PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/52, 15/62, 9/10, C12P 17/06, 19/62, C07K 19 /00

A2

(11) International Publication Number: **WO 00/00618**

(43) International Publication Date: 6 January 2000 (06.01.00)

(21) International Application Number: PCT/GB99/02044

(22) International Filing Date: 29 June 1999 (29.06.99)

(30) Priority Data:
9814006.4 29 June 1998 (29.06.98) GB

(71) Applicant *(for all designated States except US)*: BIOTICA TECHNOLOGY LIMITED [GB/GB]; 112 Hills Road, Cambridge CB2 1PH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LEADLAY, Peter, Francis [GB/GB]; 17 Clarendon Road, Cambridge CB2 2BH (GB). STAUNTON, James [ES/GB]; 29 Porson Road, Cambridge CB2 ET (GB). CORTES, Jesus [GB/GB]; 26 Cambanks, Union Lane, Cambridge CB4 1PZ (GB). McARTHUR, Hamish, Alastair, Irvine [GB/US]; 19 Pheasant Run Drive, Gales Ferry, CT 06335 (US).

(74) Agents: STUART, Ian et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

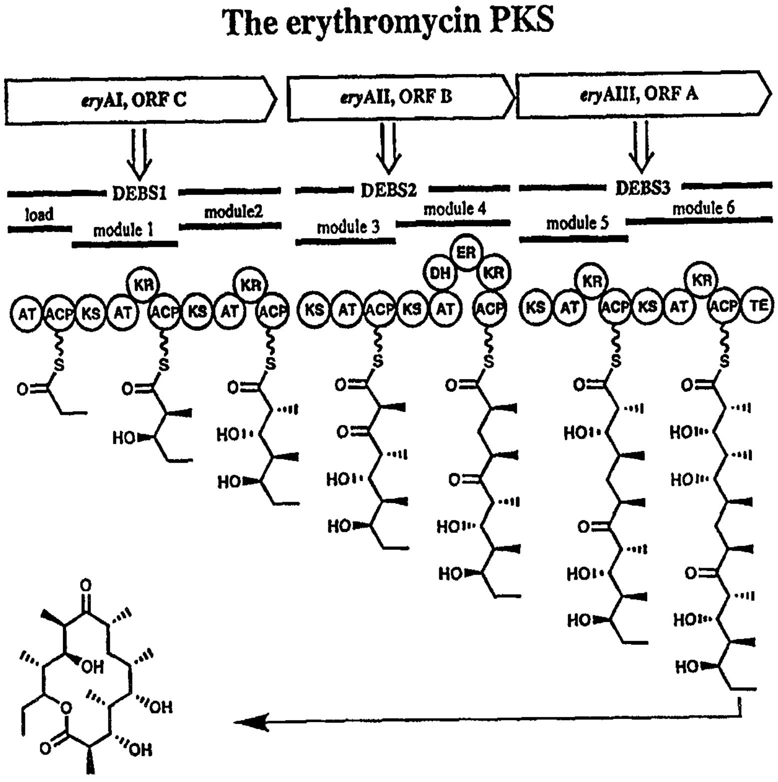
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: POLYKETIDES AND THEIR SYNTHESIS

(57) Abstract

A polyketide synthase ("PKS") of Type I is a complex multienzyme including a loading domain linked to a multiplicity of extension domains. The first extension module receives an acyl starter unit from the loading domain and each extension module adds a further ketide unit which may undergo processing (e.g. reduction). We have found that the Ksq domain possessed by some PKS's has decarboxylating activity, e.g. generating (substituted) acyl from (substituted) malonyl. The CLF domain of type II PKS's has similar activity. By inserting loading modules including such domains into PKS's not normally possessing them it is possible to control the starter units used.


The erythromycin PKS

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

Polyketides and their Synthesis

The present invention relates to processes and materials (including enzyme systems, nucleic acids, vectors and cultures) for preparing novel polyketides, particularly 12-, 14- and 16-membered ring macrolides, by recombinant synthesis and to the novel polyketides so produced. Polyketide biosynthetic genes or portions of them, which may be derived from different polyketide biosynthetic gene clusters are manipulated to allow the production of specific novel polyketides, such as 12-, 14- and 16-membered macrolides, of predicted structure. The invention is particularly concerned with the replacement of genetic material encoding the natural starter unit with other genes in order to prepare macrolides with preferentially an acetate starter unit; or preferentially a propionate unit; or preferentially with an unusual starter unit, in each case minimising the formation of by-products containing a different starter unit.

Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin, monensin, epothilones and FK506.

In particular, polyketides are abundantly produced by *Streptomyces* and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β -hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension.

The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS) have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, oleandomycin, avermectin and rapamycin, consists of a different set or "module" of enzymes for each cycle of polyketide chain extension. For an example see Figure 1 (Cortés, J. *et al.* Nature (1990) 348:176-178; Donadio, S. *et al.* Science (1991) 2523:675-679;

Swan, D.G. *et al.* Mol. Gen. Genet. (1994) 242:358-362; MacNeil, D. J. *et al.* Gene (1992) 115:119-125; Schwecke, T. *et al.* Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843).

The term "extension module" as used herein refers to the set of contiguous domains, from a β-ketoacyl-ACP synthase ("KS") domain to the next acyl carrier protein ("ACP") domain, which accomplishes one cycle of polyketide chain extension. The term "loading module" is used to refer to any group of contiguous domains which accomplishes the loading of the starter unit onto the PKS and thus renders it available to the KS domain of the first extension module. The length of polyketide formed has been altered, in the case of erythromycin biosynthesis, by specific relocation using genetic engineering of the enzymatic domain of the erythromycin-producing PKS that contains the chain releasing thioesterase/cyclase activity (Cortés *et al.* Science (1995) 268:1487-1489; Kao, C.M. *et al.* J. Am. Chem. Soc. (1995) 117:9105-9106).

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin-producing PKS (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3-α-mycarosyl-5-

oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy, 6 β-epoxy-5-oxoerythronolide B (Donadio, S. *et al.* Science (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into *Saccharopolyspora erythraea*, led to the production of 6,7-anhydroerythromycin C (Donadio, S. *et al.* Proc Natl. Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However many such attempts are reported to have been unproductive (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p. 231). The complete DNA sequence of the genes from *Streptomyces hygroscopicus* that encode the modular Type I PKS governing the biosynthesis of the macrocyclic immunosuppressant polyketide rapamycin has been disclosed (Schwecke, T. *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843). The DNA sequence is deposited in the EMBL/Genbank Database under the accession number X86780.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used as appropriate in successive cycles (Bibb, M. J. *et al.* EMBO J. (1989) 8:2727-2736; Sherman, D. H. *et al.* EMBO J. (1989) 8:2717-2725; Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II pKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained by the introduction of clones Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from *Streptomyces coelicolor*, into an anthraquinone polyketide-producing strain of *Streptomyces galileus* (Bartel, P. L. *et al.* J. Bacteriol. (1990) 172:4816-4826).

The minimal number of domains required for polyketide chain extension on a Type II PKS when expressed in a *Streptomyces coelicolor* host cell (the "minimal PKS") has been defined for example in International Patent Application Number WO 95/08548 as containing the following three polypeptides which are products of the *act I* genes: first KS; secondly a polypeptide termed the CLF with end-to-end amino acid sequence similarity to the KS but in which the essential active site residue of the KS, namely a cysteine residue, is substituted either by a glutamine residue, or in the case of the PKS for a spore pigment such as the *whiE* gene product (Chater, K. F. and Davis, N. K. Mol. Microbiol. (1990) 4:1679-1691) by a glutamic acid residue (Figure 2); and finally an ACP. The CLF has been stated for example in International Patent Application Number WO 95/08548 to be a factor that determines the chain length of the polyketide chain that is produced by the minimal PKS. However it has been found (Shen, B. *et al.* J. Am. Chem. Soc. (1995) 117:6811-6821) that when the CLF for the octaketide actinorhodin is used to replace the CLF for the decaketide tetracenomycin in host cells of *Streptomyces glaucescens*, the polyketide product is not found to be altered from a decaketide to an octaketide, so the exact role of the CLF remains unclear. An alternative nomenclature has been proposed in which KS is designated KSα and CLF is designated KSβ, to reflect this lack of knowledge (Meurer, G. *et al.* Chemistry and Biology (1997) 4:433-443). The mechanism by which acetate starter units and acetate extender units are loaded onto the Type II PKS is not known, but it is speculated that the malonyl-CoA: ACP acyltransferase of the fatty acid synthase of the host cell can fulfil the same function for the Type II PKS (Revill, W. P. *et al.* J. Bacteriol. (1995) 177:3946-3952).

International Patent Application Number WO 95/08548 describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS gene clusters, to obtain hybrid polyketides. The same International Patent Application WO 95/08548 describes the construction of a strain of *Streptomyces coelicolor* which substantially lacks the native gene cluster for actinorhodin, and the use in that strain of a plasmid vector pRM5 derived from the low-copy number vector SCP2* isolated from *Streptomyces coelicolor* (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and in which heterologous PKS-encoding DNA may be expressed under the control of the divergent *act I/ act* III promoter region of the actinorhodin gene cluster (Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The plasmid pRM5 also contains DNA from the actinorhodin biosynthetic gene cluster encoding the gene for a specific activator protein, ActII-orf4. The Act II-orf4 protein is required for transcription of the genes placed under the control of the actI/ act II bidirectional promoter and activates gene expression during the transition from growth to stationary phase in the vegetative mycelium (Hallam, S. E. *et al.* Gene (1988) 74:305-320).

Type II clusters in *Streptomyces* are known to be activated by pathway-specific activator genes (Narva, K. E. and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333; Stutzman-Engwall, K. J. *et al.* J. Bacteriol. (1992) 174:144-154; Fernandez-Moreno, M.A. *et al.* Cell (1991) 66:769-780; Takano, E. *et al.* Mol. Microbiol. (1992) 6:2797-2804; Takano, E. *et al.* Mol. Microbiol. (1992) 7:837-845), The DnrI gene product complements a mutation in the *actII*-orf4 gene of *S. coelicolor*, implying that DnrI and ActII-orf4 proteins act on similar targets. A gene (*srmR*) has been described (EP 0 524 832 A2) that is located near the Type I PKS gene cluster for the macrolide polyketide spiramycin. This gene specifically activates the production of the macrolide antibiotic spiramycin, but no other exampples have been found of such a gene. Also, no homologues of the ActII-orf4/DnrI/RedD family of activators have been described that act on Type I PKS genes.

Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or possess completely novel bioactivity. The complex polyketides produced by Type I PKSs are particularly valuable, in that they include compounds with known utility as anthelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents. Because of their structural complexity, such novel polyketides are not readily obtainable by total chemical synthesis, or by chemical modifications of known polyketides.

There is also a need to develop reliable and specific ways of deploying individual modules in practice so that all, or a large fraction, of hybrid PKS genes that are constructed, are viable and produce the desired polyketide product.

Pending International Patent Application number PCT/GB97/01819 discloses that a PKS gene assembly (particularly of Type I) encodes a loading module which is followed by at least one extension module. Thus Figure 1 shows the organisation of the DEBS genes. The first open reading frame encodes the first multi-enzyme or cassette (DEBS 1) which consists of three modules: the loading module (ery-load) and two extension modules (modules 1 and 2). The loading module comprises an acyltransferase and an acyl carrier protein. This may be contrasted with Fig. 1 of WO 93/13663 (referred to above). This shows ORF1 to consist of only two modules, the first of which is in fact both the loading module and the first extension module.

PCT/GB97/01819 describes in general terms the production of a hybrid PKS gene assembly comprising a loading module and at least one extension module. PCT/GB97/01818 also describes (see also Marsden, A. F. A. *et al.* Science (1998) 279:199-202) construction of a hybrid PKS gene assembly by grafting the wide-specificity loading module for the avermectin-producing polyketide synthase onto the first multienzyme component (DEBS 1) for the erythromycin PKS in place of the normal loading module. Certain novel polyketides can be prepared using the hybrid PKS gene assembly, as described for example in pending International Patent Application number (PCT/GB97/01810). Patent Application PCT/GB97/01819 further describes the construction of a hybrid PKS gene assembly by grafting the loading module for the rapamycin-producing polyketide synthase onto the first multienzyme component (DEBS 1) for the erythromycin PKS in place of the normal loading module. The loading module of the rapamycin PKS differs from the loading modules of DEBS and the avermectin PKS in that it comprises a CoA ligase domain, an enoylreductase ("ER") domain and an ACP, so that suitable organic acids including the natural starter unit 3,4-

dihydroxycyclohexane carboxylic acid may be activated in situ on the PKS loading domain, and with or without reduction by the ER domain transferred to the ACP for intramolecular loading of the KS of extension module 1 (Schwecke, T. *et al.* Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843).

The DNA sequences have been disclosed for several Type I PKS gene clusters that govern the production of 16-membered macrolide polyketides, including the tylosin PKS from *Streptomyces fradiae* (EP 0 791 655 A2), the niddamycin PKS from *Streptomyces caelestis* (Kavakas, S. J. *et al.* J. Bacteriol. (1998) 179:7515-7522) and the spiramycin PKS from *Streptomyces ambofaciens* (EP 0791 655 A2). All of these gene sequences have in common that they show the loading module of the PKS to differ from the loading module of DEBS and of the avermectin PKS in that they consist of a domain resembling the KS domains of the extension modules, an AT domain and an ACP (Figure 3). The additional N-terminal KS-like domain has been named KSq because it differs in each case from an extension KS by the specific replacement of the active site cysteine residue essential for β-ketoacyl-ACP synthase activity by a glutamine (Q in single letter notation) residue. The function of the KSq domain is unknown (Kavakas, S. J. *et al.* J. Bacteriol. (1998)

179:7515-7522), but its presence in these PKSs for 16-membered macrolides is surprising because the starter units of tylosin, niddamycin and spiramycin appear to be propionate, acetate and acetate respectively, that is, the same type of starter unit as in DEBS. The AT adjacent to the KSq domain is named here the ATq domain.

When the entire loading module of the tylosin PKS was used to replace the analogous loading module in the spiramycin PKS in *S. ambofaciens* (Kuhstoss *et al.* Gene (1996) 183:231-236), the nature of the starting unit was stated to be altered from acetate to propionate. Since the role of the KSq domain was not understood, no specific disclosure was made that revealed either the importance of the KSq domain, or the possible utility of these KSq-containing loading modules in ensuring the purity of the polyketide product in respect of the starter unit, even at high levels of macrolide production. The interpretation for their results was stated as: "Therefore we believe that the experiments described here provide strong experimental support for the hypothesis that the AT domains in Type I PKS systems select the appropriate substrate at each step in synthesis" (Kuhstoss *et al.* Gene (1996) 183:231-236, at p. 235). These authors noted the analogy with the CLF protein in Type II PKS systems and that the latter protein is thought to be involved in determining the chain length. They state: "KSq may serve a similar function, although it is unclear why such a function would be necessary in the synthesis of these 16-membered polyketides when it is not needed for the synthesis of other complex polyketides such as 6-DEB or rapamycin. In any case the KSq is unlikely to be involved in substrate choice at each step of synthesis." (Kuhstoss *et al.* Gene (1996) 183:231-236).

It has been shown that when genetic engineering is used to remove the loading module of DEBS, the resulting truncated DEBS in *S. erythraea* continues to produce low levels of erythromycins containing a propionate starter unit (Pereda, A. *et al.* Microbiology (1995) 144:543-553) . The same publication shows that when in this truncated DEBS the methylmalonyl-CoA -specific AT of extension module 1 was replaced by a malonyl-CoA-specific AT from an extension module of the rapamycin PKS, the products were also low levels of erythromycins containing a propionate starter unit, demonstrating that the origin of the starter units is not decarboxylation of the (methyl)malonyl groups loaded onto the enzyme by the AT of module 1, but from direct acylation of the KS of extension module 1 by propionyl-CoA. This is in contrast to a previous report, using partially purified DEBS1+TE, a truncated bimodular PKS derived from DEBS (Kao, C. M. *et al.* J. Am. Chem. Soc. (1995) 117:9105-9106) and functionally equivalent to DEBS1-TE (Brown, M. J. B. *et al.*, J. Chem. Soc. Chem. Commun. (1995) 1517-1518; Cortés, J. *et al.* Science (1991) 2523:675-679), which stated that the origins of the starter units for DEBS can include methylmalonate units which are loaded onto module 1 and are decarboxylated by the KS of module 1 (Pieper, R. *et al.* Biochemistry (1997) 36:1846-1851). It has now been found that when the DEBS1-TE protein is fully purified from extracts of recombinant *S. erythraea* it contains no such specific decarboxylase activity (Weissmann, K. et al. Biochemistry, (1998) 37, 11012-11017), further confirming that starter units do not in fact arise from decarboxylation of extension units mediated by the KS of extension module 1 .

