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(54) **Titre : GENE DE BIOSYNTHESE DE PYRIPYROPENE A**
(54) **Title: PYRIPYROPENE A BIOSYNTHETIC GENE**

(57) **Abrégé/Abstract:**

An isolated novel polynucleotide comprising a nucleotide sequence encoding at least one polypeptide involved in biosynthesis of pyripyropene A, a recombinant vector comprising the polynucleotide and a transformant comprising the polynucleotide are disclosed. By the present invention, a pyripyropene A biosynthetic gene useful for production of a novel pyripyropene analog, improvement of productivity of a pyripyropene A-producing bacterium, production of an insecticidal agent for microorganisms, creation of a plant resistant to insect pests or the like are provided.

ABSTRACT

An isolated novel polynucleotide comprising a nucleotide sequence encoding at least one polypeptide involved in biosynthesis of pyripyropene A, a recombinant vector comprising the polynucleotide and a transformant comprising the polynucleotide are disclosed. By the present invention, a pyripyropene A biosynthetic gene useful for production of a novel pyripyropene analog, improvement of productivity of a pyripyropene A-producing bacterium, production of an insecticidal agent for microorganisms, creation of a plant resistant to insect pests or the like are provided.

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DESCRIPTION

[TITLE OF INVENTION] PYRIPYROPENE A BIOSYNTHETIC GENE

[CROSS-REFERENCE TO RELATED APPLICATION]

[0001] This patent application claims priority to Japanese Patent Application No. 190862/2008 that was filed on July 24, 2008, Japanese Patent Application No. 270294/2008 that was filed on October 20, 2008 and Japanese Patent Application No. 20591/2009 that was filed on January 30, 2009.

[BACKGROUND OF THE INVENTION]

[0002] Field of Invention

The present invention relates to a pyripyropene A biosynthetic gene.

[0003] Background Art

As disclosed in Japanese Patent Laid-Open Publication No. 360895/1992 (Patent Document 1) and Journal of Antibiotics (1993), 46(7), 1168-9 (Non-patent Document 1), pyripyropene A has an inhibitory activity against ACAT (acyl CoA cholesterol acyltransferase). Application thereof to treatment of diseases caused by cholesterol accumulation or the like is expected.

[0004] Additionally, in Journal of Synthetic Organic Chemistry, Japan (1998), Vol. 56, No. 6, 478-488 (Non-patent Document 2), WO94/09147 (Patent Document 2), Japanese Patent Laid-Open Publication No. 184158/1994 (Patent Document 3), Japanese Patent Laid-Open Publication No. 239385/1996 (Patent Document 4), Japanese Patent Laid-Open Publication No. 259569/1996 (Patent Document 5), Japanese Patent Laid-Open Publication No. 269062/1996 (Patent Document 6), Japanese Patent Laid-Open Publication No. 269063/1996 (Patent Document 7), Japanese Patent Laid-Open Publication No. 269064/1996 (Patent Document 8), Japanese Patent Laid-Open Publication No. 269065/1996 (Patent Document 9), Japanese Patent Laid-Open Publication No. 269066/1996 (Patent Document 10), Japanese Patent Laid-Open

Publication No. 291164/1996 (Patent Document 11) and Journal of Antibiotics (1997), 50(3), 229-36 (Non-patent Document 3), pyripyropene analogs and derivatives, as well as ACAT inhibitory activities thereof have been disclosed.

5 [0005] Further, Applied and Environmental Microbiology (1995), 61(12), 4429-35 (Non-patent Document 4) has disclosed that pyripyropene A has an insecticidal activity against *Helicoverpa armigera* larva. Still further, WO2004/060065 (Patent Document 12) has disclosed that pyripyropene A has
10 insecticidal activities against Diamondback moth larva and *Tenebrio molitor*.

[0006] In addition, WO2006/129714 (Patent Document 13) and WO2008/066153 (Patent Document 14) have disclosed that pyripyropene analogs have insecticidal activities against
15 aphids.

[0007] Furthermore, as a pyripyropene A-producing bacterium, *Aspergillus fumigatus* FO-1289 strain is disclosed in Japanese Patent Laid-Open Publication No. 360895/1992 (Patent Document 1); *Eupenicillium reticulosporum* NRRL-3446 strain is
20 in Applied and Environmental Microbiology (1995), 61(12), 4429-35 (Non-patent Document 4); and *Penicillium griseofulvum* F1959 strain is in WO2004/060065 (Patent Document 12); and *Penicillium coprobium* PF1169 strain is in Journal of Technical Disclosure 500997/2008 (Patent Document
25 15).

[0008] Also, as a biosynthetic route of pyripyropene A, Journal of Organic Chemistry (1996), 61, 882-886 (Non-patent Document 5) and Chemical Review (2005), 105, 4559-4580 (Non-patent Document 6) have disclosed a putative biosynthetic
30 route in *Aspergillus fumigatus* FO-1289 strain. These documents have disclosed that, in *Aspergillus fumigatus* FO-1289 strain, partial structures individually synthesized by polyketide synthase and prenyltransferase are linked to synthesize pyripyropene A by a cyclase.

35 [PRIOR ART REFERENCES]
[PATENT DOCUMENTS]

[0009]

[Patent Document 1] Japanese Patent Laid-Open
Publication No. 360895/1992

[Patent Document 2] WO94/09147

5 [Patent Document 3] Japanese Patent Laid-Open
Publication No. 184158/1994

[Patent Document 4] Japanese Patent Laid-Open
Publication No. 239385/1996

10 [Patent Document 5] Japanese Patent Laid-Open
Publication No. 259569/1996

[Patent Document 6] Japanese Patent Laid-Open
Publication No. 269062/1996

[Patent Document 7] Japanese Patent Laid-Open
Publication No. 269063/1996

15 [Patent Document 8] Japanese Patent Laid-Open
Publication No. 269064/1996

[Patent Document 9] Japanese Patent Laid-Open
Publication No. 269065/1996

20 [Patent Document 10] Japanese Patent Laid-Open
Publication No. 269066/1996

[Patent Document 11] Japanese Patent Laid-Open
Publication No. 291164/1996

[Patent Document 12] WO2004/060065

[Patent Document 13] WO2006/129714

25 [Patent Document 14] WO2008/066153

[Patent Document 15] Journal of Technical Disclosure
500997/2008

[NON-PATENT DOCUMENTS]

[0010]

30 [Non-patent Document 1] Journal of Antibiotics (1993),
46(7), 1168-9

[Non-patent Document 2] Journal of Synthetic Organic
Chemistry, Japan (1998), Vol. 56, No. 6, 478-488

35 [Non-patent Document 3] Journal of Antibiotics (1997),
50(3), 229-36

[Non-patent Document 4] Applied and Environmental

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Microbiology (1995), 61(12), 4429-35

[Non-patent Document 5] Journal of Organic Chemistry (1996), 61, 882-886

[Non-patent Document 6] Chemical Review (2005), 105,
5 4559-4580

[SUMMARY OF THE INVENTION]

[0011] The present inventors have now found out a nucleotide sequence encoding at least one polypeptide involved in biosynthesis of pyripyropene A. The present invention has
10 been made based on such finding..

[0012] Accordingly, an object of the present invention is to provide an isolated novel polynucleotide having a nucleotide sequence encoding at least one polypeptide involved in biosynthesis of pyripyropene A, a recombinant vector
15 comprising the polynucleotide, and a transformant comprising the polynucleotide.

[0013] Further, according to one embodiment of the present invention, an isolated polynucleotide which is

(a) a polynucleotide having a nucleotide sequence of
20 SEQ ID NO:266,

(b) a polynucleotide having a nucleotide sequence which is capable of hybridizing with the nucleotide sequence of SEQ ID NO:266 under stringent conditions, or

(c) a polynucleotide having a polynucleotide sequence
25 encoding at least one amino acid sequence selected from SEQ ID NOs:267 to 274 or a substantially equivalent amino acid sequence thereto; is provided.

[0014] Also, according to another embodiment of the present invention, an isolated polynucleotide which has at least
30 one nucleotide sequence selected from the nucleotide sequence in any of (1) or (2) below:

(1) a nucleotide sequence in any of (a) to (h) below:

(a) a nucleotide sequence from 3342 to 5158 of a nucleotide sequence shown in SEQ ID NO:266,

35 (b) a nucleotide sequence from 5382 to 12777 of a nucleotide sequence shown in SEQ ID NO:266,

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(c) a nucleotide sequence from 13266 to 15144 of a nucleotide sequence shown in SEQ ID NO:266,

(d) a nucleotide sequence from 16220 to 18018 of a nucleotide sequence shown in SEQ ID NO:266,

5 (e) a nucleotide sequence from 18506 to 19296 of a nucleotide sequence shown in SEQ ID NO:266,

(f) a nucleotide sequence from 19779 to 21389 of a nucleotide sequence shown in SEQ ID NO:266,

(g) a nucleotide sequence from 21793 to 22877 of a nucleotide sequence shown in SEQ ID NO:266,

(h) a nucleotide sequence from 23205 to 24773 of a nucleotide sequence shown in SEQ ID NO:266;

(2) a nucleotide sequence which is capable of hybridizing with a nucleotide sequence in (1) under stringent conditions; is provided.

[0015] Further, according to another embodiment of the present invention, a polynucleotide encoding at least one polypeptide involved in biosynthesis of pyripyropene A is provided.

20 [0016] In addition, according to another embodiment of the present invention, a polynucleotide encoding a polypeptide having any one or more activities of polyketide synthase activity, prenyltransferase activity, hydroxylase activity, acetyltransferase activity or adenylate synthetase activity is provided.

[0017] Still further, according to another embodiment of the present invention, a polynucleotide which is derived from *Penicillium coprobium* PF1169 strain is provided.

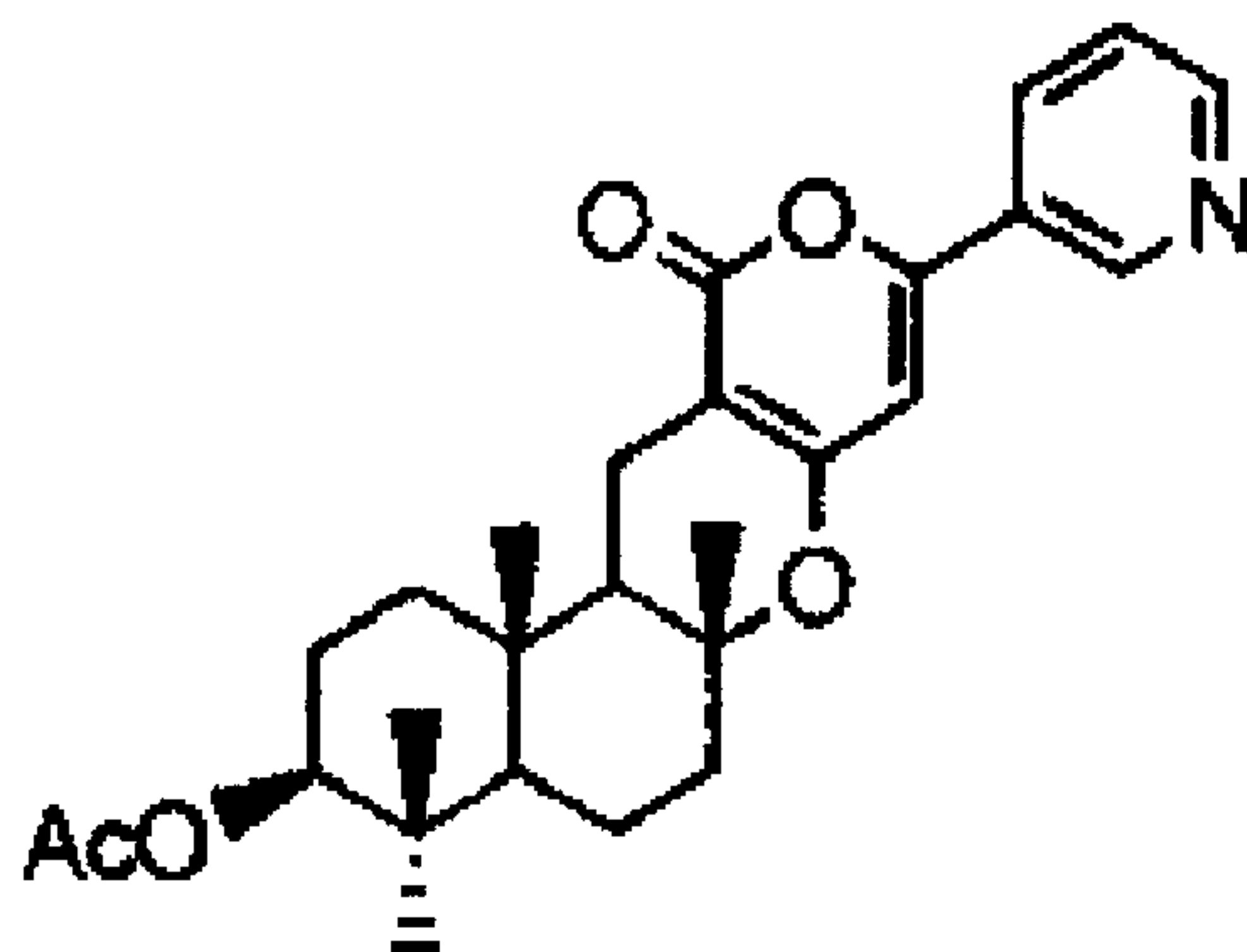
[0018] Additionally, according to another embodiment of the present invention, a recombinant vector comprising the above-mentioned polynucleotide is provided.

[0019] Still further, according to another embodiment of the present invention, a transformant comprising the above-mentioned polynucleotide is provided.

35 [0020] In addition, according to one embodiment of the present invention, a method for producing a pyripyropene A

precursor, characterized by culturing a transformant in which a polynucleotide having nucleotide sequence of the above-mentioned (c) or (d) is incorporated simultaneously or separately, and isolating the pyripyropene A precursor from
 5 pyripyropene E represented by the following formula is provided:

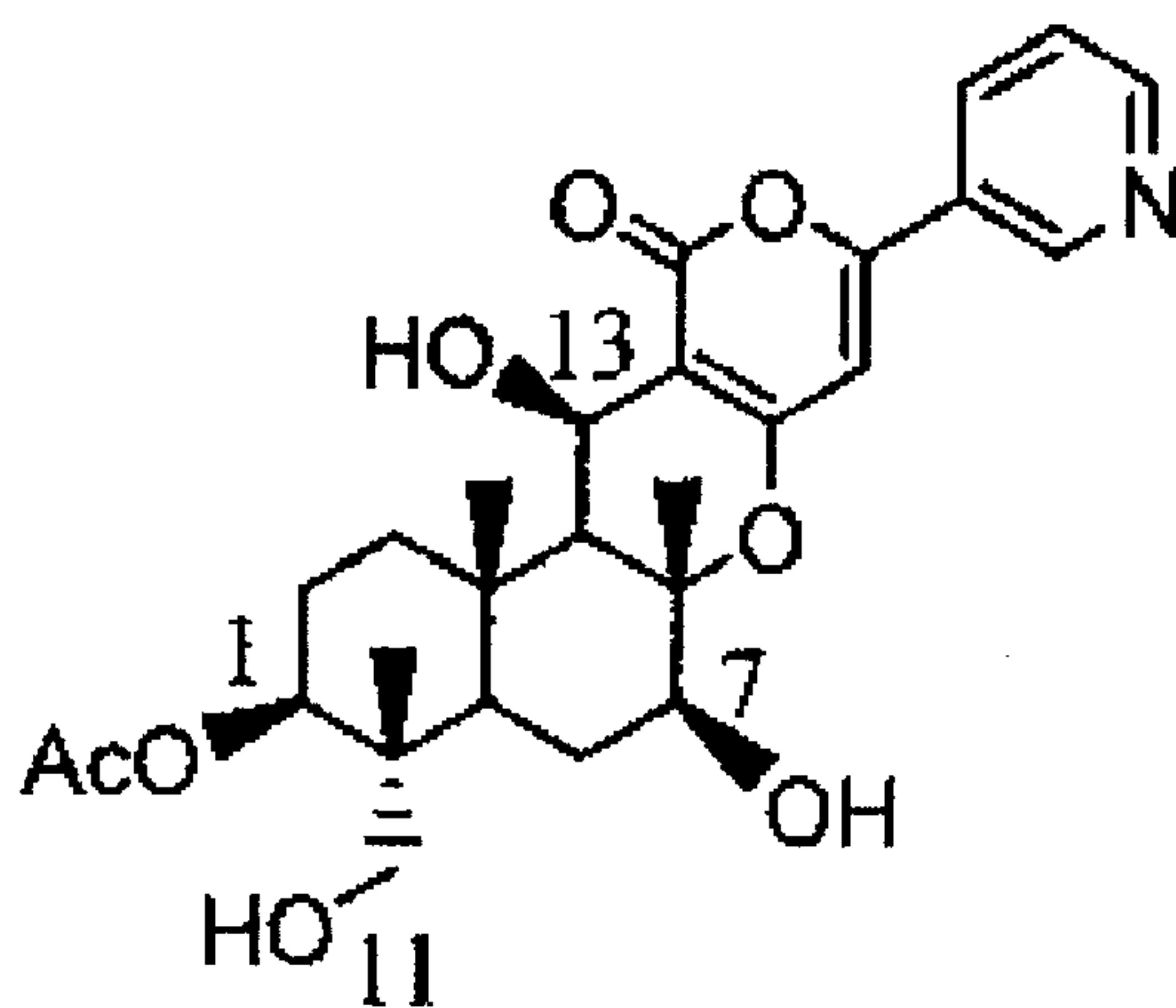
[Chemical formula 1]



Pyripyropene E

10 [0021] Still further, a production method wherein the above-mentioned pyripyropene A precursor is one represented by the following formula (I) is provided:

[Chemical formula 2]

