be only 40% in the holo-protein form under the same expression conditions. The phosphopantetheinyl transferase, Sfp, was used to convert the partially apo forms of the ACP to holo-ACP. The phosphopantetheinyl transfer reactions was performed as described previously *in vitro* and was monitored by HPLC (Quadri, L. E. *et al.*, *Biochemistry* (1998) 37, 1585-95). CoASH and Sfp were removed with anion-exchange chromatography upon reaction completion. Holo-ACP proteins were buffer exchanged into Buffer A containing 20% glycerol, flash frozen and stored at -80°C. All protein concentrations were determined by the Bradford method with the Biorad Protein Kit.

Example 5

MAT Labeling Assay.

[0055] The conversion of holo-ACP to malonyl-ACP catalyzed by MAT was assayed *in vitro* using [¹⁴C]malonyl-CoA. Reactions were performed in reaction buffer (100 mM NaH₂PO₄, pH 7.0, 1 mM DTT) at 25°C. Each reaction contained 200 μM [2-¹⁴C]malonyl-CoA (55 mCi/mmol) and 100 μM holo-ACP in a final volume of 35 μL. Reactions were initiated by adding 1 nM (final concentration) of MAT. Aliquots (10 μL) were removed at 30, 60 and 120 seconds, and were quenched by adding 3X SDS-PAGE sample buffer. The quenched fractions were applied to a 4-20% SDS gel and ACPs were separated from MAT and malonyl-CoA by electrophoresis. [¹⁴C]malonyl-labeled ACPs were visualized and quantified by using a phosphoimager (InstantImager 2024, Packard).

Example 6

KS-CLF Titration Assay

[0056] This assay detects the amount of polyketides synthesized by the minimal PKS from radiolabeled malonyl-CoA. The products are either SEK4/4b for *act* KS-CLF or SEK15/15b

for *tcm* KS-CLF. Assays were performed at 30°C in 100 µL reaction buffer containing 10% glycerol. Holo-ACP concentrations were between 2 and 100 µM. All reactions contained 100 nM MAT and 1.5 mM malonyl-CoA. For reactions involving FrenN, ZhuN, Gra ACP and DpsG, the specific activities of malonyl-CoA were 1 mCi/mmol and the concentration of the KS-CLF heterodimer was 0.7 µM. To facilitate detection of products in reactions containing poor substrates such as ZhuG and FrenJ, the specific activities of CoA used were increased to 4.5 mCi/mmol and the concentrations of KS-CLF were doubled. The reaction was initiated by adding malonyl-CoA. Six aliquots (15 µL) were taken within 40 minutes and each was added to 12 µL of quench buffer (12.5% SDS). Each quenched mixture was vortexed and extracted with 300 µL ethyl acetate twice. The combined organic phases were evaporated to dryness and redissolved in 20 µL of ethyl acetate. The reaction products were separated by thin-layer chromatography (TLC) and quantified with a phosphorimager as described before.

Example 7

KSIII Assay

[0057] This assay detects the condensation between radiolabeled acyl-CoA and malonyl-ACP catalyzed by either ZhuH or FrenI. Malonyl-ACP is generated *in situ* from holo-ACP, malonyl-CoA and MAT. Reactions were performed in a final volume of 40 µL at 25°C. Each reaction contained 100 µM [¹⁴C]-acyl-CoA, 300 µM malonyl-CoA, and 50 µM holo-ACP. In reactions containing ZhuH, [1-¹⁴C]propionyl-CoA (11 mCi/mmol) was used as the acyl donor, while [1-¹⁴C]acetyl-CoA (10 mCi/mmol) was used in assays containing FrenI. Reaction mixtures were pre-incubated together with 100 nM of MAT for 5 minutes. Condensation was initiated by adding 0.05 µM (final concentration) of ZhuH or FrenI. Aliquots (6 µL) taken within 30 minutes were quenched with 1 mL 10% cold trichloroacetic acid (TCA), which precipitates the β-ketoacyl-ACP. The solution was incubated on ice for 10 min after the addition of 20 µL of BSA (10 mg/mL), followed by centrifugation (14,000 g, 5 min). The pellet was washed with 0.5 mL of 10% cold TCA and dissolved in 0.5 mL of 98% formic acid. Each sample tube was washed with an additional 0.5 mL of water and the two fractions were combined with 4 mL of scintillation fluid and the amount of radioactivity was counted by a liquid scintillation counter (Beckman LS3801).

Example 8Assay Results

[0058] To assess the protein-protein interactions between ACPs and ketosynthases from different PKS clusters, we employed two types of assays. The PKS activity assay reconstitutes the minimal PKS *in vitro* and monitors the kinetics of full-length polyketide synthesis (Carreras, C. W. *et al.*, *Biochemistry* (1998) 37:2084-8). The KSIII-dependent assay determines the initial rates of the acyl-transfer reaction between acyl-CoA and malonyl-ACP (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). We purified two KS-CLF heterodimers, two KSIII homodimers and six different ACPs for use in these assays (Figure 6). KS-CLF proteins from the actinorhodin and tetracenomycin clusters were expressed in *S. coelicolor* CH999 containing plasmids pSEK38 and pSEK33, respectively. A FLAG tag was fused to the *N*-terminus of *act* CLF, which facilitated its purification along with stoichiometric *act* KS by using an anti-FLAG affinity column. The high specificity of the column resulted in recovery of the heterodimeric complex in > 95% purity. The *tcm* KS-CLF was purified in two steps as described before (Zawada, R. J. *et al.*, *Chem. Biol.* (1999) 6:607-15) to > 80% purity as judged by SDS-PAGE. Typical yields of purified KS-CLF proteins were between 0.5 ~ 1 mg/L culture volume.

[0059] The KSIII homologs FrenI and ZhuH were expressed in *E. coli* as *N*-termini Hexa-His fusion proteins. Purification, activity (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61) and structural (Pan, H. *et al.*, *Structure* (2002) 10:1559-68) studies of ZhuH have been previously described. FrenI was purified using a similar protocol to > 90% homogeneity.

[0060] The six ACPs studied in this work are listed in Table 1.

Table 1
Proteins studied in this wor

Acyl-Carrier Proteins	
FrenN	Frenolicin minimal PKS
FrenJ	Frenolicin PKS initiation module
ZhuN	R1128 minimal PKS
ZhuG	R1128 PKS initiation module
Gra-ORF3	Granaticin minimal PKS
DpsG	Daunorubicin minimal PKS
Ketosynthases	

actI ORF1-ORF2	actinorhodin KS-CLF
tcmKL	tetracenomycin KS-CLF
ZhuH	R1128 KSIII
FrenI	Frenolicin KSIII
MAT	Malonyl-CoA:ACP acyltransferase

[0061] Among the six, FrenJ and ZhuG have been proposed to participate in the priming of *fren* and *R1128* PKSs, respectively. Each ACP was expressed in the engineered *E. coli* host BAP1 (Pfeifer, B. A. *et al.*, *Science* (2001) 291:1790-2), which encodes the phosphopantetheinyl transferase, *sfp*, on its chromosome. ZhuN, ZhuG and FrenJ contained His₆ affinity tags and were each batch purified using Ni-NTA resin, followed by an anion-exchange chromatography step. FrenN, Gra ACP and DpsG were expressed in native forms and were purified using two chromatography steps. All ACPs were purified to >95% homogeneity. Holo-ACPs are required for the *in vitro* assays described in this report. The relative amounts of holo- and apo- forms of the proteins were determined by MALDI-TOF mass spectroscopy. All ACPs except ZhuG and FrenJ were in nearly 100% holo forms when expressed in BAP1. Apo-ZhuG and apo-FrenJ (~60%) were converted to the corresponding holo forms *in vitro* by using purified Sfp protein (Quadri, L. E. *et al.*, *Biochemistry* (1998) 37:1585-95). Holo-ZhuG and holo-FrenJ were further purified using anion-exchange chromatography upon reaction completion.

[0062] *A) MAT Malonyl Transfer Assay.* Both the PKS and KSIII assays require *in situ* generation of malonyl-ACP, which can be accomplished by the addition of malonyl-CoA and catalytic amounts of MAT. To deconvolute the protein-protein interactions between ACP and ketosynthases from potentially interfering MAT:ACP interactions, we must first evaluate the kinetics of malonyl transfer for different MAT:ACP pairs. Towards this end, we determined the initial rates of the malonylation reaction by SDS-PAGE and autoradiography. As shown in figure 4, at 1 nM MAT concentration, we were able to observe a linear increase in the intensity of the labeled ACP band within the first two minutes for all ACPs. In the absence of MAT, no ACP was labeled (data not shown). All six ACPs were labeled to a comparable extent within the assay time period. The k_{cat} difference between the best substrate (ZhuG, $k_{cat} \sim 8330 \text{ min}^{-1}$) and the least preferred substrate (Gra ACP, $k_{cat} = 3340 \text{ min}^{-1}$) is less than three-fold. Under PKS and KSIII assay conditions where the MAT concentrations are 100 nM and the rates of product formation for both KS-CLF and KSIII catalyzed reactions are significantly lower than

that of MAT (see below), we do not expect the rates of malonyl-ACP formation to affect the apparent KS:ACP interactions.

