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(54) LANTIBIOTIC BIOSYNTHETIC GENE CLUSTERS FROM A. GARBADINENSIS AND A. LIGURIAE

BIOSYNTHETISCHE GENCLUSTER FÜR LANTIBIOTIKUM AUS A. GARBADINENSIS UND A. LIGURIAE

GROUPES DE GENES BIOSYNTHETIQUES LANTIBIOTIQUES D A. GARBADINENSIS ET A. LIGURIAE

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Description

Field Of The Invention

[0001] This invention relates to characterisation of the biosynthetic gene cluster for the lantibiotic actagardine, identification of a novel variant of actagardine and its biosynthetic cluster, and methods of production and use of actagardine, a novel actagardine variant produced in a strain of A. *liguriae,* and variants of both of these produced according to this invention, utilizing genes from the characterised biosynthetic gene clusters.

Background Of The Invention

[0002] Lantibiotics are peptides having antibiotic and other activities, produced by Gram-positive bacteria. They contain, among other modified residues, the thioether amino acids lanthionine and methyllanthionine, which cross- link the peptide chain into a polycyclic structure. They have been classified into two classes, type-A and type-B, though such classification is not unproblematic. Type-A lantibiotics are generally elongate amphiphiles that are capable of forming pores in bacterial and other plasma membranes. Examples are nisin and subtilin. Type-B lantibiotics, by contrast, are globular, conformationally defined peptides that inhibit enzyme functions. Examples are cinnamycin and duramycin.

[0003] Activities ascribed to type-B lantibiotics such as cinnamycin include antimicrobial activity (providing potential application as antibiotics), inhibition of angiotensin-converting enzyme (providing a potential application in blood pressure regulation), immunomodulation via inhibition of phospholipase A2 (providing a potential application as anti-inflammatories), and interference with prostaglandin and leucotriene biosynthesis.

[0004] Type-B lantibiotics appear to exert their activity by interfering with enzyme activities by blocking the respective substrates. For example, type B lantibiotics such as mersacidin and actagardine have been found to inhibit biosynthesis of peptidoglycan; transglycosylation was identified as the target reaction. The substrate for this reaction is the lipid-bound cell wall precursor lipid II. While this is a target for the lantibiotic vancomycin, the site of action is different and is a new target binding site not used by any current antibacterial drug.

[0005] For the cinnamycin class of type B lantibiotics antibacterial activity has been observed, in particular with *Bacillus* strains, with effects described on membrane functions, ATP-dependent proton translocation and Ca^{2+} -uptake, and on ATPases. Also, the formation of defined pores in phosphatidylethanolamine-containing planar membranes has been reported. These effects can be attributed to the specific binding of these type-B lantibiotics to phosphatidylethanolamine.

[0006] Lantibiotics have been shown to have efficacy and utility as food additives and antibacterial agents against *Propionibacterium acnes* and problematic pathogens, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), which has or is developing resistance to many commonly used antibiotics, and *Streptococcus pneumoniae.* For reviews, see Sahl and Bierbaum (1998) Annual Rev. Microbiol. 52:41-79; Jack and Sahl (1995) TIBTECH 13:269 278; Gasson (1995) Chapter 10, Lantibiotics, in Vining and Stuttard (eds) Biotechnology Series: Genetics and Biochemistry of Antibiotic Production, Biotechnological I 30 Series 28, pages 283-306.

[0007] Within the field of antibiotics, there is a continuing need for the provision of new antibiotic compounds, to overcome issues such as resistance, bio-compatibility, toxicity and the like. Accordingly, methods of producing lantibiotics, and the production of variant forms of lantibiotics (which may have a different activity profile compared to native forms), are desirable.

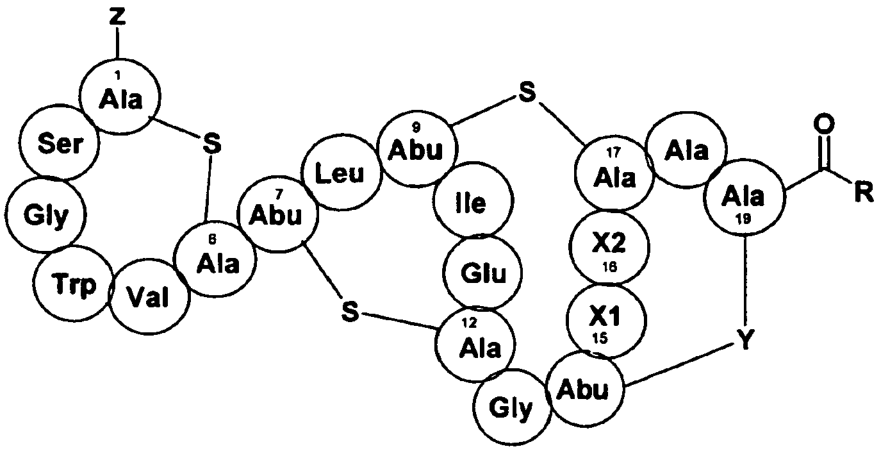
[0008] Actagardine is a known type B tetracyclic lantibiotic, 19 amino acids in length (1890 Da). It has potent activity against important Gram positive pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes* both in vitro and in in vivo animal models. The structure of actagardine is shown in Figure 4. The compound is produced from a pre-pro-peptide, the C-terminal portion of which has the polypeptide sequence of SSGWVCTLTIECGTVICAC (SEQ ID NO: 4). The polypeptide of SEQ ID NO:4 is modified by the following crosslinks, creating secondary and tertiary structure: CROSSLINK 1-6, Lanthionine (Ser-Cys); CROSSLINK 7-12, Beta-methyllanthionine (Thr-Cys); CROSSLINK 9-17, Beta-methyllanthionine (Thr-Cys); CROSSLINK 14-19, Beta-methyllanthionine sulfoxide (Thr-Cys).

[0009] Actagardine has been reported to be produced by two species of Actinoplanes; *A. garbadinensis* and *A. liguriae.* Also co-produced is an analogue in which the CROSSLINK 14-19 is not oxidized i.e. it is a beta-methyllanthionine not betamethyllanthionine sulfoxide which is named herein deoxy-actagardine.

[0010] US 6,022,851 describes the isolation of actagardine from isolated strains of *A. garbadinensis* and *A. liguriae.*

Disclosure of the Invention

[0011] The present invention relates to a compound of the formula:



wherein:

-X1-X2- represents -Leu-Val-;
-Y- is -S-;
Z is either an amino acid or $-NH_2$ wherein the latter represents the N-terminus of the Ala at position 1;
R represents -OH or $-NR^1R^2$, wherein R^1 and R^2 independently represent:

(i) hydrogen;
(ii) a group of formula $-(CH_2)_nNR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or C_{1-4}alkyl, or
R^3 and R^4 taken together represents a group $-(CH_2)_3-$, $-(CH_2)_4-$, $(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2$ or $-(CH_2)_5-$; or
R^1 and R^2 taken together with the adjacent nitrogen atom represent a piperazine moiety which may be substituted at position 4 with a substituent selected from:

(a) C_{1-4}alkyl;
(b) C_{5-7}cycloalkyl;
(c) pyridyl,
(d) $-(CH_2)_p-NR^5R^6$ in which p represents an integer from 1 to 8 and R^5 and R^6 independently represent hydrogen or C_{1-4}alkyl;
(e) piperidinyl;
(f) substitute piperidinyl, wherein the substituted piperindinyl bears a N-substituent which is C_{1-4}alkyl;
(g) benzyl; and
(h) substituted benzyl, wherein the phenyl moiety bears 1 or 2 substituents selected from chloro, bromo, nitro, C_{1-4}alkyl and C_{1-4}alkoxy,

or a pharmaceutically acceptable salt thereof.