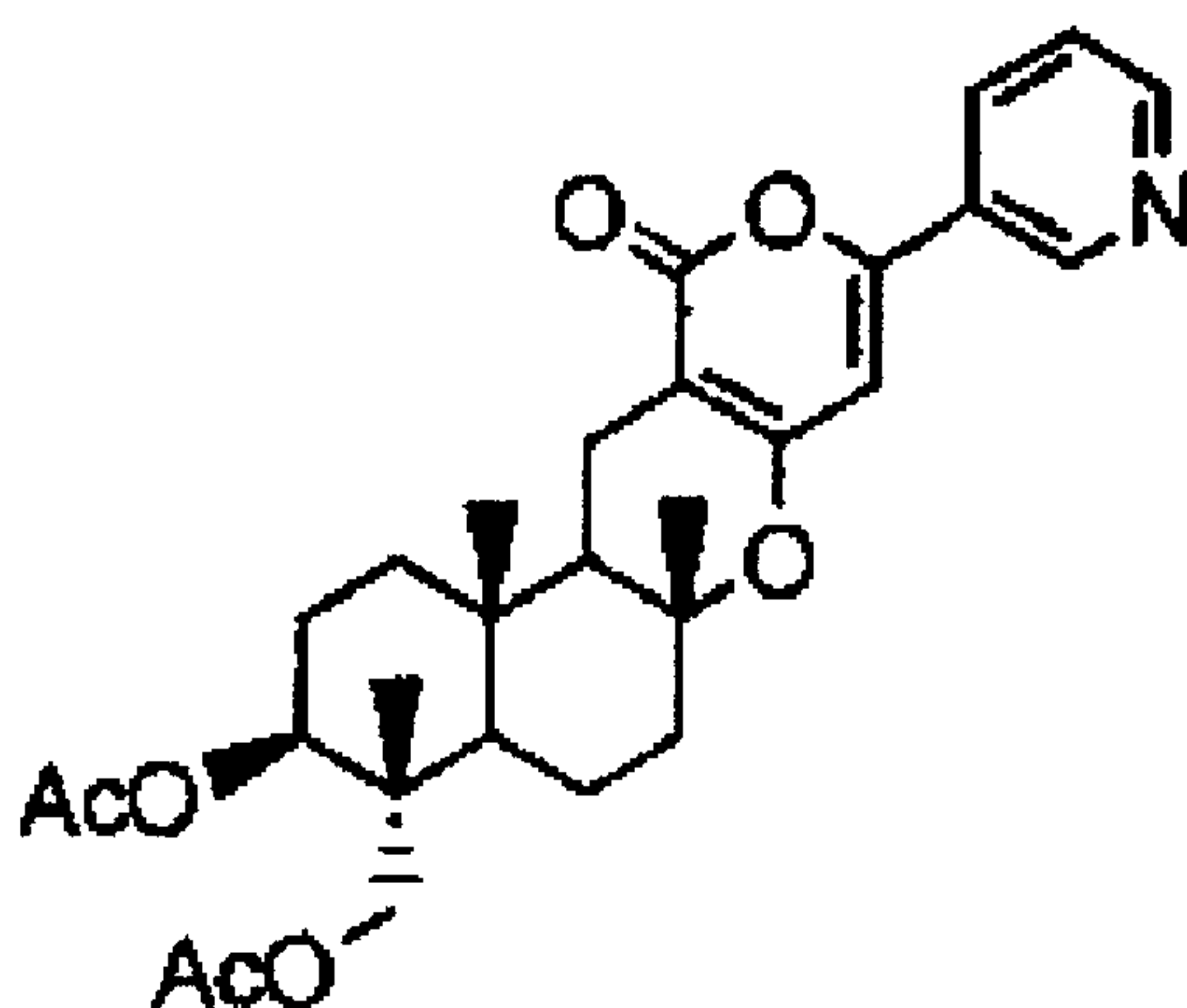


15 formula (I)

[0022] Also, a method for producing a pyripyropene A precursor characterized by culturing the above-mentioned

transformant and isolating the pyripyropene A precursor from pyripyropene O represented by the following formula is provided:

[Chemical formula 3]

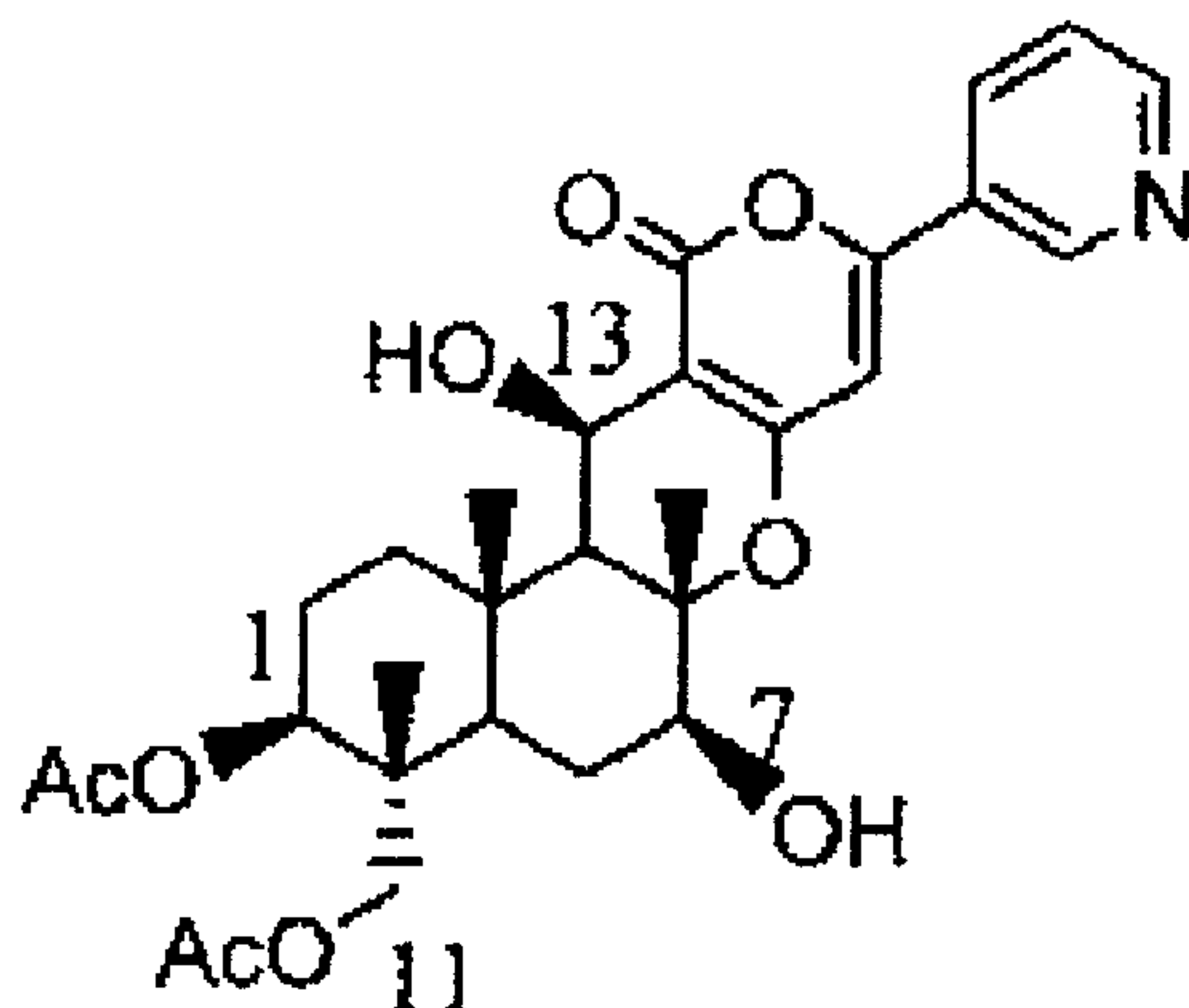


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Pyripyropene O

[0023] Still further, a production method wherein the above-mentioned pyripyropene A precursor is a compound represented by the following formula (II) is provided:

10 [Chemical formula 4]



formula (II)

[0024] According to one embodiment of the present invention, production of a novel pyripyropene analog, improvement of productivity of a pyripyropene A-producing bacterium, production of a novel insecticidal agent for microorganisms, creation of a novel plant resistant to insect

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pests or the like are provided.

[0024a] The present invention as claimed relates to:

- an isolated polynucleotide, which is selected from the polynucleotide defined in any of (a) to (f) below:

- 5 (a) a polynucleotide the nucleotide sequence of which is set forth as SEQ ID NO:266,
(b) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is selected from SEQ ID NOs:269, 270, 273 and 274,
(c) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:269 in which 1 to 40 amino acids are substituted,
10 deleted, added or inserted, and which has hydroxylase activity,
(d) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:270 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has hydroxylase activity,
(e) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:273 in which 1 to 40 amino acids are substituted,
15 deleted, added or inserted, and which has prenyltransferase activity, and
(f) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:274 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has acetyltransferase activity; and

- 20 - an isolated polynucleotide, which has a nucleotide sequence selected from the nucleotide sequence in any of (i), (ii), (iii) and (iv) below:

- (i) a nucleotide sequence from 13266 to 15144 of the nucleotide sequence shown in SEQ ID NO:266,
(ii) a nucleotide sequence from 16220 to 18018 of the nucleotide
25 sequence shown in SEQ ID NO:266,
(iii) a nucleotide sequence from 21793 to 22877 of the nucleotide sequence shown in SEQ ID NO:266,
(iv) a nucleotide sequence from 23205 to 24773 of the nucleotide sequence shown in SEQ ID NO:266.

30 [BRIEF DESCRIPTION OF DRAWINGS]

[0025]

[Figure 1] Figure 1 shows an electrophoresis pattern of PCR

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ladder), lane 1: primers of SEQ ID NOs:1 and 2, lane 2: primers of SEQ ID NOs:239 and 240, lane 3: primers of SEQ ID NOs:237 and 238, lane 4: primers of SEQ ID NOs:241 and 242, lane 5: primers of SEQ ID NOs:247 and 248, lane 6: primers of SEQ ID NOs:251 and 252, lane 7: primers of SEQ ID NOs:245 and 246, lane 8: primers of SEQ ID NOs:243 and 244, lane 9: primers of SEQ ID NOs:249 and 250, lane 10: primers of SEQ ID NOs:235 and 236, lane 11: primers of SEQ ID NOs:233 and 234, lane 12: primers of SEQ ID NOs:227 and 228, lane 13: primers of SEQ ID NOs:229 and 230, lane 14: primers of SEQ ID NOs:231 and 232.

[Figure 2] Similarly to Figure 1, Figure 2 shows an electrophoresis pattern of PCR products by agarose gel. For the electrophoresis, the PCR products amplified using the following primers were used: M: molecular weight marker (100 bp ladder), lane 1: primers of SEQ ID NOs:253 and 254, lane 2: primers of SEQ ID NOs:257 and 258, lane 3: primers of SEQ ID NOs:259 and 260, lane 4: primers of SEQ ID NOs:255 and 256, lane 5: primers of SEQ ID NOs:261 and 262.

[Figure 3] Similarly to Figure 1, Figure 3 shows an electrophoresis pattern of PCR products by agarose gel. For the electrophoresis, the PCR products amplified using the following primers were used: lane 1: molecular weight marker (100 bp ladder), lane 2: primers of SEQ ID NOs:264 and 265 (400 bp amplified fragment).

[Figure 4] Figure 4 shows the plasmid map of pUSA.

[Figure 5] Figure 5 shows the plasmid map of pPP2.

[Figure 6] Figure 6 shows a scheme of P450-2 cDNA amplification.

[Figure 7] Figure 7 shows the plasmid map of pPP3.

[Figure 8] Figure 8 shows ^1H -NMR spectrum of pyripyropene E in deuterated acetonitrile.

[Figure 9] Figure 9 shows ^1H -NMR spectrum in deuterated acetonitrile of a product of the culture of *Aspergillus oryzae*
5 transformed with plasmid pPP2.

[Figure 10] Figure 10 shows ^1H -NMR spectrum of pyripyropene O in deuterated acetonitrile.

[Figure 11] Figure 11 shows ^1H -NMR spectrum in deuterated acetonitrile of a product of the culture of *Aspergillus oryzae*
10 transformed with plasmid pPP3.

[DETAILED DESCRIPTION OF THE INVENTION]

[0026] Deposition of Microorganisms

Escherichia coli (*Escherichia coli* EPI300TM-T1^R)
transformed with plasmid pCC1-PP1 has been deposited with
15 International Patent Organism Depositary, National Institute of
Advanced Industrial Science and Technology (Address: AIST
Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan,
305-8566), under accession No. FERM BP-11133 (converted
from domestic deposition under accession No. FERM P-21704)
20 (identification reference by the depositors: *Escherichia coli*
EPI300TM-T1^R/pCC1-PP1) as of October 9, 2008 (original
deposition date).

[0027] *Aspergillus oryzae* transformed with plasmid pPP2
has been deposited with International Patent Organism
25 Depositary, National Institute of Advanced Industrial Science
and Technology (Address: AIST Tsukuba Central 6, 1-1-1
Higashi, Tsukuba, Ibaraki, Japan, 305-8566), under accession
No. FERM BP-11137 (identification reference by the depositors:
Aspergillus oryzae PP2-1) as of June 23, 2009.

30 [0028] *Aspergillus oryzae* transformed with plasmid pPP3
has been deposited with International Patent Organism
Depositary, National Institute of Advanced Industrial Science
and Technology (Address: AIST Tsukuba Central 6, 1-1-1
Higashi, Tsukuba, Ibaraki, Japan, 305-8566), under accession
35 No. FERM BP-11141 (identification reference by the depositors:
Aspergillus oryzae PP3-2) as of July 3, 2009.

[0029] Isolated Polynucleotide

The present invention is an isolated polynucleotide. The isolated polynucleotide according to the present invention is (a) a polynucleotide having the nucleotide sequence of SEQ ID NO:266; (b) a polynucleotide having a nucleotide sequence which is capable of hybridizing with the nucleotide sequence of SEQ ID NO:266 under stringent conditions, or (c) a polynucleotide having a polynucleotide sequence encoding at least one amino acid sequence selected from SEQ ID NOs:267 to 274 or a substantially equivalent amino acid sequence thereto. The above-mentioned isolated polynucleotide preferably has a nucleotide sequence encoding at least one polypeptide which has an enzyme activity involved in biosynthesis of pyripyropene A.

[0030] In the present invention, "a substantially equivalent amino acid sequence" means an amino acid sequence which does not affect an activity of a polypeptide despite the fact that one or more amino acids are altered by substitution, deletion, addition or insertion. The number of the altered amino acid residues is preferably 1 to 40 residues, more preferably 1 to several residues, still more preferably 1 to 8 residues, most preferably 1 to 4 residues.

[0031] Further, an example of the alteration which does not affect the activity includes conservative substitution. The term, "conservative substitution" means substitution of one or more amino acid residues with other chemically similar amino acid residues such that the activity of a polypeptide is not substantially altered. Examples thereof include cases where a certain hydrophobic amino acid residue is substituted with another hydrophobic amino acid residue and cases where a certain polar amino acid residue is substituted with another polar amino acid residue having the same charges. Functionally similar amino acids capable of such a substitution are known in the art for each amino acid. Concretely, examples of non-polar (hydrophobic) amino acids include alanine, valine, isoleucine, leucine, proline, tryptophan, phenylalanine,

methionine and the like. Examples of polar (neutral) amino acids include glycine, serine, threonine, tyrosine, glutamine, asparagine, cysteine and the like. Examples of positively charged (basic) amino acids include arginine, histidine, lysine and the like. Examples of negatively charged (acidic) amino acids include aspartic acid, glutamic acid and the like.

[0032] Also, the isolated polynucleotide of the present invention may be a polynucleotide having at least one nucleotide sequence selected from the nucleotide sequence in any of (1) or (2) below:

(1) a polynucleotide sequence in any of (a) to (h) below:

(a) a nucleotide sequence from 3342 to 5158 of a nucleotide sequence shown in SEQ ID NO:266,

(b) a nucleotide sequence from 5382 to 12777 of a nucleotide sequence shown in SEQ ID NO:266,

(c) a nucleotide sequence from 13266 to 15144 of a nucleotide sequence shown in SEQ ID NO:266,

(d) a nucleotide sequence from 16220 to 18018 of a nucleotide sequence shown in SEQ ID NO:266,

(e) a nucleotide sequence from 18506 to 19296 of a nucleotide sequence shown in SEQ ID NO:266,

(f) a nucleotide sequence from 19779 to 21389 of a nucleotide sequence shown in SEQ ID NO:266,

(g) a nucleotide sequence from 21793 to 22877 of a nucleotide sequence shown in SEQ ID NO:266,

(h) a nucleotide sequence from 23205 to 24773 of a nucleotide sequence shown in SEQ ID NO:266;

(2) a nucleotide sequence which is capable of hybridizing with a nucleotide sequence in (1) under stringent conditions.

[0033] A polynucleotide having at least one nucleotide sequence selected from the nucleotide sequence in any of the above-mentioned (1) or (2) preferably encodes at least one polypeptide having an enzyme activity involved in biosynthesis of pyripyropene A.

[0034] The term, "stringent conditions" in the present

invention means conditions where a washing operation of membranes after hybridization is carried out at high temperatures in a solution with low salt concentrations, for example, conditions of washing in a solution with 2×SSC concentration (1×SSC: 15 mM trisodium citrate, 150 mM sodium chloride) and 0.5% SDS at 60°C for 20 minutes.

[0035] The polynucleotide having at least one nucleotide sequence selected from the nucleotide sequence in any of the above-mentioned (1) or (2) according to the present invention is one encoding a polypeptide having any one or more activities of polyketide synthase activity, prenyltransferase activity, hydroxylase activity, acetyltransferase activity or adenylate synthetase activity; and, in particular, one encoding a polypeptide having the hydroxylase activity.

[0036] Further, according to one embodiment of the present invention, the above-mentioned polynucleotide is one encoding a polypeptide having an activity to hydroxylate the 7-position and/or 13-position of the above-mentioned pyripyropene E or O, or one encoding a polypeptide having an activity to hydroxylate the 11-position of the above-mentioned pyripyropene E.

[0037] Obtainment of Isolated Polynucleotide

The method for obtaining the isolated polynucleotide of the present invention is not particularly restricted. For instance, the polynucleotide can be isolated from *Penicillium coprobium* PF1169 strain or filamentous bacterium by the following method.

[0038] Based on a homology sequence obtained by the method of Example 9 below or the like, primers capable of specifically amplifying a polyketide synthase gene are synthesized. PCR is carried out for a fosmid genomic library of *Penicillium coprobium* PF1169 strain which is separately prepared, followed by colony hybridization. A recombinant vector is thereby obtained and the base sequence of an inserted DNA thereof is determined.

[0039] Also, based on the homology sequence obtained by

the method of Example 9 below or the like, primers capable of specifically amplifying a prenyltransferase gene are synthesized. Further, the base sequence of an inserted DNA is determined in the same manner as above.

5 [0040] Further, based on the homology sequence obtained by the method of Example 9 below or the like, primers capable of specifically amplifying any one or both of a polyketide synthase gene and prenyltransferase gene are synthesized. Further, the base sequence of an inserted DNA is determined in
10 the same manner as above.

