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CA 92130-2332 (US).
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: BIOSYNTHESIS OF POLYKETIDE SYNTHASE SUBSTRATES

(57) Abstract: The use of enzymes which catalyze the production of starter and extender units for polyketides is described. These enzymes may be used to enhance the yield of polyketides that are natively produced or polyketides that are rationally designed. By using these techniques, the synthesis of a complete polyketide has been achieved in *E. coli*. This achievement permits a host organism with desirable characteristics to be used in the production of such polyketides and to assess the results of gene shuffling.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P11/00 C12P19/62 C12N1/21 //(C12N1/21,
C12R1:19),(C12P11/00,C12R1:19),(C12P19/62,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STASSI D L ET AL.: "Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 95, no. 13, 23 June 1998 (1998-06-23), pages 7305-7309, XP002131683 ISSN: 0027-8424 abstract page 7308, left-hand column, line 4 -right-hand column, line 17 --- -/--	1,4,9, 10,13, 24,30,33

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANG L ET AL.: "Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in <i>Streptomyces ambofaciens</i> and <i>Streptomyces fradiae</i> " JOURNAL OF BACTERIOLOGY, vol. 176, no. 19, October 1994 (1994-10), pages 6107-6119, XP001009878 abstract page 6107, left-hand column, line 1 -right-hand column, line 24 page 6116, left-hand column, line 18-65 ---	1,2,4,9, 10,13, 24,30
X	WO 98 27203 A (KOSAN BIOSCIENCES) 25 June 1998 (1998-06-25)	42
A	page 2, line 29 -page 4, line 28 examples 1-12 claims 1-39 ---	1-10,12, 13, 24-30,43
X	KAO C M ET AL.: "Engineered biosynthesis of a complete macrolactone in a heterologous host" SCIENCE, vol. 265, no. 5171, 22 July 1994 (1994-07-22), pages 509-512, XP002073974 ISSN: 0036-8075	33-35
A	abstract page 510, left-hand column, line 65 -right-hand column, line 8 ---	1,2,4,6, 9,14,15, 24-26
A	HSIEH Y-J ET AL.: "Inhibition of erythromycin synthesis by disruption of malonyl-coenzyme A decarboxylase gene <i>eryM</i> in <i>Saccharopolyspora erythraea</i> " JOURNAL OF BACTERIOLOGY, vol. 176, no. 3, February 1994 (1994-02), pages 714-724, XP001009880 ISSN: 0021-9193 page 722, left-hand column, line 43-51 ---	1,10,13, 24,30
A	KHOSLA C ET AL.: "Tolerance and specificity of polyketide synthases" ANNUAL REVIEW OF BIOCHEMISTRY, vol. 68, 1999, pages 219-253, XP000884453 page 222, line 35-37 page 222, line 5-8 ---	1,10,13, 24,30
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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	--- UMEYAMA T ET AL.: "Expression of the Streptomyces coelicolor A3(2) ptpA gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in S. lividans" FEMS MICROBIOLOGY LETTERS, vol. 144, no. 2-3, 1 November 1996 (1996-11-01), pages 177-184, XP001009805 abstract	1,2,4, 10,13, 24,25,30
A	--- DONADIO S ET AL: "Erythromycin production in Saccharopolyspora erythraea does not require a functional propionyl-CoA carboxylase." MOLECULAR MICROBIOLOGY, vol. 19, no. 5, 1996, pages 977-984, XP001009721 ISSN: 0950-382X abstract page 982, right-hand column, line 27-47	12,14, 15,34,35
A	--- ZIERMANN R ET AL.: "Recombinant polyketide synthesis in Streptomyces: engineering of improved host strains" BIOTECHNIQUES, vol. 26, no. 1, January 1999 (1999-01), pages 106-110, XP002170304 abstract	1,2,4,6, 7,9, 24-26
A	--- WO 95 08548 A (JOHN INNES CENTRE ;UNIV LELAND STANFORD JUNIOR (US)) 30 March 1995 (1995-03-30) page 4, line 26 -page 7, line 12 page 21, line 32 -page 31, line 15 examples 1-3 claims 1-30	1,2,4,7, 24-26
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/28573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 19518 A (DIVERSA INC ;SHORT JAY M (US)) 22 April 1999 (1999-04-22) page 4, line 12 -page 6, line 19 page 11, line 22 -page 12, line 25 page 24, line 1-6 page 25, line 7-20 ---	1-5,7,8, 10,11, 13, 24-28, 30,33, 40,42
A	WO 99 03986 A (KOSAN BIOSCIENCES INC) 28 January 1999 (1999-01-28) page 2, line 7 -page 3, line 15 page 4, line 7-28 examples 1-3 ---	24, 30-33, 40,41
P,X	WO 00 20601 A (KOSAN BIOSCIENCES INC ;CHU DANIEL (US); KHOSLA CHAITAN (US); SANTI) 13 April 2000 (2000-04-13) page 6, line 29 -page 7, line 11 page 67, line 30 -page 70, line 2 claim 17 ---	1,2,10, 13, 24-26, 30,33
P,X	ZHANG W ET AL.: "Molecular analysis and heterologous expression of the gene encoding methylmalonyl-coenzyme A mutase from rifamycin SV-producing strain Amycolatopsis mediterranei U32" APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 82, no. 3, December 1999 (1999-12), pages 209-225, XP001009874 abstract page 222, line 12 -page 223, line 2; figure 6; table 1 ---	1,2,4, 10,13, 24,25,30
P,A	RODRIGUEZ E ET AL.: "Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of Streptomyces coelicolor A3(2)." MICROBIOLOGY (READING), vol. 145, no. 11, November 1999 (1999-11), pages 3109-3119, XP001009722 ISSN: 1350-0872 cited in the application abstract ---	12,14, 15,34,35
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Internat. Application No.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	PFEIFER B A ET AL.: "Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli" SCIENCE, vol. 291, no. 5509, 2 March 2001 (2001-03-02), pages 1790-1792, XP002170306 the whole document	1-10, 13-15, 24-30, 33-35, 42,43
T	--- PFEIFER B A ET AL.: "Biosynthesis of polyketides in heterologous hosts" MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, vol. 65, no. 1, March 2001 (2001-03), pages 106-118, XP000997626 the whole document page 108, left-hand column, line 13 -page 111, left-hand column, line 8 page 113, left-hand column, line 3 -page 116, left-hand column, line 8 -----	