[0012] In another aspect the invention relates to the cloned, sequenced and elucidated structural and regulatory information relevant to the biosynthetic gene cluster for the type-B lantibiotic, actagardine, from *Actinoplanes garbadinensis* and *A. liguriae.*

[0013] We have also surprisingly found that in an isolate of *A. liguriae,* designated herein as A. *liguriae* NCIMB 41362, a novel form of actagardine is produced which we have termed actagardine B or, in the non-oxidised form, deoxy-actagardine B. These forms have similar anti-microbial activity to actagardine and are generated from the primary polypeptide sequence of SEQ ID NO:1, which undergoes similar cross-linking to actagardine. The variants provide new and useful alternatives to actagardine. In addition, the identification of residues in actagardine B which are different from actagardine leads to the provision of further lantibiotics based on these differences.

[0014] We have also isolated gene clusters from both actagardine-producing *A. garbadinensis* and *A. liguriae* NCIMB 41362 which comprise the genes for the production of actagardine and actagardine B.

[0015] In one aspect, the present invention provides the novel actagardine B and variants thereof, including variants based on the primary polypeptide sequences of SEQ ID NO:2 and SEQ ID NO:3, as well as variants thereof.

[0016] In a further aspect, the invention provides nucleic acids encoding actagardine B and its variants, sets of nucleic acids and variants thereof derived from the above-mentioned gene clusters, methods of making actagardine B and its variants, and methods of generating novel variants of actagardine B.

Description Of The Drawings

[0017]

Figure 1 provides a map of the actagardine encoding and regulatory gene cluster isolated from *A. garbadinensis.*

Figure 2 provides a map of the encoding and regulatory gene cluster isolated from *A. liguriae* which encodes a novel variant of actagardine, herein referred to as actagardine B

Figure 3 provides a schematic showing a method disclosed herein for generation of actagardine variants utilizing nucleic acid sequences isolated from *A. garbadinensis* or from *A. liguriae.*

Figure 4 is a representation of the primary structure of mature actagardine where X1-X2 represent Val-Ile, Y is -S (O)- and Z is NH_2. "Deoxy-actagardine B" is the Val15Leu Ile16Val variant with a non-oxidised methyllanthionine bridge between AbuS14 and AlaS19.

Description of the Sequences

[0018] For the convenience of the reader, the sequences of the present application have been numbered non-contiguously as follows:

SEQ ID NO:1 is the primary polypeptide sequence of Actagardine B:

SSGWVCTLTIECGTLVCAC.

SEQ ID NO:2 is the primary polypeptide sequence of Actagardine B variant VV:

SSGWVCTLTIECGTVVCAC.

SEQ ID NO:3 is the primary polypeptide sequence of Actagardine B variant LI

SSGWVCTLTIECGTL1CAC.

SEQ ID NO:4 is the primary polypeptide sequence of Actagardine:

SSGWVCTLTIECGTVICAC;

SEQ ID NO:11 is the primary polypeptide sequence of Ala-Actagardine B:

ASSGWVCTLTI ECGTLVCAC.

SEQ ID NO:12 is the primary polypeptide sequence of Ala-Actagardine B variant VV:

ASSGWVCTLTIECGTVVCAC.

SEQ ID NO:13 is the primary polypeptide sequence of Ala-Actagardine B variant LI

ASSGWVCTLTIECGTLICAC.

SEQ ID NO:14 is the primary polypeptide sequence of Ala-Actagardine:

ASSGWVCTLTIECGTVICAC.

SEQ ID NO:212 is the primary polypeptide sequence of pre-pro-Actagardine B:

MSAITVETTWKNTDLREDLTAHPAGLGFGELSFEDLREDRTIYAASSGWVCTLTIECG TLVCAC.

SEQ ID NO:22 is the primary polypeptide sequence of pre-pro-Actagardine B variant VV:

MSAITVETTWKNTDLREDLTAHPAGLGFGELSFEDLREDRTIYAASSGWVCTLTIECG TWCAC.

SEQ ID NO:23 is the primary polypeptide sequence of pre-pro-Actagardine B variant LI

MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAASSGWVCTLTIECG TLICAC.

SEQ ID NO:119 is the primary polypeptide sequence of pre-pro-Actagardine:

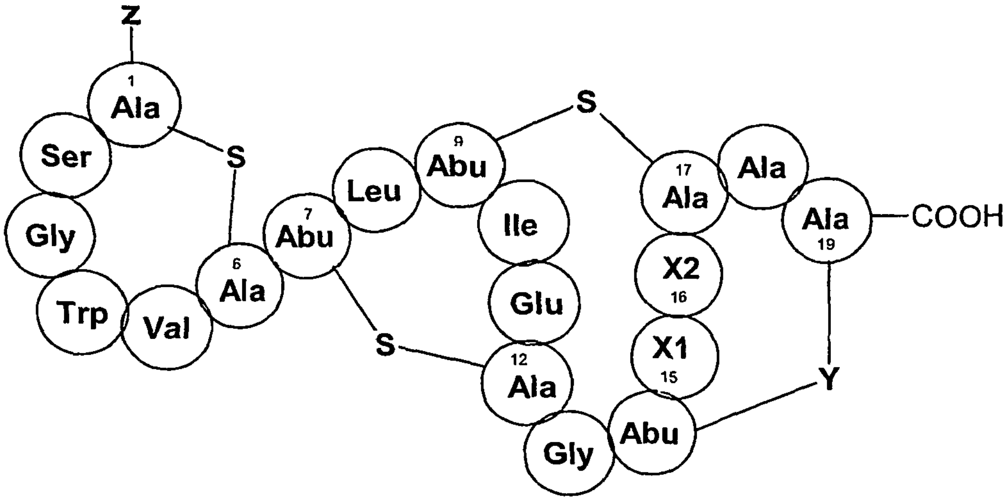
MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAASSGWVCTLTIECG TVICAC.

SEQ ID NO:100 is the non-vector, *A. garbadinensis*-derived, nucleotide sequence of the cosmid CosAG14.
***SEQ ID NOs:101 -* 132** are the polypeptide sequences of the open reading frames *orf1* - *orf32* of SEQ ID NO:100 respectively.
SEQ ID NO:200 is the non-vector, *A. liguriae*-derived, nucleotide sequence of the cosmid CosAL02.
SEQ ID NOs:201 - 231 are the polypeptide sequences of the open reading frames *orf1* - *orf31* of SEQ ID NO:200 respectively.
SEQ ID NOs:300-312 are primer sequences described herein below.