[0063] *B) PKS Activity Assay.* The abilities of the ACPs to support polyketide synthesis were assessed using an assay that included reconstituted *act* or *tcm* KS-CLF. The expected products of *act* and *tcm* minimal PKSs are SEK4/4B and SEK15/15B, respectively (McDaniel, R. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1994) 91:11542-6). The levels of polyketide accumulation were monitored by radio-TLC and were used to derive the kinetic parameters of ACP:KS-CLF interactions. Under the conditions of high malonyl-CoA concentration, linear increase in polyketide accumulation was observed within forty minutes. The results of titrating different holo-ACPs into the *act* KS-CLF catalyzed reaction are shown in Figure 11. Consistent with *in vivo* studies (McDaniel, R. *et al.*, *Science* (1993) 262:1546-50; Khosla, C. *et al.*, *Mol. Microbiol.* (1992) 6:3237-49), ACPs derived from all minimal PKSs (FrenN, ZhuN, Gra ACP, DpsG) supported polyketides synthesis effectively. The k_{cat} values based on combined SEK4/SEK4B levels ranged between 0.17-0.27 min^{-1} . Similar results were observed for titrations of the same set of ACPs into the *tcm* KS-CLF catalyzed reaction (Table 2).

Table 2
Activities of *act* and *tcm* KS-CLF towards different ACPs^a.

		FrenN	ZhuN	Gra ACP	DpsG	FrenJ	ZhuG
<i>act</i> KS-CLF ^b	K_m (μM)	5.8 ± 0.40	2.7 ± 0.18	6.4 ± 1.5	6.5 ± 1.2	ND ^c	ND
	k_{cat} (min^{-1})	0.27 ± 0.005	0.18 ± 0.002	0.17 ± 0.011	0.18 ± 0.008	ND	ND
	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	47	66	27	27	1	1
	k_{rel} ^d	1.0	1.4	0.57	0.58	0.02	0.02
<i>tcm</i> KS-CLF ^e	K_m (μM)	2.3 ± 0.34	1.41 ± 0.22	1.6 ± 0.32	2.6 ± 0.77	17.3 ± 3.3	ND
	k_{cat} (min^{-1})	0.32 ± 0.010	0.31 ± 0.008	0.36 ± 0.012	0.37 ± 0.022	0.30 ± 0.018	ND
	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	139	219	225	142	17.3	5.2
	k_{rel}	1.0	1.6	1.6	1.02	0.12	0.04

[0064] Procedures of KS-CLF titration assays are given in the experimental section. ^b For *act* KS-CLF minimal PKS, the polyketides quantified in each assay are SEK4 and SEK4B (figure 2). ^c Given the low activities of these substrates, individual K_m and k_{cat} cannot be accurately determined. The k_{cat}/K_m values given for FrenJ and ZhuG represent upper-bound estimates. ^d k_{rel} is defined as the ratio of k_{cat}/K_m value relative to that of FrenN. ^e For *tcm* KS-

CLF minimal PKS, the polyketides quantified in each assay are SEK15 and SEK15B (figure 2).

[0065] These results suggest that ACPs derived from minimal PKSs can be interchanged without significant kinetic penalties, regardless of the source of the ACP.

[0066] Three of the six ACPs investigated contain N-terminal hexa-His sequences (ZhuG, ZhuN and FrenJ). To confirm that KS-CLF:ACP interactions were unaffected by these histidine-tags, we performed *act* minimal PKS activity assays with ZhuG and ZhuN after treating the two ACPs with thrombin. Under these conditions the histidine-tags were completely cleaved after thrombin digestion, as indicated by MALDI-TOF mass spectrometry (data not shown). The titration results were identical to those observed with his-tagged variants of ZhuG and ZhuN (data not shown).

[0067] C) *KSIII Activity Assay*. The recognition of different ACPs by KSIII homologs FrenI and ZhuH can be assessed using an acyl-transfer assay. Meadows et al have shown that ZhuH catalyzes chain elongation between acetyl-, propionyl-, isobutyryl- or butyryl-CoA and malonyl-ZhuG, with propionyl-CoA being the best substrate (Meadows, E. S. *et al.*, *Biochemistry* (2001) 40:14855-61). The formation of radiolabeled β -ketopentanoyl-ACP was followed using a TCA precipitation assay. Figure 12A shows the time course of ACP labeling in the presence of ZhuH and propionyl-CoA. ZhuG is the best substrate with a k_{cat} value of 69 min^{-1} , consistent with the previous report. ZhuH showed high selectivity of the ACP substrate: the k_{cat} value of ZhuG is nearly 10-fold higher than the next best substrate, FrenJ. Among the minimal PKS ACPs, only Gra ACP exhibited detectable labeling. FrenN, ZhuN and DpsG were completely unlabeled.

[0068] To compare the ACP specificities of KSIII homologs, we performed the same assay with FrenI. We first determined the substrate specificity of FrenI towards acetyl-, propionyl- and butyryl-CoA. We expected acetyl-CoA to be the preferred acyl donor group, since a butyryl group is found at C16 of frenolicin (Figure 2). We reasoned that the butyryl group arises from the sequential reduction of acetoacetyl-FrenJ by homologs of FabG, FabA and FabI, in the same fashion as the R1128 starter units. Our findings concurred the hypothesis: the k_{cat} for acetyl-CoA in this assay was 32.8 min^{-1} (Figure 12B), approximately three-fold higher than that of propionyl-CoA, and 30-fold higher than that of butyryl-CoA (data not shown). We therefore used acetyl-CoA as the acyl donor in the FrenI:ACP assay and the results are shown in Figure 12B. FrenI displayed less selectivity towards priming ACPs, k_{cat} of ZhuG (17.6 min^{-1}) is reduced by less than two-fold compared to that of FrenJ. Consistent with

the results from the ZhuH:ACP titration assay, Gra ACP was the only minimal PKS ACP that was labeled by FrenI.

Example 9

Sequence Alignment and ACP Mutagenesis.

[0069] Sequence alignments of ACPs from different PKS clusters and FASs are shown in Figure 13. No major primary *sequence* differences are apparent between ZhuG, FrenJ and the rest of the PKS ACPs. We note subtle sequence features unique only to the pair of priming ACPs. Helix-II of ACPs (approximately spanning residues 40 ~ 50) has previously been noted to interact extensively with ketosynthases (Crump, M. P. *et al.*, *Biochemistry* (1997) 36:6000-8; Li, Q. *et al.*, *Biochemistry Submitted* (2003); Zhang, Y. M. *et al.*, *J. Biol. Chem.* (2001) 276:8231-8). The universally conserved serine (Ser41 in ZhuG) that carries the phosphopantetheinyl prosthetic arm is located within this helix. Residue 45 in both ZhuG and FrenJ is a tyrosine, whereas leucine (the consensus amino acid) or methionine is usually found at the corresponding position in all known minimal PKS ACPs. In the ACP from either the *E. coli* or *S. coelicolor* fatty acid synthase, this residue is occupied by a valine. Introduction of the bulky tyrosine residue within helix-II may lead to a significantly altered secondary structure that facilitates recognition by PKS KSIII exclusively. To assess the importance of this residue, we performed site-directed mutagenesis on both ZhuG and ZhuN. The mutants ZhuG-Y45L and ZhuN-M42Y were expressed, purified and phosphopantetheinylated as described for the wild-type ACPs. In the PKS assay, activity of neither mutant significantly changed when compared to the wild type ACP. However, the mutations affected the activities of the ACPs in the ZhuH-catalyzed condensation assay as shown in Table 3.

Table 3
Activities of ZhuG, ZhuN and mutant ACPs in ZhuH catalyzed acyl-transfer assay^a.

ACP	Initial velocity (min ⁻¹) ^b
ZhuG	69
ZhuG-Y45L	42
ZhuN	No activity
ZhuN-M42Y	14.7

^a [¹⁴C] labeled propionyl-CoA was used as the acyl donor.

^b Initial velocities were calculated from time points taken in the first 10 minutes of assay.

[0070] Replacement of tyrosine with leucine in ZhuG resulted in a less than two-fold decrease in the initial velocity. More notably, introduction of tyrosine within the helix-II of ZhuN allowed the ACP to be acylated at a rate twice that of FrenJ. The ZhuH:ACP assay demonstrates that while this residue is important in modulating KSIII:ACP interactions, its identity is not essential to facilitate KS-CLF:ACP recognition.

Example 10

New polyketide products (Figure 14, 15)

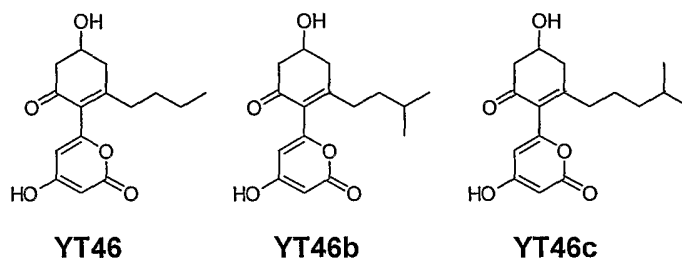
[0071] We constructed the following plasmids:

pYT44:	act KR – act KS/CLF – ZhuN – ZhuH
pYT46:	act KR – act KS/CLF – ZhuN – ZhuG – ZhuH
pSEK33:	tcm KS/CLF – act ACP
pYT82:	tcm KS/CLF – ZhuN – ZhuG – ZhuH

[0072] The PKS genes included are shown to the right of the plasmid names. As negative controls, pYT44 and pSEK33 were constructed. No priming module ACP (ZhuG) was included in these two constructs. The plasmids pYT46 and pYT82 contained both the minimal PKS and the priming module. Act KS/CLF restricts the total polyketide chain to 16 carbon units, while tcm KS/CLF restricts chain length to 20 carbon units (see Figure 3). Each of these plasmids was transformed into an engineered strain of *S. coelicolor*, CH999. This strain has been optimized for the biosynthesis, detection and isolation of aromatic polyketides. Transformants containing each of the plasmids were restreaked on R5 plates. After 10 days of culturing, polyketide products were recovered from agar plates, purified and characterized.