It is known that the DEBS loading module has a slightly broader specificity than propionate only, and in particular acetate starter units are used both in vitro and in vivo, when the PKS containing this loading module is part of a PKS that is expressed either in *S. erythraea* the natural host for erythromycin production (see for example Cortés, J. *et al.* Science (1995) 268:1487-1489), or in a heterologous host such as *S. coelicolor* (Kao, C. M. *et al.* J. Am. Chem. Soc. (1994) 116:11612-11613;

Brown, M. J. B. *et al.* J. Chem. Soc. Chem. Commun. (1995) 1517-1519). In vitro experiments using purified DEBS1-TE have demonstrated that propionyl-CoA and acetyl-CoA are alternative substrates that efficiently supply propionate and acetate units respectively to the loading module (Wiessmann, K. E. H. *et al.* Chemistry and Biology (1995) 2:583-589; Pieper, R. *et al.* J. Am. Chem. Soc. (1995) 117:11373-11374). The outcome of the competition between acetate and propionate starter units is influenced by the respective intracellular concentrations of propionyl-CoA and acetyl-CoA prevailing in the host cell used (see for example Kao, C. M. *et al.* Science (1994) 265:509-512; Pereda, A. *et al.* Microbiology (1995) 144:543-553). It is also determined by the level of expression of the host PKS, so that as disclosed for example in Pending International Patent Application number PCT/GB97/01819, when recombinant DEBS or another hybrid PKS containing the DEBS loading module is over-expressed in *S. erythraea*, the products are generally mixtures whose components differ only in the presence of either an acetate or a propionate starter unit.

There is a need to develop reliable methods for avoiding the formation of mixtures of polyketides with both acetate and propionate starter units, and to allow the specific incorporation of unusual starter units. It has now been found, surprisingly, that the role of the loading domains in the PKSs for the 16-membered macrolides tylosin, niddamycin and spiramycin is different from that of the loading domains of the avermectin PKS and of DEBS. It has been realised that the KSq domain of the tylosin PKS and the associated AT domain, which is named here ATq, together are responsible for the highly specific production of propionate starter units because the ATq is specific for the loading of methylmalonyl-CoA and not propionyl-CoA as previously thought; and the KSq is responsible for the highly specific decarboxylation of the enzyme-bound methylmalonate unit to form propionate unit attached to the ACP domain of the loading module and appropriately placed to be transferred to the KS of extension module 1 for the initiation of chain extension. In a like manner the ATq of the spiramycin and niddamycin PKSs, and the adjacent KSq, are responsible for the specific loading of malonate units rather than acetate units as previously believed, and for their subsequent specific decarboxylation to provide acetate starter units for polyketide chain extension.

It has also now been found here that not only the PKSs for the above-mentioned 16-membered macrolides, but also the PKSs for certain 14-membered macrolides particularly the oleandomycin PKS from *Streptomyces antibioticus* (Figure 4) and also the PKSs for certain polyether ionophore polyketides particularly the putative monensin PKS from *Streptomyces cinnamonensis* (Figure 4), possess a loading domain comprising a KSq domain, an ATq domain, and an ACP. In Figure 4 is shown a sequence alignment of the KSq domains and of the adjacent linked ATq domains that have been identified, showing the conserved active site glutamine (Q) residue in the KSq domains, and an arginine residue which is conserved in all extension AT domains and is also completely conserved in ATq domains. This residue is characteristically not arginine in the AT domains of either DEBS or of the avermectin PKS loading modules, where the substrate for the AT is a non-carboxylated acyl-CoA ester (Haydock, S. F. *et al.* FEBS Letters (1995) 374:246-248) . The abbreviation ATq is used here to simply to distinguish the AT domains found immediately C-terminal of Ksq from extension ATs, and the label has no other significance.

In one aspect the invention provides a PKS multienzyme or part thereof, or nucleic acid (generally DNA) encoding it, said multienzyme or part comprising a loading module and a plurality of extension modules, wherein

(a) the loading module is adapted to load a malonyl or substituted malonyl residue and then to effect decarboxylation of the loaded residue to provide an acetyl or substituted acetyl (which term encompasses propionyl) residue for transfer to an extension module; and

(b) the extension modules, or at least one thereof (preferably at least the one adjacent the loading module), are not naturally associated with a loading module that effects decarboxylation of an optionally substituted malonyl residue.

Generally the loading module will also include an ACP (acyl carrier protein) domain.

Preferably the decarboxylating functionality of the loading module is provided by a KS (ketosynthase)-type domain. Suitably this differs from a KS of a conventional extension module by possessing a glutamine residue in place of the essential cysteine residue in the active site. It is termed Ksq. It may be "natural" or genetically engineered, e.g. resulting from site-directed mutagenesis of nucleic acid encoding a different KS such as a KS of an extension module.

Alternatively the decarboxylating functionality can be provided by a CLF-type domain of the general type occuring in Type II PKS systems.

Preferably the loading functionality is provided by an AT (acyltransferase)-type domain which resembles an AT domain of a conventional extension module in having an arginine residue in the active site, which is not the case with the AT domains of loader modules which load acetate or propionate, e.g. in DEBS or avermectin PKS systems. It may be termed Atq. Once again, it may be "natural" or genetically engineered, e.g. by mutagenesis of an AT of an extension module.

Usually the loading module will be of the form:

Ksq-ATq-ACP

where ACP is acyl carrier protein.

In another aspect the invention provides a method of synthesising a polyketide having substantially exclusively a desired starter unit by providing a PKS multienzyme incorporating a loading module as defined above which specifically provides the desired starter unit. This may comprise providing nucleic acid encoding the multienzyme and introducing it into an organism where it can be expressed.

In further aspects the invention provides vectors and transformant organisms and cultures containing nucleic acid encoding the multienzyme. A preferred embodiment is a culture which produces a polyketide having a desired starter unit characterised by the substantial absence of polyketides with different starter units. Thus, for example, erythromycin can be produced substantially free from analogues resulting from the incorporation of acetate starter units in place of propionate.

Preferably the hybrid PKS encodes a loading module and from 2 to 7 extension modules and a chain terminating enzyme (generally a thioesterase).

It is particularly useful to provide a loading module of the type KSq - ATq-ACP for a PKS gene assembly which produces a 12-, 14- or 16-membered macrolide in order to prepare a 12-, 14- or 16-membered macrolide which contains exclusively or almost exclusively an acetate starter unit, even when such PKS gene assembly is expressed at high levels in an actinomycete host cell. Particularly suitable PKSs for this purpose are the components of PKSs for the biosynthesis of erythromycin, methymycin, oleandomycin, tylosin, spiramycin, midecamycin, and niddamycin for all of which the gene and modular organisation is known at least in part. Particularly suitable sources of the genes encoding a loading module of the type KSq - ATq-ACP are the loading modules of oleandomycin, spiramycin, niddamycin, methymycin and monensin which are specific for the loading of malonate units which are then decarboxylated to acetate starter units.

Similarly it is particularly useful to provide a loading module of the type KSq - ATq-ACP for a PKS gene assembly which produces the macrolides rifamycin, avermectin, rapamycin, immunomycin and FK506 whose loading modules possess an unusual specificity (and for all of which the gene and modular organisation is known at least in part) in order to prepare such macrolides containing exclusively or almost exclusively an acetate starter unit, even when such PKS gene assembly is expressed at high levels in an actinomycete host cell. Particularly suitable sources of the genes encoding a loading module of the type KSq - ATq-ACP are the loading modules of oleandomycin, spiramycin, niddamycin, methymycin and monensin which are specific for the loading of malonate units which are decarboxylated to acetate starter units.

It is similarly useful to provide a loading module of the type KSq - ATq-ACP for a PKS gene assembly which produces a 12-, 14- or 16-membered macrolide in order to prepare a 12-, 14- or 16-membered macrolide which contains exclusively or almost exclusively a propionate starter unit, even when such PKS gene assembly is expressed at high levels in an actinomycete host cell. Particularly suitable PKSs for this purpose are the components of PKSs for the biosynthesis of erythromycin, methymycin, oleandomycin, tylosin, spiramycin, midecamycin, and niddamycin for all of which the gene and modular organisation is known at least in part. A particularly suitable source of the genes encoding a loading module of the type KSq-ATq-ACP is the loading module of tylosin which is specific for the loading of methylmalonate units which are decarboxylated to propionate starter units.

Similarly it is particularly useful to provide a loading module of the type KSq - ATq-ACP for a PKS gene assembly which produces the macrolides rifamycin, avermectin, rapamycin, immunomycin and FK506 whose loading modules possess an unusual specificity (and for all of which the gene and modular organisation is known at least in part) in order to prepare such macrolides containing exclusively or almost exclusively a propionate starter unit, even when such PKS gene assembly is expressed at high levels in an actinomycete host cell. A particularly suitable source of the genes encoding a loading module of the type KSq - ATq-ACP is the loading module of tylosin which is specific for the loading of methylmalonate units which are decarboxylated to propionate starter units.

In the loading module of the type KSq - ATq-ACP the domains or portions of them may be derived from the same or from different sources, and comprise either natural or engineered domains. For example the ATq domain can be replaced by an AT domain derived from any extension module of a Type I PKS, having specificity either for loading of malonate units or for loading of methylmalonate units respectively, so long as the KSq domain is chosen to have a matching specificity towards either methylmalonate or malonate units respectively.

Alternatively, the KSq domain in the loading module provided of the type KSq - ATq-ACP may be substituted by the CLF polypeptide of a Type II PKS. It is now apparent that in contrast to its previous identification as a factor uniquely determining chain length, the CLF, in addition to any other activities that it may possess, is the analogue of the KSq domain and can act as a decarboxylase towards bound malonate units.

The appreciation that the CLF domain of Type II PKS's has decarboxylating activity has led us to devise useful interventions in Type II systems, e.g. to enhance the yields obtainable in some fermentations. Many high-yielding industrial fermentations tend to give mixtures, owing to the incorporation of undesired starters. This is particularly the case in systems which have auxiliary genes for generating unusual starters. CLF genes may act to produce undesired acyl species, leading to products incorporating the undesired acyl units.

For example the production of oxytetracycline involves an unusual malonamido starter. However the undesired activity of a CLF domain causes some decarboxylation, leading to the incorporation of acetyl instead. Daunomycin synthesis likewise involves an unusual starter which is liable to the "parasitic" activity of a CLF domain.

The active site (for decarboxylation) of a CLF domain generally includes a glutamine residue. We find that the decarboxylating activity of the domain can be removed by a mutation by which the Gln residue is converted into (for example) Ala.

Thus in a further aspect the invention provides a system and process for synthesis of a type II (aromatic) polyketide, in which a gln residue of a CLF domain of the type II PKS is mutated to suppress decarboxylation activity. Techniques of site-specific mutagenesis by which this can be achieved are by now well known to those skilled in the art.

The loading module of the type KSq - ATq-ACP may be linked to a hybrid PKS produced for example as in PCT/GB97/01819 and PCT/GB97/01810. It is particularly useful to link such a loading module to gene assemblies that encode hybrid PKSs that produce novel derivatives of

14-membered macrolides as described for example in PCT/GB97/01819 and PCT/GB97/01810.

The invention further provides such PKS assemblies furnished with a loading module of the type KSq - ATq-ACP, vectors containing such assemblies, and transformant organisms that can express them. Transformant organisms may harbour recombinant plasmids, or the plasmids may integrate. A plasmid with an *int* sequence will integrate into a specific attachment site (*att*) of the host's chromosome. Transformant organisms may be capable of modifying the initial products, eg by carrying out all or some of the biosynthetic modifications normal in the production of erythromycins (as shown in Figure 5) and for other polyketides. Use may be made of mutant organisms such that some of the normal pathways are blocked, e.g. to produce products withut one or more "natural" hydroxy-groups or sugar groups. The invention further provides novel polyketides as producible, directly or indirectly, by transformant organisms. This includes polyketides which have undergone enzymatic modification.

In a further aspect the invention provides both previously-obtained polyketides and novel polyketides in a purer form with respect to the nature of the starter unit, than was hitherto possible. These include 12- , 14- and 16-membered ring macrolides which are either "natural" or may differ from the corresponding "natural" compound:

a) in the oxidation state of one or more of the ketide units (ie selection of alternatives from the group: -CO-, -CH(OH)-, alkene -CH-, and $-CH_2-$) where the stereochemistry of any -CH(OH)- is also independently selectable;

b) in the absence of a "natural" methyl side-chain; or

c) in the stereochemistry of "natural" methyl; and/or ring substituents other than methyl.

It is also possible to prepare derivatives of 12-, 14- and 16-membered ring macrolides having the differences from the natural product identified in two or more of items a) to c) above.

Derivatives of any of the afore-mentioned polyketides which have undergone further processing by non-PKS enzymes, eg one or more of hydroxylation, epoxidation, glycosylation and methylation may also be prepared.

The present invention provides a novel method of obtaining both known and novel complex polyketides without the formation of mixtures of products differing only in having either an acetate or a propionate starter unit. In addition the present invention provides a method to obtain novel polyketides in which the starter unit is an unusual starter unit which is derived by the action of a KSq domain on the enzyme-bound product of an AT of unusual specificity derived from an extension module of a natural Type I PKS. In particular the AT of extension module 4 of the FK506 PKS gene cluster preferentially incorporates an allyl side-chain; the AT of extension module 6 of the niddamycin PKS gene cluster preferentially incorporates a sidechain of structure $HOCH_2$-; and the ATs of extension module 5 of spiramycin and of extension module 5 of monensin incorporate an ethyl side chain. In each case the KSq domain is preferentially one that is naturally propionate-specific. Alternatively, any KS from an extension module of a Type I PKS may be converted into a KSq domain capable of decarboxylating a bound carboxylated acyl thioester, by site-directed mutagenesis of the active site cysteine residue to replace it by another residue, preferably glutamine. It is known that the animal fatty acid synthase, which shares many mechanistic features with Type I PKS, in the absence of acetyl-CoA, has a demonstrable malonyl-CoA decarboxylase activity (Kresze,

G. B. *et al.* Eur. J. Biochem. (1977) 79:191-199). When treated with an alkylating agent such as iodoacetamide the fatty acid synthase is inactivated by specific modification of the active site cysteine of the KS, and the resulting protein has an enhanced malonyl-CoA decarboxylase activity. The conversion of a fatty acid KS domain into a decarboxylase mirrors the genetically-determined change between the KS domains and the KSq domain in Type I PKSs. Indeed, the size and polarity characteristics of a glutamine side chain very closely approximate those of carboxamido-cysteine. The KSq to be used for decarboxylation of an unusual alkylmalonate unit is preferably selected from the same extension module of the same Type I PKS that provides the unusual AT, in order to optimise the decarboxylation of the unusual alkylmalonate, and the ACP to be used is preferably also the ACP of the same extension module.

Suitable plasmid vectors and genetically engineered cells suitable for expression of PKS genes incorporating an altered loading module are those described in PCT/GB97/01819 as being suitable for expression of hybrid PKS genes of Type I. Examples of effective hosts are *Saccharopolyspora erythraea*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Streptomyces fradiae*, *Streptomyces longisporoflavus*, *Streptomyces hygroscopicus*, *Micromonospora griseorubida*, *Streptomyces lasaliensis*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus*, *Streptomyces albus*, *Amycolatopsis mediterranei*, and *Streptomyces tsukubaensis*. These include hosts in which SCP2*-derived plasmids are known to replicate autonomously, such as for example *S. coelicolor*, *S. avermitilis* and *S. griseofuscus*; and other hosts such as *Saccharopolyspora erythraea* in which SCP2*-derived plasmids become integrated into the chromosome through homologous recombination between sequences on the plasmid insert and on the chromosome; and all such vectors which are integratively transformed by suicide plasmid vectors.

Some embodiments of the invention will now be described with reference to the accompanying drawings in which:

Fig 1 is a diagram showing the functioning of 6-deoxyerythronolide B synthase (DEBS), a modular PKS producing 6-deoxyerythronolide B (6-DEB) a precursor of erythromycin A.

Fig 2 gives the amino acid sequence comparison of the KS domains and the CLF domains of representative Type II PKS gene clusters. The active site Cysteine (C) of the KS domains is arrowed in the Figure and aligns with the Glutamine (Q) or glutamic acid (E) of the CLF domains. The abbreviations used, and the relevant Genbank/EMBL accession numbers are: GRA: granaticin from *Streptomyces violaceoruber* (X63449); HIR: unknown polyketide from *Saccharopolyspora hirsuta* (M98258); ACT, actinorhodin from *Streptomyces coelicolor* (X63449); CIN: unknown polyketide *from Streptomyces cinnamonensis* (Z11511); VNZ: jadomycin from *Streptomyces venezuelae* (L33245); NOG: anthracyclines from *Streptomyces nogalater* (Z48262); TCM: tetracenomycin from *S. glaucescens* (M80674); DAU: daunomycin from *Streptomyces sp.* C5 (L34880); PEU, doxorubicin from *Streptomyces peucetius* (L35560); WHI: WhiE spore pigment from *Streptomyces coelicolor* (X55942).

Fig 3 shows the gene organisation of the PKSs for three 16-membered ring macrolides, tylosin, spiramycin and niddamycin.

Fig 4 shows the amino acid sequence alignment of KSq-ATq loading didomains of the PKSs for niddamycin, platenolide(spiramycin), monensin, oleandomycin and tylosin. The sequences for the monensin and oleandomycin loading didomains have not been previously disclosed.

Fig. 5 The enyzmatic steps that convert 6-deoxyerythronolide B into erythromycin A *in Saccharopolyspora erythraea*

Fig. 6 is a diagram showing the construction of plasmid pJLK117.

Fig. 7 shows the structures of two oligonucleotides.

The present invention will now be illustrated, but is not intended to be limited, by means of some examples. All NMR spectra were measured in $CDCl_3$ using a Bruker 500mHz DMX spectrometer unless otherwise indicated and peak positions are expressed in parts per million (ppm) downfield from tetramethysilane. The atom number shown in the NMR structure is not representative of standard nomenclature, but correlates NMR data to that particular example.