Detailed Description of the Invention

Compounds

[0019] In one aspect, the present invention provides a compound of the formula (I):



wherein:

-X1-X2- represent -Leu-Val-; -Val-Val- or -Leu-Ile-;
Y is -S- or -S(O)-; and
Z is either H_2N- or Ala-,

or a pharmaceutically acceptable salt thereof. In a further aspect, the invention provides variants and biologically active derivatives of these compounds.

[0020] Where -X1-X2- represent -Leu-Val-, Y is -S(O)- and Z is NH_2 the compound of the invention is also referred to as actagardine B.

[0021] Where -X1-X2- represent -Leu-Val-, Y is -S(O)- and Z is Ala- the compound of the invention is also referred to as ala-actagardine B.

[0022] Where -X1-X2- represent -Leu-Val-, Y is -S- and Z is NH_2 the compound of the invention is also referred to as deoxy-actagardine B.

[0023] Where -X1-X2- represent -Leu-Val-, Y is -S- and Z is Ala- the compound of the invention is also referred to as ala-deoxy-actagardine B.

[0024] It will be understood by reference to Z being a group H_2N-, that this moiety represents the N-terminus of the alanine residue at position 1 of the above compound. By reference to the group Z being Ala-, it will be understood that this moiety represents an alanine, conventionally referred to in the art as Ala(0), linked to the alanine at position 1 via an amide bond.

[0025] In one embodiment Z is an amino acid, for example selected from Ala, Ile-, Lys-, Phe-, Val-, Glu-, Asp-, His-, Leu, Arg-, Ser- and Trp- and said amino acids are in the L- or D-configuration. In one embodiment, the amino acid is Ala.

Variants

[0026] A variant of a compound of formula (I) is a compound which one of more, for example from 1 to 5, such as 1, 2, 3 or 4 amino acids are substituted by another amino acid. Preferably the amino acid is at a position selected from positions 2, 3, 4, 5, 8, 10, 11, 13 or 18 of the compound of formula (I).

[0027] A variant may also comprise a substitution at position 15 or 16, provided that when both positions 15 and 16 are substituted and none of the other positions are changes, 15 and 16 are not Val and Ile respectively.

[0028] Where Z is Ala-, variants of compounds of the invention include those in which Ala- is replaced by another amino acid (particularly a naturally occurring amino acid encoded by the genetic code or its D- isoform), more particularly an amino acid selected from the group Ile-, Lys-, Phe-, Val-, Glu-, Asp-, His-, Leu, Arg-, Ser- and Trp-. In one aspect, the amino acid may be selected from the group Ile-, Lys-, Phe-, Val-, Glu-, Asp-, His-, Leu-, Arg- and Ser-. Such variants may be produced by chemical addition of the residue to compounds where Z = H_2N , as described in US 6,022,851. It will be appreciated that the chemical addition of an amino acid allows the amino acid to be in the L- or D- configuration. This includes D-Ala, in addition to the D- forms of other amino acids such as those mentioned above.

Derivatives

[0029] Derivatives of compounds of the invention (including variants) are those in which one or more amino acid side chain of the compound of the invention has been modified, for example by esterification, amidation or oxidation.

[0030] Derivatives of compounds of the invention may be monoamide derivatives at one of the carboxy functions of actagardine, particularly at the C-terminal. More particularly, a derivative may be a compound in which the C-terminal of the compound of the invention is of the formula -COR, in which R represents the group $-NR^1R^2$, wherein R^1 and R^2 independently represent:

(i) hydrogen;
(ii) a group of formula $-(CH_2)_n-NR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or (C_1-C_4) alkyl or R^3 and R^4 taken together represent a group $-(CH_2)_3-$, $-(CH_2)_4-$, $(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2-$ or $-(CH_2)_5-$; or R^1 and R^2 taken together with the adjacent nitrogen atom represent a piperazine moiety which may be substituted in position 4 with a substituent selected from:

  (a) (C_1-C_4)alkyl;
  (b) (C_5-C_7)-cycloalkyl,
  (c) pyridyl,
  (d) $-(CH_2)_p-NR^5R^6$ in which p represents an integer from 1 to 8 and R^5 and R^6 independently represent hydrogen or (C_1-C_4) alkyl;
  (e) piperidinyl;
  (f) substituted piperidinyl, wherein the substituted piperidinyl bears a N-substituent which is (C_{1-4})alkyl;
  (g) benzyl; and
  (h) substituted benzyl, wherein the phenyl moiety bears 1 or 2 substituents selected from chloro, bromo, nitro, (C_1-C_4)alkyl and (C_1-C_4)alkoxy.

[0031] In one embodiment, in the formula -COR, R represents the group $-NR^1R^2$, wherein R^1 and R^2 independently represent hydrogen, a group of formula $-(CH_2)_n-NR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or (C_1-C_4) alkyl or R^3 and taken together represent a group $-(CH_2)_3-$, $-(CH_2)_4-$, $(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2-$ or $-(CH_2)_5-$, or R^1 and R^2 taken together with the adjacent nitrogen atom represent a piperazine moiety which may be substituted in position 4 with a substituent selected from (C_1-C_4)alkyl, (C_5-C_7)-cycloalkyl, pyridyl, benzyl, and substituted benzyl wherein the phenyl moiety bears 1 or 2 substituents selected from chloro, bromo, nitro, (C_1-C_4)alkyl and (C_1-C_4)alkoxy.

[0032] Further, a derivative may include a compound in which the carboxy function of a side chain of an internal residue, e.g. that of the residue Glu11, is modified from -COOH to a group $-COOR^5$ in which R^5 represents hydrogen, (C_1-C_4)alkyl or (C1-C_4)alkoxy (C_2-C_4)alkyl.

[0033] N-terminal derivatives of compounds of the invention may be those in which the N-terminal amino group $-NH_2$ is instead a group $-NHR^6$ wherein R^6 represents C_{1-4}alkyl.

[0034] In one embodiment R is OH.

[0035] In one embodiment R^1 and R^2 of the amino acid in the compound independently represent:

(i) hydrogen;
(ii) a group of formula $-(CH_2)_nNR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or C_{1-4}alkyl.

[0036] In one embodiment the compound is selected from the group consisting of:

deoxyactagardine B *N*-[3-dimethylaminopropyl]monocarboxamide;
deoxyactagardine B *N*-[1-(1-methyl-4-piperidinyl)piperazine]monocarboxamide;
deoxyactagardine B [1-(3-dimethylaminopropyl)piperazine]monocarboxamide;
deoxyactagardine B;
D-Ala(0)deoxyactagardine B;
L-Ile(0)deoxyactagardine B;
L-Val(0)deoxyactagardine B;
L-Phe(0)deoxyactagardine B;
L-Lys(0)deoxyactagardine B; or
L-Tryp(0)deoxyactagardine B.