[0041] In addition, based on the homology sequence of at least one nucleotide sequence selected from SEQ ID NO:266 and the nucleotide sequence in any of the above-mentioned (1) or (2) according to the present invention, primers capable of
15 specifically amplifying any one or more of a polyketide synthase gene, prenyltransferase gene, hydroxylase gene, acetyltransferase gene or adenylate synthetase gene, preferably the hydroxylase gene are synthesized. Further, the base sequence of an inserted DNA is determined in the same manner
20 as above.

[0042] Still further, based on an amino acid sequence conserved among various filamentous bacterium polyketide synthases, degenerate primers for amplification were synthesized and the base sequence of an inserted DNA is
25 determined.

[0043] Transformant

In general, examples of a method for improving productivity of a secondary metabolism product by gene recombination include improving expression of a gene encoding
30 a protein catalyzing a biosynthetic reaction which is a rate limiting reaction, improving expression of or disrupting a gene regulating expression of a biosynthetic gene, blocking an unnecessary secondary metabolism system, and the like. Therefore, specifying the biosynthetic gene makes it possible to
35 improve the productivity of the secondary metabolism product by ligating the gene to an appropriate vector and introducing

the vector into a production bacterium.

[0044] Meanwhile, in order to create a novel active substance by gene recombination, domain alteration of polyketide synthase [Ikada and Ohmura, "PROTEIN, NUCLEIC
5 ACID AND ENZYME" Vol. 43, p. 1265-1277, 1998], [Carreras, C.W. and Santi, D.V., "Current Opinion in Biotechnology", (UK), 1998, Vol. 9, p. 403-411], [Hutchinson, C. R., "Current Opinion in Microbiology", (UK), 1998, Vol. 1, p. 319-329], [Katz, L. and McDaniel, R., "Medicinal Research Reviews", (USA), 1999, Vol.
10 19, p. 543-558]; disruption of a biosynthetic gene; introduction of a modification enzyme gene from other organisms [Hutchinson, C. R., "Bio/Technology", (USA), 1994, Vol. 12, p. 375-380]; and the like are carried out. Thus, specifying the biosynthetic gene makes it possible to create the novel active
15 substance by ligating the gene to an appropriate vector and introducing the vector into a bacterium producing a secondary metabolism product.

[0045] Therefore, pyripyropene A can be produced or productivity thereof can be improved by ligating the isolated
20 polynucleotide according to the present invention to the appropriate vector, introducing the vector into a host, expressing it, enhancing expression thereof, or carrying out gene disruption of part of the isolated polynucleotide using homologous recombination and impairing functions thereof.

25 [0046] Gene disruption using homologous recombination can be carried out in accordance with a conventional method. Preparation of a vector used for the gene disruption and introduction of the vector into a host are apparent for those skilled in the art.

30 [0047] The recombinant vector according to the present invention preferably comprises any one or more of polynucleotides having the nucleotide sequence in SEQ ID NO:266 and the above-mentioned (1); a polynucleotide having a nucleotide sequence which is capable of hybridizing with the
35 nucleotide sequence in SEQ ID NO:266 and the above-mentioned (1) under stringent conditions, or a

polynucleotide having a polynucleotide sequence encoding at least one amino acid sequence selected from SEQ ID NOs:267 to 274 or a substantially equivalent amino acid sequence thereto. More preferably, the recombinant vector according to
5 the present invention is one wherein the above-mentioned polypeptide comprises a polynucleotide hydroxylating the 7-position and/or 13-position of the pyripyropene E or O, and the above-mentioned polypeptide comprises a polynucleotide hydroxylating the 11-position of the pyripyropene E.

10 [0048] A recombinant vector for gene introduction can be prepared by modifying the polynucleotide provided by the present invention into an appropriate form depending on an object and ligating it to a vector in accordance with a conventional method, for example, gene recombination
15 techniques described in [Sambrook, J. et al., "Molecular cloning: a laboratory manual", (USA), 2nd Edition, Cold Spring Harbor Laboratory, 1989].

[0049] The recombinant vector used in the present invention can be appropriately selected from virus, plasmid,
20 fosmid, cosmid vectors or the like. For instance, when a host cell is *Escherichia coli*, examples thereof include λ phage-based bacteriophage and pBR and pUC-based plasmids. In the case of a *Bacillus subtilis*, examples include pUB-based plasmids. In the case of yeast, examples include YEp, YRp, YCp and
25 YIp-based plasmids.

[0050] In addition, it is preferred that at least one plasmid among the used plasmids comprise a selection marker for selecting a transformant. As the selection marker, a gene encoding drug resistance and gene complementing auxotrophy
30 can be used. Concrete preferred examples thereof include when a host to be used is bacterium, ampicillin resistant genes, kanamycin resistant genes, tetracycline resistant gene and the like; in the case of yeast, tryptophan biosynthetic gene (TRP1), uracil biosynthetic gene (URA3), leucine biosynthetic gene (LEU2) and the like; in the case of a fungus, hygromycin
35 resistant genes, bialaphos resistant genes, bleomycin resistant

genes, aureobasidin resistant genes and the like; and in the case of a plant, kanamycin resistant genes, bialaphos resistant genes and the like.

[0051] Further, DNA molecules serving as an expression
5 vector used in the present invention preferably has DNA sequences necessary to express each gene, transcription regulatory signals and translation regulatory signals such as promoters, transcription initiation signals, ribosome binding sites, translation stop signals, terminators. Preferred examples
10 of the promoters include promoters of lactose operon, tryptophan operon and the like in *Escherichia coli*; promoters of alcohol dehydrogenase gene, acid phosphatase gene, galactose metabolizing gene, glyceraldehyde 3-phosphate dehydrogenase gene or the like in yeast; promoters of α -amylase gene,
15 glucoamylase gene, cellobiohydrolase gene, glyceraldehyde 3-phosphate dehydrogenase gene, abp1 gene or the like in fungi; a CaMV 35S RNA promoter, a CaMV 19S RNA promoter or a nopaline synthetase gene promoter in plants.

[0052] A host in which the isolated polynucleotide
20 according to the present invention is introduced may be appropriately selected, depending on the type of the used vector, from actinomycetes, *Escherichia coli*, *Bacillus subtilis*, yeast, filamentous bacteria, plant cells or the like.

[0053] A method of introducing a recombinant vector into
25 a host may be selected, depending on a host cell under test, from conjugal transfer, transduction by phage, as well as methods of transformation such as a calcium ion method, a lithium ion method, an electroporation method, a PEG method, an *Agrobacterium* method or a particle gun method.

30 [0054] In cases where a plurality of genes is introduced into host cells in the present invention, the genes may be contained in a single DNA molecule or individually in different DNA molecules. Further, when a host cell is a bacterium, each gene can be designed so as to be expressed as polycistronic
35 mRNA and made into one DNA molecule.

[0055] The transformant according to the present

invention preferably comprises any one or more of polynucleotides having the nucleotide sequence in SEQ ID NO:266 and the above-mentioned (1); a polynucleotide having a nucleotide sequence which is capable of hybridizing with the
5 nucleotide sequence in SEQ ID NO:266 and the above-mentioned (1) under stringent conditions, or a polynucleotide having a polynucleotide sequence encoding at least one amino acid sequence selected from SEQ ID NOs:267 to 274 or a substantially equivalent amino acid sequence
10 thereto.

[0056] The transformant obtained can be cultured by a conventional method and newly characteristics obtained can be studied. As the medium, commonly used components, for example, as carbon sources, glucose, sucrose, starch syrup,
15 dextrin, starch, glycerol, molasses, animal and vegetable oils or the like can be used. Also, as nitrogen sources, soybean flour, wheat germ, corn steep liquor, cotton seed meal, meat extract, polypeptone, malto extract, yeast extract, ammonium sulfate, sodium nitrate, urea or the like can be used. Besides, as
20 required, addition of sodium, potassium, calcium, magnesium, cobalt, chlorine, phosphoric acid (dipotassium hydrogen phosphate or the like), sulfuric acid (magnesium sulfate or the like) or inorganic salts which can generate other ions is effective. Also, as required, various vitamins such as thiamin (thiamine
25 hydrochloride or the like), amino acids such as glutamic acid (sodium glutamate or the like) or asparagine (DL-asparagine or the like), trace nutrients such as nucleotides, or selection agents such as antibiotics can be added. Further, organic substances or inorganic substances which help the growth of a
30 bacterium and promote the production of pyripyropene A can be appropriately added.

[0057] The pH of the medium is, for example, about pH 5.5 to pH 8. As the method for culturing, solid culturing under aerobic conditions, shake culturing, culturing with bubbling
35 under stirring or deep part aerobic culturing can be employed and, in particular, the deep part aerobic culturing is most

appropriate. The appropriate temperature for the culturing is 15°C to 40°C and, in many cases, the growth takes place around 22°C to 30°C. The production of pyripyropene A varies depending on the medium and culturing conditions, or the used host. In any method for culturing, the accumulation usually reaches a peak in 2 days to 10 days. The culturing is terminated at the time when the accumulation of pyripyropene A in the culture reaches the peak and a desired substance is isolated and purified from the culture.

10 [0058] To isolate pyripyropene A from the culture, it can be extracted and purified by a usual separation means using properties thereof, such as a solvent extraction method, an ion exchange resin method, an adsorption or distribution column chromatography method, a gel filtration method, dialysis, a
15 precipitation method, a crystallization method, which may be individually used or appropriately used in combination.

[0059] Method for Producing Pyripyropene A Precursor

In order to isolate pyripyropene A, pyripyropene A can be isolated from a pyripyropene A precursor using a known
20 method. An example of the known method includes the method of WO2009/022702. By culturing a microorganism containing a vector containing one or more of the above, the pyripyropene A precursor can be isolated from pyripyropene E. The pyripyropene A precursor may be, for example, the
25 compound represented by the above-mentioned formula (I).

[0060] Also, by culturing a microorganism comprising a vector containing one or more, the pyripyropene A precursor can be isolated from pyripyropene O. An example may be the compound represented by the above-mentioned formula (II).

30 [EXAMPLES]

[0061] The present invention will be further illustrated in detail by the following examples, which are not intended to restrict the present invention.

[0062] Example 1: Preparation of Genomic DNA of
35 *Penicillium coprobium* PF1169 Strain

Sterilized NB medium (500 ml) was placed in an

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Erlenmeyer flask (1 L). *Penicillium coprobium* PF1169 strain (Journal of Technical Disclosure No. 500997/2008 (Patent Document 15)) precultured in 1/2 CMMY agar medium at 28°C for 4 days was added to the above-mentioned medium and subjected to liquid culture at 28°C for 4 days. Filtration was carried out with Miracloth to obtain 5 g of bacterial cells. From these bacterial cells, 30 µg of genomic DNA was obtained in accordance with the manual attached to genomic DNA purification kit Genomic-tip 100/G (manufactured by Qiagen K.K.).

[0063] Example 2: Degenerate primers for Amplification of Polyketide Synthase (PKS) and Amplified Fragment Thereof

Based on an amino acid sequence conserved among various filamentous bacterium polyketide synthases, the following primers were designed and synthesized as degenerate primers for amplification:

LC1: GAYCCIMGITTYTTYAAAYATG (SEQ ID NO:1)

LC2c: GTICCGTICCRTGCATYTC (SEQ ID NO:2)

(wherein R=A/G, Y=C/T, M=A/C, I=inosine).

Using these degenerate primers, the genomic DNA prepared in Example 1 and ExTaqTM polymerase (manufactured by Takara Bio Inc.) were allowed to react in accordance with the attached manual. An amplified fragment of about 700 bp was detected (see Figure 1). Further, the above-mentioned amplified fragment was analyzed to specify the sequence of its internal 500 bp (SEQ ID NO:3).

[0064] Example 3: Large-scale Sequencing of Genomic DNA and Amino Acid Sequence Homology Search

The genomic DNA of *Penicillium coprobium* PF1169 strain obtained in Example 1 was subjected to large-scale sequencing and homology search for amino acid sequences. Specifically, part of 50 µg of genomic DNA was pretreated and thereafter subjected to Roche 454TMFLX DNA sequencer to obtain about 250 bp, 103 thousands of fragment sequences (in total, 49 Mb of sequence).

[0065] For theses sequences, as known sequences among

polyketide synthases and prenyltransferases, the following five sequences (sequences derived from polyketide synthases: *Aspergillus*(A.) *fumigatus* PKS 2146 a.a. and *Penicillium*(P.) *griseofluvum* 6-methylsalicylic acid synthase 1744 a.a.; as well
 5 as prenyltransferases: *Aspergillus* (A.) *fumigatus* Prenyltransferase, *Aspergillus*(A.) *fumigatus* Prenyltransferase (4-hydroxybenzoate octaprenyltransferase) and *Penicillium*(P.) *marneffe* Prenyltransferase) were selected and search by
 10 homology sequence search software blastx was carried out, thereby obtaining 89, 86, 2, 1 and 3 of homology sequences, respectively (see Table 1). Further, from the homology sequences of A. *fumigatus* PKS 2146 a.a. and P. *griseofluvum* 6-methylsalicylic acid synthase 1744 a.a., 19 and 23 of contig sequences were respectively obtained (the contig sequences of
 15 A. *fumigatus* PKS 2146 a.a.: SEQ ID NOs:179 to 197; the contig sequences of P. *griseofluvum* 6-methylsalicylic acid synthase 1744 a.a.: SEQ ID NOs:198 to 220) (see Table 1).

[0066] [Table 1]

Table 1

Enzyme Name	Origin	Number of Homology Sequences	SEQ ID NO.
Polyketide Synthases	<i>A. fumigatus</i> PKS 2146 a.a.	89	4-92
	<i>P. griseofluvum</i> 6-methylsalicylic acid synthase 1744 a.a.	86	93-178
	<i>A. fumigatus</i> PKS 2146 a.a.	19 (Contig sequences)	179-197
	<i>P. griseofluvum</i> 6-methylsalicylic acid synthase 1744 a.a.	23 (Contig sequences)	198-220
Prenyltransferases	<i>A. fumigatus</i> Prenyltransferase	2	221, 222
	<i>A. fumigatus</i> Prenyltransferase (4-hydroxybenzoate octaprenyltransferase)	1	223
	<i>P. marneffe</i> Prenyltransferase	3	224-226

[0067] Example 4: PCR Amplification from Genomic DNA

From the search results of blastx obtained in Example 3, for polyketide synthases, 13 types of primer pairs shown in SEQ ID NOs:227 to 252 were synthesized. Similarly,
5 for prenyltransferases, 5 types of primer pairs shown in SEQ ID NOs:253 to 262 were synthesized. When PCR was carried out for the genomic DNA using these primers, amplified fragments with the expected size were seen for all of the primer pairs (see Figure 1 and Figure 2).

10 [0068] Example 5: Construction of Phage Genomic Library

A λ phage genomic library of *Penicillium coprobium* PF1169 strain was constructed using λ BlueSTAR *Xho* I Half-site Arms Kit (manufactured by Takara Bio Inc., Cat. No. 69242-3) in
15 accordance with the attached manual. That is, genomic DNA was partially digested using a restriction enzyme, *Sau*3A1. The DNA fragment with about 20 kb (0.5 μ g) was ligated to 0.5 μ g of λ BlueSTAR DNA attached to the kit. This ligation solution was subjected to in vitro packaging using Lambda INN
20 Packaging kit (manufactured by Nippon Gene Co., Ltd.) based on the manual attached to the kit to obtain 1 ml of a solution. This solution with packaged phages (10 μ l) was infected into 100 μ l of *E. coli* ER1647 strain and cultured on a plaque-forming medium at 37°C overnight, thereby obtaining about 500 clones
25 of plaques. Thus, the genomic library composed of about 50000 clones of phages in which 10 to 20 kb genomic DNA of *Penicillium coprobium* PF1169 strain were introduced by infection was constructed.

[0069] Example 6: Screening from Phage Library

30 For 10000 clones of the phage library prepared in Example 5, the primary screening was carried out by plaque hybridization using, as a probe, the PCR product amplified by LC1-LC2c primer pair prepared above. For labeling and detection of the probe, AlkPhos Direct Labelling and Detection
35 System with CDP-Star (manufactured by GE Healthcare, Cat. No. RPN3690) was used. The above-mentioned hybridization was

carried out in accordance with the attached manual.

[0070] By the primary screening, 6 clones remained as candidates. Further, as the result of the secondary screening by plaque hybridization, 4 clones were obtained. These positive clones were infected into *E. coli* BM25.8 strain and the phages were converted to plasmids in accordance with the attached manual, thereby obtaining 4 types of plasmids containing a desired region.

10 [0071] Example 7: Preparation of Fosmid Genome Library

A genomic library of *Penicillium coprobium* PF1169 strain was constructed using CopyControl Fosmid Library Production Kit (manufactured by EPICENTRE, Cat. No. CCFOS110) in accordance with the manual attached thereto. That is, 0.25 µg of DNA fragment of about 40 kb genomic DNA was blunt-ended and then incorporated into fosmid vector pCCFOS (manufactured by Epicentre). This ligation solution was subjected to in vitro packaging using MaxPlax Lambda Packaging Extract attached to the kit based on the manual attached to the kit. This solution with packaged virus (10 µl) was infected into 100 µl of *E. coli* EPI300TM-T1^R strain and cultured on a medium containing chloramphenicol at 37°C overnight and selected,, thereby obtaining about 300 clones of plaques. Thus, about 30000 clones of the fosmids in which 40 kb of the genomic DNA of *Penicillium coprobium* PF1169 strain were introduced by infection were obtained. They were aliquoted in a 96 well plate so as to be about 50 clones per well. Thus, the genomic library composed of 96 pools, about 4800 clones was constructed.

30 [0072] Example 8: Fosmid Library Screening

In accordance with the manual attached to the fosmid, plasmid DNAs were individually prepared from 96 pools of the library prepared in Example 7. Using the degenerate primers for polyketide synthase amplification synthesized in Example 2, PCR was carried out for 96 pools of these plasmid DNA samples. As a result, DNA fragments of about 700 bp

were amplified from 9 pools. Further, a petri dish containing colonies of about 300 clones or more was prepared from the positive pools and re-screening was carried out by colony hybridization. As a result, using by LC1-LC2c primer pair, 9
5 types of fosmids were obtained from about 4800 clones.

[0073] Example 9: Large-Scale Sequencing of Genomic DNA and Amino Acid Sequence Homology Search

Genomic DNA of *Penicillium coprobium* PF1169 strain obtained in Example 1 was subjected to large-scale
10 sequencing and homology search for amino acid sequences. Specifically, part of 50 µg of genomic DNA was pretreated and then subjected to Roche 454FLX DNA sequencer to obtain 1405 fragment sequences with an average contig length of 19.621 kb (sequence of a total base length of 27.568160 Mb).