[0073] Comparison of HPLC analyses of products synthesized by CH999/YT44 and CH999/YT46 revealed that several new polyketides were present in the CH999/YT46 extract. Mass spectrometry and NMR characterization were used to elucidate the structures of these compounds. The two major compounds are YT46 and YT46b. Both of these compounds are primed by non-acetate starter units. YT46 is primed by a propionyl starter unit while YT46b is primed by an isobutyryl starter unit. In addition, small amounts of YT46c, which is identical to YT46, except for an isovaleryl priming unit, is also isolated. The YT46 series of compounds incorporate one starter molecule and five malonyl-CoA extender units. The total chain length is between 15~17 carbons, which agrees with the previous C16 assignment of act KS-CLF. The YT46 series of compounds are novel molecules previously not isolated from polyketide producing organisms. These molecules are biosynthesized by the combined

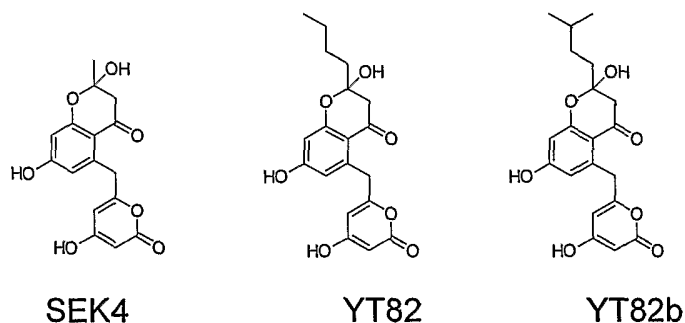
priming/minimal PKS modules in good yields (>30 mg/L). These results confirmed that an alkylacyl-ZhuG is able to suppress decarboxylative priming by the act KS/CLF and the KS/CLF is subsequently able to process the nascent polyketide chain despite a different "head group".



Example 11

Existing polyketides with new starter units (Figure 16)

[0074] To generalize the above observations to other minimal PKSs we characterized the product profile of CH999/pYT82. Compared to the control CH999/pSEK33, which primarily produces SEK15, two additional products were observed in large quantities (> 30 mg/L). These two compounds were shown to be YT82 and YT82b. YT82 is a variant of the previously known compound SEK4. YT82 contains a four carbon alkyl units instead of a methyl group found in SEK4, while YT82b contains a five carbon alkyl unit.



[0075] This represents the first successful incorporation of non-acetate starter units into existing polyketides. SEK4 was originally produced by the *act* minimal PKS. However in this case, the *tcm* minimal PKS cannot extend the chain further after the addition of seven malonyl units to the starter molecule, resulting in the release of a polyketide chain with a total of eight malonyl units plus a C4 starter unit (for a total of C20). The cyclization pattern thus becomes identical to that of SEK4, a product of the *act* minimal PKS alone (eight malonyl units with no starter unit). Therefore, by incorporating a starter unit with four backbone carbons, the *tcm* minimal PKS effectively becomes an octaketide synthase with respect to the rest of the

polyketide molecule. These results therefore indicate that all polyketides produced by aromatic PKSs such as the *act*, *fren*, *tcm*, and *whiE* PKSs (in combination with different accessory protein and tailoring enzymes) can now be outfitted with these novel starter units by coexpression with the ZhuH/ZhuG priming module and any other post PKS modification enzymes.

[0076] Our results with the host strains CH999/ pYT46 and Ch999/pYT82 imply that ZhuH-ZhuG may serve as a universal priming module for the biosynthesis of new aromatic polyketides. In an attempt to further expand the range of primer units utilized by ZhuH, we have verified that amino acid catabolism in *S. coelicolor* is the primary source of primer units such as isobutyryl-CoA (valine) and isovaleryl-CoA (leucine). These pathways involve transamination to convert the amino acid into the corresponding α -ketoacid, followed by decarboxylation to synthesize the corresponding acyl-CoA. Given the tolerance of these catabolic pathways in the actinomycetes for unnatural amino acids, it should be possible to expand the repertoire of primer units by feeding the recombinant strains constructed as above with unnatural amino acids such as allylglycine, norvaline, norleucine and fluorinated derivatives thereof. Successful elaboration of the corresponding acyl-CoA primers into full-length polyketides can yield additional polyketide variants.

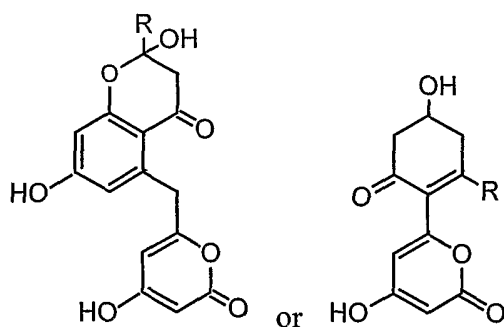
[0077] The foregoing examples are intended to illustrate but not to limit the invention. All references cited herein are incorporated herein by reference.

Claims

What is claimed:

1. A nucleic acid molecule comprising a minimal aromatic polyketide synthase (PKS) gene and a heterologous gene encoding a priming module.
2. The nucleic acid molecule defined in claim 1 wherein the heterologous gene encodes a R1128 or frenolicin priming module.
3. The nucleic acid molecule defined in claim 1 wherein the heterologous gene comprises a nucleic acid molecule encoding a first and a second polypeptide, wherein the first polypeptide has ketosynthase (KS) III analog activity and a second polypeptide has acyl carrier protein (ACP) activity.
4. The nucleic acid molecule defined in claim 2 wherein the heterologous gene comprises genes for encoding ZhuH and ZhuG, or Fren I and Fren J.
5. The nucleic acid molecule defined in any one of claims 1-4 wherein the minimal polyketide synthase gene is a minimal actinomyces polyketide synthase gene.
6. The nucleic acid molecule defined in claim 5 wherein the minimal actinomyces polyketide synthase gene is selected from the group of actinorhodin, tetracenomycin, frenolicin, R1128, granaticin, and daunorubicin synthase genes.
7. The nucleic acid molecule defined in any one of claims 1-6 wherein the minimal PKS gene comprises a gene selected from the group consisting of genes encoding FrenN, Gra-ACP, DpsG, ZhuN, Act and TcmKL.
8. A host cell comprising the nucleic acid molecule defined in any one of claims 1-7.
9. The host cell defined in claim 8 which is CH999.

10. A plasmid comprising the nucleic acid molecule defined in any one of claims 1-7.
11. The plasmid defined in claim 10 which is pYT46 or pYT82.
12. A process for producing a polyketide comprising coexpressing proteins encoded by the nucleic acid molecule of any one of claims 1-7 in the presence of starter units and extender units under conditions to produce an aromatic polyketide.
13. The process defined in claim 12 wherein the starter units are non-acetate starter units.
14. The process defined in claim 13 wherein the non-acetate starter units are alkyl starter units.
15. The process defined in any one of claims 12-14 wherein an alkylacyl-ACP intermediate synthesized by the priming module suppresses decarboxylative priming by the minimal PKS.
16. A polyketide produced by the method defined in any one of claims 12-15.
17. A compound having the formula:

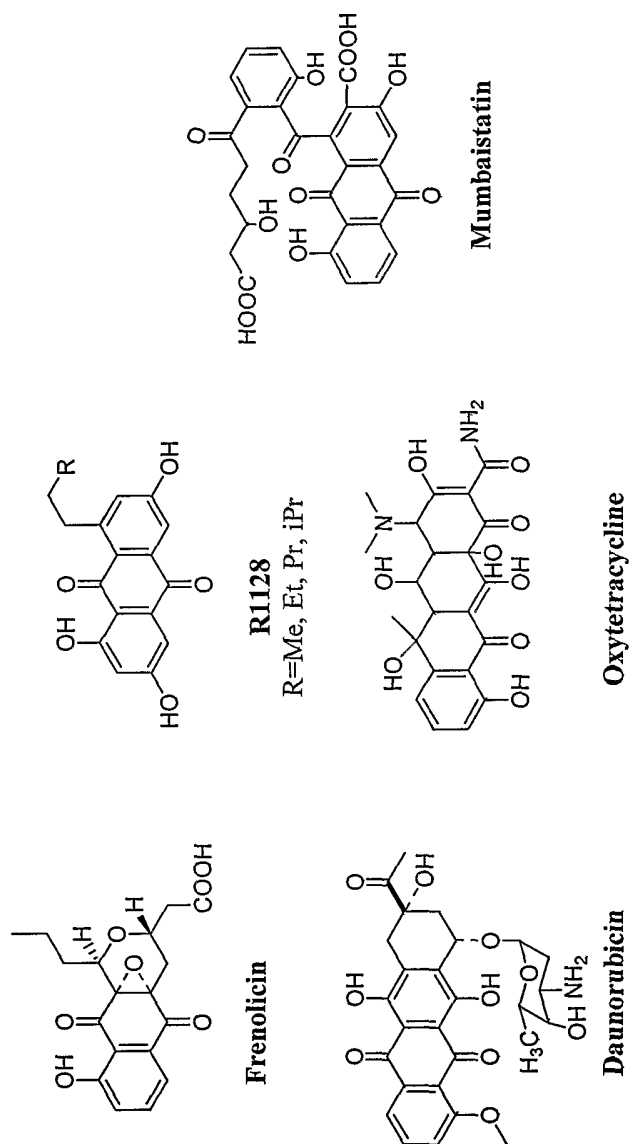


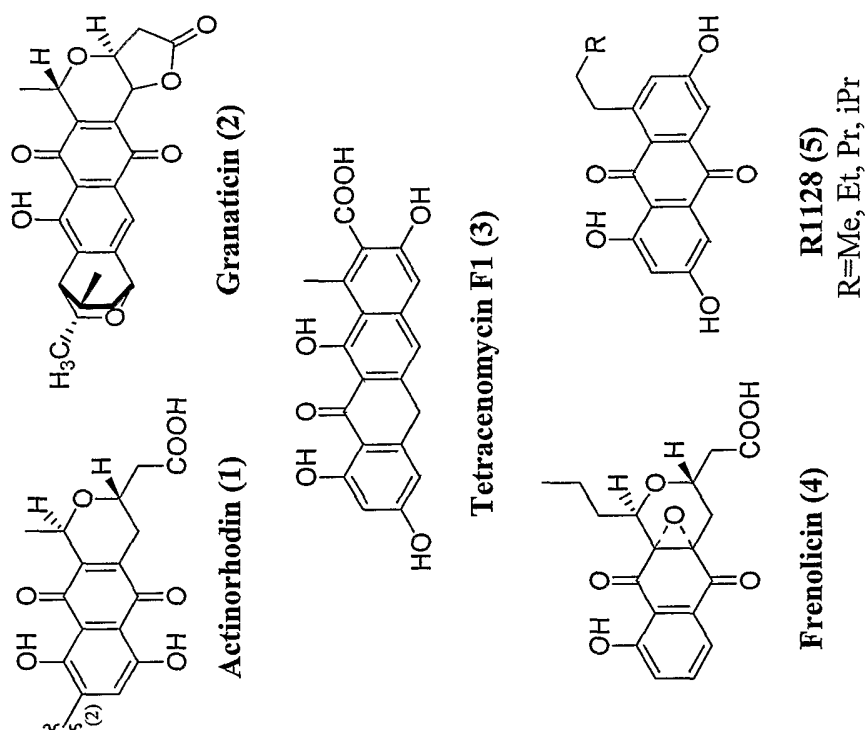
or pharmaceutically acceptable salt or prodrug thereof,

wherein R is a hydrocarbonyl group greater than C₂ optionally containing a heteroatom.

18. The compound of claim 17
wherein the optional heteroatom is O or halo.
19. The compound of claim 18
wherein R is selected from the group consisting of alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, aryl, haloalkyl, haloalkenyl, hydroxyalkyl, and hydroxyalkenyl.
20. A compound selected from the group consisting of: YT82, YT82b, YT46, YT46b, YT46c, or pharmaceutically acceptable salt or prodrug thereof.

Pharmaceutically Important Aromatic Polyketides



**Figure 2**

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Minimal PKS

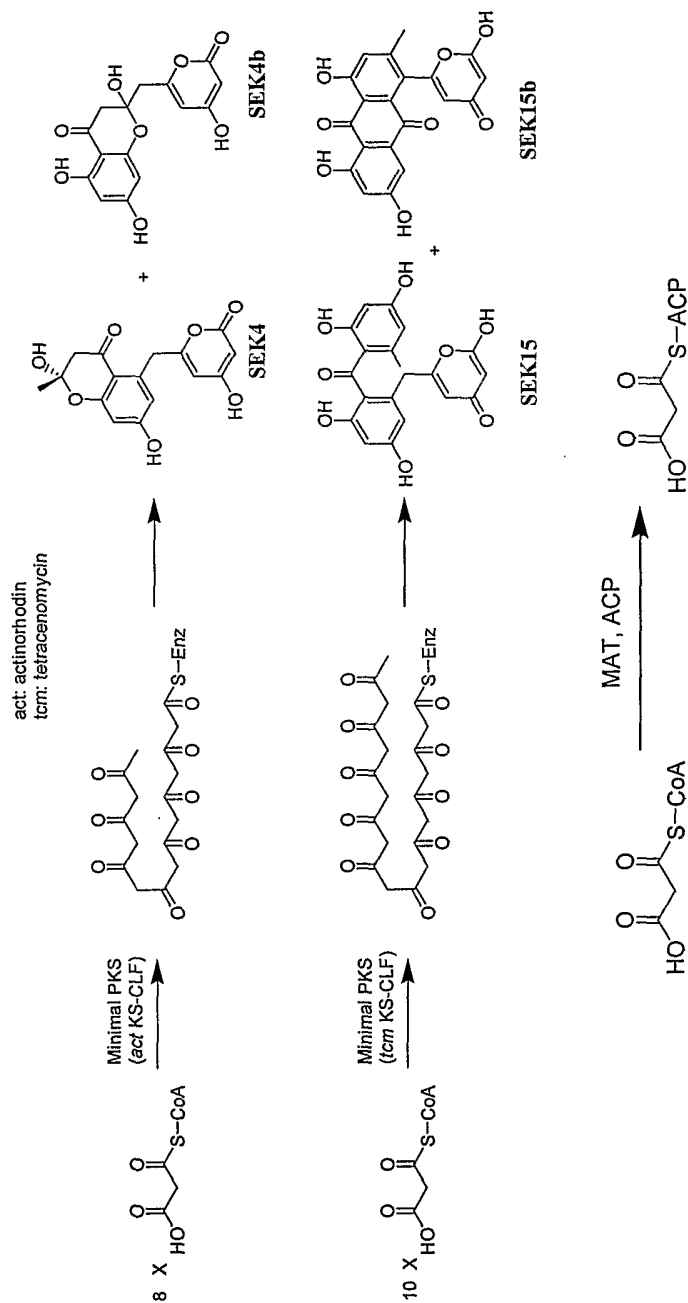
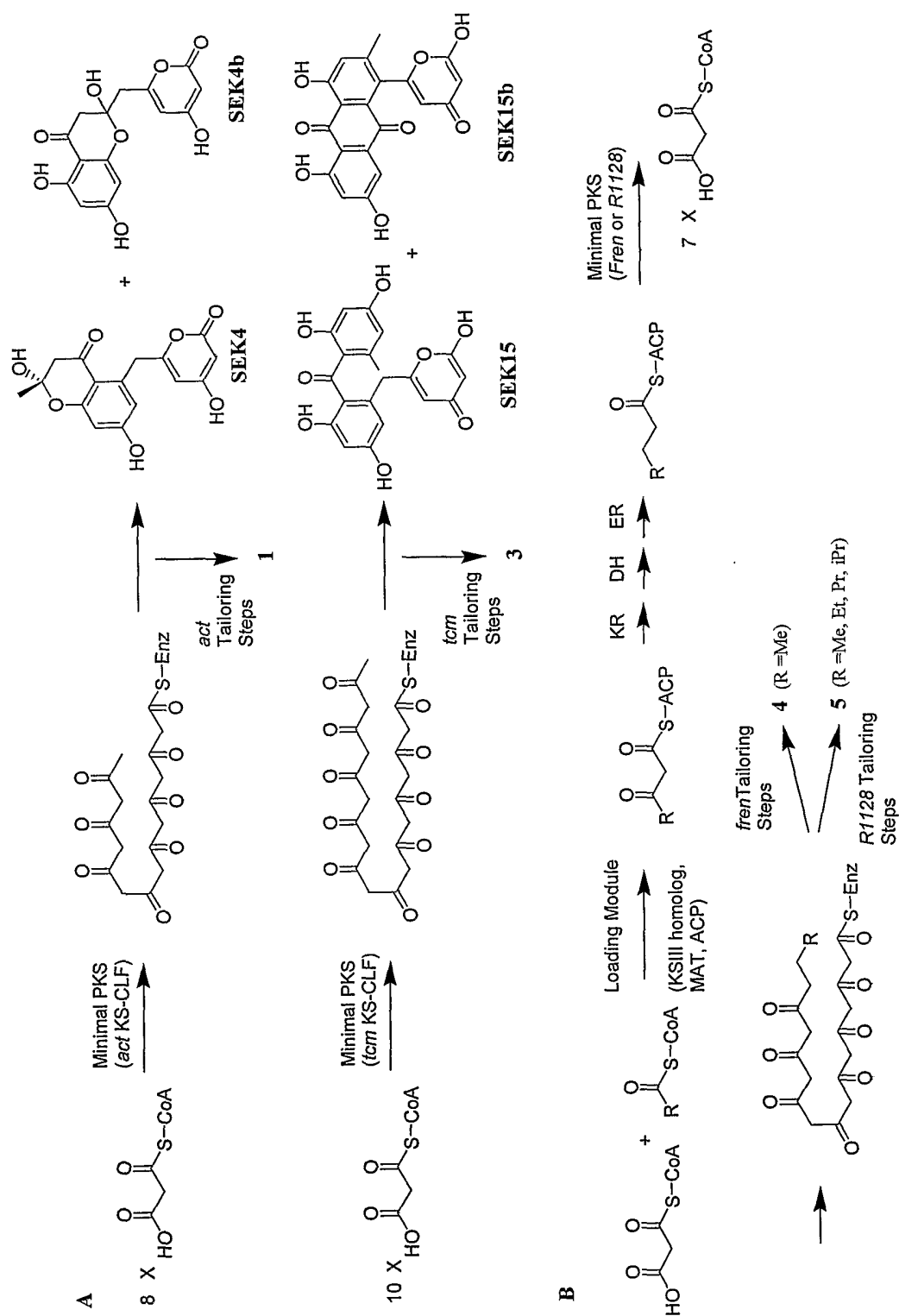