[0037] The term "(C_1-C_4)alkyl" represents straight or branched alkyl chains of from 1 to 4 carbon atoms, such as: methyl, ethyl, propyl, 1-methylethyl, butyl, 1-methylpropyl or 1,1-dimethylethyl while the term "(C_2-C_4)alkyl" represents straight or branched alkyl chains of from 2 to 4 carbon atoms such as: ethyl, propyl, 1-methylethyl, butyl, 1-methylpropyl or 1,1-dimethylethyl. The term "(C_5-C_7)cycloalkyl" represents a cycloalkyl group selected from cyclopentyl, cyclohexyl and cycloheptyl.

[0038] The term "(C_1-C_4)alkoxy" represents a straight or branched alkoxy chain of 1 to 4 carbon atoms such as methoxy, ethoxy, propoxy, 1-methylethoxy, butoxy, 1-methylpropoxy and 1,1-dimethylethoxy.

[0039] Derivatives according to the present invention may be made according to the methods described for the manufacture of derivatives of actagardine in EP-0195359, the disclosure of which is incorporated herein by reference.

Further Embodiments

[0040] Where the derivative is a compound where the C-terminal is of the formula -COR, in which R represents the group $-NR^1R^2$, in some embodiments, R^1 is H and R^2 represents a group of formula $-(CH_2)_n-NR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or (C_1-C_4) alkyl or R^3 and R^4 taken together represent a group $-(CH_2)_3-$, $-(CH_2)_4-$, $(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2-$ or $-(CH_2)_5-$. In these embodiments, R^3 and R^4 preferably represent hydrogen or (C_1-C_4) alkyl. More preferably R^3 and R^4 represent (C_1-C_2) alkyl, e.g. methyl. Integer n may be preferably from 2 to 5, and more preferably 2 to 4, e.g. 3.

[0041] In other embodiments, R^1 and R^2 taken together with the adjacent nitrogen atom represent a piperazine moiety. The N-substituent in the 4 position may preferably be selected from:

(a) (C_1-C_4)alkyl;
(b) (C_5-C_7)-cycloalkyl,
(d) $-(CH_2)_p-NR^5R^6$ in which p represents an integer from 1 to 8 and R^5 and R^6 independently represent hydrogen or (C_1-C_4) alkyl;
(e) piperidinyl; and
(f) substituted piperidinyl, wherein the substituted piperidinyl bears a N-substituent which is (C_{1-4})alkyl.

[0042] The piperdinyl and substituted piperidinyl groups preferably have their nitrogen atom at the 4-position.

[0043] The N-substituent may more preferably be selected from:

(d) $-(CH_2)_p-NR^5R^6$ in which p represents an integer from 1 to 8 and R^5 and R^6 independently represent hydrogen or (C_1-C_4) alkyl; and

(f) substituted piperidinyl, wherein the substituted piperidinyl bears a N-substituent which is (C_{1-4})alkyl.

[0044] If the N substituent is $-(CH_2)_p-NR^5R^6$, then R^5 and R^6 may be preferably (C_1-C_4)alkyl, more preferably (C_1-C_2) alkyl, e.g. methyl. Integer p is preferably 1 to 4, e.g. 3.

[0045] If the N substituent is substituted piperidinyl, then the N-substituent is preferably (C_1-C_2) alkyl, e.g. methyl. As mentioned above, the N is preferably in the 4-position.

[0046] The present invention relates to the gene clusters of SEQ ID NO:100 and SEQ ID NO:200 and the polypeptides encoded by these clusters and variants thereof. The polypeptide of SECT ID NO:119 is pre-pro-actagardine and the polypeptide of SEQ ID NO:212 is pre-pro-actagardine B. The remaining polypeptides and their variants (as defined herein) are referred to herein generically as "cluster polypeptides". Cluster polypeptides derived from SEQ ID NO:100 are referred to as "1xx polypeptides" and those derived from SEQ ID NO:200 are referred to as "2xx polypeptides". Polypeptides which are 100% identical in both sequence and length to a cluster polypeptide are referred to as "wild-type" polypeptides. A cluster polypeptide derived from SEQ ID NO:100 or SEQ ID NO:200 may be wild type or variant.

[0047] A polypeptide may be in substantially isolated form. Isolated polypeptides of the invention will be those as defined above in isolated form, free or substantially free of material with which it is naturally associated such as other polypeptides with which it is found in the cell. For example, the polypeptides may of course be formulated with diluents or adjuvants and still for practical purposes be isolated.

[0048] A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

Lantibiotic Polypeptide and LantibioticA gene

[0049] In the present invention, reference to a lantibioticA or LanA polypeptide, the *LantibioticA* or *LanA* gene refers generically to a type B lantibiotic polypeptide or the gene encoding such a peptide. Thus reference to these includes reference to cinnamycin, mersacidin, actagardine and actagardine B and the genes encoding these products. Reference to a lantibiotic producing host cell refers to any host cell which in its native form produces a LanA polypeptide, as further defined herein below.

[0050] A LanA polypeptide is a polypeptide with anti-microbial activity. Anti-microbial activity may be examined by determining the MIC value against a reference organism, e.g. *Micrococcus luteus.* A LanA polypeptide is considered to exhibit anti-microbial activity if it has a MIC value of less than or equal to 16-fold higher than that of actagardine against the same strain of the reference microorganism. In the present invention, the *A. garbadinensis LanA* gene is referred to as actA and the *A. liguriae LanA* gene is referred to as *LigA.*

Other Lan Polypeptides

[0051] As used herein, reference to a "LanM" polypeptide is a polypeptide derived from a Lantibiotic gene cluster which is a modification factor required for the conversion of a precursor polypeptide to a lantibiotic compound. LanM polypeptides include those of SEQ ID N0:120 (ActM) or a variant thereof, SEQ ID NO:213 (LigM) or a variant thereof, a cinM polypeptide as defined in WO02/0883 a mrsM polypeptide as disclosed in Altena *et al*, 2000, or a homologous polypeptide from another gene cluster of a bacteria which produces a type B lantibiotic.

[0052] Reference to a "LanR" polypeptide is a polypeptide derived from a Lantibiotic gene cluster which is a regulatory factor required for the regulation of production of a precursor polypeptide. LanR polypeptides include those of SEQ ID NO:122 (ActR) or a variant thereof, SEQ ID NO:216 (LigR) or a variant thereof, a cinR1 polypeptide as defined in WO02/088367, a mrsR1 polypeptide as disclosed in Altena et *al*, 2000, or a homologous polypeptide from another gene cluster of a bacteria which produces a type B lantibiotic.

[0053] Reference to a "LanT" polypeptide is a polypeptide derived from a Lantibiotic gene cluster which is a transporter factor required for the production of a precursor polypeptide to a lantibiotic compound. LanT polypeptides include those of SEQ ID NO:123 (ActT) or a variant thereof, SEQ ID NO:214 (LigT) or a variant thereof, a cinT polypeptide as defined in WO02/088367, a mrsT polypeptide as disclosed in Altena *et al*, 2000, or a homologous polypeptide from another gene cluster of a bacteria which produces a type B lantibiotic.

[0054] Reference to a "LanO" polypeptide is a polypeptide derived from a Lantibiotic gene cluster which is a factor believed to be involved in the oxidation of the deoxy-form of actagardine and compounds of the invention to actagardine or to compounds of the invention in which Y is - S(O)-.