15 For these sequences, as known sequences among polyketide synthases and prenyltransferases, the following five sequences (sequences derived from polyketide synthases: *Penicillium(P.) griseoflukum* 6-methylsalicylic acid synthase 1744 a.a. (P22367) and *Aspergillus(A.) fumigatus* PKS 2146 a.a. (Q4WZA8); as well as prenyltransferases: *Penicillium(P.) marneffe*
20 *i* Prenyltransferase (Q0MRO8), *Aspergillus (A.) fumigatus* Prenyltransferase (Q4WBI5) and *Aspergillus(A.) fumigatus* Prenyltransferase (4-hydroxybezoate octaprenyltransferase) (Q4WLD0)) were selected and search by
25 homology sequence search software blastx was carried out, thereby obtaining 22 (P22367), 21 (Q4WZA8), 2 (Q0MRO8), 3 (Q4WBI5) and 3 (Q4WLD0) of the homologous sequences, respectively.

[0074] Example 10: Fosmid Library Screening and
30 Sequence Analysis of Cluster Genes

In accordance with the manual attached to a fosmid kit (manufactured by EPICENTRE, CopyControl Fosmid Library Production Kit), plasmid DNAs were individually prepared from 96 pools of the library prepared in Example 7.
35 Based on base sequences determined by Roche 454FLX DNA sequencer, homology search for amino acid sequences was

carried out to search regions adjacent to polyketide synthase and prenyltransferase. Based on the base sequence of prenyltransferase of the obtained region, a primer pair (No. 27) capable of amplifying 400 bp DNA fragment was synthesized.

5 Using the primers, PCR was carried out for these 48 pools of plasmid DNA samples. As a result, expected DNA fragments of about 400 bp (SEQ ID NO:263) were amplified from 11 pools (see Figure 3). Further, a petri dish containing colonies of about 300 clones or more was prepared from 6 pools of the

10 positive pools and re-screening was carried out by colony hybridization. As a result, by using 27F + 27R primer pair (27F primer: SEQ ID NO:264, 27R primer: SEQ ID NO:265), 4 types of fosmids were obtained from about 4800 clones. One of them was named pCC1-PP1 and the entire sequence of the inserted

15 fragment was determined (SEQ ID NO:266).

The obtained pCC1-PP1 was transformed into *Escherichia coli* EPI300TM-T1^R strain (included in the fosmid kit), thereby obtaining *Escherichia coli* EPI300TM-T1^R strain/pCC1-PP1.

20 When a homology search was carried out between the above-mentioned sequence of SEQ ID NO:266 and each of Adenylate-forming enzyme; LovB-like polyketide synthase; Cytochrome P450 monooxygenase, Integral membrane protein, FAD-dependent monooxygenase, which are hydroxylases;

25 UbiA-like prenyltransferase; Acetyltransferase, Toxin biosynthesis protein Tri7, which are acetyltransferases; and Cation transporting ATPase (the above-mentioned enzymes are all derived from *Aspergillus fumigatus* Af293 strain), a high homology of 70% or more was seen in any search.

30 The nucleotides 3342 to 5158 of SEQ ID NO:266 encode Adenylate-forming enzyme and the corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:267; the nucleotides 5382 to 12777 of SEQ ID NO:266 encode LovB-like polyketide synthase and the

35 corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:268; the nucleotides 13266 to

15144 of SEQ ID NO:266 (hereinafter, a protein encoded by this polynucleotide sequence (P450-1) is referred to as Cytochrome P450 monooxygenase (1)) and the nucleotides 16220 to 18018 (hereinafter, a protein encoded by this polynucleotide sequence
5 (P450-2) is referred to as Cytochrome P450 monooxygenase (2)) encode Cytochrome P450 monooxygenases and the corresponding polypeptides are shown with the amino acid sequences depicted in SEQ ID NOs:269 and 270, respectively; the nucleotides 18506 to 19296 of SEQ ID NO:266 encode
10 Integral membrane protein and the corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:271; the nucleotides 19779 to 21389 of SEQ ID NO:266 encode FAD-dependent monooxygenase and the corresponding polypeptide is shown with the amino acid sequence depicted in
15 SEQ ID NO:272; the nucleotides 21793 to 22877 of SEQ ID NO:266 encode UbiA-like prenyltransferase and the corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:273; the nucleotides 23205 to 24773 of SEQ ID NO:266 encode Acetyltransferase and the
20 corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:274; the nucleotides 25824 to 27178 of SEQ ID NO:266 encode Toxin biosynthesis protein Tri7 and the corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:275; and the nucleotides
25 27798 to 31855 of SEQ ID NO:266 encode Cation transporting ATPase and the corresponding polypeptide is shown with the amino acid sequence depicted in SEQ ID NO:276.

[0075] Example 11: Hydroxylation of Pyripyropene E or Pyripyropene O by Transformation of *Aspergillus Oryzae*

30 Pyripyropene E used below can be produced by, for example, a method for culturing a microorganism based on the method described in Japanese Patent Laid-Open Publication No. 239385/1996 (Patent Document 4), WO94/09147 or U.S. Patent No. 5597835, or the total synthesis method described in
35 Tetrahedron Letters, vol. 37, No. 36, 6461-6464, 1996. Also, pyripyropene O used below can be produced by, for example, a

method for culturing a microorganism based on the method described in J. Antibiotics 49, 292-298, 1996 or WO94/09147.

[0076] (1) Preparation of Expression Vector for Introducing into Filamentous Bacterium

5 pUSA (Figure 4) and pHSG399 (Takara Bio Inc.) were individually digested with *KpnI* and ligated, thereby obtaining pUSA-HSG. This plasmid was digested with *SmaI* and *KpnI* in the order mentioned, and subjected to gel purification, thereby obtaining a linear vector DNA having a *KpnI* cohesive
10 end and *SmaI* blunt end.

[0077] (2) Preparation of Plasmid pPP2

With fosmid pCC1-PP1 as a template, the polynucleotide of the above-mentioned P450-1 was amplified using a primer pair P450-1 with Kpn F (SEQ ID NO:277)/P450-1
15 with Swa R (SEQ ID NO:278). The purified DNA fragment was cloned into pCR-Blunt (Invitrogen, Cat. No. K2700-20). The plasmid obtained was digested with *KpnI* and *SwaI*. The above-mentioned P450-1 fragment was ligated to the above-described vector pUSA-HSG. thereby obtaining a plasmid
20 pPP2 shown in Figure 5.

[0078] (3) Preparation of Plasmid pPP3

With fosmid pCC1-PP1 as a template, in accordance with the flow shown in Figure 6, exons alone were first amplified using primer pairs F1(SEQ ID NO:279)/R1(SEQ ID
25 NO:280), F2(SEQ ID NO:281)/R2(SEQ ID NO:282), F3(SEQ ID NO:283)/R3(SEQ ID NO:284), F4(SEQ ID NO:285)/R4(SEQ ID NO:286), F5(SEQ ID NO:287)/R5(SEQ ID NO:288) and F6(SEQ ID NO:289)/R6(SEQ ID NO:290), thereby obtaining six fragments. Next, amplification was carried out with these
30 fragments as templates using primer pairs of F1/R2, F3/R4 and F5/R6, thereby obtaining longer fragments. Further, by repeating amplification using primer pairs of F1/R4 and F1/R6, cDNA which did not contain introns of the polynucleotide of the above-mentioned P450-2 was prepared. This cDNA fragment
35 was inserted into pCR-Blunt (Invitrogen, Cat. No. K2700-20) and the obtained plasmid was used as a template for

amplification by a primer pair, infusion F of P450-2-cDNA (SEQ ID NO:291)/infusion R of P450-2-cDNA (SEQ ID NO:292). Based on the manual of the kit, a plasmid pPP3 shown in Figure 7 was obtained using In-Fusion Advantage PCR Cloning Kit (Clontech).

[0079] (4) Transformation of *Aspergillus Oryzae* (*A. oryzae*)

In a CD-Met (containing L-Methionine 40µg/ml) agar medium, *A. oryzae* (HL-1105 strain) was cultured at 30°C for one week. From this petri dish, conidia ($>10^8$) were collected and seeded in 100 ml of YPD liquid medium in a 500 ml-flask. After 20-hour culturing (30°C, 180 rpm), bacterial cells having a moss ball shape were obtained. The bacterial cells were collected with a 3G-1 glass filter, washed with 0.8 M NaCl, and water was removed well. The resultant was suspended with TF solution I (protoplast formation solution) and then shook at 30°C, at 60 rpm for 2 hours. At a 30-minute interval, observation under the microscope was carried out and the presence of protoplasts was checked. Thereafter, the culture medium was filtered and subjected to centrifugation (2000 rpm, 5 minutes) to collect protoplasts, which were then washed with TF solution II. After washing, 0.8 volume of TF solution II and 0.2 volume of TF solution III were added and mixed, thereby obtaining a protoplast suspension.

[0080] To 200 µl of this suspension, 10 µg of plasmid DNA (pPP2 or pPP3) was added. The mixture was left to stand on ice 30 minutes and added with TF solution III (1 mL). The resulting mixture was gently mixed and then left to stand at room temperature for 15 minutes. Thereafter, the plasmid DNA was introduced into the above-mentioned protoplasts. To this, TF solution II (8 mL) was added and subjected to centrifugation (at 2000 rpm for 5 minutes). Further, protoplasts were then recovered with 1 to 2 ml being left over. The recovered protoplast solution was dropped to a regeneration medium (lower layer) and a regeneration medium (upper layer) was poured. The resultant was mixed by turning a petri dish and

then cultured at 30°C for 4 to 5 days. Generated clones were isolated in the regeneration medium (lower layer), subcultured and purified, thereby obtaining a transformant (*Aspergillus oryzae* PP2-1 and *Aspergillus oryzae* PP3-2).

5 [0081] The above-mentioned TF solution I (protoplast formation solution) was prepared with the following compositions.

	Name of Compound	Concentration
	Yatalase (manufactured by Takara Bio Inc.)	20 mg/ml
10	Ammonium sulfate	0.6 M
	Maleic acid-NaOH	50 mM

After the above-mentioned compositions (pH5.5) were prepared, filter sterilization was carried out.

15 [0082] The above-mentioned TF solution II was prepared with the following compositions.

	Name of Compound	
	1.2 M Sorbitol (MW=182.17)	43.72 g
	50 mM CaCl ₂	10 ml 1 M CaCl ₂ (1/20)
	35 mM NaCl	1.4 ml 5 M NaCl
20	10 mM Tris-HCl	2 ml 1 M Tris-HCl (1/100)
	Up to total volume	200 ml

After the above-mentioned compositions were prepared, autoclave sterilization was carried out.

25 [0083] The above-mentioned TF solution III was prepared with the following compositions.

	Name of Compound	
	60% PEG4000	6 g
	50 mM CaCl ₂	500 µl 1 M CaCl ₂ (1/20)
	50 mM Tris-HCl	500 µl 1 M Tris-HCl (1/100)
30	Up to total volume	10 ml

After the above-mentioned compositions were prepared, filter sterilization was carried out.

[0084] The above-mentioned regeneration medium was prepared with the following compositions.

	Name of Compound	Concentration
35	Sorbitol (MW=182.17)	218.6 g 1.2 M

	NaNO ₃	3.0 g	0.3% (w/v)
	KCl	2.0 g	0.2% (w/v)
	KH ₂ PO ₄	1.0 g	0.1% (w/v)
	MgSO ₄ ·7H ₂ O	2 ml of 1 M MgSO ₄	0.05% 2 mM
5	Trace elements solution	1 ml	
	Glucose	20.0 g	2% (w/v)
	Up to the total volume 1 L		

After the above-mentioned compositions (pH5.5) were prepared, autoclave sterilization was carried out.

10 [0085] In addition, the Trace elements solution used above was prepared with the following composition.

	Name of Compound	
	FeSO ₄ ·7H ₂ O	1.0 g
	ZnSO ₄ ·7H ₂ O	8.8 g
15	CuSO ₄ ·5H ₂ O	0.4 g
	Na ₂ B ₄ O ₇ ·10H ₂ O	0.1 g
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.05 g
	Up to the total volume	1 L

20 After the above-mentioned compositions were prepared, autoclave sterilization was carried out.

[0086] (5) Function Analysis and Addition Culture Test of P450-1

To a YPD medium (1% (w/v) Yeast Extract, 2% (w/v) Peptone, 2% (w/v) Dextrose) containing 1% (w/v) maltose, a 1/100 volume of 2 mg/mL dimethyl sulfoxide solution of pyripyropene E was added to provide medium A. From flora of *Aspergillus oryzae* PP2-1 cultured in Czapek Dox agar medium, conidia thereof were collected and suspended in sterilized water. This conidia suspension was adjusted to 10⁴ spores/mL. Further, 100 µL of this adjusted conidia suspension was added to 10 mL of medium A and cultured with shaking at 25°C for 96 hours. To this culture solution, 10 mL of acetone was added and the mixture was mixed well. Thereafter, acetone was removed using a centrifugal concentrator. To this, 10 mL of ethyl acetate was added and the resulting mixture was mixed well and then only the ethyl acetate layer was recovered.

A dried product obtained by removing ethyl acetate using the centrifugal concentrator was dissolved in 1000 μ L of methanol. This was used as a sample and analyzed by LC-MS (Waters, Micromass ZQ, 2996PDA, 2695 Separation module, Column: Waters XTerra C18 (Φ 4.5 $\times$ 50 mm, 5 μ m)) and LC-NMR (Avance500 manufactured by Burker Daltonik).

[0087] As the results of the above-mentioned LC-MS measurement, it was confirmed that the obtained compound was single compound A which increased by a molecular weight of 16 compared with pyripyropene E. In addition, as the results of the LC-NMR measurement, it was confirmed that this compound A was an 11-position hydroxide of pyripyropene E. It was confirmed that the above-mentioned Cytochrome P450 monooxygenase (1) was an enzyme hydroxylating the 11-position of pyripyropene E with pyripyropene E as a substrate.

[0088] Physicochemical properties of the above-mentioned compound A are shown below:

1. Mass spectrum: ES-MS 468M/Z ($M+H$)⁺
2. Molecular formula: C₂₇H₃₃NO₆
3. HPLC: Column: Waters XTerra Column C18 (5 μ m, 4.6 mm $\times$ 50 mm), 40°C, Mobile phase: From 20% aqueous acetonitrile solution to 100% acetonitrile in 10 minutes (linear gradient), Flow rate: 0.8 ml/min, Detection: Retention time 6.696 minutes at UV 323 nm
4. ¹H-NMR spectrum (CD₃CN, 2H: 3.134, 3.157 H-11)

The charts of the ¹H-NMR spectrum of pyripyropene E and ¹H-NMR spectrum according to 4 described above are shown in Figure 8 and Figure 9, respectively.

[0089] (6) Function Analysis and Addition Culture Test of P450-2

To a YPD medium (1% (w/v) Yeast Extract, 2% (w/v) Peptone, 2% (w/v) Dextrose) containing 1% (w/v) maltose, a 1/100 volume of 2 mg/mL dimethyl sulfoxide solution of pyripyropene E was added to provide medium B, and similarly a 1/100 volume of 2 mg/mL dimethyl sulfoxide solution of

pyripyropene O was added to provide medium C. From flora of *Aspergillus oryzae* PP3-2 cultured in Czapek Dox agar medium, conidia thereof were collected and suspended in sterilized water. This conidia suspension was adjusted to 10^4 spores/mL. Further, 500 μ L of the adjusted conidia suspension was added to 50 mL of medium B or medium C and cultured with shaking at 25°C for 96 hours. To this culture solution, 50 mL of acetone was added and the mixture was mixed well. Thereafter, acetone was removed using a centrifugal concentrator. To this, 50 mL of ethyl acetate was added and the resulting mixture was mixed well and then only the ethyl acetate layer was recovered. A dried product obtained by removing ethyl acetate using the centrifugal concentrator was dissolved in 1500 μ L of methanol. This was used as a sample and analyzed by LC-MS (manufactured by Waters, Micromass ZQ, 2996PDA, 2695 Separation module, Column: Waters XTerra C18 (Φ 4.5 $\times$ 50 mm, 5 μ m)) and LC-NMR (manufactured by Burker Daltonik, Avance500). As the results of the LC-MS measurement, from a sample obtained from the medium B, compound B which increased by a molecular weight of 32 compared with pyripyropene E was detected. Also, from a sample obtained from the medium C, compound C which increased by a molecular weight of 32 compared with pyripyropene O was detected. Further, as the results of the LC-NMR measurement, it was confirmed that the compound C was a 7-position and 13-position hydroxide of pyripyropene O. It was confirmed that the above-mentioned Cytochrome P450 monooxygenase (2) was an enzyme hydroxylating the 7-position and 13-position of each of pyripyropene E or pyripyropene O.

[0090] Physicochemical properties of the above-mentioned compound B are shown below:

1. Mass spectrum: ES-MS 484M/Z ($M+H$)⁺
2. Molecular formula: C₂₇H₃₃NO₇
3. HPLC: Column: Waters XTerra Column C18 (5 μ m, 4.6 mm $\times$ 50 mm), 40°C, Mobile phase: From 20% aqueous acetonitrile solution to 100% acetonitrile in 10 minutes (linear

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gradient), Flow rate: 0.8 ml/min, Detection: Retention time 5.614 minutes at UV 323 nm

[0091] Physicochemical properties of the above-mentioned compound C are shown below:

- 5 1. Mass spectrum: ES-MS 542M/Z (M+H)⁺
2. Molecular formula: C₂₉H₃₅NO₉
3. HPLC: Column: Waters XTerra Column C18 (5 µm, 4.6 mm×50 mm), 40°C, Mobile phase: From 20% aqueous acetonitrile solution to 100% acetonitrile in 10 minutes (linear
- 10 gradient), Flow rate: 0.8 ml/min, Detection: Retention time 5.165 minutes at UV 323 nm
4. ¹H-NMR spectrum (CD₃CN, 1H 4.858 H-13), (CD₃CN, 1H 3.65 H-7)

15 The charts of the ¹H-NMR spectrum of pyripyropene O and the above-mentioned compound C are shown in Figure 10 and Figure 11, respectively.