HPLC methods

Method 1

Column	Waters Symmetry 5_C18 2.1mm x 150mm
Flow	0.29 ml/min
Mobile phase	Gradient: A:B (22.78) to A:B (38:62) over 12 minutes, then to A:B (80:20) by minute 15. Maintain for 1 minute. Re-equilibrate before next sample. Where A = acetonitrile and B = 0.01M ammonium acetate in 10% acetonitrile and 0.02% TFA

Method B

Column	Waters Symmetry 5_ C18 2.1mm X 150mm
Flow	0.29 ml/min
Mobile phase	Gradient:28:72 acetonitrile: 10mM NH40Ac to 50:50 in 18 minutes. 50:50 until 25 minutes. back to 28:72, re-equilibrate for 7 minutes
Instrument	Acquired with Hewlett Packard 1100 LC/MS with APCI source

Tap Water medium

glucose	5g/litre
tryptone	5g/litre
yeast extract	2.5g/litre
EDTA	36mg/litre
Tap water to 1L total volume	

ERY - P medium

dextrose	50g/litre
Nutrisoy™ flour	30g/litre
$(NH_4)_2SO_4$	3g/litre
NaCl	5g/litre
$CaCO_3$	6g/litre
Tap water to 1L total volume	
pH adjusted to 7.0	

Example 1

Construction of the Recombinant Vector pPFL43

Plasmid pCJR24 was prepared as described in PCT/GB97/01819. pPFL43 is a pCJR24-based plasmid containing the gene encoding a hybrid polyketide synthase that contains the putative monensin PKS loading module (isolated from *S. cinnamonensis*) the DEBS extension modules 1 and 2 and the chain-terminating thioesterase. Plasmid pPFL43 was constructed as follows:

The following synthetic oligonucleotides: 5'-CCATATGGCCGCATCCGCGTCAGCGT-3' and 5'-GGCTAGCGGGTCCTCGTCCGTGCCGAGGTCA-3' are used to amplify the DNA encoding the putative monensin-producing loading module using a cosmid that contains the 5' end of the putative monensin-producing PKS genes from *S. cinnamonensis* or chromosomal DNA of *S. cinnamonensis* as template. The PCR product of 3.3 kbp is purified by gel electrophoresis, treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which has been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL40. Plasmid pPFL40 was identified by restriction pattern and sequence analysis.

Plasmid pHD30His is a derivative of pNEWAVETE (PCT/GB97/01810) which contains the avermectin loading module, erythromycin extension modules 1 and 2 and the ery thioesterase domain. Plasmid pNEWAVETE was cut with EcoRI and HinDIII and a synthetic oligonucleotide linker was inserted that encodes the addition of a C-terminal polyhistidine tail to the polypeptide. The following oligonucleotides:

5'-AATTCACATCACCATCACCATCACTAGTAGGAGGTCTGGCCATCTAGA-3'

and

5'-AGCTTCTAGATGGCCAGACCTCCTACTAGTGATGGTGATGGTGATGTG-3'

were annealed together and the duplex was ligated to EcoRI-and HinDIII-cut pNEWAVETE. The resulting plasmid was cut with NdeI and XbaI and ligated into plasmid pCJR24 that had been previously cut with same two enzymes, to produce plasmid pND30His.

Plasmid pPFL40 was digested with *Nde* I and *Nhe* I and the 3.3 kbp fragment was purified by gel electrophoresis and ligated to pND30-His previously digested with *Nde* I and *Nhe* I and treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL43. Plasmid pPFL43 was identified by restriction analysis.

Example 2

Construction of*S. erythraea* JC2/ pPFL43

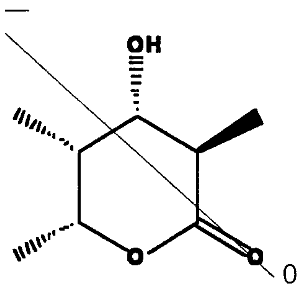
Plasmid pPFL43 was used to transform *S.erythraea* JC2 protoplasts. The construction of strain JC2 from which the resident DEBS genes are substantially deleted is given in Pending Patent Application PCT/GB97/01819. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL43 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *mon* PKS fragment encoding for the loading module.

Example 3

Production of polyketides using *S. erythraea* JC2/pPFL43

A frozen suspension of strain *S. erythraea* JC2/

pPFL43 was inoculated in eryP medium, containing 5 μg/ml of thiostrepton. The inoculated culture was allowed to grow for seven days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH=3.0. The broth was extracted twice with two volumes of ethyl acetate and the combined extracts were washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give crude product. The product was shown to have the structure shown below, and by MS, GC-MS and ^{1}H NMR was found to be identical to an authentic sample.



Example 4

Construction of *S. erythraea* NRRL2338/pPFL43

Plasmid pPFL43 was used to transform *S. erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL43 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *mon* PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL43 was selected in this way.

Example 5a

Production of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL 2338/pPFL43

The culture *Saccharopolyspora erythraea* NRRL2338(pPFL43), constructed with the wild-type loading domain displaced by a monensin loader-D1TE DNA insert, produced as described in Example 2, was inoculated into 30ml Tap Water medium with 50 ug/ml thiostrepton in a 300ml Erlenmeyer flask. After three days incubation at 29°C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.0625 volumes methanol to concentrate the extract 16-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.0 min retention time peak was observed as the major component, with *m/z* value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A. A second peak was observed with a retention time of 6.4 min and with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B.

Example 5b

Production and Recovery of 13-methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL43) at 8L scale

Saccharopolyspora erythraea NRRL2338 (pPFL43) was inoculated into 1000mls Tap Water medium with 50 ug/ml thiostrepton in a 2.8l Fernbach flask. After three days incubation at 29°C, this flask was used to inoculate 8l of ERY-P medium in a 14l Microferm fermentor jar (New Brunswick Scientific Co., Inc., Edison, NJ). The broth was incubated at 28°C with an aeration rate of 8l/min, stirring at 800 rpm and with pH maintained between 6.9 and 7.3 with NaOH or H_2SO_4 (15%). Water was added to maintain volume at the 24 hour volume level. The fermentation was continued for 167 hours. After this time, presence of 13-methyl- erythromycin A and B were confirmed by adjusting a broth sample from the fermentor to pH 8.5 with NaOH, then extracting with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.25

volumes methanol to concentrate the extract 4-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.1 min retention time peak was observed as the major component, with *m/z* value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A. A second peak was observed with a retention time of 6.6 min and with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B.

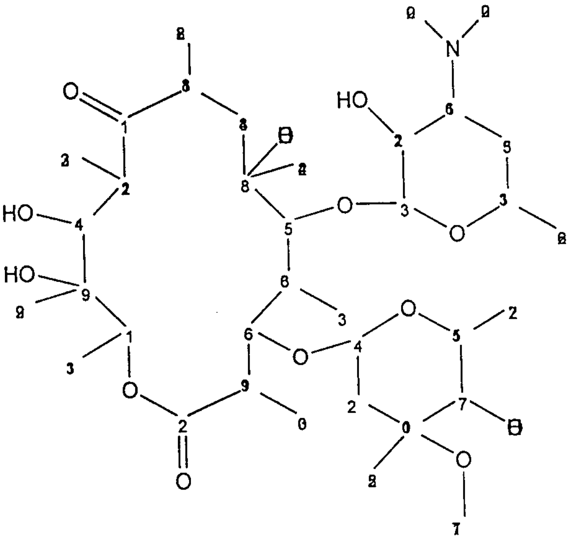
About 35 liters of broth containing approximately 2.8 grams of 13-methyl- erythromycin A were processed for recovery of product. Broth was filtered through a pilot sized Ceraflo ceramic unit and loaded onto a 500ml XAD-16 resin column. The product was eluted using 100% methanol. A 175ml CG-161 adsorption column was prepared and equilibrated with 20% methanol/water. A portion of the product solution was adjusted to 20% methanol and loaded onto the column, no breakthrough of product was observed. Washing of the column with up to 40% methanol/water failed at removing any significant level of impurities. Elution with 50% methanol/water achieved chromatographic separation of the product from the two major impurities, 13-methyl-erythromycin B and a degradation product, 13-methyl-dehydroerythromycin A. The purest cuts were combined and reduced in volume by approximately 75% using evaporation to achieve <10% methanol concentration. To enhance 13-methyl-erythromycin A extraction, solid sodium bicarbonate was added until a total concentration of 250mM was obtained.

The aqueous product layer was extracted 2x with methylene chloride, using one-half the total volume each time. The volume was reduced to light yellow solids by evaporation. The 13-methyl-erythromycin A was purified by dissolving the crude crystals into methylene chloride at ambient temperature and diluting to 15% methylene chloride with hexane. The cloudy solution is placed at -10°C for ~30 minutes when the liquid is decanted to a 2nd flask, leaving the majority of impurities behind as an oil. The flask is left overnight at -10°C, followed by filtration of off-white13-methyl-erythromycin A crystals the next day. Approximately 300 milligrams of 13-methyl-erythromycin A were isolated from the partial work-up of the 351 broth volume.

Approximately 100 grams of evaporated mother liquor were utilized further to isolate 13-methyl-erythromycin B. Residual 13-methyl-erythromycin A was removed with repetitive extraction of the initial sample with aqueous acetic acid (pH 5). The subsequent methylene chloride layer was chromatographed on 700 g of silica gel using 20% methanol in methylene chloride. The 13-methyl-erythromycin B enriched fractions, as determined by LC/MS, were combined and evaporated to yield ~11.0 grams of dark oil. The oil was dissolved in a minimal amount of methanol and loaded onto 500 ml of Amberchrom CG-161 resin. The 13-methyl-erythromycin B was eluted at 2 bed volumes per hour with 40% methanol in deionized water. One bed volume fractions were collected and assayed by LC/MS. Fractions 42 through 62 were combined, diluted to ~20% methanol with deionized water, and neutralized to pH 7.5 with sodium bicarbonate. The resulting solution was extracted once with 41 of methylene chloride, concentrated to ~500 ml, and dried over anhydrous magnesium sulfate. After removal of the MgSO4 by filtration the filtrate was evaporated to give ~110 mg of light brown solids. The 110 mg of crude 13-methyl-erythromycin B was dissolved in ~ 3.0 milliliters of HPLC grade acetonitrile and loaded onto a 20cm x 20cm, 2mm thick, silica gel preparative thin layer chromatography (PTLC) plate. The plate was developed with 60:40 methanol:acetonitrile. The desired portion of silica from the PTLC plate (iodine visualisation) was removed and extracted with HPLC grade acetone. The acetone extract was evaporated to give 12.1 mg of clear solid.

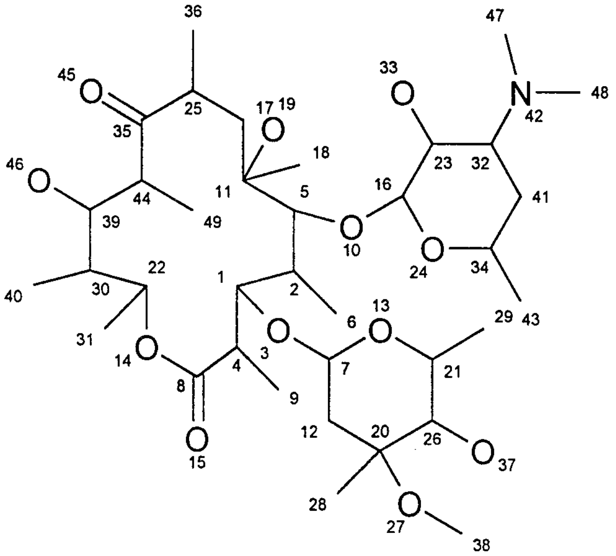
Identification of the 13-methyl-erythromycin A and 13-methyl-erythromycin B samples were confirmed by mass spectroscopy (LC/MS Method B) and NMR spectroscopy. The 13-methyl-erythromycin A sample peak had a 4.7 min retention time, with *m/z* value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A. The 13-methyl-erythromycin B sample peak had a 7.6 min retention time, with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B.

<u>NMR, 13-methyl-erythromycin A</u>:



#	13C - ppm	#H	1H - ppm
1	221.91	0	
2	175.99	0	
3	103.63	1	4.45
4	96.81	1	4.88
5	83.76	1	3.60
6	79.86	1	4.10
7	78.36	1	3.05
8	75.50	0	
9	74.87	0	
10	73.07	0	
11	72.25	1	5.19
12	71.25	1	3.26
13	69.53	1	3.53
14	69.24	1	3.97
15	66.16	1	4.06
16	65.96	1	2.48
17	49.96	3	3.36
18	45.36	1	2.79
19	45.07	1	2.81
20	40.73	3	2.32
21	39.00	1	3.15
22	35.30	2	2.42/1.61
24	27.20	3	1.50
25	21.92	3	1.28
26	21.82	3	1.27
27	18.99	3	1.32
28	18.60	3	1.22
29	16.07	3	1.19
30	15.08	3	1.19
31	14.23	3	1.26
32	12.12	3	1.19
33	9.60	3	1.15
34	39.00	2	1.98/1.75
35	28.90	2	1.72/1.27
36	40.94	1	2.05

NMR, 13-methyl-erythromycin B:



#	13C - PPM	#H attached	1H - PPM
1	80.50	1	4.15
2	40.62	1	2.15
4	45.17	1	2.84
5	84.08	1	3.62
6	9.86	3	1.18
7	97.26	1	4.88
8	176.48	0	
9	15.25	3	1.22
11	75.98	0	
12	35.43	2	2.42/1.61
16	103.75	1	4.46
17	38.77	2	2.09/1.72
18	27.67	3	1.51
20	73.09	0	
21	66.20	1	4.06
22	70.27	1	5.58
23	71.24	1	3.28
25	45.49	1	2.81
26	78.29	1	3.06
28	21.91	3	1.28
29	19.03	3	1.33
30	41.61	1	1.65

31	18.73	3	1.29
32	65.94	1	2.53
34	69.52	1	3.55
35	219.92	0	
36	19.03	3	1.21
38	49.97	3	3.36
39	70.17	1	3.88
40	9.27	3	0.95
41	29.12	2	1.73/1.28
43	21.80	3	1.27
44	39.87	1	3.07
47	40.74	3	2.35
48	40.74	3	2.35
49	9.62	3	1.04

Example 6

Construction of the Recombinant Vector pPFL42

Plasmid pPFL42 is a pCJR24-based plasmid containing the gene encoding a hybrid polyketide synthase that contains the tylosin-producing PKS loading module, the erythromycin extension modules 1 and 2 and the chain-terminating thioesterase. Plasmid pPFL42 was constructed as follows:

The following synthetic oligonucleotides:
5'-CCATATGACCTCGAACACCGCTGCACAGAA-3' and
5'-GGCTAGCGGCTCCTGGGCTTCGAAGCTCTTCT-3'
were used to amplify the DNA encoding the tylosin-producing loading module using either cos6T (a cosmid that contains the tylosin-producing PKS genes from *S. fradiae*) or chromosomal DNA from *S. fradiae* as template. The PCR

product of 3.3 kbp was purified by gel electrophoresis, treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL39. Plasmid pPFL39 was identified by restriction and sequence analysis.

Plasmid pPFL39 was digested with *Nde* I and *Nhe* I and the 3.3 kbp fragment was purified by gel electrophoresis and ligated to pND30 previously digested with *Nde* I and *Nhe* I and treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL42. Plasmid pPFL42 was identified by restriction analysis.

Example 7

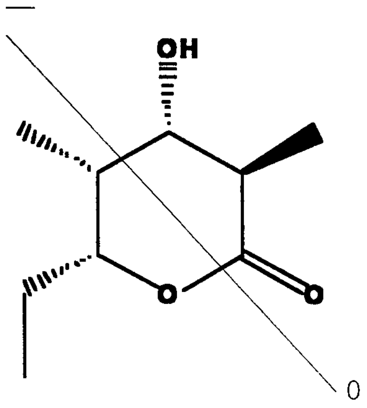
Construction of *S. erythraea* JC2/pPFL42

Plasmid pPFL42 was used to transform *S. erythraea* JC2 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL42 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the tyl PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL42 was identified in this way,

Example 8

Production of polyketides using *S. erythraea* JC2/pPFL42

A frozen suspension of strain *S. erythraea* JC2/pPFL42 was used to inoculate eryP medium containing 5 μg/ml of thiostrepton and allowed to grow for seven days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH=3. The broth was extracted twice with two volumes of ethyl acetate and the combined extracts were washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give crude product. The product was shown to have the structure shown below, and was identical, as judged by MS, GC-MS, and ^{1}H NMR with an authentic sample:.



Example 9

Construction of *S. erythraea* NRRL2338/pPFL42

Plasmid pPFL42 was used to transform *S. erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL42 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *tyl* PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL42 was identified in this way.