[0055] LanO polypeptides include those of SEQ ID NO:122 (ActO) or a variant thereof, SEQ ID NO:215 (LigO) or a variant thereof, or a homologous polypeptide from another gene cluster of a bacteria which produces a type B lantibiotic.

Cluster Polypeptides

[0056] In one aspect, the invention provides an isolated cluster polypeptide selected from any one of SEQ ID NOs: 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230 and 231. In another aspect, the invention provides a cluster polypeptide which is a variant of any of the above-mentioned sequences.

[0057] Cluster polypeptides of particular interest include 1xx and 2xx polypeptides which are LanM, LanR, LanT or LanO polypeptides.

[0058] A "variant", in relation to a cluster polypeptide, denotes: any polypeptide having an amino acid sequence which is different from, but which shows significant amino acid sequence identity with, the amino acid sequence of a reference polypeptide (in this case any wild type cluster polypeptide), or a fragment of that polypeptide.

[0059] Unless otherwise specified, significant amino acid sequence identity is preferably at least 80%, more preferably 85%, 90% or 95%, still more preferably 98% or 99%. A variant is preferably of a length which is the same as, or at least 70%, preferably at least 80%, more preferably at least 90% and most preferably at least 95% of the length of the wild type cluster polypeptide.

[0060] "Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the sequence with which it is being compared, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. The % identity values used herein are generated by BLAST- 2 which was obtained from Altschul et al. (1996); http://blast. wustl/edu/blast/README. html. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSPS and HSPS2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer’ sequence in the aligned region, multiplied by 100. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU BLAST-2 to maximize the alignment score are ignored).

[0061] Desirably, a variant will retain a biological function of the reference polypeptide. In the present invention, biological function is retained wherein the variant, when present in a host cell with the other members of its cluster, is capable of producing a lantibiotic. This may be determined, for example, by providing a host cell containing SEQ ID NO: 100 in the case of a 1xx cluster polypeptide variant, or SEQ ID NO:200 in the case of a 2xx polypeptide variant, wherein the host cells produce actagardine or actagardine B respectively, modifying the sequence to encode the variant, and determining whether a lantibiotic polypeptide is still produced.

Precursor Polypeptides

[0062] In another aspect, the invention provides polypeptides, preferably in isolated form, which are precursors of the compounds of the present invention or of actagardine. The precursor polypeptides include the polypeptides of any one of SEQ ID NOs:1-4, SEQ ID NOs:11-14, SEQ ID NOs:212, 22, 23 and 119, as well as variants or derivatives thereof which can be converted to a lantibiotic polypeptide.

[0063] A variant of a precursor polypeptide of any one of SEQ ID NOs:1-4 is a polypeptide in which one or more, for example from 1 to 5, such as 1, 2, 3 or 4 amino acids are substituted by another amino acid. Preferably the amino acid is at a position selected from positions 2, 3, 4, 5, 8, 10, 11, 13 or 18 of any one of SEQ ID NOs:1-4.

[0064] A variant of a precursor polypeptide of any one of SEQ ID NOs:11-14 is a polypeptide in which one or more, for example from 1 to 5, such as 1, 2, 3 or 4 amino acids are substituted by another amino acid. Preferably the amino acid is at a position selected from positions 3, 4, 5, 6, 9, 11, 12, 14 or 19 of any one of SEQ ID NOs:11-14.

[0065] A variant of a precursor polypeptide of any one of SEQ ID NOs:212, 22, 23 and 119 is a polypeptide in which one or more, for example from 1 to 5, such as 1, 2, 3 or 4 amino acids of the C-terminal region (residues 46-64) corresponding to SEQ ID NOs:1-4 respectively are substituted by another amino acid. Preferably the amino acid is at a position selected from positions corresponding to positions 2, 3, 4, 5, 8, 10, 11, 13 or 18 of any one of SEQ ID NOs: 1-4. Such variants may further include changes to the N-terminal region which retain at least 70%, for example at least 80%, preferably at least 90%, for example at least 95% of the N-terminal regions (residues 1-45). For example, a variant of the N-terminal region of SEQ ID NO:212 or SEQ ID NO:119 may comprise one or more substitutions (e.g. from 1 to 12, such as from 1 to 5, e.g. 1, 2 or 3 substitutions at positions 4, 5, 6, 8, 9, 12, 13, 17, 18, 19, 21 and 32 which our data shows are varied between SEQ ID NO:212 and 119.

[0066] Substitutions may be of one amino acid by another naturally occurring amino acid and may be conservative or non-conservative substitutions. Conservative substitutions include those set out in the following table, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non- polar	GA
		ILV
	Polar - uncharged	CSTM
		NQ
	Polar - charged	D E
		KR
AROMATIC		HFWY

[0067] For SEQ ID NO:212, the substitutions may be of an amino acid which differs from the amino acid residue located in the corresponding location of SEQ ID NO:119, or *vice versa*. In either case, the substitution may be to introduce the SEQ ID NO:119 amino acid into SEQ ID NO:212, or *vice versa* (e.g. Ile at position 4 of SEQ ID NO:212 may be substituted by Leu, and so on).

[0068] A precursor polypeptide may be obtained by expression of a nucleic acid encoding the polypeptide in a cell which is a non-producer of a lantibiotic.

Nucleic Acid

[0069] A nucleic acid of the invention may be a DNA or RNA, though preferably a DNA. A nucleic acid of the invention may be single- or double-stranded. In one aspect, the invention provides an isolated nucleic acid encoding a cluster polypeptide. In another aspect, the invention provides an isolated nucleic acid encoding a precursor polypeptide or variant or fragment thereof.

[0070] In a further aspect, the invention provides an isolated nucleic acid which may comprise all or a fragment of SEQ ID NO:100 or SEQ ID NO:200, including a fragment comprising an intergenic region disclosed herein. Such regions may include a promoter or other regulatory element for the expression of a cluster polypeptide or a precursor polypeptide of the present invention.

[0071] Twenty-five nucleotides is recognised by those skilled in the art as a sufficient number of nucleotides to be specific to the particular gene or gene cluster or sub-sequence thereof as disclosed herein. Thus fragments include fragments of SEQ ID NO:100 or SEQ ID NO:200, or variants thereof having significant sequence identity, which are at least 25, e.g. at least 30, e.g. at least 50, e.g. at least 100, e.g. at least 250 nucleotides in length.

[0072] Promoters that are variants of those intergenic sequences are also included and the specific intergenic sequences (or parts thereof) are preferred embodiments. In all cases, where a preferred embodiment of an orf, gene, nucleic acid, polypeptide or promoter is defined by reference to a specific sequence, the invention in its broader sense is intended to include embodiments having variants of that specific sequence.