Figure 3



Figures 4A and 4B

R1128 Biosynthesis

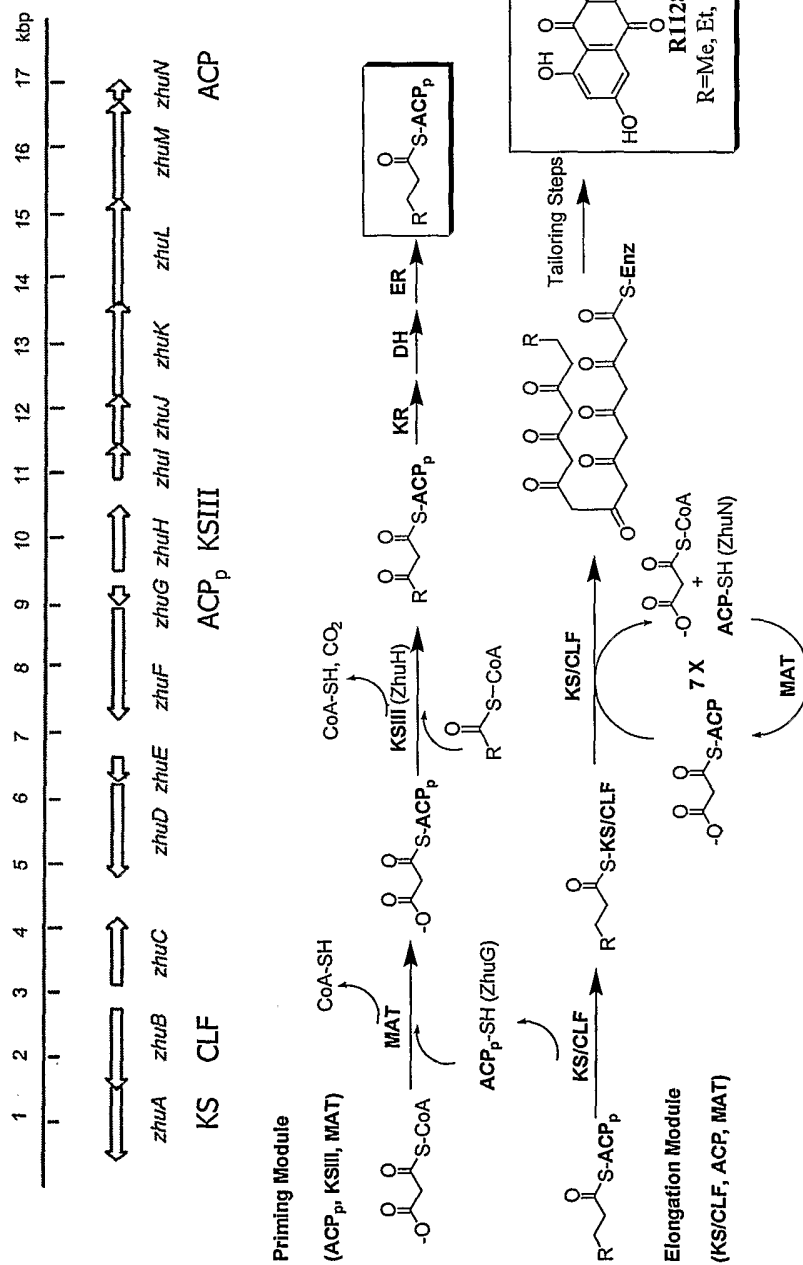


Figure 5

10

9

8

7

6

5

4

3

2

1

66 kD

56

43

36

27

20

14

7

Figure 6

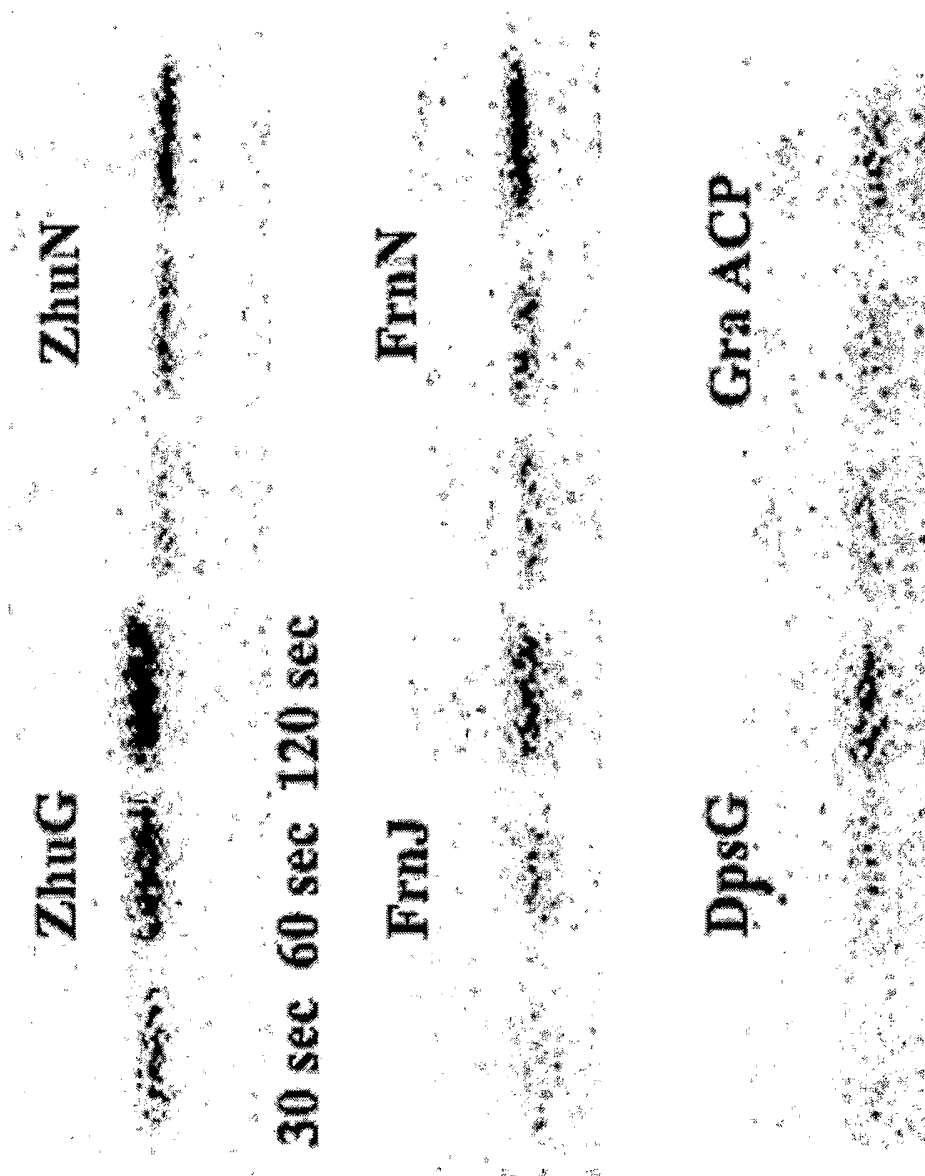
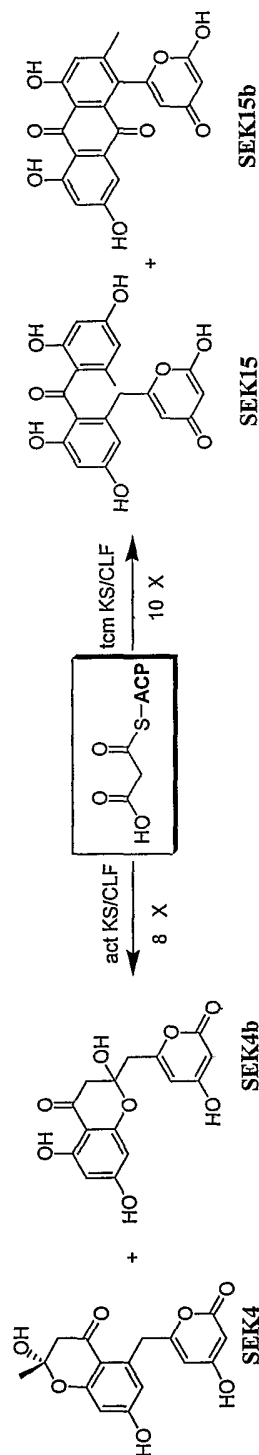