Example 10

Production of polyketides using *S. erythraea* NRRL2338/pPFL42

A frozen suspension of strain *S. erythraea* NRRL2338/pPFL42 was used to inoculate eryP medium containing 5 μg/ml of thiostrepton and allowed to grow for seven days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH=9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS and a macrolide was identified with the following structure, identical with that of authentic erythromycin A (together with other products, which were identified as the corresponding erythromycins B and D, the result of incomplete post-PKS processing):

Example 11

Construction of plasmid pPFL35

Plasmid pPFL35 is a pCJR24-based plasmid containing a PKS gene comprising a loading module, the first and second extension modules of DEBS and the chain terminating thioesterase. The loading module comprises the KSq domain DNA from the loading module of the oleandomycin PKS fused to the malonyl-CoA-specific AT of module 2 of the rapamycin PKS, in turn linked to the DEBS loading domain ACP. Plasmid pPFL35 was constructed via several intermediate plasmids as follows:

A 411 bp DNA segment of the *eryAI* gene from *S.erythraea* extending from nucleotide 1279 to nucleotide 1690 (Donadio, S. *et al.*, Science (1991) 2523:675-679) was amplified by PCR using the following synthetic oligonucleotide primers:-

5'-TGGACCGCCGCCAATTGCCTAGGCGGGCCGAACCCGGCT-3' and

5'-CCTGCAGGCCATCGCGACGACCGCGACCGGTTCGCC-3'

The DNA from a plasmid designated pKSW, derived from pT7-7 and DEBS1-TE in which new *Pst* I and *Hin*dIII sites had been introduced to flank the KS1 of the first extension module, was used as a template. The 441 bp PCR product was treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL26. The new *Mfe* I/*Avr* II sites bordering the insert are adjacent to the *Eco* RI site in the polylinker of pUC18. Plasmid pPFL26 was identified by restriction pattern and sequence analysis.

An *Mfe* I restriction site is located 112 bp from the 5' end of the DNA encoding the propionyl-CoA:ACP transferase of the loading module of DEBS. Plasmid pKSW was digested with *Mfe* I and *Pst* I and ligated with the 411 bp insert obtained by digesting plasmid pPFL26 with *Mfe* I and *Pst* I. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL27. Plasmid pPFL27 contains a PKS gene comprising the DEBS loading module, the first and second extension modules of DEBS and the DEBS chain terminating thioesterase. Plasmid pPFL27 was identified by its restriction pattern.

Plasmid pPFL27 was digested with *Nde* I and *Avr* II and ligated to a 4.6kbp insert derived from digesting plasmid pMO6 (PCT/GB97/01819) with *Nde* I and *Avr* II. Plasmid pMO6 contains a PKS gene comprising the DEBS loading module, the first and second extension modules of DEBS and the DEBS chain terminating thioesterase, except that the DNA segment encoding the methylmalonate-specific AT within the first extension module has been specifically substituted by the DNA encoding the malonate-specific AT of module 2 of the *rap* PKS. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL28. Plasmid pPFL28 contains a hybrid PKS gene comprising the DEBS loading module, the malonate-specific AT of module 2 of the *rap* PKS, the ACP of the DEBS loading module, followed by the first and second extension modules of DEBS and the DEBS chain terminating thioesterase. Plasmid pPFL28 was identified by restriction analysis.

A DNA segment encoding the KSq domain from the *ole*AI gene of *S. antibioticus* extending from nucleotide 1671 to nucleotide 3385 was amplified by PCR using the following synthetic oligonucleotide primers:-

5'-CCACATATGCATGTCCCCGGCGAGGAA-3' and

5'-CCCTGTCCGGAGAAGAGGAAGGCGAGGCCG-3'

and chromosomal DNA from *Streptomyces antibioticus* as a template. The PCR product was treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL31. The new *Nde* I site bordering the insert is adjacent to the *Eco* RI site of the pUC18 polylinker while the new *Bsp* EI site borders the *Hin* dIII site of the linker region. Plasmid pPFL31 was identified by restriction and sequence analysis.

Plasmid pPFL31 was digested with *Nde* I and *Avr* II and the insert was ligated with plasmid pPFL28 that had been digested with *Nde* I and *Avr* II. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL32. Plasmid pPFL32 was identified by restriction analysis.

Plasmid pPFL32 was digested with *Nde* I and *Xba* I and the insert was ligated to plasmid pCJR24, which had been digested with *Nde* I and *Xba* I and purified by gel electrophoresis. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid, pPFL35. Plasmid pPFL35 was identified by restriction analysis.

Example 12

Construction of *S. erythraea* JC2 / pPFL35

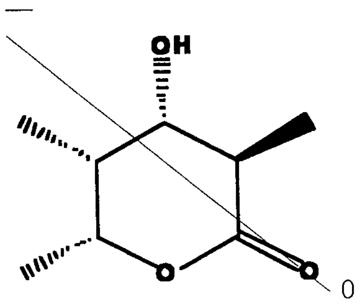
Plasmid pPFL35 was used to transform *S. erythraea* JC2 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton.

Several clones were tested for the presence of pPFL35 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *rap* PKS fragment encoding for module 2 acyltransferase. A clone with an integrated copy of pPFL35 was identified in this way.

Example 13

Production of polyketides using *S. erythraea* JC2 / pPFL35

A frozen suspension of strain *S. erythraea* JC2 / pPFL35 was used to inoculate eryP medium containing 5 μg/ml of thiostrepton and allowed to grow for seven days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH=3. The broth was extracted twice with two volumes of ethyl acetate and the combined extracts were washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give crude product. The product was shown to have the following structure, and was found by MS, GC-MS and ^{1}H NMR to be identical to authentic material:



Example 14

Construction of *S. erythraea* NRRL2338/pPFL35

Plasmid pPFL35 was used to transform *S.erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium (Yamamoto *et al.*) containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL35 integrated into the chromosome by DIG-Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the *rap* PKS fragment encoding for module 2 AT. A clone with an integrated copy of pPFL35 was identified in this way.

Example 15

Production of 13-methyl-erythromycin A and B using *Sacch.erythraea* NRRL-2338 (pPFL35)

The culture *Saccharopolyspora erythraea* NRRL2338 (pPFL35), constructed with the wild-type loading domain displaced by an oleandomycin KSQ-rapamycin AT2- D1TE DNA insert, prepared as described in Example 14, was inoculated into 30ml Tap Water medium with 50 μg/ml thiostrepton in a 300ml Erlenmeyer flask. After two days incubation at 29°C, this flask was used to inoculate 300 ml of ERY-P medium in a 300ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using a Zymark TurboVap LV Evaporator, then reconstituted in 0.25 volumes methanol to concentrate the extract 4-fold. The structure of the products were confirmed by LC/MS, Method A. A peak was observed with a retention time of 4.0 min and with an m/z value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A ($C_{36}H_{65}NO_{13}$). A second peak was observed with a retention time of 6.4 min and with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B ($C_{36}H_{65}NO_{12}$).

Example 16

Construction of Recombinant Vector pPFL44

Plasmid pPFL44 is a pCJR24- based plasmid containing the gene encoding a hybrid polyketide synthase that contains the spiramycin PKS loading module, the erythromycin extension modules 1 and 2 and the chain-terminating thioesterase. Plasmid pPFL44 was constructed as follows:

The following synthetic oligonucleotides:
5'-CCATATGTCTGGAGAACTCGCGATTTCCCGCAGT-3' and
5'-GGCTAGCGGGTCGTCGTCGTCCCGGCTG-3'
were used to amplify the DNA encoding the spiramycin-producing loading module using chromosomal DNA from the spiramycin producer *S. ambofaciens* prepared according to the method described by Hopwood *et al.* (1985). The PCR

product of 3.3 kbp was purified by gel electrophoresis, treated with T4 polynucleotide kinase and ligated to plasmid pUC18, which had been linearised by digestion with *Sma* I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones were checked for the desired plasmid pPFL41. Plasmid pPFL41 was identified by restriction pattern and sequence analysis.

Plasmid pPFL41 was digested with *Nde* I and *Nhe* I and the 3.3 kbp fragment was purified by gel electrophoresis and ligated to pND30 (a plasmid derived from plasmid pCJR24 having as insert the *ave* PKS loading module and extension modules 1 and 2 or DEBS and the DEBS thioesterase) (PCTGB97/01810) previously digested with *Nde* I and *Nhe* I and treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E.coli* DH10B cells and individual clones checked for the desired plasmid pPFL44. Plasmid pPFL44 was identified by restriction analysis.

Example 17

Construction of *S. erythraea* JC2/pPFL44

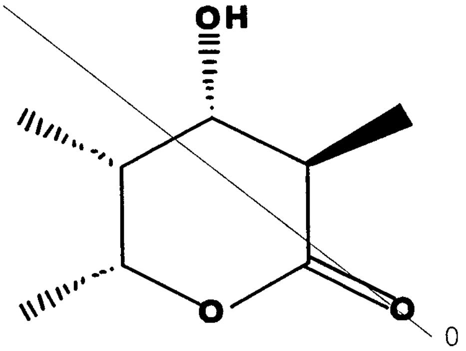
Plasmid pPFL44 was used to transform *S.erythraea* JC2 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton.

Several clones were tested for the presence of pPFL44 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the srm PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL44 was identified in this way.

Example 18

Production of polyketides using *S. erythraea* JC2/pPFL44

A frozen suspension of strain *S. erythraea* JC2/pPFL44 was used to inoculate eryP medium containing 5 μg/ml of thiostrepton and allowed to grow for seven days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH=3. The broth was extracted twice with two volumes of ethyl acetate and the combined extracts were washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give crude product. The product was shown to have the structure shown below and by GC-MS and ^{1}H NMR analysis was identical to authentic material:



Example 19

Construction of *S. erythraea* NRRL2338/pPFL44

Plasmid pPFL44 was used to transform *S.erythraea* NRRL2338 protoplasts. Thiostrepton resistant colonies were selected in R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pPFL44 integrated into the chromosome by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA containing the spiramycin PKS fragment encoding for the loading module. A clone with an integrated copy of pPFL44 was identified in this way.

Example 20

Production of 13 methyl-erythromycin A and B using *Sacch. erythraea* NRRL-2338 (pPFL44)

The culture *Saccharopolyspora etythraea* NRRL2338 (pPFL44), constructed with the wild-type loading domain displaced by spiramycin loader-D1TE DNA insert, was inoculated into 30ml Tap Water medium with 50 μg/ml thiostrepton in a 300ml Erlenmeyer flask. After three days incubation at 29°C, this flask was used to inoculate 300 ml of ERY-P medium in a 300 ml flask. The broth was incubated at 29°C at 200 rpm for 6 days. After this time, the whole broth was adjusted to pH 8.5 with NaOH, then extracted with equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness at 45°C under a nitrogen stream using Zymark TurboVap LV Evaporator, then reconstituted in 0.0625 volumes methanol to concentrate the extract 16-fold. The structures of the products were confirmed by LC/MS, Method A. A 4.0 min retention time peak was observed as the major component, with *m/z* value of 720 $(M+H)^+$, required for 13-methyl-erythromycin A ($C_3H_{65}NO_{13}$). A second peak was observed with a retention time of 6.4 min and with *m/z* value of 704 $(M+H)^+$, required for 13-methyl-erythromycin B ($C_{36}H_{65}NO_{12}$).

Example 21

Construction of plasmid pJLK114

Plasmid pJLK114 is a pCJR24 based plasmid containing a PKS gene comprising the ery loading module, the first and the second extension modules of the ery PKS and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by a synthetic oligonucleotide linker containing the recognition sites of the following restriction enzymes: AvrII, BglII, SnaBI, PstI, SpeI, NsiI, Bsu36I and HpaI. It was constructed via several intermediate plasmids as follows (Figure 6).

Construction of plasmid pJLK02

The approximately 1.47 kbp DNA fragment of the eryAI gene of S. erythraea was amplified by PCR using as primers the synthetic oligonucleotides:
5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3' and
5'-ATGTTAACCGGTCGCGCAGGCTCTCCGTCT-3' and plasmid pNTEP2 (Oliynyk, M. et al., Chemistry and Biology (1996) 3:833-839; WO98/01546) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and

then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK02 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK03

The approximately 1.12 kbp DNA fragment of the eryAI gene of S. erythraea was amplified by PCR using as primers the synthetic oligonucleotides:
5'-ATGTTAACGGGTCTGCCGCGTGCCGAGCGGAC-3' and
5'-CTTCTAGACTATGAATTCCCTCCGCCCAGC-3' and plasmid pNTEPH as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK03 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK04

Plasmid pJLK02 was digested with PstI and HpaI and the 1.47 kbp insert was ligated with plasmid pJLK03 which had been digested with PstI and HpaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK04 was identified by its restriction pattern.

Construction of plasmid pJLK05

Plasmid pJLK01 (PCT/GB97/01819) was digested with PstI and AvrII and the 460 bp insert was ligated with plasmid pJLK04 which had been digested with PstI and AvrII. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK05 was identified by its restriction pattern.

Construction of plasmid pJLK07

Plasmid pJLK05 was digested with ScaI and XbaI and plasmid pNTEPH was digested with NdeI and ScaI and these two fragments were ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK07 was identified by its restriction pattern.

Construction of plasmid pJLK114

The two synthetic oligonucleotides Plf and Plb (Figure 7) were each dissolved in TE-buffer. 10 μl of each solution (0.5nmol/μl) were mixed and heated for 2 minutes to 65C and then slowly cooled down to room temperature. Plasmid pJLK07 was digested with AvrII and HpaI and ligated with the annealed oligonucleotides. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK114 was identified by its restriction pattern.

Plasmid pJLK117 is a pCJR24 based plasmid containing a PKS gene comprising the ery loading module, the first and the second extension modules of the ery PKS and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by a synthetic oligonucleotide linker containing the recognition sites of the following restriction enzymes. AvrII, BglII, SnaBI, PstI, SpeI, NsiI, Bsu36I and NheI.

It was constructed via several intermediate plasmids as follows (Figure 6).

Construction of plasmid pJLK115

Plasmid pJLK114 was digested with NdeI and XbaI and the approximately 9.9 kbp insert was ligated with plasmid pUC18 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK115 was identified by its restriction pattern.

Construction of plasmid pJLK116

Plasmid pJLK13 (PCT/GB97/01819) was digested with Bsu36I and XbaI and the 1.1 kbp fragment was ligated with plasmid pJLK115 which had been digested with Bsu36I and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK116 was identified by its restriction pattern.

Construction of plasmid pJLK117

Plasmid pJLK116 was digested with NdeI and XbaI and the 9.9 kbp fragment was ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK117 was identified by its restriction pattern.

Example 11

Construction of plasmid pJLK29

Plasmid pJLK29 is a pJLK117 based plasmid except that the DNA fragment encoding the reductive loop of module 10 of the rap PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows. (Figure 5)

Construction of plasmid pJLK121.1

The approximately 2.2 kbp DNA segment of the rapB gene of S. hygroscopicus encoding the reductive loop of module 10 was amplified by PCR using as primers the synthetic oligonucleotides:
5'-TAAGATCTTCCGACGTACGCGTTCCAGC-3' and
5'-ATGCTAGCCACTGCGCCGACGAATCACCGGTGG-3' and as template an approximately 7 kbp fragment, which has been obtained by digestion of cosmid cos 26 (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843) with ScaI and SphI. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content.

The desired plasmid pJLK121.1 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK29

Plasmid pJLK121.1 was digested with BglII and NheI and the 2.2 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK29 was identified by its restriction pattern.

Example 24

Construction of Plasmid pJLK50

The approximately 6.1 kbp DNA segment of the erythromycin PKS gene cluster of S. erythraea encoding the DNA fragment from the beginning of the ACP of module 2 to the beginning of the ACP of module 3 was amplified by PCR using as primers the synthetic oligonucleotides:
5'-TACCTGAGGGACCGGCTAGCGGGTCTGCCGCGTG-3' and
5'-ATGCTAGCCGTTGTGCCGGCTCGCCGGTCGGTCC-3' and plasmid pBAM25 (published pBK25 by Best, D J et al. Eur J Biochem (1992) 204: 39-49) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid

pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK50 was identified by its restriction pattern and DNA sequencing.

Example 25

Construction of *S.erythraea* strain JLK10

Strain JLK10 is a variant of strain NRRL2338 in which the reductive loop of *ery* module 2 (i.e. the KR domain) is replaced by the reductive loop of the rapamycin module 10. It was constructed using plasmid pJLK54 which was constructed as follows.

Construction of plasmid pJLK54

Plasmid pJLK54 is a pJLK29 based plasmid containing a PKS gene comprising the ery loading module, the first, the second and the third extension modules of the ery cluster and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by the equivalent segment of module 10 of the rapamycin PKS.

It was constructed as follows.

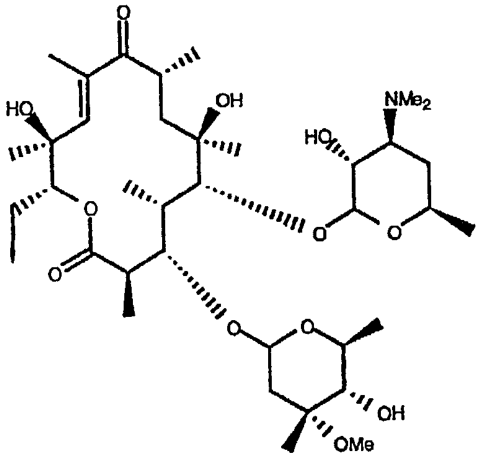
Plasmid pJLK50 was digested with NheI and the 6.1 kbp insert was ligated with plasmid pJLK29 which had been digested with NheI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK54 was identified by its restriction pattern.

Use of plasmid pJLK54 for construction of S. erythraea NRRL2338/pJLK54 and the production of TKL derivatives

Approximately 5 µg plasmid pJLK54 were used to transform protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA is obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE.