[0073] The term "variant" as used herein in relation to a particular nucleic acid (the reference nucleic acid) denotes: any nucleic acid having a sequence which is different from that of the reference nucleic acid, but which is its complement or which shows significant nucleic acid sequence identity with, or hybridization under stringent conditions to, the reference nucleic acid or its complement or a fragment of the reference nucleic acid or its complement; or any nucleic acid which encodes an amino acid sequence having significant amino acid sequence identity with the amino acid sequence encoded by the reference nucleic acid, or a fragment of that nucleic acid. The term "variant" also refers to nucleic acids which differ from each other due only to the degeneracy of the genetic code, and which therefore encode identical deduced amino acid sequences. Variant nucleic acids of the invention are further defined as follows. If a variant nucleic acid of the invention is introduced into the gene clusters identified herein, in place of the sequence of which it is a variant, and the recombinant fragment is introduced into a suitable host cell under suitable conditions for lantibiotic production (e.g. as shown in the Examples), then production of a molecule having one or more activities of a lantibiotic (especially antibiotic activity) will result. Preferably production will be regulated to occur at high cell density.

[0074] Significant nucleic acid sequence identity is preferably at least 50%, more preferably 60%, 70%, 80% or 90%, still more preferably 95%, 98% or 99%. Significant nucleic acid sequence identity is preferably shown between the variant nucleic acid (or a portion thereof) and a fragment of at least 30 residues of the reference nucleic acid, more preferably a fragment of at least 60, 90 or 120 residues, still more preferably a fragment of 180, 240 or 300 residues, more preferably the entire reference nucleic acid.

[0075] "Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the sequence under comparison. The identity values used herein were generated by the BLASTN module of WU BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

[0076] In relation to variants of the promoters used in the present invention, nucleic acid sequence identity is preferably assessed over a sequence of at least 30 residues, more preferably 40 or 50 residues, still more preferably 60 residues.

[0077] "Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0. 0015 M sodium citrate/ 0.1% sodium dodecyl sulfate at 50˚C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42˚C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1 % SDS, and 10% dextran sulfate at 42˚C, with washes at 42˚C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55˚C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55˚C.

[0078] When a nucleic acid of interest is said to be in "operative association" with a promoter or regulatory sequence, this means that the promoter/regulatory sequence is able to direct transcription of the nucleic acid of interest in an appropriate expression system, with the nucleic acid of interest in the correct reading frame for translation. Preferably when a nucleic acid of interest is in operative association with a promoter / regulatory sequence, the transcript of the nucleic acid of interest contains an appropriately located ribosome binding site for expression in an appropriate expression system of the polypeptide encoded by the nucleic acid of interest. See for example Sambrook et al. (1989) and Ausubel et al. 25 (1995).

[0079] When a nucleic acid is referred to as "isolated", this may mean substantially or completely isolated from some or all other nucleic acid normally present in *A. garbadinensis* and/or *A. liguriae,* especially nucleic acid from outside the gene cluster segments identified herein.

[0080] In light of the foregoing disclosure, it will be appreciated that this invention provides nucleotide sequences or a set of nucleotide sequences encoding the actagardine or actagardine B biosynthetic gene cluster. Accordingly, the entire gene cluster or portions thereof of at least twenty-five contiguous nucleotides may be used for a wide variety of applications, including but not limited to: expression of actagardine or actagardine B; use as probes to screen other organisms for related molecules and the like; use to induce gene silencing and the like.

Expression Construct

[0081] In a further aspect of the invention, there is provided an expression construct comprising a nucleic acid encoding a cluster polypeptide or a lantibiotic polypeptide of the invention operably linked to a promoter.

[0082] In a further aspect, there is provided a set of expression constructs. A set of expression constructs comprises two or more polypeptide coding sequences of the present invention and at least one promoter suitable for the expression of said sequences. The promoter(s) may be a promoter with which the polypeptide gene is naturally associated with (or in the case of a variant, the promoter of the gene from which the variant is derived), or may be a constitutive or inducible promoter functional in the host cell. Promoters thus include intergenic regions of SEQ ID NO:100 or SEQ ID NO:200 upstream of any of the open reading frames listed in Tables 1 and 2.

[0083] The promoter(s) will be operably linked to the nucleic acids of the set of expression constructs. By "operably linked" it will be understood that the promoter will be able to direct transcription of the nucleic acid of interest in an appropriate expression system, with the nucleic acid of interest in the correct reading frame for translation. Preferably when a nucleic acid of interest is in operative association with a promoter / regulatory sequence, the transcript of the nucleic acid of interest contains an appropriately located ribosome binding site for expression in an appropriate expression system of the polypeptide encoded by the nucleic acid of interest. See for example Sambrook et al. (1989), Ausubel et al. (2002) and Kieser (2000).

[0084] Sets of expression constructs according to the invention include numerous permutations of genes encoding precursor and cluster polypeptides of the invention as defined above. In various aspects of the invention, the set will include at least a *LanA* gene. Examples of such sets are set out as "Set 1" to "Set 7" below, though these sets should be understood to be merely illustrative and not limiting.

Set 1: A *LanA* gene encoding a precursor polypeptide, preferably a precursor polypeptide capable of being converted to a compound of the invention, plus a *LanM* gene encoding a LanM polypeptide. The LanM polypeptide is preferably a LanM of SEQ ID NO:120 or a variant thereof, or SEQ ID NO:213 or a variant thereof.

Set 2: A *LanA* gene encoding a precursor polypeptide, preferably a precursor polypeptide capable of being converted to a compound of the invention, plus a *LanR* gene encoding a LanR polypeptide. The LanR polypeptide is preferably a LanR of SEQ ID NO:122 or a variant thereof, or SEQ ID NO:216 or a variant thereof.

Set 3: A *LanA* gene encoding a precursor polypeptide, preferably a precursor polypeptide capable of being converted to a compound of the invention, plus a *LanM* gene encoding a LanM polypeptide, plus a *LanR* gene endcoding a LanR polypeptide. The LanM polypeptide is preferably a LanM of SEQ ID NO:120 or a variant thereof, or SEQ ID NO:213 or a variant thereof. The LanR polypeptide is preferably a LanR of SEQ ID NO:122 or a variant thereof, or SEQ ID NO:216 or a variant thereof.

Set 4: The genes of Set 3 together with a *LanO* gene encoding a LanO polypeptide. The LanO polypeptide is preferably SEQ ID NO:122 or a variant thereof, or SEQ ID NO:215 or a variant thereof.

Set 5: The genes of Set 3 or Set 4 together with a *LanT* gene encoding a LanT polypeptide. The LanT polypeptide is preferably SEQ ID NO:123 or a variant thereof, or SEQ ID NO:214 or a variant thereof.

Set 6: The genes of SEQ ID NOs:116 to 127 or variants thereof.

Set 7: The genes of SEQ ID NOs:206 to 220 or variants thereof.

[0085] In one aspect, a set will comprises sequences which all encode 1xx polypeptides or which all encode 2xx polypeptides. However sets which are made up of both 1xx and 2xx polypeptides are not excluded from the present invention.