[Accession Numbers]

- [0092] FERM BP-11133
- FERM BP-11137
- 20 FERM BP-11141

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 20375-1016 Seq 14-02-11 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

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SEQ ID NO: 266

DNA 39008 nucleotides

Biosynthetic locus involved in synthesis of pyripyropene A
from *Penicillium coprobium* PF1169

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gaagcattca	gaatgcgggc	caaccggaag	tgacaatcta	ggagatcagc	acttggcaga	34800
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tgagtcattg	atcttaatga	ggatccgaga	acttactcgc	cggggatccc	agactgcata	35220
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gactttggtc	cagcgccgcc	tgggatagac	ttgacagaga	accaaactgg	cgacttgcta	37080
ggagcagtga	ttcctgtagc	ggtggtcgcg	acgactgcgg	tgatattgcg	gacgattgcg	37140

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ccgacgagga tcaaagagat ccgacaaaca gctattgatg actatctcat tgttgcgggcg 37200
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aagccgatgg gccagtact cacaacagac tcttttaggca ttccatatgg caacggttat 37320
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tctagcagag gaaagtttcg atgtgcatgc cgagccctaa aaagtcccag tgcggagaat 38760
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acccctctgg taatcatgta gatcctcttg cttactgcgt tggctccttt gtatcgtact 38880
ttccgcgtcg cagcattata agaggataga gagaccgcat gagagaatac acaagagaaa 38940
tcactaatte actacctgat cccccaattc actcaacatg tctcacattc acacttccag 39000
attgcaaa                                     39008

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SEQ ID NO: 269

Polypeptide 509 amino acids (P450-1)

From *Penicillium coprobium* PF1169

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Met Asp Asn Met Asp Asn Met Asn Asn Thr Pro Leu Gly Phe Asn Trp
1          5          10          15
Ala Trp Ala Val Ile Ile Ser Phe Leu Gly Leu Leu Thr Phe Ser Phe
          20          25          30
Val Ser Pro His Leu Phe Pro Ser Arg Leu Thr Val Ile Asn Gly Gly
          35          40          45
Arg Ala Trp Asp Ile Phe Arg Thr Lys Ala Lys Lys Arg Phe Arg Ser
          50          55          60
Asp Ala Ala Arg Leu Ile Lys Asn Gly Phe Glu Glu Ser Pro Asp Ala
65          70          75          80
Phe Arg Ile Ile Thr Asp Asn Gly Pro Leu Leu Val Leu Ser Pro Gln
          85          90          95
Tyr Ala Arg Glu Val Arg Ser Asp Asp Arg Leu Ser Leu Asp His Phe
          100          105          110

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Ile	Ala	Ser	Glu	Phe	His	Pro	Asn	Ile	Pro	Gly	Phe	Glu	Pro	Phe	Lys
		115					120					125			
Leu	Ile	Leu	Asp	Pro	Lys	Asn	Pro	Leu	Asn	Thr	Ile	Leu	Lys	Ser	Asn
	130					135					140				
Leu	Thr	Gln	Ala	Leu	Glu	Asp	Leu	Ser	Ala	Glu	Val	Thr	Glu	Ala	Leu
145					150					155					160
Ser	Ala	Thr	Cys	Thr	Asp	Asp	Pro	Glu	Trp	His	Glu	Val	Ser	Val	Ser
			165						170					175	
Gln	Thr	Ala	Leu	Lys	Ile	Ile	Ala	Gln	Met	Ala	Ser	Lys	Ala	Phe	Ile
			180					185					190		
Gly	Gln	Glu	Arg	Cys	Arg	Asp	Ala	Lys	Trp	His	Asn	Ile	Ile	Ile	Thr
		195					200					205			
Tyr	Thr	His	Asn	Val	Tyr	Gly	Ala	Ala	Gln	Ala	Leu	His	Phe	Trp	Pro
	210					215					220				
Ser	Phe	Leu	Arg	Pro	Ile	Val	Ala	Gln	Phe	Leu	Pro	Ala	Cys	Arg	Thr
225					230					235					240
Leu	Gln	Ala	Gln	Ile	Ala	Glu	Ala	Arg	Glu	Ile	Leu	Glu	Pro	Leu	Val
				245					250					255	
Ala	Gln	Arg	Arg	Ala	Glu	Arg	Ala	Thr	Arg	Ala	Ala	Gln	Glu	Lys	Pro
			260					265					270		
His	Pro	Ser	Gly	Gly	Asp	Ile	Ile	Asp	Trp	Leu	Glu	Gln	Phe	Tyr	Gly
		275					280					285			
Asp	Gln	Pro	Tyr	Asp	Pro	Val	Ala	Ala	Gln	Leu	Leu	Leu	Ser	Phe	Ala
	290					295					300				
Ala	Ile	His	Gly	Thr	Ser	Asn	Leu	Leu	Ala	Gln	Ala	Leu	Ile	Asp	Leu
305					310					315					320
Cys	Gly	Gln	Pro	Glu	Leu	Val	Gln	Asp	Leu	Arg	Glu	Glu	Ala	Val	Ser
				325					330					335	
Val	Leu	Gly	Lys	Glu	Gly	Trp	Thr	Arg	Ala	Ala	Leu	Tyr	Gln	Leu	Lys
			340					345					350		
Leu	Met	Asp	Ser	Ala	Leu	Lys	Glu	Ser	Gln	Arg	Leu	Ala	Pro	Asn	Arg
		355					360					365			
Leu	Leu	Ser	Met	Gly	Arg	Ile	Ala	Gln	Gly	Asp	Met	Asp	Leu	Ser	Asp
	370					375					380				
Gly	Leu	Arg	Ile	His	Arg	Gly	Thr	Thr	Leu	Met	Val	Ser	Ala	His	Asn
385					390					395					400
Met	Trp	Asp	Pro	Glu	Ile	Tyr	Pro	Asp	Pro	Arg	Lys	Tyr	Asp	Gly	Tyr
				405					410					415	
Arg	Phe	His	Lys	Leu	Arg	Gln	Thr	Ser	Gly	Gln	Glu	Gly	Gln	His	Gln
			420					425					430		
Leu	Val	Ser	Ser	Thr	Pro	Asp	His	Met	Gly	Phe	Gly	Tyr	Gly	Lys	His
		435					440					445			
Ala	Cys	Pro	Gly	Arg	Phe	Phe	Ala	Ala	Ala	Gln	Ile	Lys	Val	Ala	Leu
	450					455					460				
C															

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SEQ ID NO: 270

Polypeptide 505 amino acids (P450-2)

From Penicillium coprobium PF1169

Met	Ile	Glu	Leu	Lys	Asp	Ala	Ser	Met	Gly	Ala	Val	Leu	Leu	Thr	Cys	1	5	10	15
Val	Leu	Val	Leu	Ala	Gly	Leu	Tyr	Leu	Ile	Arg	Leu	Thr	Leu	Ser	Ser	20	25	30	
Asp	Gln	Leu	Asp	Lys	Phe	Pro	Ser	Ile	Asn	Pro	Arg	Lys	Pro	Trp	Glu	35	40	45	
Ile	Val	Asn	Val	Phe	Ala	Gln	Arg	Arg	Phe	Gln	Gln	Asp	Gly	Pro	Arg	50	55	60	
Tyr	Leu	Glu	Ala	Gly	Tyr	Ala	Lys	Ser	Pro	Ile	Phe	Ser	Val	Val	Thr	65	70	75	80
Asp	Leu	Gly	Pro	Lys	Leu	Val	Val	Ser	Gly	Ala	Phe	Ile	Glu	Glu	Phe	85	90	95	
Lys	Asp	Glu	Lys	Leu	Leu	Asp	His	Tyr	Arg	Ser	Met	Ile	Glu	Asp	Phe	100	105	110	
Met	Ala	Glu	Val	Pro	Gly	Phe	Glu	Ser	Met	Phe	Leu	Gly	Asn	Leu	His	115	120	125	
Asn	Thr	Val	Leu	Arg	Asp	Val	Ile	Ser	Val	Ile	Thr	Arg	Glu	Leu	Glu	130	135	140	
Gln	Leu	Leu	Ala	Pro	Leu	Ser	Asp	Glu	Val	Ser	Ala	Ala	Leu	Val	Asp	145	150	155	160
Thr	Trp	Thr	Asp	Ser	Pro	Asp	Trp	His	Glu	Val	Ala	Leu	Leu	Pro	Ser	165	170	175	
Met	Leu	Gly	Leu	Ile	Ala	Lys	Val	Ser	Ser	Leu	Val	Phe	Val	Gly	Glu	180	185	190	
Pro	Leu	Cys	Arg	His	Pro	Val	Trp	Leu	Glu	Thr	Val	Ile	Asn	Phe	Thr	195	200	205	
Leu	Ile	Arg	His	Asn	Ala	Ile	Leu	Ala	Leu	His	Gln	Cys	Pro	Ala	Val	210	215	220	
Leu	Arg	Pro	Val	Leu	His	Trp	Val	Leu	Pro	Pro	Cys	Gln	Lys	Leu	Arg	225	230	235	240
Arg	Glu	Ile	Arg	Thr	Ala	Arg	Thr	Leu	Ile	Asp	Ser	Ala	Leu	Glu	Lys	245	250	255	
Ser	Arg	Lys	Asn	Pro	Gln	Thr	Glu	Lys	Phe	Ser	Ser	Val	Ala	Trp	Val	260	265	270	
Asp	Ala	Phe	Ala	Lys	Gly	Asn	Lys	Tyr	Asn	Ala	Ala	Met	Val	Gln	Leu	275	280	285	
Arg	Leu	Ala	Asn	Ala	Ser	Ile	His	Ser	Ser	Ala	Asp	Leu	Leu	Val	Lys	290	295	300	
Ile	Leu	Ile	Asn	Leu	Cys	Glu	Gln	Pro	Glu	Leu	Ile	Arg	Asp	Leu	Arg	305	310	315	320
Asp	Glu	Ile	Ile	Ser	Val	Leu	Gly	Glu	Asn	Gly	Trp	Arg	Ser	Ser	Thr	325	330	335	
Leu	Asn	Gln	Leu	Lys	Leu	Leu	Asp	Ser	Val	Leu	Lys	Glu	Ser	Gln	Arg	340	345	350	
Leu	His	Pro	Val	Thr	Thr	Gly	Ala	Phe	Ser	Arg	Phe	Thr	Arg	Gln	Asp	355	360	365	
Ile	Lys	Leu	Thr	Asn	Gly	Thr	Glu	Ile	Pro	Ser	Gly	Thr	Pro	Ile	Met	370	375	380	

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Val	Thr	Asn	Asp	Val	Ala	Gly	Asp	Ala	Ser	Ile	Tyr	Asp	Asp	Pro	Asp
385					390					395					400
Val	Phe	Asp	Gly	Tyr	Arg	Tyr	Phe	Arg	Met	Arg	Glu	Gly	Ala	Asp	Lys
			405						410					415	
Ala	Arg	Ala	Pro	Phe	Thr	Thr	Thr	Gly	Gln	Asn	His	Leu	Gly	Phe	Gly
			420					425					430		
Tyr	Gly	Lys	Tyr	Ala	Cys	Pro	Gly	Arg	Phe	Phe	Ala	Ala	Thr	Glu	Ile
		435					440					445			
Lys	Ile	Ala	Leu	Cys	His	Met	Leu	Leu	Lys	Tyr	Glu	Trp	Arg	Leu	Val
	450					455					460				
Lys	Asp	Arg	Pro	His	Gly	Ile	Val	Thr	Ser	Gly	Phe	Ala	Ala	Phe	Arg
465					470					475					480
Asp	Pro	Arg	Ala	Ser	Ile	Glu	Val	Arg	Arg	Arg	Ala	Val	Ala	Gly	Glu
				485					490					495	
Glu	Leu	Glu	Val	Leu	Thr	Gly	Lys	Lys							
			500					505							

SEQ ID NO: 273

Polypeptide 317 amino acids (UbiAPT)

From Penicillium coprobium PF1169

Met	Ala	Gly	Ser	Gln	Ser	Thr	Ala	Gln	Leu	Ala	Arg	Leu	Leu	Ile	Asp
1				5					10					15	
Ile	Ser	Arg	Phe	Asp	Lys	Tyr	Asn	Cys	Leu	Phe	Ala	Ile	Phe	Pro	Gly
			20					25					30		
Val	Trp	Ser	Ile	Phe	Leu	Ala	Ala	Ala	Ser	Arg	His	Ala	Asp	Gly	Asp
		35				40						45			
Pro	Val	Pro	Leu	Asp	Phe	Val	Leu	Gly	Arg	Ala	Gly	Leu	Ala	Phe	Met
	50					55					60				
Tyr	Thr	Tyr	Met	Leu	Ser	Gly	Ala	Gly	Met	Val	Trp	Asn	Asp	Trp	Ile
65					70					75					80
Asp	Arg	Asp	Ile	Asp	Ala	Gln	Val	Ala	Arg	Thr	Lys	Asn	Arg	Pro	Leu
				85					90					95	
Ala	Ser	Gly	Arg	Leu	Ser	Thr	Arg	Ala	Ala	Leu	Ile	Trp	Met	Leu	Val
			100					105					110		
Gln	Tyr	Ala	Ala	Ser	Val	Trp	Leu	Met	Asp	Arg	Met	Val	Ser	Gly	Gln
		115					120					125			
Asp	Val	Trp	Thr	Tyr	Met	Leu	Pro	Leu	Thr	Thr	Gly	Ile	Ile	Leu	Tyr
	130					135					140				
Pro	Phe	Gly	Lys	Arg	Pro	Thr	Ser	Arg	Lys	Leu	Gly	Val	Tyr	Pro	Gln
145					150					155					160
Tyr	Ile	Leu	Gly	Ala	Ser	Ser	Ala	Leu	Thr	Ile	Leu	Pro	Ala	Trp	Ala
			165					170						175	
Ser	Val	Tyr	Thr	Gly	Arg	Ile	Ser	Leu	Lys	Asp	Leu	Gly	Met	Arg	Cys
			180					185					190		
Leu	Pro	Leu	Cys	Leu	Phe	Leu	Phe	Leu	Trp	Thr	Ile	Tyr	Phe	Asn	Thr
		195				200						205			
Ala	Tyr	Ser	Tyr	Gln	Asp	Ile	Lys	Asp	Asp	Cys	Lys	Leu	Asn	Val	Asn
	210					215									
Ser	Ser	Tyr	Val	Leu	Ala	Gly	Ser	His	Val	Arg	Gly	Met	Leu	Leu	Leu
225					230					235					240
Gln	Ala	Ile	Ala	Val	Val	Leu	Val	Ile	Pro	Trp	Ile	Leu	Tyr	Thr	Ser

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				245					250					255			
Ala	Ser	Thr	Trp	Leu	Trp	Val	Ser	Trp	Leu	Gly	Val	Trp	Thr	Ala	Ser		
			260					265					270				
Leu	Gly	Glu	Gln	Leu	Tyr	Leu	Phe	Asp	Val	Lys	Asp	Pro	Ser	Ser	Gly		
		275					280					285					
Gly	Lys	Val	His	Arg	Arg	Asn	Phe	Ala	Leu	Gly	Ile	Trp	Asn	Val	Leu		
	290					295					300						
Ala	Cys	Phe	Val	Glu	Leu	Leu	Tyr	Ala	Ser	Gly	Ser	Leu					
305					310					315							

SEQ ID NO: 274

Polypeptide 522 amino acids (Acetyltransferase [AT])

From Penicillium coprobium PF1169

Met	Ser	Thr	Gln	Glu	Val	Cys	Leu	Pro	Val	Ser	Gln	Arg	Asp	Gln	Val		
1			5					10					15				
Lys	Glu	Gly	Pro	Val	Arg	Leu	His	Gly	Leu	Cys	Glu	Asp	Gly	Met	Cys		
			20					25					30				
Asp	Ala	Arg	Arg	Thr	Gly	Asp	Arg	Ser	Ala	Tyr	Pro	Leu	Ser	Ser	Leu		
		35					40					45					
Asp	His	Asn	Pro	Leu	Gly	Met	Asn	Val	Thr	Phe	Leu	Leu	Phe	Phe	Gln		
	50					55					60						
Thr	Thr	Gln	Pro	Glu	Lys	Ser	Ile	Gly	Val	Leu	Glu	Asn	Gly	Ile	Glu		
65					70						75				80		
Leu	Leu	Leu	Lys	Val	His	Pro	Phe	Leu	Ala	Gly	Asp	Val	Thr	Arg	Arg		
			85						90					95			
Thr	Glu	Ser	Ser	Gln	Thr	Lys	Tyr	Thr	Trp	Gln	Ile	Glu	Pro	Glu	Ala		
			100					105						110			
Ser	Glu	Ser	Leu	Val	Gln	Phe	Pro	Ile	Leu	Arg	Ile	Arg	His	Tyr	Gln		
		115					120					125					
Ala	Glu	Ser	Phe	Lys	Glu	Ile	Gln	Ser	Lys	Cys	Leu	Leu	Thr	Gly	Thr		
	130					135					140						
Glu	Glu	Gln	Glu	Ile	Ile	Ser	Arg	Leu	Ala	Pro	Leu	Pro	Ile	Asp	Met		
145					150					155					160		
Asp	Ile	Ser	Leu	Pro	Arg	Arg	Pro	Ile	Leu	Arg	Phe	Gln	Ala	Asn	Val		
			165						170					175			
Met	Arg	Asp	Gly	Ile	Ile	Leu	Ala	Met	Thr	Phe	His	His	Ser	Ala	Met		
		180						185						190			
Asp	Gly	Ala	Gly	Ala	Ala	Arg	Val	Leu	Gly	Leu	Leu	Ala	Asp	Cys	Cys		
		195					200					205					
Arg	Asp	Pro	Thr	Ala	Met	Ser	Ser	Ala	Ser	Val	Ser	Pro	Asp	Arg	Gln		
	210					215						220					
Leu	Arg	Ser	Glu	Ile	Glu	Arg	Leu	Val	Pro	Glu	Ser	Ser	Ser	Gly	Leu		
225					230					235					240		
Ser	Arg	Met	Asp	Phe	Ser	Lys	His	Tyr	Cys	Gly	Leu	Gly	Asp	Trp	Ala		
			245						250					255			
Ala	Leu	Leu	Ala	Lys	Asn	Trp	Ser	Gly	Phe	Val	Arg	Ala	Arg	Ala	Thr		
			260					265						270			
Glu	Leu	Val	Thr	Trp	Arg	Leu	Lys	Ile	Pro	Gly	Pro	Lys	Ile	Glu	Tyr		
		275					280					285					
Leu	Lys	Glu	Ala	Cys	Asn	Thr	Leu	Ile	Lys	Gly	Gln	Thr	Ser	Phe	Gln		
	290					295					300						

Ala	Asp	Gly	Arg	Pro	Ser	Pro	Gly	Phe	Leu	Ser	Ser	Asn	Asp	Ile	Val
305					310					315					320
Ser	Ala	Leu	Leu	Ala	Met	Ile	Leu	Arg	Gln	Ala	Gly	Gln	Leu	Ala	Gly
				325					330					335	
Lys	Ser	Thr	Glu	Leu	Ser	Ile	Ala	Val	Asp	Met	Arg	Gly	Asn	Phe	Lys
			340					345					350		
Thr	Pro	Ala	Phe	Asp	Asp	Tyr	Leu	Gly	Asn	Met	Val	Leu	Leu	Thr	Tyr
		355					360					365			
Thr	Pro	Ile	Gln	Ala	Gly	Arg	Asn	Glu	Ala	Leu	Val	Asp	Gly	Thr	Asp
	370					375					380				
Pro	Ser	Val	Glu	Leu	Arg	Gln	Glu	Cys	Leu	Glu	Asp	Leu	Thr	Gln	Ile
385					390					395					400
Ala	Ala	Arg	Ile	Arg	Gln	Ser	Leu	Leu	Ala	Val	Asp	Ala	Glu	Tyr	Ile
				405					410					415	
Gln	Asp	Ala	Leu	Ser	His	Leu	His	Ser	Gln	Pro	Asp	Trp	Ala	Asp	Ile
			420					425					430		
Gly	Phe	Arg	Gly	Val	Pro	Ile	Pro	Leu	Ser	Ser	Phe	Arg	Asn	Phe	Glu
		435					440					445			
Ile	Phe	Gly	Leu	Asp	Phe	Gly	Glu	Ser	Leu	Gly	Ala	Gln	Pro	Arg	Gly
	450					455					460				
Phe	Gln	Leu	His	Leu	Pro	Val	Leu	Gly	Gly	Met	Cys	Phe	Ile	Leu	Pro
465					470					475					480
Lys	Gly	Gln	Asp	Asp	Val	Ala	Ser	Thr	Glu	Pro	Trp	Asp	Leu	His	Leu
				485					490					495	
Thr	Leu	Asn	Arg	Asp	Asp	Gln	Leu	Leu	Leu	Ala	Lys	Asp	Pro	Leu	Phe
			500					505					510		
Cys	Trp	Ala	Ile	Gly	Ala	Gln	Ala	Lys	Glu						
		515					520								

CLAIMS:

[1] An isolated polynucleotide, which is selected from the polynucleotide defined in any of (a) to (f) below:

- (a) a polynucleotide the nucleotide sequence of which is set forth as SEQ ID NO:266,
- (b) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is selected from SEQ ID NOs:269, 270, 273 and 274,
- (c) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:269 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has hydroxylase activity,
- (d) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:270 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has hydroxylase activity,
- (e) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:273 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has prenyltransferase activity, and
- (f) a polynucleotide encoding a polypeptide, wherein the amino acid sequence of the polypeptide is set forth as SEQ ID NO:274 in which 1 to 40 amino acids are substituted, deleted, added or inserted, and which has acetyltransferase activity.