Construction of S.erythraea strain JLK10 and its use in production of 13-methyl-10,11-dehydro-erythromycin A

S. erythraea strain JLK10 is a mutant of S. erythraea NRRL2338 in which the 'reductive loop' of ery module 2 i.e. the ketoreductase domain is substituted by the 'reductive loop' of rapamycin module 10. It was constructed starting from S. erythraea NRRL2338 into which plasmid pJLK54 had been integrated. S. erythraea NRRL2338/pJLK54 was subjected to several rounds of non-selective growth which resulted in second crossover concomitant with the loss of the integrated plasmid. Clones in which replacement of the erythromycin gene coding for DEBS1 with the mutant version had occurred, were identified by Southern blot hybridisation. One of these was named S. erythraea strain JLK10 and was used to inoculate SM3 medium (eryP medium gave similar results), and allowed to grow for seven to ten days at 28-30°C. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH 9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The following macrolide C-13 methyl erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes).



Example 26

Construction of plasmid pPFL50

Plasmid pPFL50 is a pPFL43-based plasmid from which a DNA fragment encoding KR1 (in part), ACP1 and module 2 of the erythromycin PKS and the erythromycin TE, has been removed. It was constructed as follows. Plasmid pPFL43 was digested with SfuI and XbaI to remove a 6.5 kb fragment. The 5' overhangs were filled in with Klenow fragment DNA Polymerase I and the plasmid was recircularised. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pPFL50 was identified by its restriction pattern.

Construction of S. erythraea JLK10/pPFL50

Approximately 5 μg plasmid pPFL50 were used to transform protoplasts of S. erythraea strain JLK10 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous chromosomal DNA region. S. erythraea strain JLK10/pPFL50 was used to inoculate SM3 medium containing 5 μg/ml thiostrepton (eryP medium containing 5 μg/ml thiostrepton gave similar results)and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH 9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C-13 methyl 10,11-dehydro-erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes)

Construction of S. erythraea NRRL2338/pPFL50

Approximately 5 μg plasmid pPFL50 were used to transform protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous region of the chromosomal DNA. S. erythraea NRRL2338/pPFL50 was used to inoculate SM3 medium containing 5 μg/ml thiostrepton (eryP medium containing 5 μg/ml thiostrepton gives similar results) and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH 9.5. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C-13 methyl erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes).

Construction of plasmid pCB121

Plasmid pCB121 is a plasmid containing the monensin loading module and KS of monensin module 1 followed by the erythromycin module 1 AT and part of the erythromycin module1 KR. It was constructed via several intermediate plasmids as follows.

Construction of plasmid pPFL45

The approximately 1.8 kbp DNA segment of the monensin PKS gene cluster of Streptomyces cinnamonensis encoding part of the ACP of the loading module and KS of module 1 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-CGTTCCTGAGGTCGCTGGCCCAGGCGTA-3'

5'-CGAAGCTTGACACCGCGGCGCGGCGCGG-5'

and a cosmid containing the 5' end of the monensin PKS genes from S. cinnamonensis or alternatively chromosomal DNA of S. cinnamonensis as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E.

coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pPFL45 was identified by its restriction pattern.

Construction of plasmid pPFL47

Plasmid pPFL45 was digested with NdeI and Bsu36I and the approximately 2.6 kbp fragment was ligated into plasmid pPFL43 which had been digested with NdeI and Bsu36I. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pPFL47 was identified by its restriction pattern.

Construction of plasmid pCB135

Plasmid pCJR24 was digested with HindIII, the 5' overhang was filled in with Klenow fragment DNA Polymerase I and religated. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pCB135 was identified by its restriction pattern, lacking the recognition site for HindIII.

Construction of plasmid pKSW1

Plasmid pKS1W is a pNTEP2 (GB97/01810)-derived vector containing a DEBS1TE-derived triketide synthase with the unique restriction sites introduced at the limits of KS1. Plasmid pKS1W is obtained via several intermediate plasmids as follows.

Construction of plasmids pMO09, pMO10 and pMO13

For the PCR amplification for plasmid pMO09, the following synthetic oligonucleotides were used as mutagenic primers, one containing a MunI site and the other a PstI site:

5' -GCGCGCCAATTGCGTGCACATCTCGAT- 3'

and 5' -CCTGCAGGCCATCGCGACGACCGCGACCGGTTCGCCG- 3'

For the PCR amplification for plasmid pMO10, the following synthetic oligonucleotides were used as mutagenic primers, one containing a HindIII site and the other an EcoRV site:

5' -GTCTCAAGCTTCGGCATCAGCGGCACCAA- 3'

and 5' -CGTGCGATATCCCTGCTCGGCGAGCGCA-3'

For the PCR amplification for plasmid pMO13, the following synthetic oligonucleotides were used as mutagenic primers, one containing a PstI site and the other a HindIII site:

5' -GATGGCCTGCAGGCTGCCCGGCGGTGTGAGCA- 3'

and 5' -GCCGAAGCTTGAGACCCCCGCCCGGCGCGGTCGC- 3'

PCR was carried out on pNTEP2 (GB97/01810) as template using Pwo DNA polymerase and one cycle of: 96°C (1min); annealing at 50°C (3min); and extension at 72°C (1min), and 25 cycles of: 96°C (1min); annealing at 50°C (1min); and extension at 72°C (1min) in the presence of 10% (vol/vol) dimethylsulphoxide. The products were end-repaired and cloned into pUC18 digested with SmaI and the ligation mixture was transformed into E. coli DH 10B. Plasmid DNA was prepared from individual colonies. The desired plasmids for pMO09 (3.8kbp), pMO10 (3.9 kbp) and pMO13 (4.3 kbp) were identified by their restriction pattern and DNA sequencing.

Construction of plasmid pMO11

Plasmid pMO13 was digested with HindIII, and the 1.2 kbp insert was cloned into pMO10 which had been digested with HindIII. The ligation mixture was transformed into E. coli DH 10B. The desired plasmid (5.0 kbp) was identified by its restriction pattern and designated pMO11.

Construction of plasmid pMO12

Plasmid pMO09 was digested with PstI, and the 1.6 kbp insert was cloned into pMO11 which had been digested with PstI. The ligation mixture was transformed into E. coli DH 10B. The desired plasmid (6.6 kbp) was identified by its restriction pattern and designated pMO12.

Construction of pKS1W

Plasmid pMO12 was digested with MunI and EcoRV, and the 3.9 kbp fragment was cloned into pNTEPH (see below) which had been digested with MunI and EcoRV. The ligation mixture was transformed into E. coli DH 10B. The desired plasmid (13. kbp) was identified by its restriction pattern and designated pKS1W.

Construction of pNTEPH

Plasmid pNTEPH was obtained from pNTEP2 by removing the HindIII site. pNTEP2 was digested with HindIII, the 5' overhang was filled in with Klenow Fragment DNA Polymerase I and religated. The desired plasmid (13.6 kbp) was identified by its restriction pattern.

Construction of plasmid pCB136

Plasmid pKSW1 was digested with NdeI and XbaI and the approximately 11.2 kbp fragment was ligated with plasmid pCB135 which had been digested with NdeI and XbaI.The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pCB136 was identified by its restriction pattern.

Construction of plasmid pCB137

Plasmid pCB136 was digested with SfuI and XbaI to remove a 6.5 kb fragment,. the 5' overhangs were filled in with Klenow Fragment DNA Polymerase I and religated. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pCB137 was identified by its restriction pattern.

Construction of plasmid pCB121

Plasmid pPFL47 was digested with NdeI and HindIII and the approximately 4.4 kbp insert was ligated with plasmid pCB137 which had been digested with NdeI and HindIII. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pCB121 was identified by its restriction pattern.

Example

Construction of S.erythraea JLK10/pCB121

Approximately 5 µg plasmid pCB121 were used to transform protoplasts of S. erythraea JLK10 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous chromosomal DNA region. S. erythraea strain JLK10/pCB121 was used to inoculate SM3 medium containing 5 µg/ml thiostrepton (eryP medium containing 5 µg/ml thiostrepton gave similar results) and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH 9. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by

HPLC/MS, MS/MS and 1H-NMR. The macrolide C13-methyl-10,11-dehydro-erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes):

Example

Construction of S. erythraea NRRL2338/pCB121

Approximately 5 µg plasmid pCB121 were used to transform protoplasts of S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid had integrated into the homologous chromosomal DNA region. S. erythraea NRRL2338/pPFL50 was used to inoculate SM3 medium containing 5 µg/ml thiostrepton (eryP medium containing 5 µg/ml thiostrepton gave similar results) and allowed to grow for seven to ten days at 28-30oC. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH=9.. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. Products were analysed by HPLC/MS, MS/MS and 1H-NMR. The macrolide C13-erythromycin A was identified (accompanied by products of incomplete processing by post-PKS enzymes):

Although the present invention is illustrated by the examples listed above, they should not be regarded as limiting the scope of the invention. The above descriptions illustrate for the first time the construction of a Type I PKS gene assembly containing a wholly or partly heterologous Ksq-containing loading module and its use to obtain polyketide products of utility as synthetic intermediates or as bioactive materials such as antibiotics. It will readily occur to the person skilled in the art that a wholly or partly heterologous Ksq-containing loading module from other PKS gene sets could be used to replace the loading module of DEBS, or indeed into a quite different PKS gene assembly. It will also readily occur to the person skilled in the art that the additional specificity provided by the more efficient discrimination made between methylmalonyl-CoA and malonyl-CoA by an Atq, followed by specific decarboxylation by a Ksq, is preferable to the imperfect discrimination between propionyl-CoA and acetyl-CoA that is a feature of the DEBS loading module and of many other PKS loading modules, in that it maximises the production of a single product rather than a mixture differing from each other in the nature of the starter unit. The avoidance of such mixtures increases yields and avoids the need for tedious and difficult separation procedures.

Although the present invention is illustrated by the examples listed above, they should not be regarded as limiting the scope of the invention. The above descriptions illustrate for the first time the construction of a Type I PKS gene assembly containing a wholly or partly heterologous KSq-containing loading module and its use to obtain polyketide products of utility as synthetic intermediates or as bioactive materials such as antibiotics. It will readily occur to the person skilled in the art that a wholly or partly heterologous KSq-containing loading module from other PKS gene sets could be used to replace the loading module of DEBS, or indeed into a quite different PKS gene assembly. It will also readily occur to the person skilled in the art that that the additional specificity provided by the more efficient discrimination made between methylmalonyl-CoA and malonyl-CoA by an ATq, followed by specific decarboxylation by a KSq, is preferable to the imperfect discrimination between propionyl-CoA and acetyl-CoA that is a feature of the DEBS loading module and of many other PKS loading modules, in that it maximises the production of a single product rather than a mixture differing from each other in the nature of the starter unit. The avoidance of such mixtures increases yields and avoids the need for tedious and difficult separation procedures.

CLAIMS:

1. A system for use in producing a polyketide having substantially exclusively a desired starter unit by providing a PKS multienzyme which comprises a loading module and a plurality of extension modules, wherein said loading module is adapted to load an optionally substituted malonyl and then to effect decarboxylation of the loaded residue to provide a corresponding optionally substituted acetyl residue for transfer to an adjacent one of said extension modules, and wherein at least one of the extension modules is not naturally associated with a loading module that effects decarboxylation; with the proviso that the target polyketide is not a 14-membered macrolide having a 13-methyl group due to incorporation of an (unsubstituted) acetate starter unit.

2. A system according to claim 1 wherein said adjacent extension module to which the acetate starter is transferred is not naturally associated with a loading module that effects decarboxylation.

3. A system according to claim 1 or 2 wherein the decarboxylating functionality of the loading module is provided by a ketosynthase-type domain having a glutamine residue in the active site or other residue other than cysteine.

4. A system according to claim 1 or 2 wherein the decarboxylating functionality of the loading module is provided by a CLF-type domain.

5. A system according to any of claims 1 to 4 wherein the loading module's loading functionality is provided by an acyltransferase-type domain having an arginine residue in the active site.

6. A system according to any of claims 1-5 wherein the loading module includes an acyl carrier protein.

7. A system according to any of claims 1-3, 5 or 6 wherein at least the Ksq domain of said loading module corresponds to the loading module of the PKS multienzyme of oleandomycin, spiramycin, niddamycin, methmycin or monensin.

8. A PKS multienzyme as expressible by the DNA of the system of any of claims 1 to 7 or a variant having the ability to synthesize a said polyketide compound.

9. Nucleic acid encoding the PKS multienzyme of claim 8.

10. A vector containing nucleic acid as defined in claim 9.

11. A transformant organism comprising a system according to any of claims 1 to 7.

12. A process for producing a polyketide which comprises culturing an organism according to claim 11 and recovering the polyketide.

13. A system, multienzyme, nucleic acid, vector, organism or process according to any preceding claim wherein said polyketide is selected from

(a) 12- and 16-membered macrolides with acetate starter units

(b) 12, 14 and 16-membered macrolides with propionate starter units

(c) variants of rifamycin, avermectin, rapamycin, immunomycin and FK506 with acetate starter units or propionate starter units

(d) a polyketide wherein the starter unit gave rise to a sidechain selected from allyl and hydroxymethyl.

14. A variant of a parent polyketide which differs from the parent polyketide in the side chain provided by the starter unit.

15. A process for preparing a type II polyketide comprising culturing an organism containing a type II polyketide synthase ("PKS") wherein the wild type synthase includes a CLF domain which tends to effect decarboxylation to produce an undesired starter; wherein said organism contains a PKS which has been genetically engineered to suppress the decarboxylating activity of said CLF domain.