Figure 7

In vitro Titration of KS:ACP Interactions

Priming Module ACP _p		Minimal PKS ACP:		Priming Module KSIII		Minimal PKS KS/CLF	
FrenJ	Frenolicin	FrenN	Frenolicin	FrenI	Frenolicin	Act	Actinorhodin
ZhuG	R1128	ZhuN	R1128	ZhuH	R1128	TcmKL	Tetracenomycin
		Gra-ACP	Granaticin				
		DpsG	Daunorubicin				

KS/CLF:ACP Titration



KSIII:ACP Titration

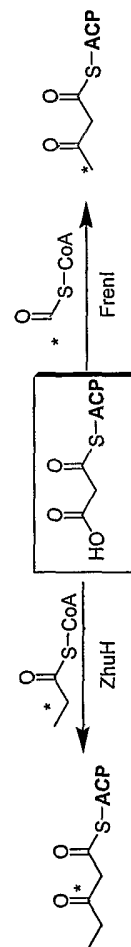


Figure 8

KS/CLF Prefers Minimal PKS ACPs

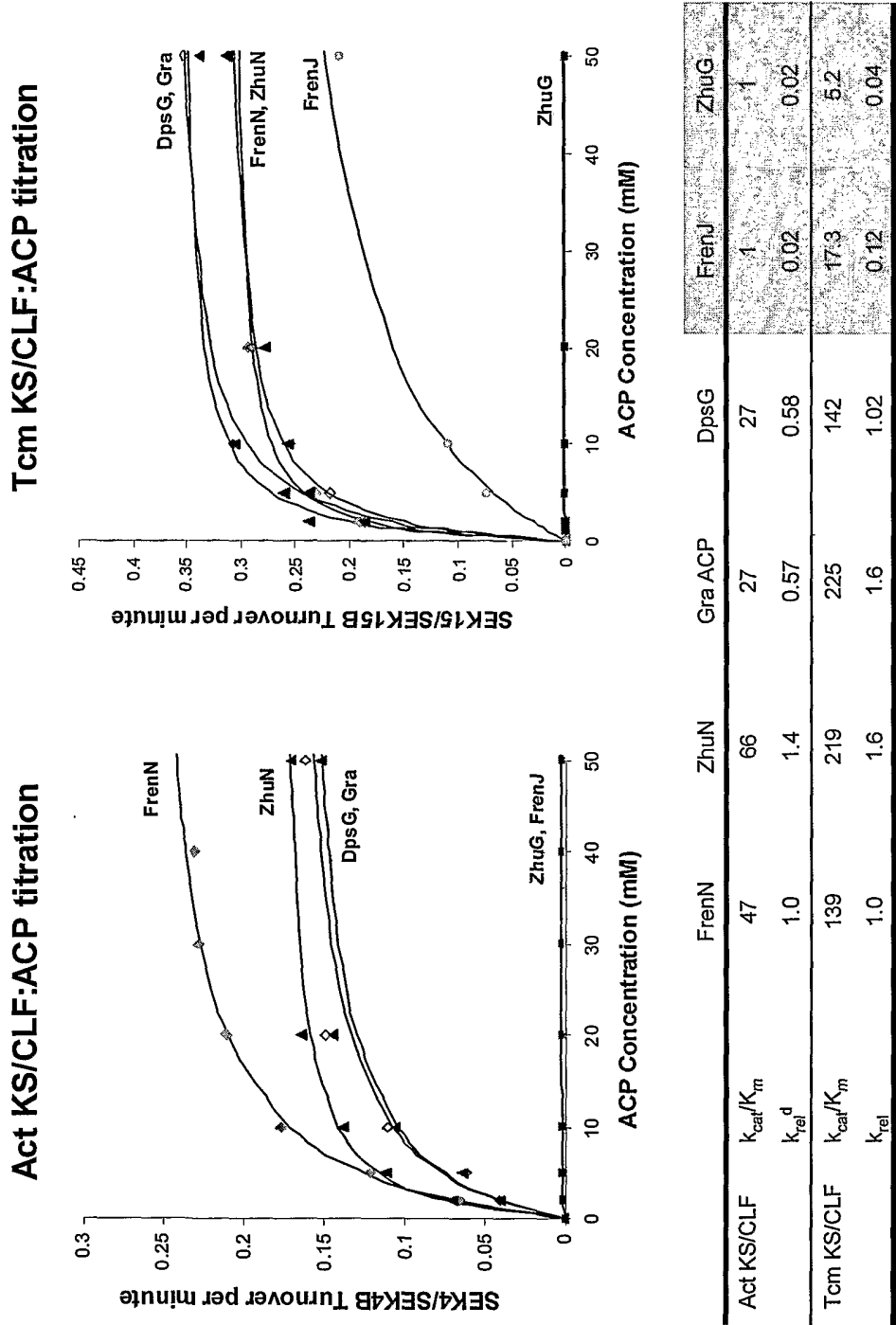
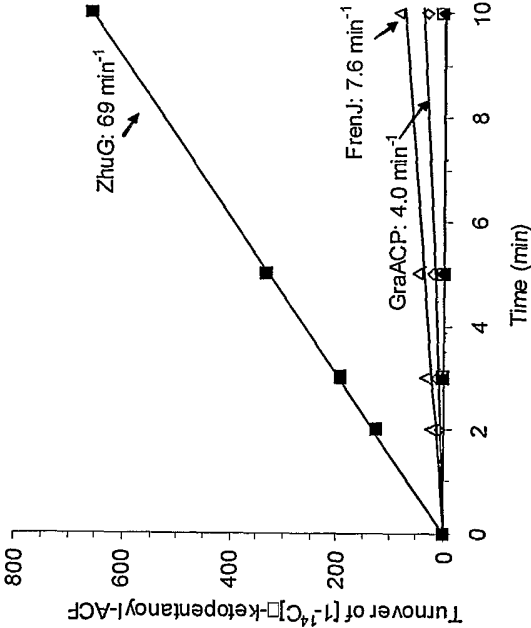