[2] The polynucleotide according to claim 1, wherein the amino acid sequences of the polypeptides encoded by the polynucleotides of said (c), (d), (e) and (f) are amino acid sequences of SEQ ID NOs:269, 270, 273 and 274 in which 1 to 8 amino acids are substituted, deleted, added or inserted, respectively.

[3] The polynucleotide according to claim 1, wherein the amino acid sequences of the polypeptides encoded by the polynucleotides of said (c), (d), (e) and (f) are amino acid sequences of SEQ ID NOs:269, 270, 273 and 274 in which 1 to 4 amino acids are substituted, deleted, added or inserted, respectively.

[4] The polynucleotide according to claim 1, wherein in any of (c) to (f), 1 to 40 amino acids are conservatively substituted.

[5] The polynucleotide according to any one of claims 1 to 4, encoding at least one polypeptide involved in biosynthesis of pyripyropene A.

[6] An isolated polynucleotide, which has a nucleotide sequence selected from the nucleotide sequence in any of (i), (ii), (iii) and (iv) below:

(i) a nucleotide sequence from 13266 to 15144 of the nucleotide sequence shown in SEQ ID NO:266,

(ii) a nucleotide sequence from 16220 to 18018 of the nucleotide sequence shown in SEQ ID NO:266,

(iii) a nucleotide sequence from 21793 to 22877 of the nucleotide sequence shown in SEQ ID NO:266,

(iv) a nucleotide sequence from 23205 to 24773 of the nucleotide sequence shown in SEQ ID NO:266.

[7] The polynucleotide according to claim 6, encoding at least one polypeptide involved in biosynthesis of pyripyropene A.

[8] The polynucleotide according to claim 6 or 7, encoding a polypeptide having activity to hydroxylate the 7-position and/or 13-position of pyripyropene E or pyripyropene O.

[9] The polynucleotide according to claim 6 or 7, encoding a polypeptide having activity to hydroxylate the 11-position of pyripyropene E.

[10] A recombinant vector comprising any one or more of said polynucleotide according to claim 1 or 6.

[11] The recombinant vector according to claim 10, comprising a polynucleotide encoding a polypeptide having activity to hydroxylate the 7-position and/or 13-position of pyripyropene E or pyripyropene O.

[12] The recombinant vector according to claim 10, comprising a polynucleotide encoding a polypeptide having activity to hydroxylate the 11-position of pyripyropene E.

[13] A cell comprising plasmid pCC1-PP1 as deposited under accession No. FERM BP-11133.

[14] A cell comprising plasmid pPP2 as deposited under accession No. FERM BP-11137.

[15] A cell comprising plasmid pPP3 as deposited under accession No. FERM BP-11141.

[16] A transformed cell comprising any one or more of said polynucleotide according to claim 1 or claim 6.

[17] The transformed cell according to claim 16, wherein said transformed cell is *Escherichia coli* comprising plasmid pCC1-PP1 as deposited under accession No. FERM BP-11133.

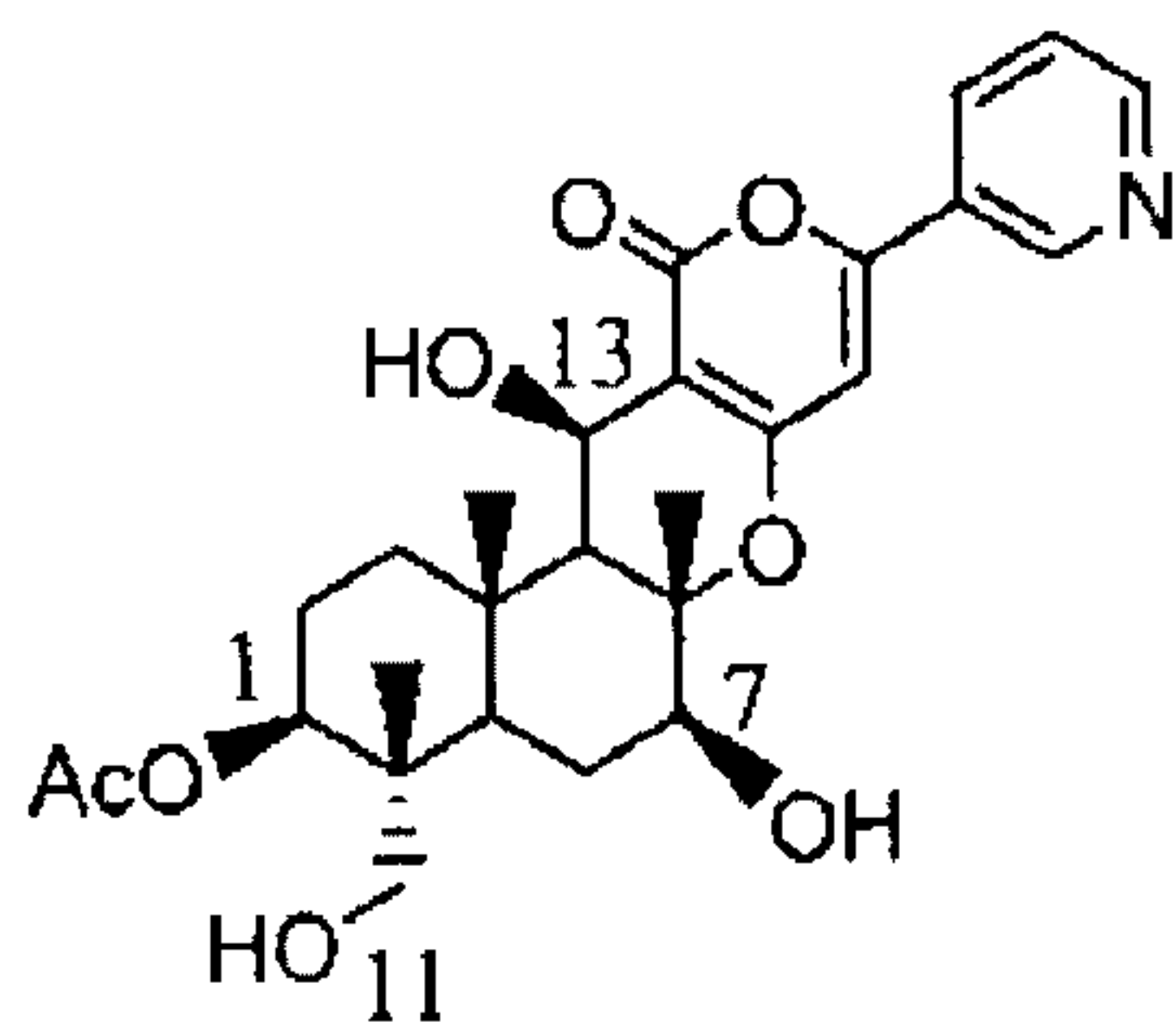
[18] The transformed cell according to claim 16, wherein said transformed cell is *Aspergillus oryzae* comprising plasmid pPP2 as deposited under accession No. FERM BP-11137.

[19] The transformed cell according to claim 16, wherein said transformed cell is *Aspergillus oryzae* comprising plasmid pPP3 as deposited under accession No. FERM BP-11141.

[20] A method for producing a pyripyropene A precursor, which comprises culturing a microorganism comprising any one or more of said vectors according to any one of claims 10 to 12 and isolating the pyripyropene A precursor from pyripyropene E.

[21] A method for producing a pyripyropene A precursor, which comprises culturing said transformed cell according to claim 18 and said transformed cell according to claim 19 simultaneously or separately, and isolating the pyripyropene A precursor from pyripyropene E.

[22] The production method according to claim 20 or 21, wherein said pyripyropene A precursor is represented by formula (I) below:

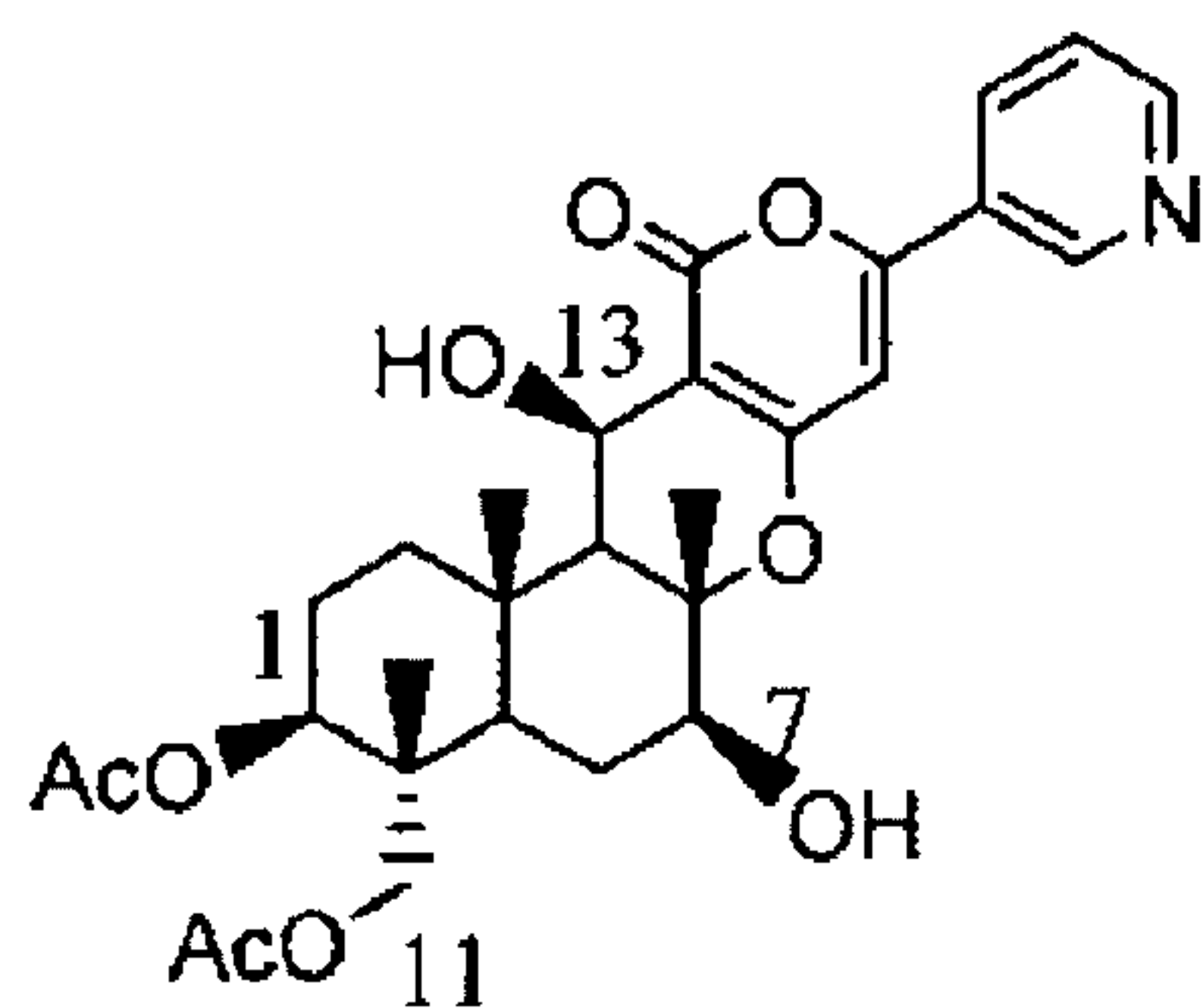


formula (I).

[23] A method for producing a pyripyropene A precursor, which comprises culturing a microorganism comprising any one or more of said vectors according to any one of claims 10 to 12 and isolating the pyripyropene A precursor from pyripyropene O.

[24] A method for producing a pyripyropene A precursor, which comprises culturing the transformed cell according to claim 19 and isolating the pyripyropene A precursor from pyripyropene O.

[25] The production method according to claim 23 or 24, wherein said pyripyropene A precursor is represented by formula (II) below:



formula (II).

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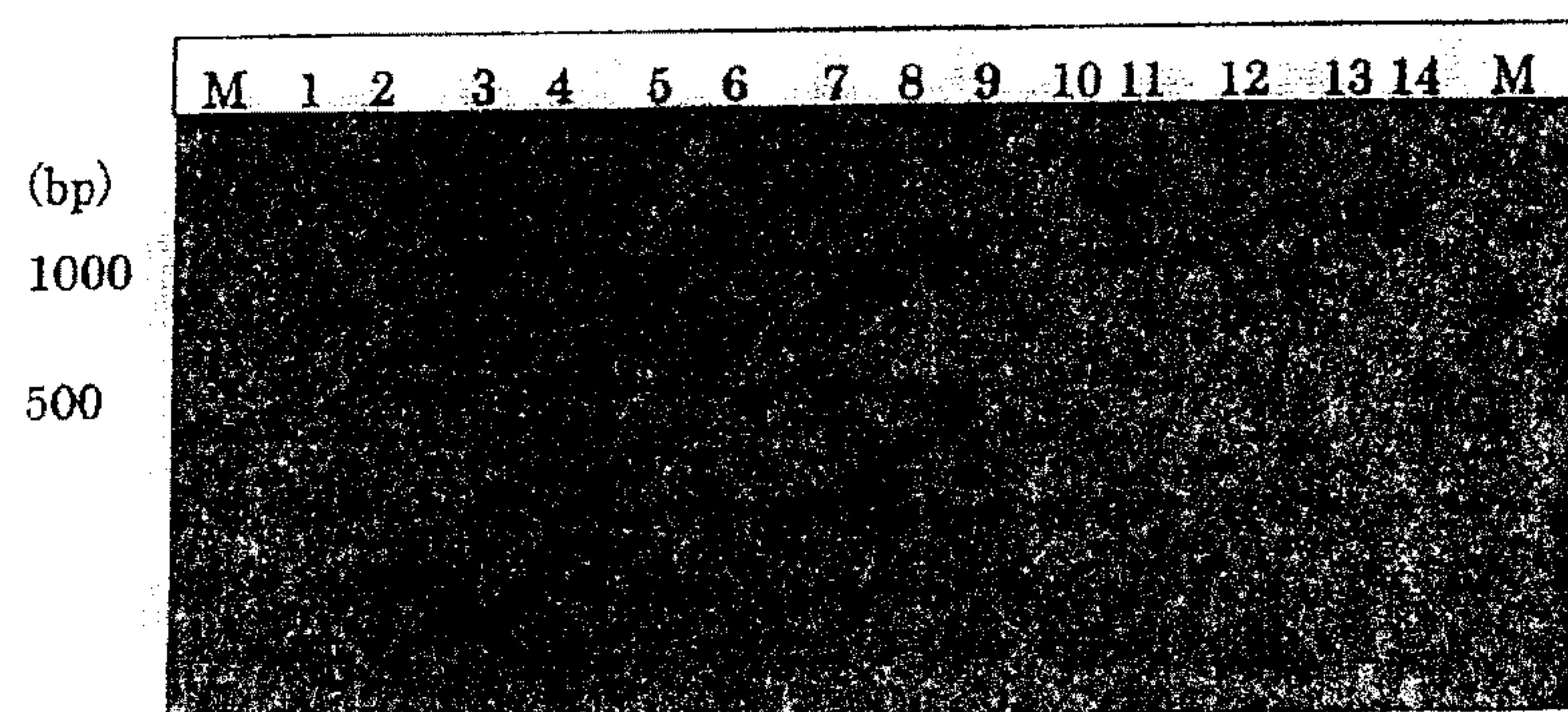