The erythromycin PKS

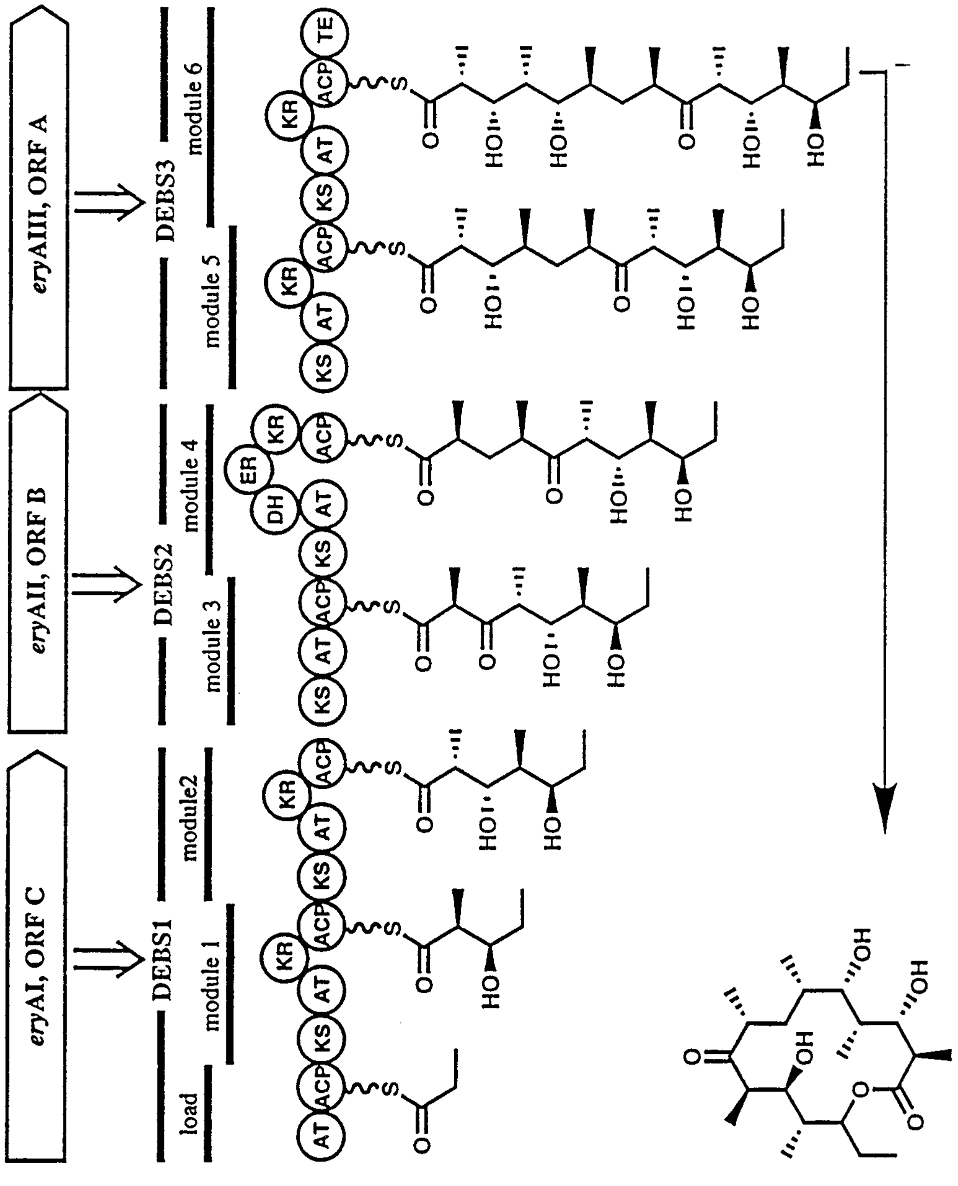


Fig. 1

```
KCLFDAU     ---------------------------MVTGLGIVAPNGLGVGAIWDAVLNGRNGIGPLR
KCLFPEU     MTGTAARTASSQLHASPAGRRGLRGRAVVTGLGIVAPNGLGVGAYWDAVLNGRNGIGPLR
KCLFACT     ------------------------MSVLITGVGVVAPNGLGLAPYWSAVLDGRHGLGPVT
KCLFHIR     ------------------------MSTWVTGMGVVAPNGLGADDHWAATLKGRHGISRLS
KCLFGRA     ------------------MSTPDRRRAVVTGLSVAAPGGLGTERYWKSLLTGENGIAELS
KCLFNOG     ----------------------MTAAVVVTGLGVVAPTGLGVREHWSSTVRGASAIGPVT
KCLFTCM     --------------------MSAPAPVVVTGLGIVAPNGTGTEEYWAATLAGKSGIDVIQ
KCLFCIN     ----------------------MTP-VAVTGMGIAAPNGLGRPTTGRPPWAPRAASAAST
KCLFVNZ     ----------------------MSASVVVTGLGVAAPNGLGREDFWASTLGGKSGIGPLT
KCLFWHIE    -----------MSGPQRTGTGGGSRRAVVTGLGVLSPHGTGVEAHWKAVADGTSSLGPVT
KSGRA       ----------------------MTRRVVITGVGVRAPGGSGTKEFWDLLTAGRTATRPIS
KSHIR       ----------------------MTRRVVITGVGVRAPGGLGAKNFWELLTSGRTATRRIS
KSACT       ----------------------MKRRVVITGVGVRAPGGNGTRQFWELLTSGRTATRRIS
KSCIN       ---------------------MTQRRVAITGIEVLAPGGLGRKEFWQLLSEGRTATRGIT
KSVNZ       ---------------------MTARRVVITGIEVLAPGGTGSKAFWNLLSEGRTATRGIT
KSNOG       ------------------MKESINRRVVITGIGIVAPDATGVKPFWDLLTAGRTATRTIT
KSTCM       ------------------MTRHAEKRVVITGIGVRAPGGAGTAAFWDLLTAGRTATRTIS
KSDAU       ----------------------MNRRVVITGMGVVAPGAIGIKSFWELLLSGTTATRAIT
KSPEU       ----------------------MNRRIVITGIGVVAPGAVGTKPFWELLLSGTTATRAIS
KSWHI       ---------------------MTRRRVAVTGIGVVAPGGIGTPQFWRLLSEGRTATRRIS
                                            :**: : :* . *             .

KCLFDAU     RFADDGRLGRLAGEVSDFVP-EDHLPKRLLVQTDPMTQMTALAAAEWALREAGCAPSS--
KCLFPEU     RFTGDGRLGRLAGEVSDFVP-EDHLPKRLLAQTDPMTQY-ALAAAEWALRESGCSPSS--
KCLFACT     RFDVSRYPATLAGQIDDFHA-PDHIPGRLLPQTDPSTRL-ALTAADWALQDAKADPES-L
KCLFHIR     RFDPTGYPAELAGQVLDFDA-TEHLPKRLLPQTDVSTRF-ALAAAAWALADAEVDPAE-L
KCLFGRA     RFDASRYPSRLAGQIDDFEA-SEHLPSRLLPQTDVSTRY-ALAAADWALADAGVGPESGL
KCLFNOG     RFDAGRYPSKLAGEVPGFVP-EDHLPSRLMPQTDHMTRL-ALVAADWAFQDAAVDPSK-L
KCLFTCM     RFDPHGYPVRVGGEVLAFDA-AAHLPGRLLPQTDRMTQH-ALVAAEWALADAGLEPEK-Q
KCLFCIN     RFDPSGYPAQLAGEIPGFRA-AEHLPGRLVPQTDRVTRL-SLAAADWALADAGVEVAA-F
KCLFVNZ     RFDPTGYPARLAGEVPGFAA-EEHLPSRLLPQTDRMTRL-ALVAADWALADAGVRPEE-Q
KCLFWHIE    REGCAHLPLRVAGEVHGFDA-AETVEDRFLVQTDRFTHF-ALSATQHALADARFGRADVD
KSGRA       FFDASPFRSRIAGEI-DFDAVAEGFSPREVRRMDRATQF-AVACTRDALADSGLDTGA-L
KSHIR       FFDPTPNRSQIAAEC-DFDPEHEGLSPREIRRMDRAAQF-AVVCTRDAVADSGLEFEQ-V
KSACT       FFDPSPYRSQVAAEA-DFDPVAEGFGPRELDRMDRASQF-AVACAREAFAASGLDPDT-L
KSCIN       FFDPAPFRSKVAAEA-DFCGLENGLSPQEVRRMDRAAQF-AVVTAR-AVEDSGAELAA-H
KSVNZ       FFDPTPFRSRVAAEI-DFDPEAHGLSPQEIRRMDRAAQF-AVVAAR-AVADSGIDLAA-H
KSNOG       AFDPSPFRSRIAAEC-DFDPLAEGLTPQQIRRMDRATQF-AVVSARESLEDSGLDLGA-L
KSTCM       LFDAAPYRSRIAGEI-DFDPIGEGLSPRQASTYDRATQL-AVVCAREALKDSGLDPAA-V
KSDAU       TFDATPFRSRIAAEC-DFDPVAAGLSAEQARRLDRAGQF-ALVAGQEALTDSGLRIGE-D
KSPEU       TFDATPFRSRIAAEC-DFDPVAAGLSAEQARRLDRAGQF-ALVAGQEALADSGLRIDE-D
KSWHI       LFDPSGLRSQIAAEC-DFEPSDHGLGLATAQRCDRYVQF-ALVAASEAVRDANLDMNR-E
                      :..:   *      .        *   :  ::     :.  :
```

Fig 2A

```
KCLFDAU     -PLEAGVITASASGGFASGQRELQNLWSKG-----PAHVSAYMSFAWFY-AVNTGQIAIR
KCLFPEU     -PLEAGVITASASGGFAFGQRELQNLWSKG-----PAHVSAYMSFAWFY-AVNTGQIAIR
KCLFACT     TDYDMGVVTANACGGFDFTHREFRKLWSEG-----PKSVSVYESFAWFY-AVNTGQISIR
KCLFHIR     PEYGTGVITSNATGGFEFTHREFRKLWAQG-----PEFVSVYESFAWFY-AVNTGQISIR
KCLFGRA     DDYDLGVVTSTAQGGFDFTHREFHKLWSQG-----PAYVSVYESFAWFY-AVNTGQISIR
KCLFNOG     PEYGVGVVTASSAGGFEFGHRELQNLWSLG-----PQYVSAYQSFAWFY-AVNTGQVSIR
KCLFTCM     DEYGLGVLTAAGAGGFEFGQREMQKLWGTG-----PERVSAYQSFAWFY-AVNTGQISIR
KCLFCIN     DPLDMGVVTASHAGGFEFGQDELQKLLGQG-----QPVLSAYQSFAWFY-AVNSGQISIR
KCLFVNZ     DDFDMGVVTASASGGFEFGQGELQKLWSQG-----SQYVSAYQSFAWFY-AVNSGQISIR
KCLFWHIE    SPYSVGVVTAAGSGGGEFGQRELQNLWGHG-----SRHVGPYQSIAWFY-AASTGQVSIR
KSGRA       DPSRIGVALGSAVASATSLENEYLVMSDSGREWLVDPAHLSPMMFDYLSPGVMPAEVAWA
KSHIR       PPERIGVSLGSAVAAATSLEQEYLVLSDGGREWQVDPAYLSAHMFDYLSPGVMPAEVAWT
KSACT       DPARVGVSLGSAVAAATSLEREYLLLSDSGRDWEVDAAWLSRHMFDYLVPSVMPAEVAWA
KSCIN       PPHRIGVVVGSAVGATMGLDNEYRVVSDGGRLDLVDHRYAVPHLYNYLVPSSFAAEVAWA
KSVNZ       DPYRVGVTVGSAVGATMGLDEEYRVVSDGGRLDLVDHAYAVPHLYDYMVPSSFSAEVAWA
KSNOG       DASRTGVVVGSAVGCTTSLEEEYAVVSDSGRNWLVDDGYAVPHLFDYFVPSSIAAEVAHD
KSTCM       NPERIGVSIGTAVGCTTGLDREYARVSEGGSRWLVDHTLAVEQLFDYFVPTSICREVAWE
KSDAU       SAHRVGVCVGTAVGCTQKLESEYVALSAGGANWVVDPHRGAPELYDYFVPSSLAAEVAWL
KSPEU       SAHRVGVCVGTAVGCTQKLESEYVALSAGGAHWVVDPGRGSPELYDYFVPSSLAAEVAWL
KSWHI       DPWRAGATLGTAVGGTTRLEHDYVLVSERGSRWDVDDRRSEPHLERAFTPATLSSAVAEE
                 *.  .   .     . :   :   *                 :        ::
                          ↓

KCLFDAU     -HDLRGPVGVVVAEQAGGLDALAHAR-RKVRGGAE-LIVSGAMDSSLCP-YGMAAQVRSG
KCLFPEU     -HDLRGPVGVVVAEQAGGLDALAHAR-RKVRGGAE-LIVSGAVDSSLCP-YGMAAQVKSG
KCLFACT     -HGMRGPSSALVAEQAGGLDALGHAR-RTIRRGTP-LVVSGGVDSALDP-WGWVSQIASG
KCLFHIR     -HGLRGPGSVLVAEQAGGLDAVGHGG--AVRNGTP-MVVTGGVDSSFDP-WGWVSHVSSG
KCLFGRA     -NTMRGPSAALVGEQAGGLDAIGHAR-RTVRRGPG-WCSAVASTRRSTR-GASSSQLSGG
KCLFNOG     -HGLRGPGGVLVTEQAGGLDALGQAR-RQLRRGLP-MVVAGAVDGSPCP-WGWVAQLSSG
KCLFTCM     -HGMRGHSSVFVTEQAGGLDAAAHAA-RLLRKGTLNTALTGGCEASLCP-WGLVAQIPSG
KCLFCIN     -HGMKGPSGVVVSDQAGGLDALAQAR-RLVRKGTP-LIVCGAVEPRSAPGAGSPSSPAGG
KCLFVNZ     -NGMKGPSGVVVSDQAGGLDAVAQAR-RQIRKGTR-LIVSGGVDASLCP-WGWVAHVASD
KCLFWHIE    -NDFKGPCGVVAADEAGGLDALAHAA-LAVRNGTD-TVVCGATEAPLAP-YSIVCQLGYP
KSGRA       -AGAEGPVTMVSDGCTSGLDSVGYAV-QGTREGSADVVVAGAADTPVSPIVVACFDAIKA
KSHIR       -VGAEGPVAMVSDGCTSGLDSLSHAC-SLIAEGTTDVMVAGAADTPITPIVVSCFDAIKA
KSACT       -VGAEGPVTMVSTGCTSGLDSVGNAV-RAIEEGSADVMFAGAADTPITPIVVACFDAIRA
KSCIN       -VGAEGPSTVVSTGCTSGIDAVGIAV-ELVREGSVDVMVAGAVDAPISPIP-CVLDAIKA
KSVNZ       -VGAEGPNTVVSTGCTSGLDSVGYARGELIREGSADVMIAGSSDAPISPITMACFDAIKA
KSNOG       RIGAEGPVSLVSTGCTSGLDAVGRAA-DLIAEGAADVMLAGATEAPISPITVACFDAIKA
KSTCM       -AGAEGPVTVVSTGCTSGLDAVGYGT-ELIRDGRADVVVCGATDAPISPITVACFDAIKA
KSDAU       -AGAEGPVNIVSAGCTSGIDSIGYAC-ELIREGTVDVMLAGGVDAPIAPITVACFDAIRV
KSPEU       -AGAEGPVNIVSAGCTSGIDSIGYAC-ELIREGTVDAMVAGGVDAPIAPITVACFDAIRA
KSWHI       -FGVRGPVQTVSTGCTSGLDAVGYAY-HAVAEGRVDVCLAGAADSPISPITMACFDAIKA
                .*    .    :.*:*: . .       *        .
                          ↑

KCLFDAU     RLSGSDDPTAGYLPFDRRAAGHVPGEG-GAILAVEDAERVAERG-GKVYGSIAGT-ASFD
KCLFPEU     RLSGSDNPTAGYLPFDRRAAGHVPGEG-GAILTVEDAERAAERG-AKVYGSIAGYGASFD
KCLFACT     RISTATDPDRAYLPFDERAAGYVPGEG-GAILVLEDSAAAEARGRHDAYGELAGCASTFD
KCLFHIR     RVSRATDPGRAYLPFDVAANGYVPGEG-GAILLLEDAESAKARG-ATGYGEIAGYAATFD
KCLFGRA     LVSTVADPERAYLPFDVDASGYVPGEG-GAVLIVEDADSARARG---AERIYVRSPLRRD
KCLFNOG     GLSTSDDPRRAYLPFDAAAGGHVPGEG-GALLVLESDESARARGVTRWYGRIDGYAATFD
KCLFTCM     FLSEATDPHDAYLPFDARAAGYVPGEG-GAMLVAERADSARERDAATVYGRIAGHASTFD
KCLFCIN     -MSDSDEPNRAYLPFDRDGRGYVPGGGRGVVPPLERAEAAPARG-AEVYGE-AGPLARL-
KCLFVNZ     RLSTSEEPARGYLPFDREAQGHVPGEG-GAILVMEAAEAARERG-ARIYGEIAGYGSTFD
KCLFWHIE    ELSRATEPDRAYRPFTEAACGFAPAEG-GAVLVVEEEAAARERG-ADVRATVAGHAATFT
```

Fig 2B

```
KSGRA           TTPRNDDPAHASRPFDGTRNGFVLAEG-AAMFVLEEYEAAQRRG-AHIYAEVGGYATRSQ
KSHIR           TTPRNDDPEHASRPFDNSRNGFVLAEG-AALFVLEELEHARARG-AHVYAEISGCATRLN
KSACT           TTARNDDPEHASRPFDGTRDGFVLAEG-AAMFVLEDYDSALARG-ARIHAEISGYATRCN
KSCIN           TTPRHDAPATASRPFDSTRNGFVLGEG-AAFFVLEELHSARRRG-AHIYAEIAGYATRSN
KSVNZ           TTNRYDDPAHASRPFDGTRNGFVLGEG-AAVFVLEELESARARG-AHIYAEIAGYATRSN
KSNOG           TTPRNDTPAEASRPFDRTRNGFVLGEG-AAVFVLEEFEHARRRG-ALVYAEIAGFATRCN
KSTCM           TSANNDDPAHASRPFDRNRDGFVLGEG-SAVFVLEELSAARRRG-AHAYAEVRGFATRSN
KSDAU           TSDHNDTPETLA-PFSRSRNGFVLGEG-GAIVVLEEAEAAVRRG-ARIYAEIGGYASRGN
KSPEU           TSDHNDTPETASRPFSRSRNGFVLGEG-GAIVVLEEAEAAVRRG-ARIYAEIGGYASRGN
KSWHI           TSPNNDDPAHASRPFDADRNGFVMGEG-AAVLVLEDLEHARARG-ADVYCEVSGYATFGN
                       *     **     *.. . * ...   *    .  *.

KCLFDAU         -PPPGSGRP---SALARAVETALADAGLDRSDIAVVFADGAA-VGELDVAEAEALASVFG
KCLFPEU         -PPPGSGRP---SALARAVETALADAGLDGSDIAVVFADGAA-VPELDAAEAEALASVFG
KCLFACT         -PAPGSGRP---AGLERAIRLALNDAGTGPEDVDVVFADGAG-VPELDAAEARAIGRVFG
KCLFHIR         -PAPGSERP---PALRRAIELALADAELRPEQVDVVFADAAG-VAELDAIEAAAIRELFG
KCLFGRA         -PAPGSGRP---PALGRAAELALAEAGLTPADISVVFADGAG-VPELDRAEADTLARLFG
KCLFNOG         -PPPGSGRP---PNLLRAAQAALDDAEVGPEAVDVVFADASG-TPDEDAAEADAVRRLFG
KCLFTCM         -ARPGTGRP---TGPARAIRLALEEARVAPEDVDVVYADAAG-VPALDRAEAEALAEVFG
KCLFCIN         -PAPHSGRG---STRAHAIRTALDDAGTAPGDIRRVFADGGGRYPN-DRAEAEAISEVFG
KCLFVNZ         -PRPGSGRE---PGLRKAIELALADAGAAPGDIDVVFADAAA-VPELDRVEAEALNAVFG
KCLFWHIE        GAGRWAESR---EGLARAIQGALAEAGCRPEEVDVVFADALG-VPEADRAEALALADALG
KSGRA           -AYHMTGLKKDGREMAESIRAALDEARLDRTAVDYVNAHGSG-TKQNDRHETAAFKRSLG
KSHIR           -AYHMTGLKTDGREMAEAIRVALDLARIDPTDIDYINAHGSG-TKQNDRHETAAFKRSLG
KSACT           -AYHMTGLKADGREMAETIRVALDESRTDATDIDYINAHGSG-TRQNDRHETAAYKRALG
KSCIN           -AYHMTGLR-DGAEMAEAIRLALDEARLNPEQVDYINAHGSG-TKQNDRHETAAFKKALG
KSVNZ           -AYHMTGLRPDGAEMAEAIRVALDEARMNPTEIDYINAHGSG-TKQNDRHETAAFKKSLG
KSNOG           -AFHMTGLRPDGREMAEAIGVALAQAGKAPADVDYVNAHGSG-TRQNDRHETAAFKRSLG
KSTCM           -AFHMTGLKPDGREMAEAITAALDQARRTGDDLHYINAHGSG-TRQNDRHETAAFKRSLG
KSDAU           -AYHMTGLRADGAEMAAAITAALDEARRDPSDVDYVNAHGTA-TRQNDRHETSAFKRSLG
KSPEU           -AYHMTGLRADGAEMAAAITAALDEARRDPSDVDYVNAHGTA-TKQNDRHETSAFKRSLG
KSWHI           -AYHMTGLTKEGLEMARAIDTALDMAELDGSAIDYVNAHGSG-TQQNDRHETAAVKRSLG
                 .   :           :   **  :      :  : *.. .     *  *: :    :*
```