INTERNATIONAL SEARCH REPORT

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13, 24-33, 40-43 (all partially); 14, 15, 34, 35 (all compl.)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-13,24-33,40-43 (all partially); 14,15,34, 35 (all completely)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a functional propionyl-CoA carboxylase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells which contain a functional propionyl-CoA carboxylase and possibly further a functional phosphopantetheinyl transferase.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for propionyl-CoA carboxylase, e.g., encoded by the *pccB* and *accA2* genes from *S. coelicolor*.

Recombinant microbial cells (e.g., *Streptomyces* (*coelicolor* CH999) or *Escherichia* (*coli*)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said propionyl-CoA carboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, said functional propionyl-CoA carboxylase, e.g., encoded by the *pccB* and *accA2* genes from *S. coelicolor*, and methods for producing a polyketide using said reaction mixture.

2. Claims: 1-12,42,43 (all partially)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises deleting a pathway responsible for propionate catabolism, e.g., the *prpA-D* operon of *E. coli*.

Said host cells which do not produce polyketide in the

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells which contain a functional phosphopantetheinyl transferase and which have been further modified to delete said *prpA-D* operon.

3. Claims: 1-11,13,24-33,40-43 (all partially); 16,17,36, 37 (all completely)

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a malonyl-CoA decarboxylase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for malonyl-CoA decarboxylase, e.g., encoded by the *matA* gene of *R. trifolii*. Recombinant microbial cells (e.g., *Streptomyces* (coelicolor CH999) or *Escherichia* (coli)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said malonyl-CoA decarboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, said malonyl-CoA decarboxylase, e.g., encoded by the *matA* gene from *R. trifolii*, and methods for producing a polyketide using said reaction mixture.

4. Claims: 1-11,13,24-33,40-43 (all partially); 18-23,38, 39 (all completely)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Prokaryotic host cells (e.g., of the genus *Escherichia* (e.g., *E. coli*), *Streptomyces*, *Bacillus*, *Pseudomonas* or *Flavobacterium*) which are genetically modified for enhanced synthesis of at least one (complete) polyketide (e.g., 6-dEB), wherein said modification comprises incorporation of an expression system for producing a malonyl-CoA synthetase that catalyzes the production of starter and/or extender units.

Said host cells which do not produce polyketide in the absence of genetic modification, and wherein said modification comprises incorporation of an expression system for a polyketide synthase (PKS) and incorporation of an expression system for a phosphopantetheinyl transferase. A method to produce a polyketide comprising culturing said cells.

A method to assess the results of a procedure effecting modification of PKS genes using said *E. coli* cells.

A method to enhance the production of a polyketide in said microbial host cells which method comprises the provision of said host cell with an expression system for malonyl-CoA synthetase, e.g., encoded by the *matB* gene of *R. trifolii*, and which possibly further includes providing the substrate (of formula $R_2C(COOH)_2$, with R according to claims 22 and 23) for malonyl-CoA synthetase and an expression system for a second enzyme that effects entry of said substrate into the cell, e.g., the malonate transporter encoded by the *matC* gene of *R. trifolii*.

Recombinant microbial cells (e.g., *Streptomyces* (coelicolor CH999) or *Escherichia* (coli)) that produce a (complete) polyketide (e.g., 6-dEB) which have been modified to contain an expression system for (a) nucleotide sequence(s) encoding said propionyl-CoA carboxylase, and methods for producing a polyketide using said cells.

A reaction mixture for the production of a polyketide comprising in addition to enzymes catalyzing the production of said polyketide, a first enzyme which catalyzes the conversion of a substrate to an extender or starter unit for said polyketide (malonyl-CoA synthetase), and possibly further a substrate therefor (of formula $R_2C(COOH)_2$, with R according to claim 39), and methods for producing a polyketide using said reaction mixture.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No

PCT/US 00/28573

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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