FIG.1

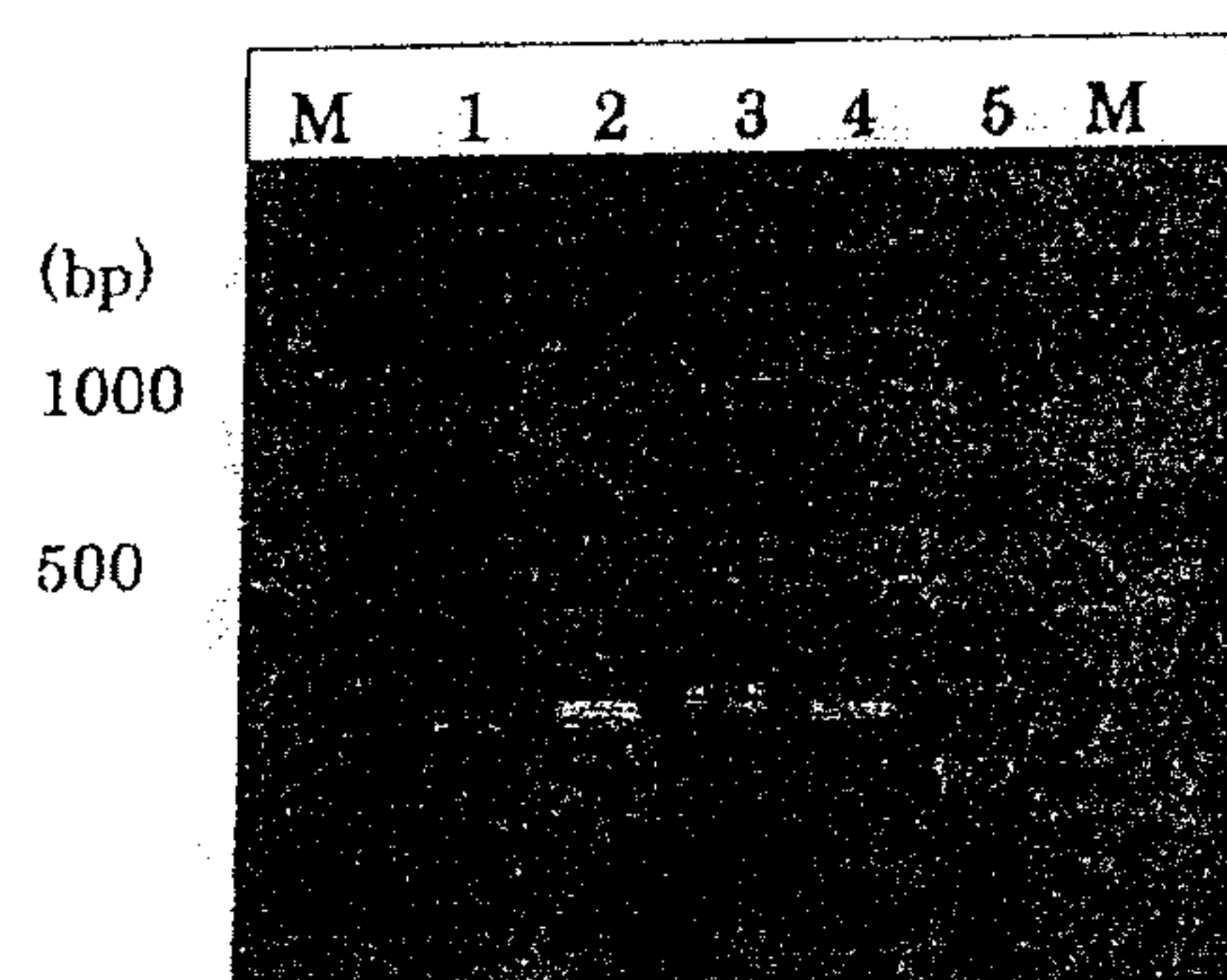


FIG.2

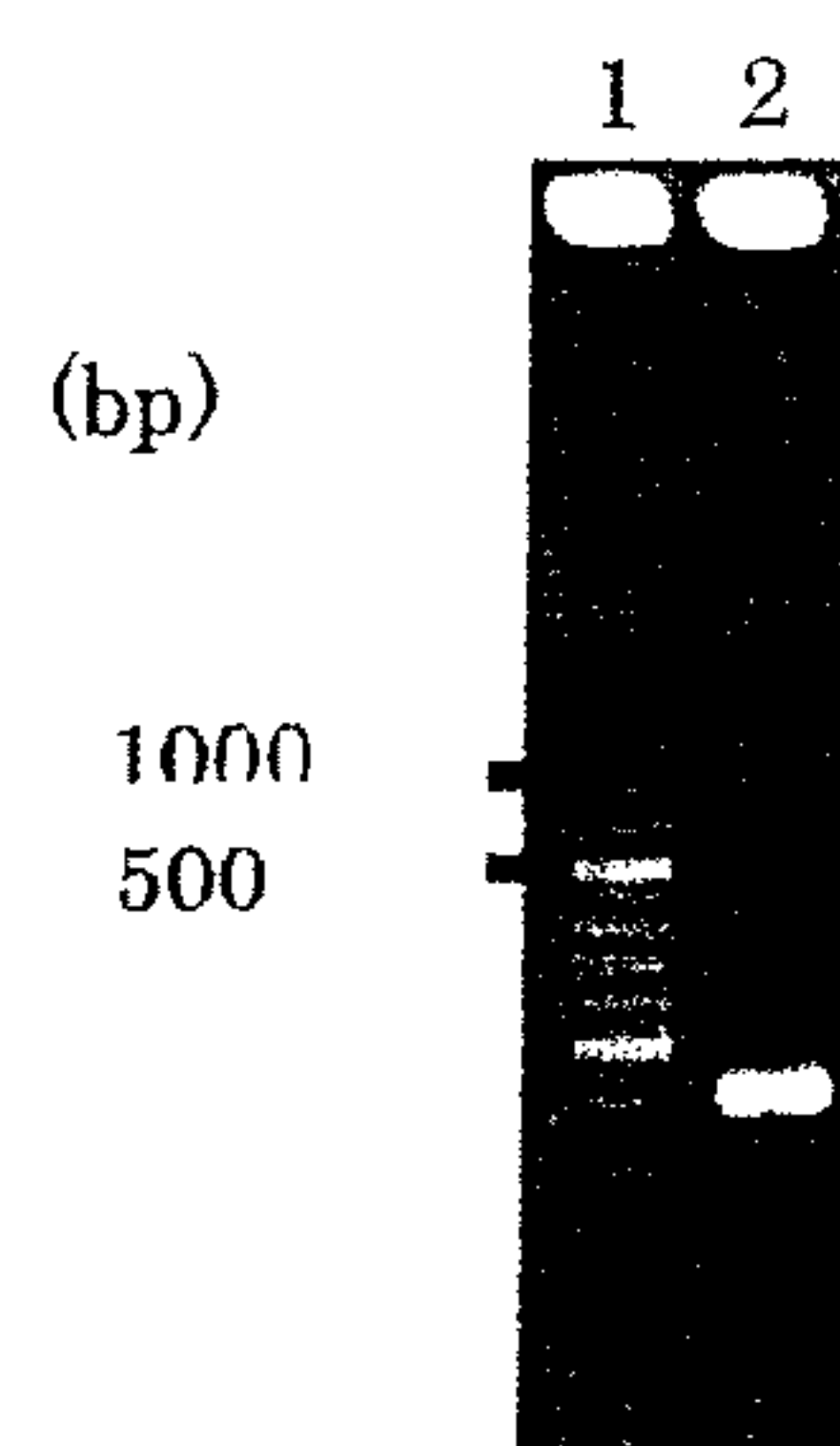


FIG.3

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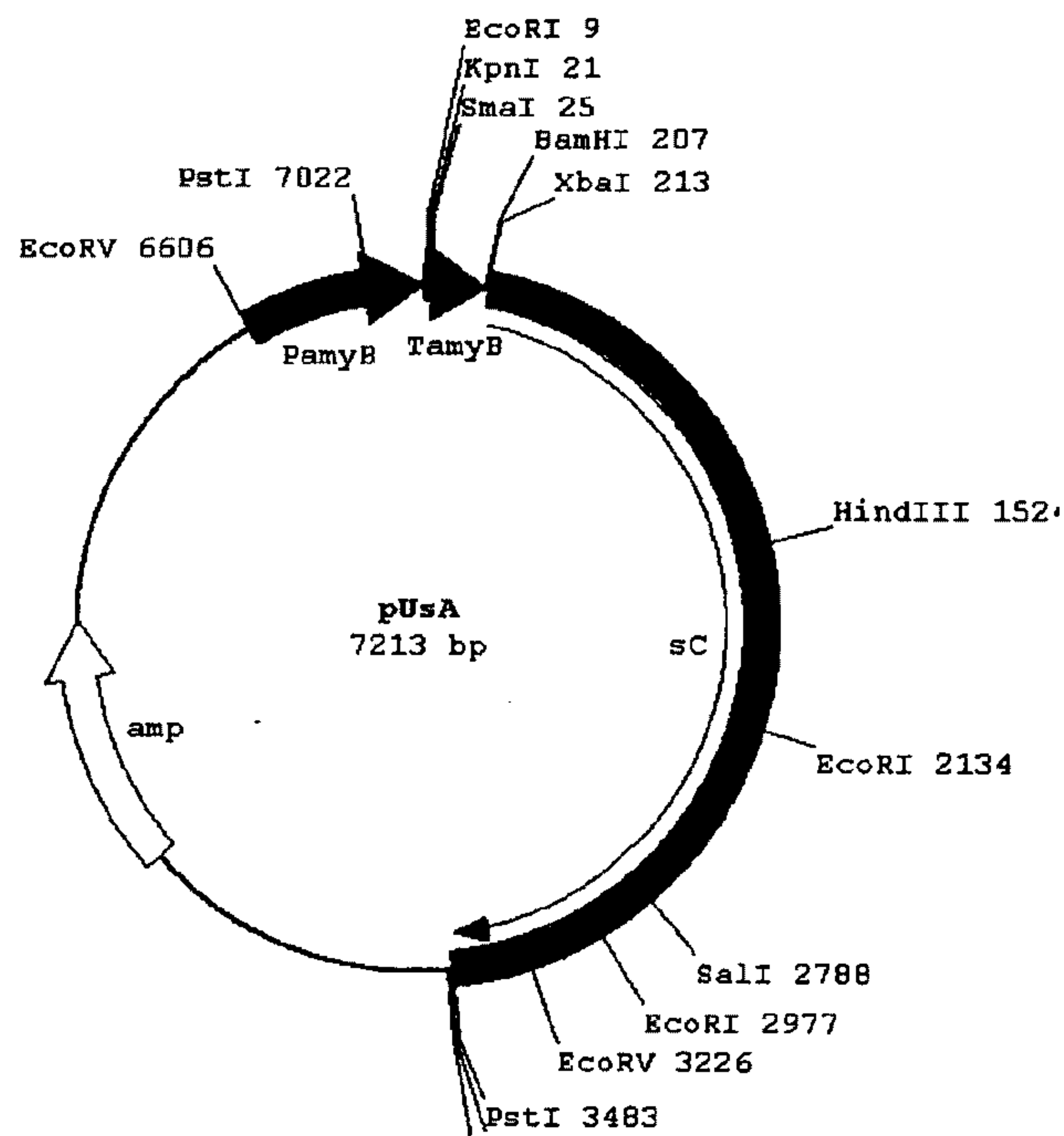


FIG.4

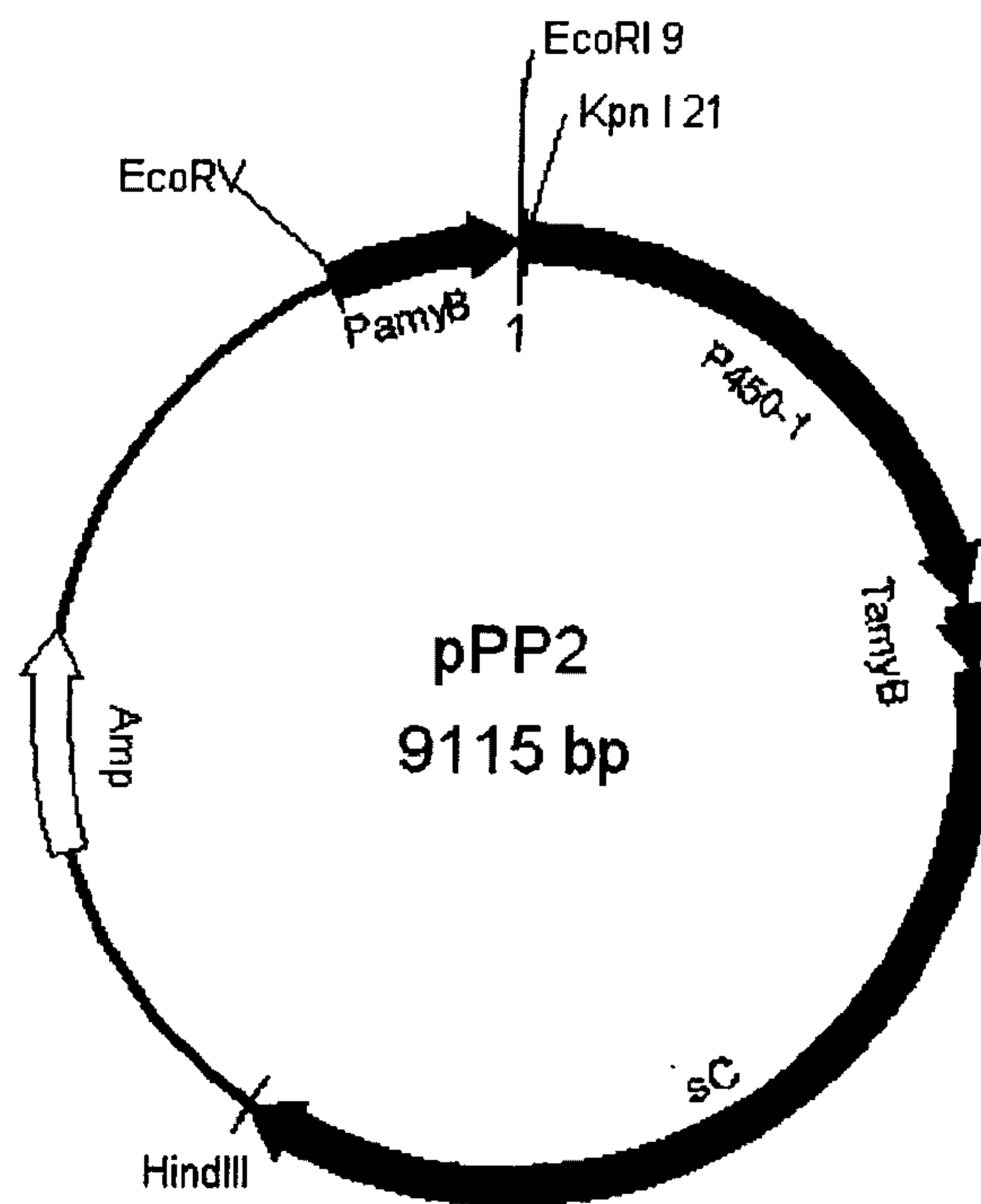


FIG.5

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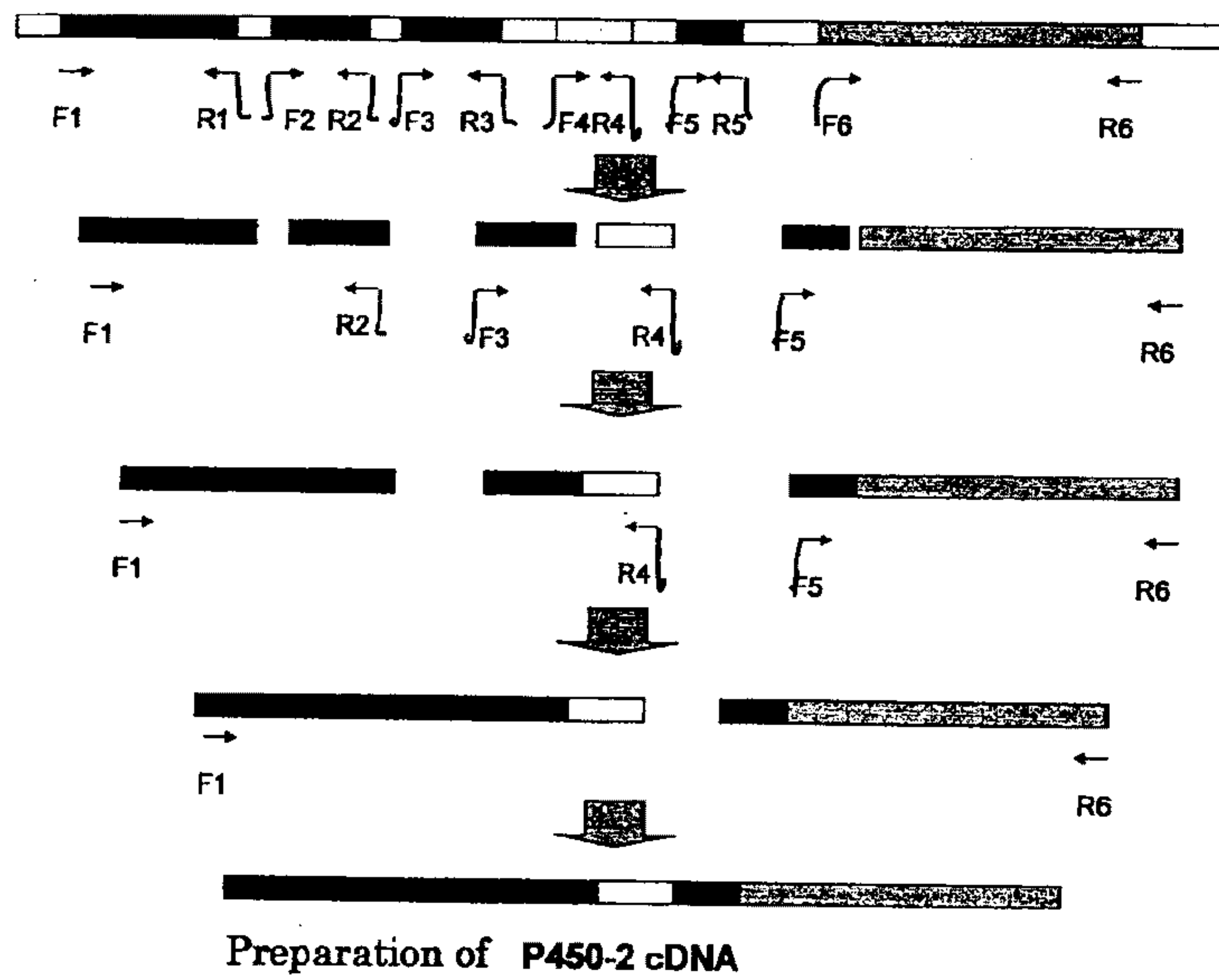