Recombinant Expression Vector

[0086] In another aspect, there is provided a recombinant vector comprising one or more expression constructs of the invention. In an alternative aspect, there is provided a set of recombinant vectors which comprise a set of expression constructs of the invention. Suitable vectors comprising nucleic acid for introduction into bacteria can be chosen or constructed, containing appropriate additional regulatory elements if necessary, including additional promoters, terminator fragments, enhancer elements, marker genes and other elements as appropriate. Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Sambrook et al (1989) Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel et al. (1995) Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995). Many aspects of the employment of these techniques in the context of *Streptomyces* spp. are described in detail in Hopwood et al (1985) Genetic manipulation of *Streptomyces* a laboratory manual (Norwich: John Innes Foundation) and Practical Streptomyces Genetics (2000) Kieser T. et *al*., The John Innes Foundation p.386. The disclosures of Sambrook et al, Ausubel et al, Hopwood et al and Kieser et al are all incorporated herein by reference for these and all other purposes.

Expression Cassettes

[0087] In another aspect, the inventors have developed a vector system useful for producing and screening lantibiotic derivatives of actagardine B. This is achieved by introducing one or more restriction endonuclease recognition sites into the *LanA* gene which encodes SEQ ID NO:1, 11 or 212 in order to produce an expression cassette system. Thus in another aspect, the invention provides a recombinant DNA cassette which comprises a nucleotide sequence encoding an actagardine B precursor polypeptide, wherein said sequence comprises
a first restriction site at or adjacent the N-terminal encoding region of the encoding sequence;
optionally a second restriction site downstream of the first restriction site and within the encoding sequence; and
a third restriction site at or adjacent the C-terminal encoding region of the encoding sequence,
wherein at least one of said restriction sites does not occur within the *LanA* coding sequence shown as SEQ ID NO:200.

[0088] In a further aspect, there is provided a recombinant DNA cassette which comprises a nucleotide sequence encoding an actagardine precursor polypeptide, wherein said sequence comprises
a first restriction site at or adjacent the N-terminal encoding region of the encoding sequence;
optionally a second restriction site downstream of the first restriction site and within the encoding sequence; and
a third restriction site at or adjacent the C-terminal encoding region of the encoding sequence,
wherein at least one of said restriction sites does not occur within the *LanA* coding sequence shown as SEQ ID NO:100.

[0089] Generally, all two or three sites will be different from each other. It is also desirable that when the cassette is carried by a vector, the sites are unique for that vector.

[0090] In a preferred aspect, the non-naturally occurring restriction enzyme site is the second restriction site and is located between codons 5 and 16, such as between 6 and 15, of the encoding sequence of SEQ ID NO:1 or SEQ ID NO:4.

[0091] The cassette will desirably also include a *LanA* leader sequence and a *LanA* promoter, and may include in addition one or more cluster genes, particularly where such a cluster gene is required to complement the loss of the equivalent host cell gene.

[0092] The cassette of the invention described above may be engineered in a variety of ways. For example, the fragment obtained by cleaving the cassette between the first and second, first and third, or second and third, restriction sites may be replaced with a variant coding sequence encoding a .lantibiotic A variant. Thus the invention provides a variant of the cassette of the invention wherein said variant has from 1 to 15 nucleotide substitutions within the encoding region of the encoding sequence.

[0093] As an intermediate to the production of such a variant, the sequence of between the first and second, first and third, or second and third, restriction sites may be replaced by a larger stuffer fragment.

[0094] In another aspect, the cassette encoding a lantibiotic derivative may be used to transform a host cell to express the derivative, for example to assess its anti-bacterial properties.

[0095] In one aspect, a multiplicity of expression cassettes may be made to provide a library of different derivatives, which may then be screened for activity.

[0096] An expression cassette of the invention may be based on any cloning and expression vector used in the art for the expression of genes in host cells. Such vectors will include one or more origins of replication, which may be temperature sensitive. The vectors may include a selectable marker, such as the chloramphenicol acetyl transferase gene, the erythromycin resistance gene, the apramycin resistance gene or the tetracycline resistance gene. The vector may also contain a targeting region, this region being homologous to a genomic sequence present in the host cell outside the LanA gene cluster. Such a vector may be used to integrate the cassette into the genomic sequence homologous to the targeting region.

[0097] The expression cassette may also comprise one or more cluster genes in addition to the *LanA* gene or derivative thereof. Where the host cell is a ΔLanA host cell in which the *LanA* gene has been inactivated in a manner which also inactivates such a cluster gene (e.g. in the strain disclosed in Altena et al, 2000), it is desirable that this gene or an equivalent gene is provided on the expression cassette.

[0098] As used herein, by "at or adjacent the N-terminal encoding region" it is meant that the first base of the restriction site is located at a position from six residues upstream of the ATG codon of the LanA leader sequence to no more than six codons downstream of the first codon of the propeptide. Preferably the first base of the restriction site is located at a position from twelve, preferably six, residues upstream to six residues downstream of the first codon of the propeptide encoding sequence.

[0099] In one aspect, the first restriction site is a *Bgl*II site.

[0100] Similarly, by "at or adjacent the C-terminal encoding region" it is meant that the first base of the restriction site either includes at least one of the nucleotides of the termination codon of the propeptide or the 5' or 3' nucleotide of the restriction site is no more than twelve, preferably six, residues downstream or upstream respectively of the termination codon.

[0101] In one aspect, the third restriction site is a *Avr*II site.

[0102] The second restriction site, when present, will lie between the first and third restriction sites. Preferably the restriction site includes at least one nucleotide present from codon 5 to codon 16, preferably codon 8 to 16 of the propeptide-encoding sequence. In the accompanying examples, a *Bsr*G1 site has been introduced by altering codons 6 and 7 of the ActA-encoding sequence. However, other changes are also contemplated by the present invention.

[0103] It is also possible to introduce more than one change such that the expression cassette includes two or more sites between the first and third restriction sites.

[0104] The cassette may include two or three non-naturally occurring restriction sites. In the accompanying example, all three sites do not normally occur in the ActA sequence encoded by of SEQ ID NO:100.

[0105] The expression cassette simplifies the rapid production of lantibiotic derivatives, as discussed further herein below.

[0106] In one aspect, the region between the first and second sites, the first and third, or the second and third sites, may be replaced by a stuffer fragment. Where two or more sites between the first and third sites are present, the region between any pair of such sites may also be replaced by a stuffer fragment. A stuffer fragment is a piece of DNA which is larger than the sequence which it replaces. The stuffer fragment may be from 50 to 5000 nucleotides in size, for example from about 500 to 2000 nucleotides in size. The value of introducing these stuffer DNA fragments is that when the region is replaced by a lantibiotic-encoding oligonucleotide there is a significant decrease in plasmid size. The resulting plasmid can thus be readily purified away from any minor population of unrestricted plasmid thus eliminating any background.

[0107] A cassette of the invention may be used to introduce specific changes to the ActA sequence in a vector which can then be introduced into a host cell for expression of a lantibiotic. To achieve this, the sequence is desirably operably linked to the LanA (e.g. ActA or LigA) leader sequence, which in turn is operably linked to the *LanA* promoter (e.g. ActA or LigA)

[0108] In addition or as an alternative, the vector comprising the cassette may also include a *LanR* gene. The *LanR* gene will be located downstream of, and in tandem with, the lantibiotic A coding sequence.