Fig 2c

```
KCLFDAU         P--HRVPVTVPKTLTGRLYSGAGPLDVATGLLALRDEVVPATGHVH-PDPDLPLDVVTGR
KCLFPEU         P--RRVPVTVPKTLTGRLYSGAGPLDVATALLALRDEVVPATAHVD-PDPDLPLDVVTGR
KCLFACT         R--EGVPVTVPKTTTGRLYSGGGPLDVVTALMSLREGVIAPTAGVTSVPREYGIDLVLGE
KCLFHIR         P--SGVPVTAPKTMTGRLYSGGGPLDLVAALLAIRDGVIPPTVHTAEPVPEHQLDLVTGD
KCLFGRA         P--RGVPVTAPKALTGRLCAGGGPADLAAALLALRDQVIPATGRHRAVPDAYALDLVTGR
KCLFNOG         P--YGVPVTAPKTMTGRLSAGGAALDVATALLALREGVVPPTVNVSRPRPEYELDLVLA-
KCLFTCM         P--GAVPVTAPKTMTGRLYAGGAALDVATALLSIRDCVVPPTVGTGAPAPGLGIDLVLHQ
KCLFCIN         P--GRVPVTCPRTMTGRLHSGAAPLDVACALLAMRAGVIPPTVHID-PCPEYDLDLVLYQ
KCLFVNZ         T--GAVPVTAPKTMTGRLYSGAAPLDLAAAFLAMDEGVIPPTVNVE-PDAAYGLDLVVGG
KCLFWHIE        PHAARVPVTAPKTGTGRAYCAAPVLDVATAVLAMEHGLIPPTPHVL--DVCHDLDLVTGR
KSGRA           EHAYAVPVSSIKSMGGHSLGAIGSIEIAASVLAIEHNVVPPTANLHTPDPECDLDYVPLT
KSHIR           EHAYRTPVSSIKSMVGHSLGAIGSIEVAACALAIEHGVVPPTANLHEPDPECDLDYVPLT
KSACT           EHARRTPVSSIKSMVGHSLGAIGSLEIAACVLALEHGVVPPTANLRTSDPECDLDYVPLE
KSCIN           EHAYRTPVSSIKSMVGHSLGAIGSIEIAASALAMEYDVVPPTANLHTPDPECDLDYVPLT
KSVNZ           DHAYRTPVSSIKSMVGHSLGAIGSIEIAASALAMEHNVVPPTGNLHTPDPECDLDYVR-S
KSNOG           DHAYRVPVSSIKSMIGHSLGAIGSLEIAASVLAITHDVVPPTANLHEPDPECDLDYVPLR
KSTCM           QRAYDVPVSSIKSMIGHSLGAIGSLELAACALAIEHGVIPPTANYEEPDPECDLDYVPNV
KSDAU           DHAYRVPISSVKSMIGHSLGAAGSLEVAATALAVEYGAIPPTANLHDPDPELDLDYVPLT
KSPEU           EHAYRVPISSIKSMIGHSLGAVGSLEVAATALAVEYGVIPPTANLHDPDPELDLDYVPLT
KSWHI           EHAYATPMSSIKSMVGHSLGAIGSIELAACVLAMAHQVVPPTANYTTPDPECDLDYVPRE
                     .*::  ::  *:   .    ::.   :::    :..*           :* *

KCLFDAU         PRAMADARAALVVARGHGGFNSALVVRGAA-------
KCLFPEU         PRSLADARAALLVARGYGGFNSALVVRGAA-------
KCLFACT         PRSTAPRTA-LVLARGRWGFNSAAVLRRFAPTP----
KCLFHIR         PRHQQLGTA-LVLARGKWGFNSAVVVRGVTG------
KCLFGRA         PREAALSAA-LVLARGRHGFNSAVVVTLRGSDHRRPT
KCLFNOG         PRRTPLARA-LVLARGRGGFNAAMVVAGPRAETR---
KCLFTCM         PRELRVDTA-LVVARGMGGFNSALVVRRHG-------
KCLFCIN         VRPAALRTA-LGGARGHGGFNSALVVRAGQ-------
KCLFVNZ         PRTAEVNTA-LVIARGHGGFNSAMVVRSAN-------
KCLFWHIE        ARPAEPRTA-LVLARGLMGSNSALVLRRGAVPPEGR-
KSGRA           AREQRVDTV-LTVGSGFGGFQSAMVLHRPEEAA----
KSHIR           AREQRVDTV-LSVGSGFGGFQSAMVLRRLGGANS---
KSACT           ARERKLRSV-LTVGSGFGGFQSAMVLRDAETAGAAA-
KSCIN           ARDQRVDSV-LTVGSGFGGFQSAMVLTSAQ---RSTV
KSVNZ           CREQLTDSV-LTVGSGFGGFQSAMVLARPE---RKIA
KSNOG           ARACPVDTV-LTVGSGFGGFQSAMVLCGPGSRGRSAA
KSTCM           AREQRVDTV-LSVGSGFGGFQSAAVLARPKETRS---
KSDAU           AREKRVRHA-LTVGSGFGGFQSAMLLSRPER------
KSPEU           AREKRVRHA-LTVGSGFGGFQSAMLLSRLER------
KSWHI           ARERTLRHV-LSVGSGFGGFQSAVVLSGSEGGLR---
                 *      . *  . *  * ::* ::
mole:~/ks2%
```

Fig 2D

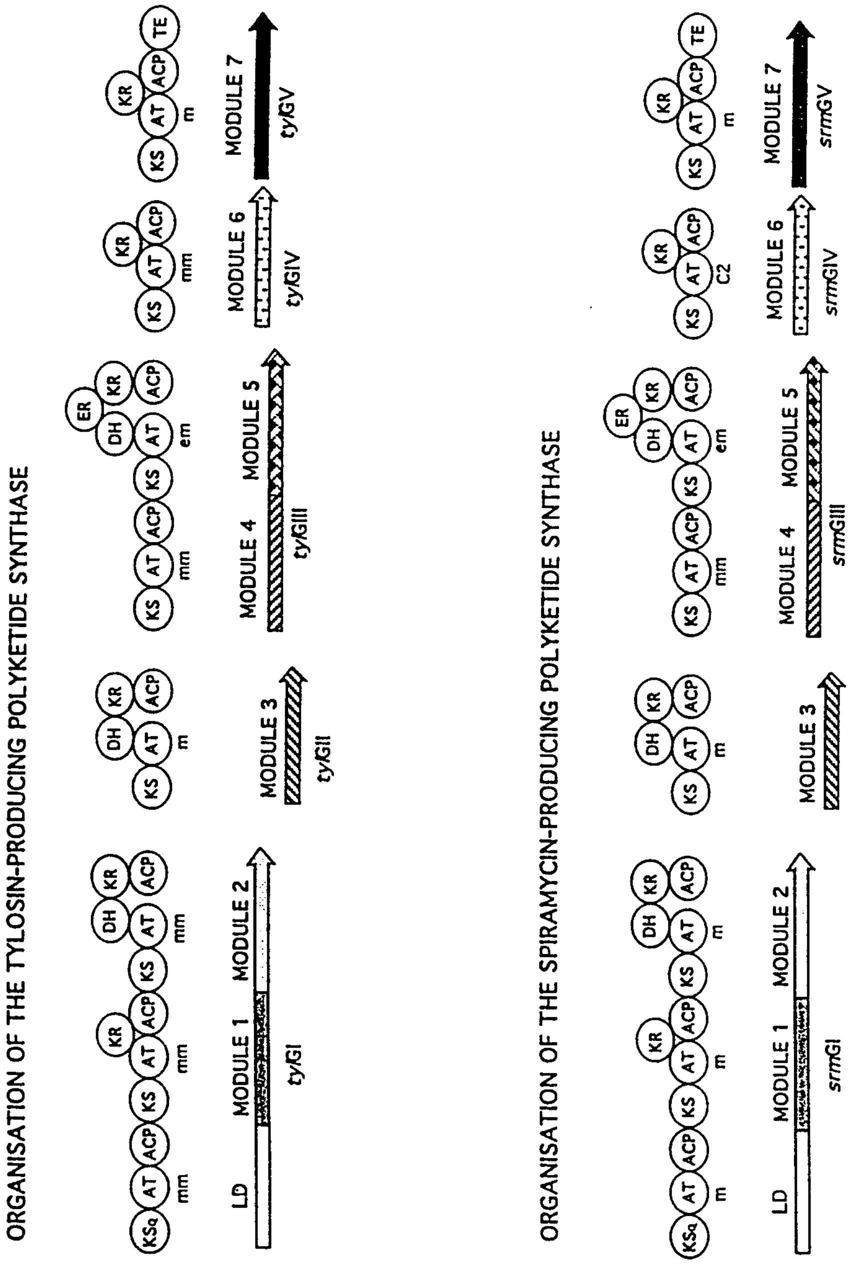
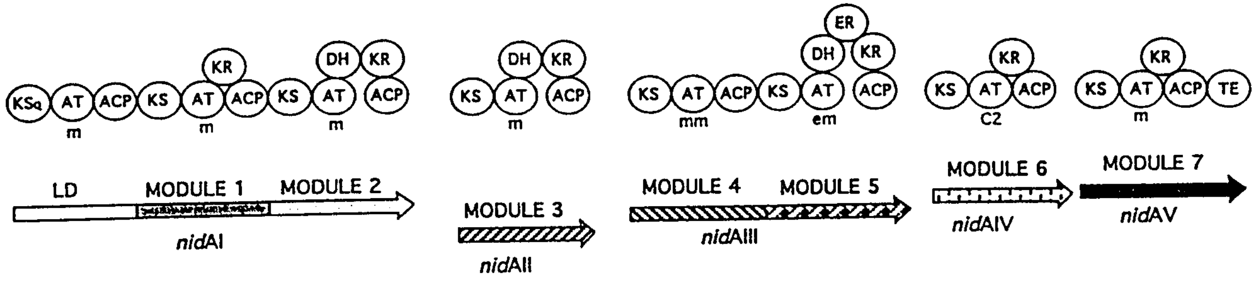

ORGANISATION OF THE TYLOSIN-PRODUCING POLYKETIDE SYNTHASE
KSq
AT
ACP
KS
AT
ACP
KR
KS
AT
ACP
DH
KR
KS
AT
ACP
DH
KR
KS
AT
ACP
KS
AT
ACP
DH
ER
KR
KS
AT
ACP
KR
KS
AT
ACP
TE
KR
mm
mm
mm
m
mm
em
mm
m
LD
MODULE 1
MODULE 2
MODULE 3
MODULE 4
MODULE 5
MODULE 6
MODULE 7
tylGI
tylGII
tylGIII
tylGIV
tylGV
ORGANISATION OF THE SPIRAMYCIN-PRODUCING POLYKETIDE SYNTHASE
KSq
AT
ACP
KS
AT
ACP
KR
KS
AT
ACP
DH
KR
KS
AT
ACP
DH
KR
KS
AT
ACP
KS
AT
ACP
DH
ER
KR
KS
AT
ACP
KR
KS
AT
ACP
TE
KR
m
m
m
m
mm
em
C2
m
LD
MODULE 1
MODULE 2
MODULE 3
MODULE 4
MODULE 5
MODULE 6
MODULE 7
srmGI
srmGII
srmGIII
srmGIV
srmGV

Fig 3A

ORGANISATION OF THE NIDDAMYCIN-PRODUCING POLYKETIDE SYNTHASE



m: malonyl transferase
mm: methylmalonyl transferase
em: ethylmalonyl transferase
C2: unknown C2 unit transferase

Fig 3B

```
               1                                                  50
niddamycin     ~~~~~~~~~~ ~~~~~~~~~~ MAGHGDATAQ KAQDAEKSED GSDAIAVIGM
platenolide    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~MS GELAISRSDD RSDAVAVVGM
monensin       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MAAS ASASPSGPSA GPDPIAVVGM
oleandomycin   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~MHVPGEE NGHSIAIVGI
tylosin        MSSALRRAVQ SNCGYGDLMT SNTAAQNTGD QEDVDGPDST HGGEIAVVGM

               51                                                100
niddam...      SCRFPGAPGT AEFWQLLSSG ADAVVTAADG RRR....... .....GTIDA
platenol.      ACRFPGAPGI AEFWKLLTDG RDAIGRDADG RRR....... .....GMIEA
monensin       ACRLPGAPDP DAFWRLLSEG RSAVSTAPPE RRRADSGLHG P...GGYLDR
oleandom       ACRLPGSATP QEFWRLLADS ADALDEPPAG RFPTGSLSSP PAPRGGFLDS
tylosin        SCRLPGAAGV EEFWELLRSG RGMPTRQDDG TWRAA..... .......LED

               101                                               150
niddam...      PADFDAAFFG MSPREAAATD PQQRLVLELG WEALEDAGIV PESLRGEAAS
platenol.      PGDFDAAFFG MSPREAAETD PQQRLMLELG WEALEDAGIV PGSLRGEAVG
monensin       IDGFDADFFH ISPREAVAMD PQQRLLLELS WEALEDAGIR PPTLARSRTG
oleandom       IDTFDADFFN ISPREAGVLD PQQRLALELG WEALEDAGIV PRHLRGTRTS
tylosin        HAGFDAGFFG MNARQAAATD PQHRLMLELG WEALEDAGIV PGDLTGTDTG

               151                                               200
niddam...      VFVGAMNDDY ATLLH.RAGA PTDTYTATGL QHSMIANRLS YFLGLRGPSL
platenol.      VFVGAMHDDY ATLLH.RAGA PVGPHTATGL QRAMLANRLS YVLGTRGPSL
monensin       VFVGAFWDDY TDVLNLRAPG AVTRHTMTGV HRSILANRIS YAYHLAGPSL
oleandom       VFMGAMWDDY AHLAHARGEA ALTRHSLTGT HRGMIANRLS YALGLQGPSL
tylosin        VFAGVASDDY A.VLTRRSAV SAGGYTATGL HRALAANRLS HFLGLRGPSL

               201                                               250
niddam...      VVDTGQSSSL VAVALAVESL RGGTSGIALA GGVNLVLAEE GS.AAMERVG
platenol.      AVDTAQSSSL VAVALAVESL RAGTSRVAVA GGVNLVLADE GT.AAMERLG
monensin       TVDTAQSSSL VAVHLACESI RSGDSDIAFA GGVNLICSPR TTELAAARFG
oleandom       TVDTGQSSSL AAVHMACESL ARGESDLALV GGVNLVLDPA GT.TGVERFG
tylosin        VVDSAQSASL VAVQLACESL RRGETSLAVA GGVNLILTEE ST.TVMERMG

               251                                               300
niddam...      ALSPDGRCHT FDARANGYVR GEGGAIVVLK PLADALADGD RVYCVVRGVA
platenol.      ALSPDGRCHT FDARANGYVR GEGGAAVVLK PLADALADGD PVYCVVRGVA
monensin       GLSAAGRCHT FDARADGFVR GEGGGLVVLK PLAAARRDGD TVYCVIRGSA
oleandom       ALSPDGRCYT FDSRANGYAR GEGGVVVVLK PTHRALADGD TVYCEILGSA
tylosin        ALSPDGRCHT FDARANGYVR GEGGGAVVLK PLDAALADGD RVYCVIKGGA

               301                                               350
niddam...      TGNDGGGPGL TVPDRAGQEA VLRAACDQAG VRPADVRFVE LHGTGTPAGD
platenol.      VGNDGGGPGL TAPDREGQEA VLRAACAQAR VDPAEVRFVE LHGTGTPVGD
monensin       VNSDGTTDGI TLPSGQAQQD VVRLACRRAR ITPDQVQYVE LHGTGTPVGD
oleandom       LNNDGATEGL TVPSARAQAD VLRQAWERAR VAPTDVQYVE LHGTGTPAGD
tylosin        VNNDGGGASL TTPDREAQEA VLRQAYRRAG VSTGAVRYVE LHGTGTRAGD
```