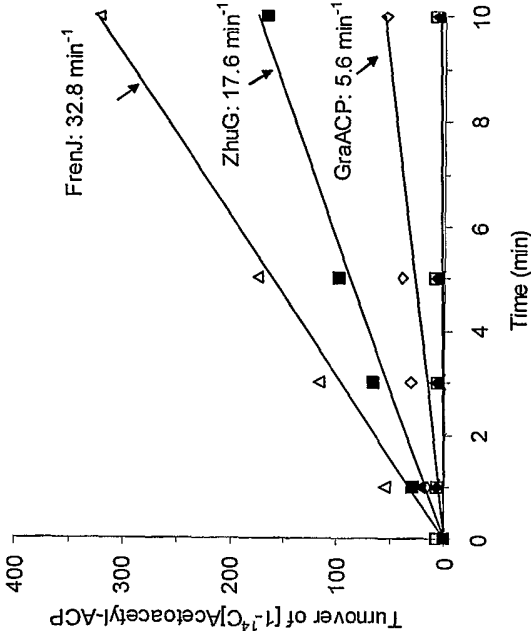
Figure 9

KSIII Prefers Priming Module ACP_ps

ZhuH:ACP titrations



FrenI:ACP titrations



**Ketosynthases in the Initiation and Elongation Modules Have
Orthogonal Acyl Carrier Protein Specificity.**

Figure 10

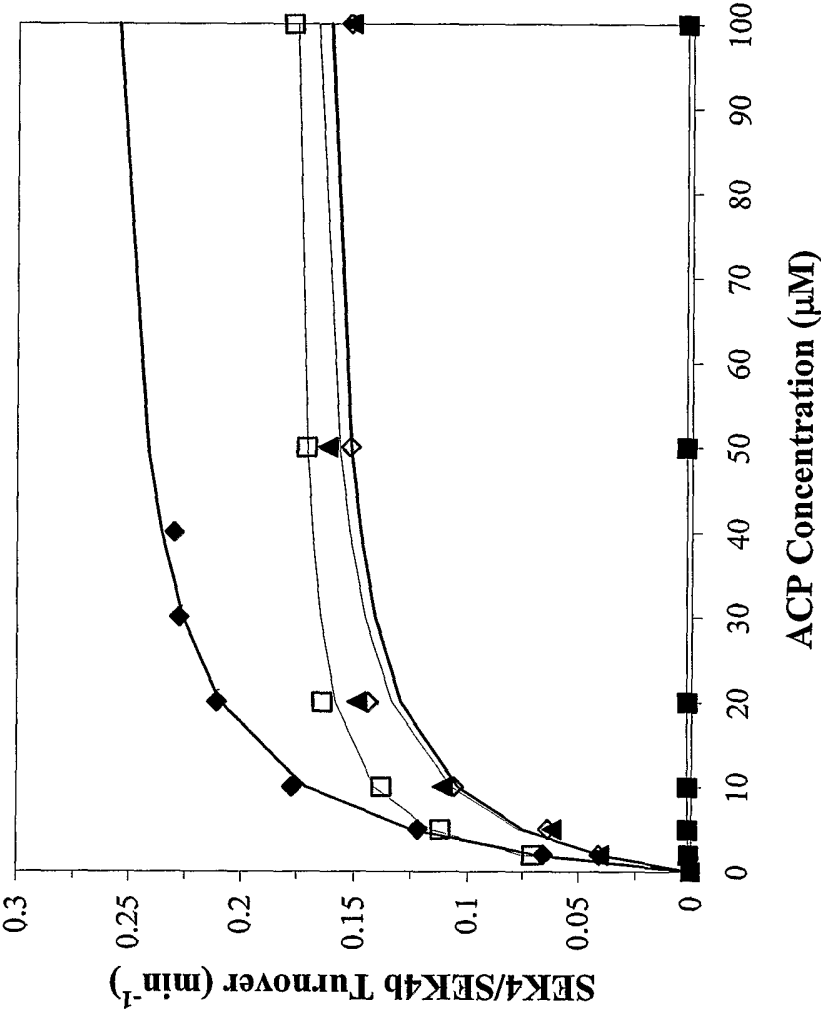


Figure 11

Figure 12A

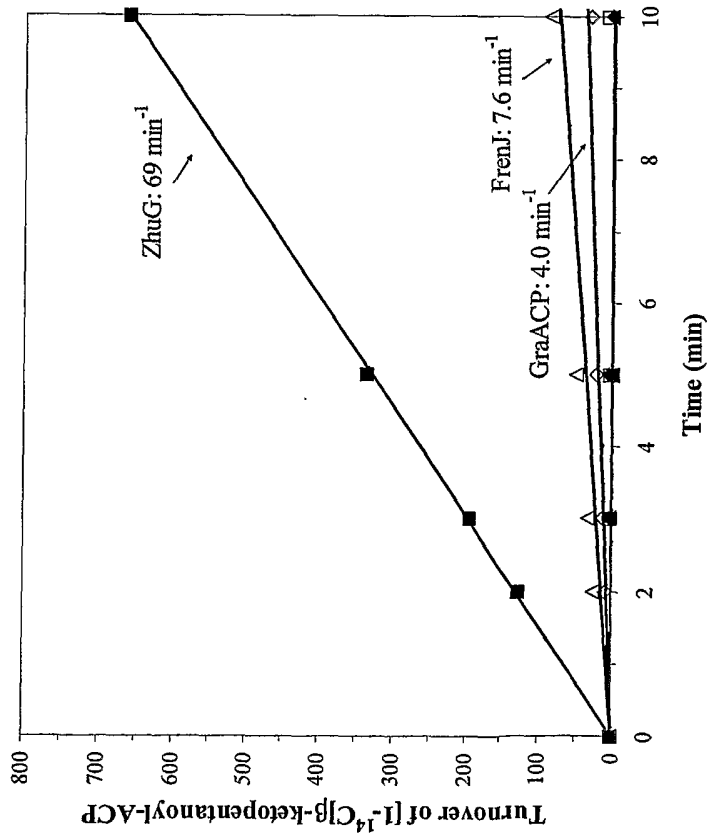
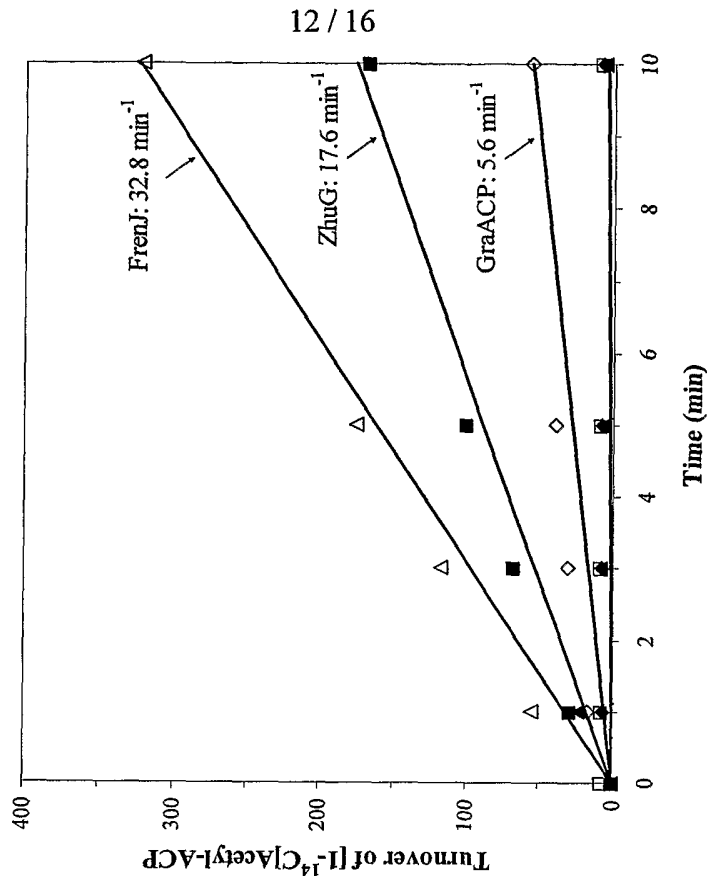


Figure 12B



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ZhuG
FrenJ
MtmS
Otc_ACP
Act_ACP
Gra_ACP
ZhuN
FrenN
Gris_ACP
SnoA3
DpsG
AknD
TcmM
Whie_ACP
Ecoli_ACPp
Scoe_ACPp

-----MDPFTLDDLKRLIDACVGTDDAVQLDETAATPF-LDLGLDSLAV Y EVVT 49
-----MSAFTLTTEFKKLVESQSYDAESAEALHGQALDTSE-TDLGYDSLTV Y EIVT 49
-----MHLEDLTAVLRECAGESEAGLTAAALDVSF-ADLGYDSVAV L ETTA 46
-----MTLLTSLDLLTLRECAGEESIDLGGDVEDVAF-DALGYDSLAL L NTVG 49
-----MATLLTTDDLRRAALVECAGETDGTDLSGDFDLRF-EDIGYDSLAL M ETAA 50
-----MAR-LTLDGLRTILVACAGEDDGLSGDILDTIF-EELGYDSLAL M ESAS 49
-----MTDDLRRIILTECAGEDSVDLGGDILDTPF-TELGYDSLAL M ETAA 46
-----MSALTVDLKKLAEETAGEDDSVLAG-ELDTPF-VDLGYDSLAL L ETAA 48
-----MSKQEFTELDLKRILLEGAGADEGVDLDGDLDTDF-EELGYESLAL L ETGG 51
-----MKQQLTTERIMEIMRECAGYGEDVDALGDTDGADF-AALGYDSLAL L ETAG 50
-----MAELSLAEELREIMRQSLGEDEVPLAD-ADTVTF-EDLGLDSLAV L ETVN 48
-----MSAFTVEELFQIMRECAGEEAAVDLAD-AAEQEF-ALLGYDSLAL M EALS 48
-----MPQIGLPRLVEIIRECAGDPDERDLGDLIDVTY-QDLGYDSLAL L EISA 49
-----MPSHEKEKEMDNQLTVOELAAALMKKAAGVTVDVKELEDRLDSGF-AEFGLDLGL L GIVG 59
-----MSTIEERVK---KIIGEQLGVKQEEVTNNASFVEDLGADSLDT V ELVM 45
-----MAATQEEIVAGLAIEIVNEIAGIPVEDVKDKSFDTDDLVDLSLM V EVVV 49

ZhuG
FrenJ
MtmS
Otc_ACP
Act_ACP
Gra_ACP
ZhuN
FrenN
Gris_ACP
SnoA3
DpsG
AknD
TcmM
Whie_ACP
Ecoli_ACPp
Scoe_ACPp

RIQDERGVAISDDDDIDGLETPRDMVAFVN---GLLVETAG----- 86
RIQDEHGVTPDEELDLDLDTPRALIAVVD---ARAGSRT----- 85
RIERDHGVVLDEEAVSEAEATLGQYLALVNEALADGTRAA----- 85
RIERYGVQLGDDAVEKATTPRALIEMTNASLTGASPSAGGAARDK 95
RLESRYGVSIPDDVAGRVDTPRELLDLIN---GALAEAA----- 86
RIERELGVALADGDIINEELTPRVLLDLVN---GAQAEAA----- 85
RIEQEFGVAIPDDEFAELATPRAVLAAVS---TAVSAAA----- 82
VLQQRYGIALTDETVGRIGTPRELLDEVN---TTPATA----- 83
RIEREYGITLDDDLVADSRTPRSLIAAINAAAFQGLVEA----- 89
RLREFGIQLGDEVVADARTPAELTALVN---RTVAEAA----- 86
HIERTYGVKLPEEELAEVRTPHSMLIFVN---ERLRAAA----- 84
RVERGLGIALPEETVGEVLTAAAFVDVN---AELARSAPVVEAAG 91
KLEQDLGVSIPGE---ELKTPRHTLHLVN---TETAGEVA----- 83
ELENGHGAVLPPD-AERCNPREFLDLVN---NNIVAGA----- 94
ALEEEFDTEIPDEEAEKITTQAAIDYIN---GHQA----- 78
AAEERFDVKIPDDDDVKNLKTVGDATKYIL---DHQA----- 82

Figure 13

Engineered Biosynthesis of Novel Aromatic Polyketides

CH999/pYT44:

Act KS-CLF, ZhuN, ZhuH, act KR

Major Products: mutactin, dehydromutactin

CH999/pYT45:

Act KS-CLF, ZhuG, ZhuH, act KR

No Polyketide product observed.