FIG.6

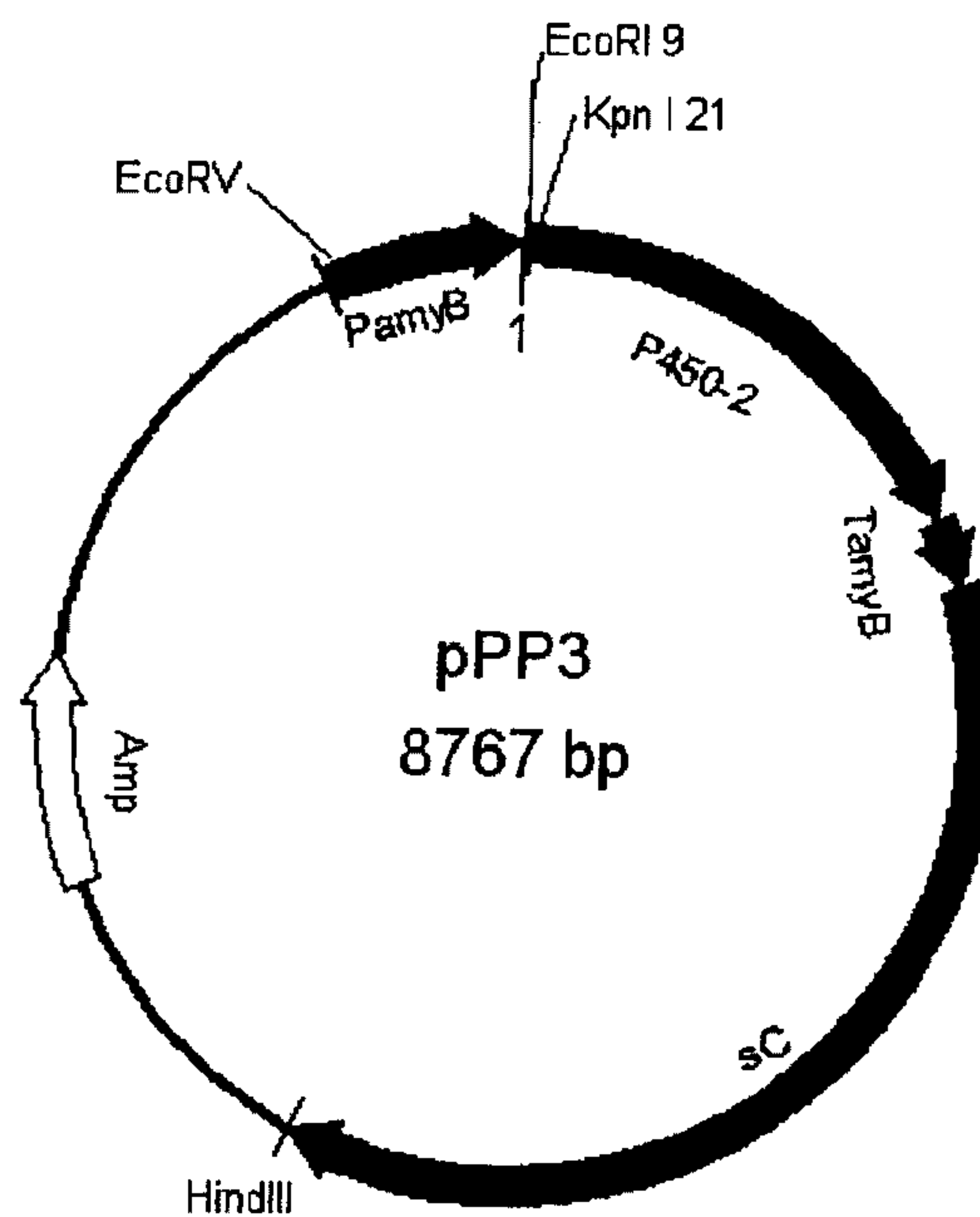


FIG.7

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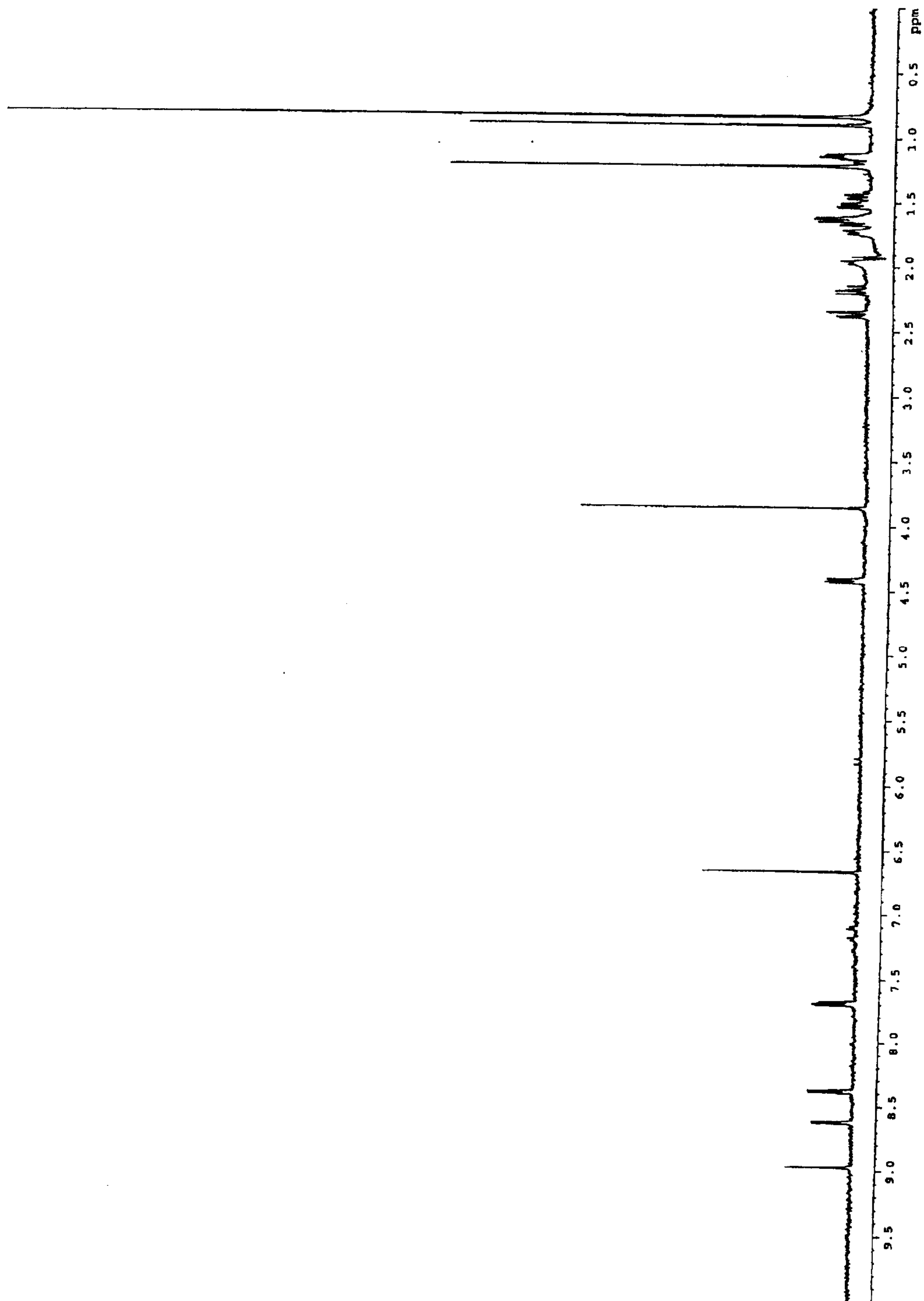


FIG.8

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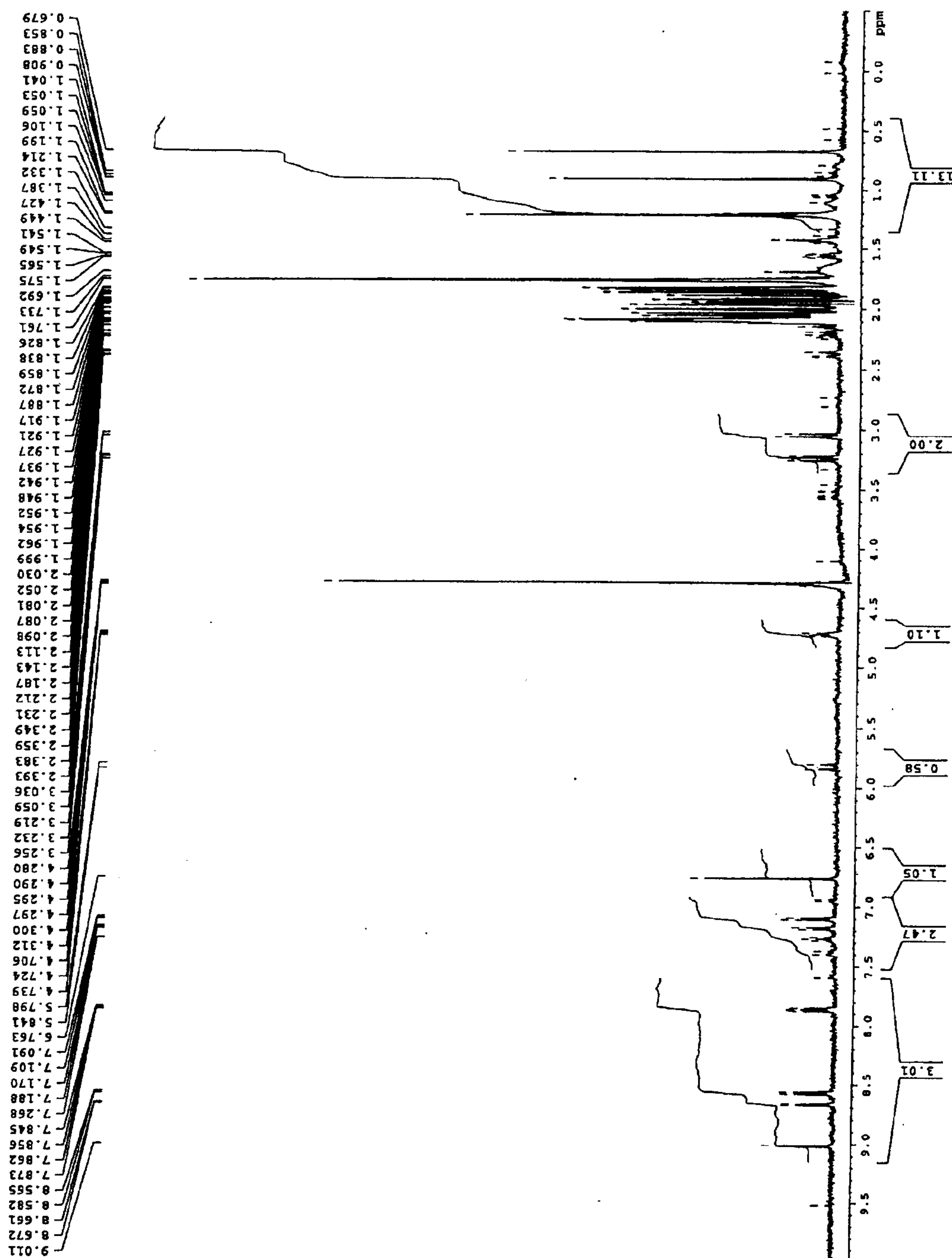


FIG.9

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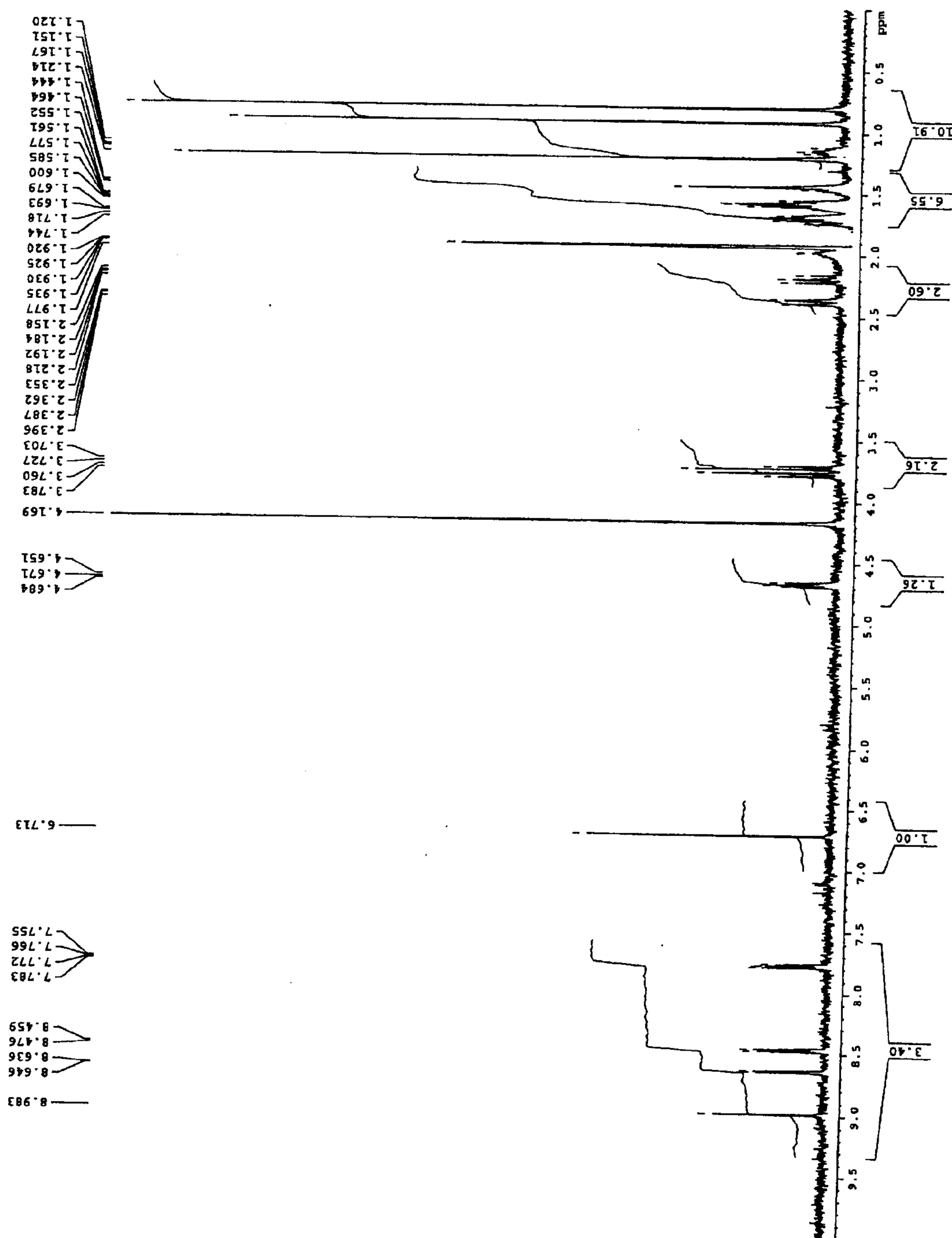


FIG.10

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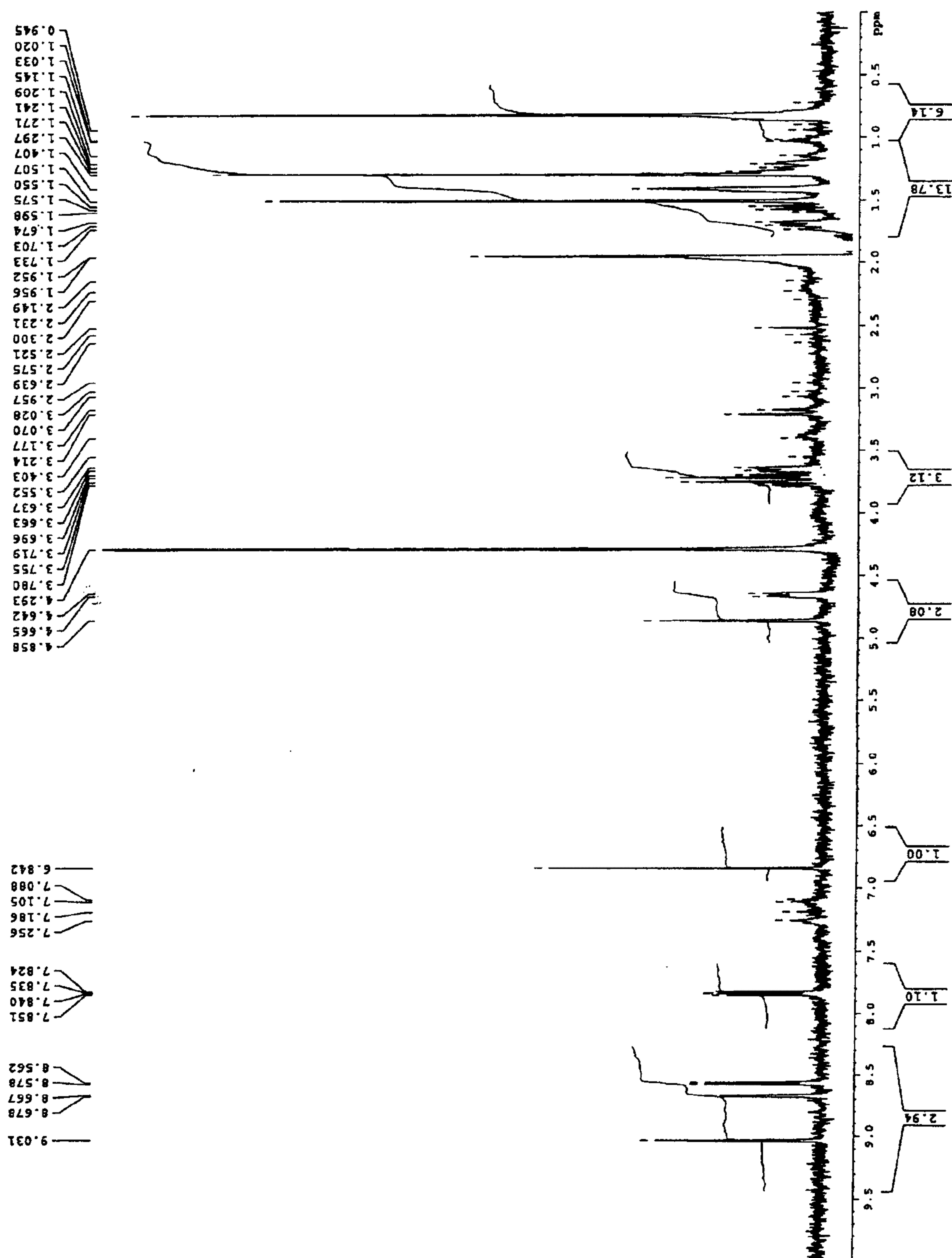


FIG.11