Fig 4A

```
            351                                                400
niddam...   PVEAEALGAV YGTGRP..AN EPLLVGSVKT NIGHLEGAAG IAGFVKAALC
platenol.   PVEAHALGAV HGSGRP..AD DPLLVGSVKT NIGHLEGAAG IAGLVKAALC
monensin    PIEAAALGAA LGQDAA..RA VPLAVGSAKT NVGHLEAAAG IVGLLKTALS
oleandom    PVEAEGLGTA LGTARP..AE APLLVGSVKT NIGHLEGAAG IAGLLKTVLS
tylosin     PVEAAALGAV LGAGADSGRS TPLAVGSVKT NVGHLEGAAG IVGLIKATLC

            401                                                450
niddam...   LHERALPASL NFETPNPAIP LERLRLKVQT AHAALQPGTG GGPLLAGVSA
platenol.   LRERTLPGSL NFATPSPAIP LDQLRLKVQT AAAELPLAPG GAPLLAGVSS
monensin    IHHRRLAPSL NFTTPNPAIP LADLGLTVQQ DLADWP..RP EQPLIAGVSS
oleandom    IKNRHLPASL NFTSPNPRID LDALRLRVHT AYGPWP..SP DRPLVAGVSS
tylosin     VRKGELVPSL NFSTPNPDIP LDDLRLRVQT ERQEW.NEED DRPRVAGVSS

            451                                                500
niddam...   FGMGGTNCHV VLEETPGG.. .......... .......... ...RQPAE.T
platenol.   FGIGGTNCHV VLEHLPSR.. .......... .......... ...PTPAV.S
monensin    FGMGGTNGHV VVA....AAP DSVAVPEPVG VPERVEVPEP VVVSEPVVVP
oleandom    FGMGGTNCHV VLSELRNAGG DGAGKGPYTG TEDRLGATEA EKRPDPATGN
tylosin     FGMGGTNVHL VIAEAPAAAG SSGAGGSGAG SGAGISAVSG VV........

            501                                                550
niddam...   GQADACLFSA SPMLLLSARS EQALRAQAAR LREHL..EDS GADPLDIAYS
platenol.   VAAS...LPD VPPLLLSARS EGALRAQAVR LGETV..ERV GADPRDVAYS
monensin    TPWP...... .....VSAHS ASALRAQAGR LRTHLAAHRP TPDAARVGHA
oleandom    GPDPAQDTHR YPALILSARS DAALRAQAER LRHHL.EHSP GQRLRDTAYS
tylosin     .......... ..PVVVSGRS RVVVREAAGR LAE..VVEAG GVGLADVAVT

            551                                                600
niddam...   LATTRTRFEH RAAVPCGDPD RLSSALAALA AGQTPRGVRI GS..TDADGR
platenol.   LASTRTLFEH RAVVPCGGRG ELVAALGGFA AGRVSGGVRS GR..A.VPGG
monensin    LATTRAPLAH RAVLLGGDTA ELLGSLDALA EGAETASIVR GEAYT..EGR
oleandom    LATRRQVFER HAVVTGHDRE DLLNGLRDLE NGLPAPQVLL GRTPTPEPGG
tylosin     MAD.RSRFGY RAVVLARGEA ELAGRLRALA GGDPDAGVVT G...AVLDGG

            601                                                650
niddam...   LALLFTGQGA QHPGMGQELY TTDPHFAAAL DEVCEELQRC GTQNLREVMF
platenol.   VGVLFTGQGA QWVGMGRGLY AGGGVFAEVL DEVLSMVGEV DGRSLRDVMF
monensin    TAFLFSGQGA QRLGMGRELY AVFPVFADAL DEAFAALDVH LDRPLREIVL
oleandom    LAFLFSGQGS QQPGMGKRLH QVFPGFRDAL DEVCAELDTH LGRLL.....
tylosin     VVVGAAPGGA GAAGGAGAAG GAGGGGVVLV FPGQGTQWVG MGAGLLGSSE

            651                                                700
niddam...   TPDQPD.... .......... .......... LLDRTEYTQP ALFALQTALY
platenol.   GDVDVDAGAG ADAGAGAGAG VGSGSGSVGG LLGRTEFAQP ALFALEVALF
monensin    GETDSGGNVS GENVIGEGA. ......DHQA LLDQTAYTQP ALFAIETSLY
oleandom    .GPEAGPPLR DVMFAERGT. ......AHSA LLSETHYTQA ALFALETALF
tylosin     VFAASMRECA RALSVHVGWD LLEVVSGGAG .LERVDVVQP VTWAVMVSLA

            701                                         ↓      750
niddam...   RTLTARGTQA HLVLGHSVGE ITAAHIAGVL DLPDAARLIT ARAHVMGQLP
platenol.   RALEARGVEV SVVLGHSVGE VAAATVAGVL SLGDAVRLVV ARGGLMGGLP
monensin    RLAASFGLKP DYVLGHSVGE IAAAHVAGVL SLPDASALVA TRGRLMQAVR
oleandom    RLLVQWGLKP DHLAGHSVGE IAAAHAAGIL DLSDAAELVA TRGALMRSLP
tylosin     RYWQAMGVDV AAVVGHSQGE IAAATVAGAL SLEDAAAVVA LRAGLIGRYL
                                                        ↑      Fig4B
```

```
             751                                                  800
niddam...    HG.GAMLSVQ AAEHDLDQLA HTHG..VEIA AVNGPTHCVL SGPRTALEET
platenol.    VG.GGMWSVG ASESVVRGVV EGLGEWVSVA AVNGPRSVVL SGDVGVLESV
monensin     AP.GAMAAWQ ATADEAAEQL AGHERHVTVA AVNGPDSVVV SGDRATVDEL
oleandom     GG.GVMLSVQ APESEVAPLL LGREAHVGLA AVNGPDAVVV SGERGHVAAI
tylosin      AGRGAMAAVP LPAGEVEAGL .AKWPGVEVA AVNGPASTVV SGDRRAVAGY

             801                                                  850
niddam...    AQHLREQNVR HTWLKVSHAF HSALMDPMLG AFRDTLNTLN Y..QPPTIPL
platenol.    VASLMGDGVE YRRLDVSHGF HSVLMEPVLG EFRGVVESLE FGRVRPGVVV
monensin     TAAWRGRGRK AHHLKVSHAF HSPHMDPILD ELRAVAAGLT FHE..PVIPV
oleandom     EQILRDRGRK SRYLRVSHAF HSPLMEPVLE EFAEAVAGLT FRA..PTTPL
tylosin      VAVCQAEGVQ ARLIPVDYAS HSRHVEDLKG ELERVLSGI. .RPRSPRVPV

             851                                                  900
niddam...    ISNLTGQIA. .....DPNHL CTPDYWIDHA RHTVRFADAV QTAHHQGTTT
platenol.    VSGVSGGVV. .....GSGEL GDPGYWVRHA REAVRFADGV GVVRGLGVGT
monensin     VSNVTGELVT ATATGSGAGQ ADPEYWARHA REPVRFLSGV RGLCERGVTT
oleandom     VSNLTG.... ..APVDDRTM ATPAYWVRHV REAVRFGDGI RALGKLGTGS
tylosin      CSTVAGEQPG EPVF...... .DAGYWFRNL RNRVEFSAVV GGLLEEGHRR

             901                                                  950
niddam...    YLEIGPHPTL TTLLHHTL.. .DNP...... .........T TIPTLHRERP
platenol.    LVEVGPHGVL TGMAGECLGA GDDV...... .........V VVPAMRRGRA
monensin     FVELGPDAPL SAMARDCFPA P......... .ADRSRPRPA AIATCRRGRD
oleandom     FLEVGPDGVL TAMARACVTA APEPGHRGEQ GADADAHTAL LLPALRRGRD
tylosin      FIEVSAHPVL V......... .....HAIEQ TAEAADRSVH ATGTLRRQDD

             951
niddam...    EPETLTQAIA AVGVRTDGID WAVLCGASRP RRVELPTYAF
platenol.    EREVFEAALA TVFTRDAGLD ATALHTGSTG RRIDLPTTPF
monensin     EVATFLRSLA QAYVRGADVD FTRAYGATAT RRFPLPTYPF
oleandom     EARSLTEAVA RLHLHGVPMD WTSVLGGDVS .RVPLPTYAF
tylosin      SPHRLLTSTA EAWAHGATLT WDPAL..PPG HLTTLPTYPF
```

niddam: niddamycin; platenol: platenolide I (spiramycin); oleandom: oleandomycin.

Fig 4c

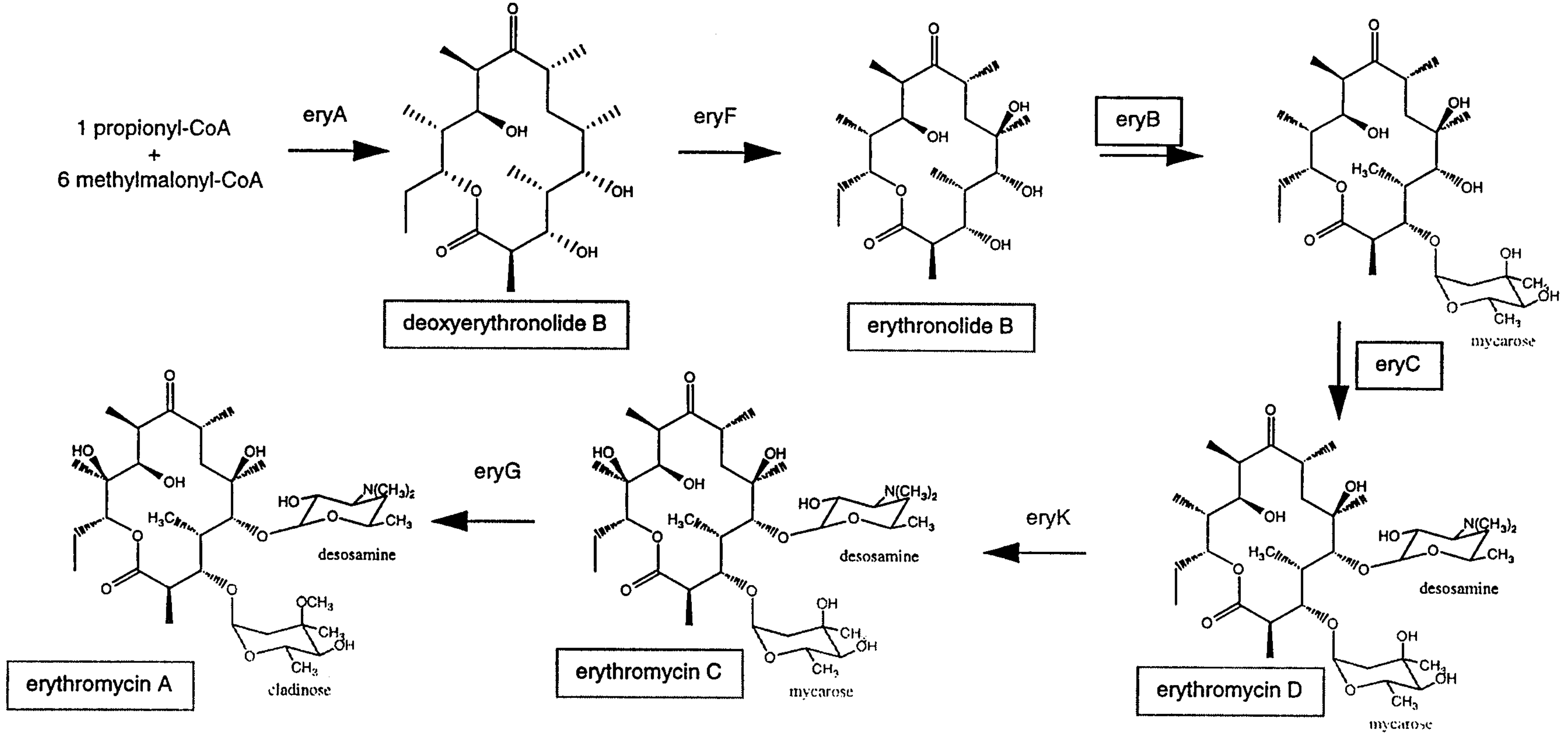

1 propionyl-CoA
+
6 methylmalonyl-CoA
eryA
deoxyerythronolide B
eryF
erythronolide B
eryB
mycarose
eryC
erythromycin D
desosamine
mycarose
eryK
erythromycin C
desosamine
mycarose
eryG
erythromycin A
desosamine
cladinose
OH
HO
O
H3C
CH3
N(CH3)2
OCH3

Fig. 5

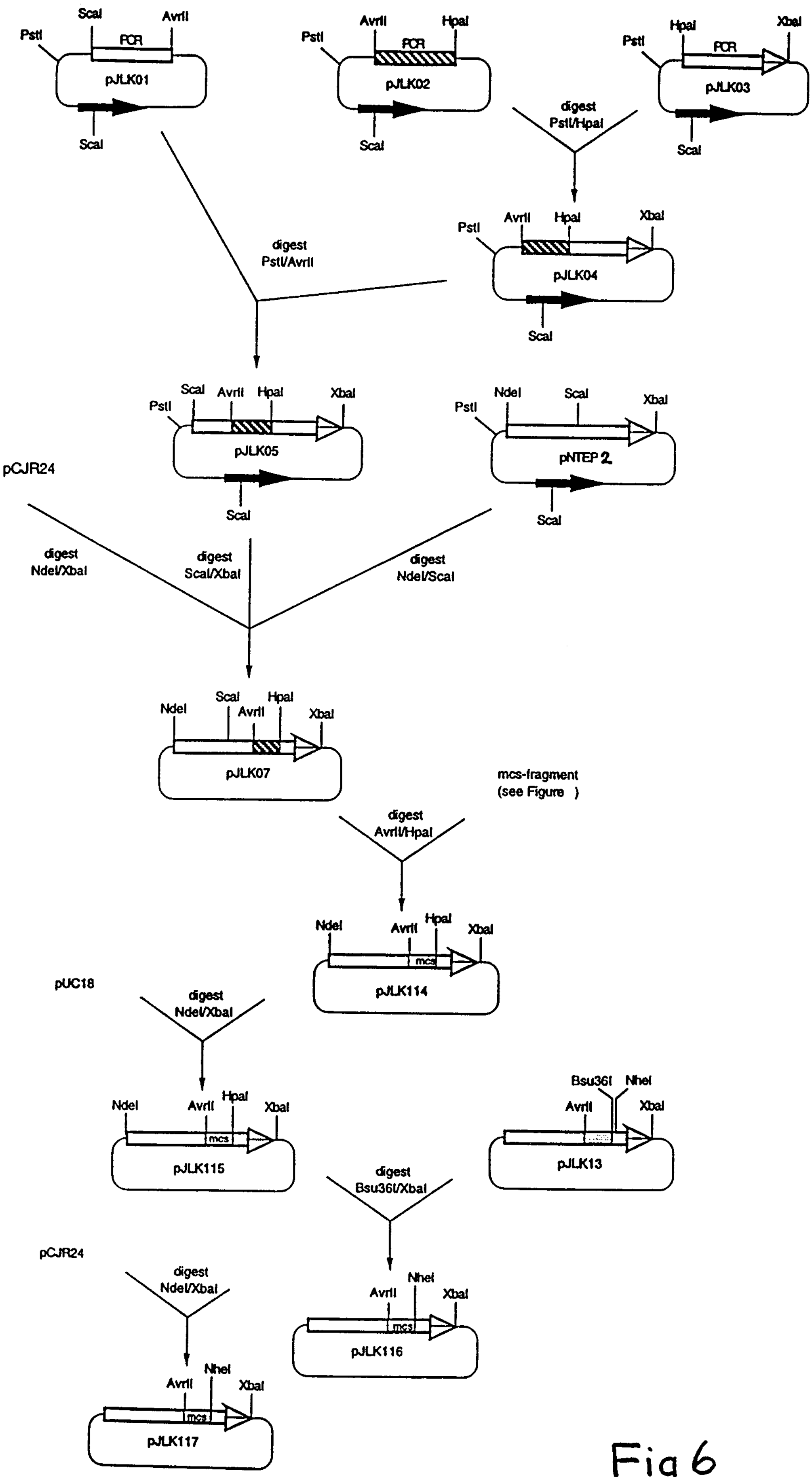

ScaI
AvrII
PstI
PCR
pJLK01
ScaI
AvrII
HpaI
PstI
PCR
pJLK02
ScaI
HpaI
XbaI
PstI
PCR
pJLK03
ScaI
digest
PstI/HpaI
PstI
AvrII
HpaI
XbaI
pJLK04
ScaI
digest
PstI/AvrII
ScaI
AvrII
HpaI
XbaI
PstI
pJLK05
ScaI
NdeI
ScaI
XbaI
PstI
pNTEP 2
ScaI
pCJR24
digest
NdeI/XbaI
digest
ScaI/XbaI
digest
NdeI/ScaI
NdeI
ScaI
AvrII
HpaI
XbaI
pJLK07
mcs-fragment
(see Figure)
digest
AvrII/HpaI
NdeI
AvrII
HpaI
XbaI
mcs
pJLK114
pUC18
digest
NdeI/XbaI
NdeI
AvrII
HpaI
XbaI
mcs
pJLK115
Bsu36I
NheI
AvrII
XbaI
pJLK13
digest
Bsu36I/XbaI
pCJR24
digest
NdeI/XbaI
NheI
AvrII
XbaI
mcs
pJLK116
NheI
AvrII
XbaI
mcs
pJLK117

Fig 6

Figure 7

forward (Plf):

5'-CTA GGC CGG GCC GGA CTG GTA GAT CTG CCT ACG TAT CCT TTC CAG GGC AAG CGG TTC TGG CTG CAG CCG GAC CGC ACT AGT CCT CGT GAC GAG GGA GAT GCA TCG AGC CTG AGG GAC CGG TT-3'

backward (Plb):

5'-AAC CGG TCC CTC AGG CTC GAT GCA TCT CCC TCG TCA CGA GGA CTA GTG CGG TCC GGC TGC AGC CAG AAC CGC TTG CCC TGG AAA GGA TAC GTA GGC AGA TCT ACC AGT CCG GCC CGG C-3'

oligos annealed:

```
CTAGGCCGGGCCGGACTGGTAGATCTGCCTACGTATCCTTTCCAGGGCAAGCGGTTCTGGCTGCAGCCGGACCGCACTAGTCCTCGTGACGAGGGAGATGCATCGAGCCTGAGGGACCGGTT
    CGGCCCGGCCTGACCATCTAGACGGATGCATAGGAAAGGTCCCGTTCGCCAAGACCGACGTCGGCCTGGCGTGATCAGGAGCACTGCTCCCTCTACGTAGCTCGGACTCCCTGGCCAA
-----               ------   ------                         ------          ------               ------    -------     ---
AvrII               BglII    SnaBI                          PstI            SpeI                 NsiI      Bsu36I      HpaI
```