CH999/pYT46:

Act KS-CLF, ZhuN, ZhuG, ZhuH, act KR

Major Products: mutactin, dehydromutactin
YT46, YT46b and YT46c

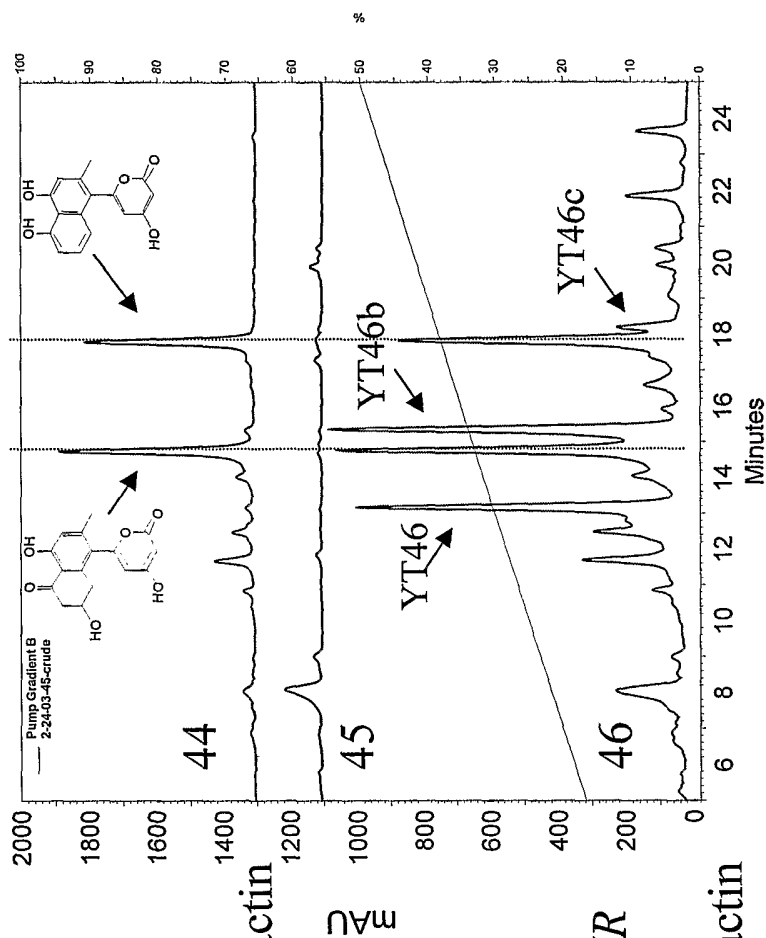


Figure 14

Aromatic Polyketides with New Structures

CH999/pYT46 Products:

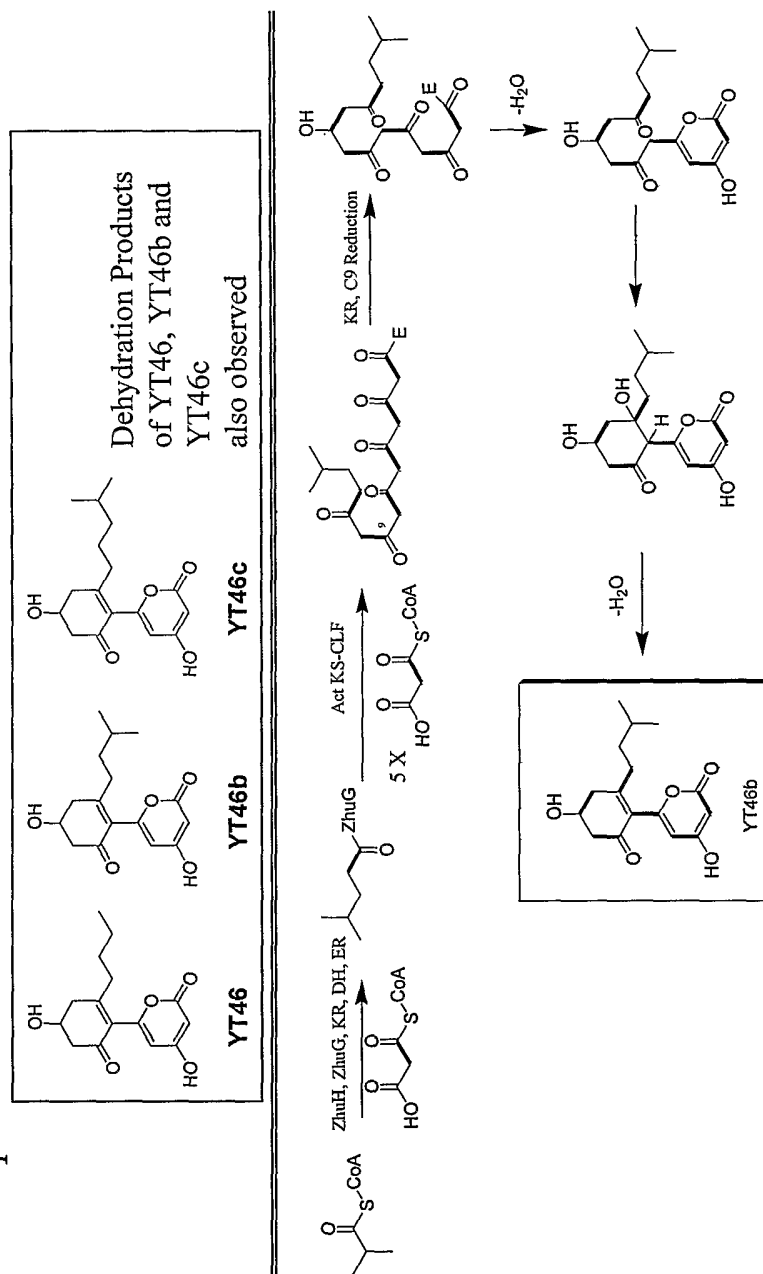


Figure 15

Engineered Biosynthesis of Existing Aromatic Polyketides Containing Novel Starter Units

Incorporation of Novel Starter Units into Aromatic Polyketides by coexpressing the Loading Module Proteins with *tcm* Minimal PKS

Novel Polyketide Synthesized by CH999/pYT82:

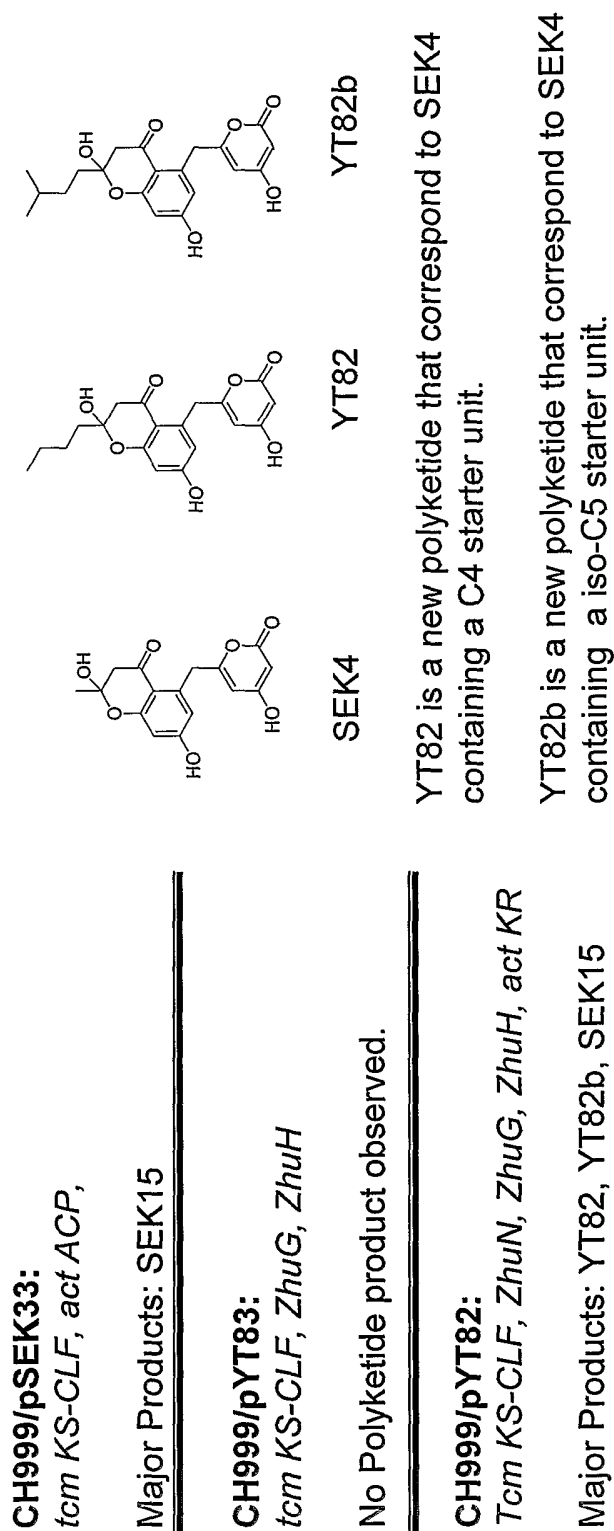


Figure 16