Expression Libraries

[0109] Expression cassettes of the invention may be used to provide libraries of lantibiotic-encoding genes. Such libraries may be made by introducing into the cassette, between the first and second restriction sites, the first and third restriction sites, or the second and third restriction sites, a multiplicity of sequences each of which corresponds to the corresponding *ActA* or *LigA* sequence apart from having from 1 to 15, for example from 1 to 10, preferably from 1 to 6, for example from 1 to 3 nucleotide changes compared to the propeptide portion of SEQ ID NOs:100 or 200. Preferably such changes result in a change of the protein encoded by the sequence. However non-coding changes are not excluded.

[0110] Libraries form a further aspect of the invention. Such libraries may comprise from 10 to 100,000, such as from 10 to 10,000 for example from 10 to 1,000 different coding sequences which are variants of the lantibiotic A coding sequence of an expression cassette.

[0111] An expression cassette encoding a lantibiotic A derivative may be introduced into a host cell for expression of the lantibiotic.

[0112] In one embodiment, the library may be transformed into host cells, and colonies isolated and/or screened for antibacterial activity. The sequences of the lantibiotic A expressed by individual colonies showing such activity can be determined. Where the lantibiotic A shows activity, the invention further provides a lantibiotic obtained by the method of the invention.

Host Cell

[0113] Two main types of host cells are envisaged by the present invention. The first type of host cell is a lantibiotic producing host cell. Alternatively the host cell may be a non-producer cell, i.e. does not contain a LanA gene or its associated cluster genes required for producing a LanA polypeptide.

[0114] In one embodiment, the invention provides a host cell transformed with a set of expression constructs of the invention. The set of constructs may be any one of Sets 1 to 7 as defined above, or a set based upon any other combination of precursor and cluster polypeptide-encoding nucleic acids. In another embodiment, the host cell may be transformed with a expression cassette of the invention.

[0115] In a further embodiment, there is provided a library of host cells, each one comprising a different expression cassette of the invention.

A Lantibiotic-producing host cell

[0116] In one embodiment, the host cell may be a lantibiotic producing host cell. A lantibiotic producing host cell is one in which an expression construct comprising a *LanA* gene, if introduced into the cell in the absence of any cluster gene, would be expressed and a LanA polypeptide produced. Such cells include any type-B lantibiotic producing cell such as any strain of a bacillus, an actinomycete, or a streptomycete, (e.g. *S. lividans* or *S. coelicolor*) which produces a lantibiotic. Examples of such cells include a cinnamycin-producing host cell (*Streptomyces cinnamoneus cinnamoneus* DSM 40005), or an actagardine-producing *Actinoplanes garbadinensis* or *A. liguriae* NCIMB 41362.

[0117] Where the invention relates to the productions of compounds of the formula (I) in which -X1-X2- represent -Leu-Val-, the host cell may be *A. liguriae* NCIMB 41362 without any further modification.

[0118] In one aspect, a host cell of this class may comprise a mutation in its endogenous *LantibioticA* gene such that the gene is not expressed or the gene product is inactive. Such a host cell may be obtained by targeted homologous recombination to delete or mutate the *LanA* gene of the host cell. Methods to achieve this are known as such in the art and are illustrated in Altena et al, (2000) and WO2005/093069, the disclosures of which are incorporated herein by reference. The resulting host cell is referred to as a Δ*LanA* host cell. In one particular embodiment, the host cell is a Δ*lignA A. liguriae* NCIMB 41362 host cell in which the *ligA* gene has been inactivated, for example by mutation or deletion, e.g. deletion brought about by homologous recombination. In another embodiment, the host cell is a Δ*ActA A. garbadinensis* host cell in which the *ActA* gene has been inactivated, for example by mutation or deletion, e.g. deletion brought about by homologous recombination.

[0119] The transformation of a host cell of this type with other cluster genes is also contemplated by the present invention, though where the host cell provides cluster genes necessary for the production of a lanA, the provision of such cluster genes is optional.

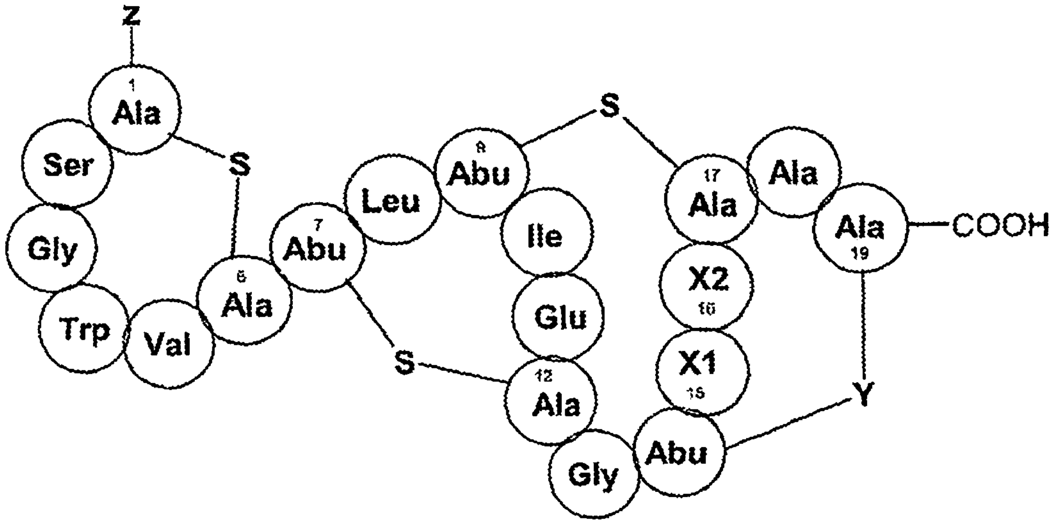
Non-producer cell

[0120] A non-producer cell may be any host cell in which expression of a LanA gene encoding a precursor polypeptide capable of being converted to actagardine or a variant thereof, or to a compound of the invention, can produce such a product provided the *LanA* gene is introduced into the cell as part of a set of expression constructs which are capable of converting a precursor polypeptide to actagardine or a variant thereof, or to a compound of the invention. A non-producer host cell may be a bacterial host cell. Bacterial host cells include an actinomycete, or a streptomycete, e.g. *S. lividans*, *S. coelicolor* or *S. cinnamoneus.*

[0121] Host cells may be those in which the *lanO* gene is inactivated by mutation or deletion (or in the case of non-producer cells, not present), or those in which the expression of the *lanO* gene is increased, e.g. by provision of two or more copies of the gene or by linking the gene to a promoter which enhances expression in the host cell. Modulation of the *lanO* gene in this manner may be desirable to alter the relative levels of oxidized (Y = -S(O)-) and reduced (Y = -S-) forms of compounds of the invention produced in the host cell.

Production of Compounds of the Invention

[0122] The invention also provides a method of preparing a compound of formula:



wherein:

- X1-X2- represents -Leu-Val-;
- Y- is -S-;

[0123] Z is Ala or -NH_2 wherein the latter represent the N-terminus of the Ala at position 1, the method comprising expressing a nucleic acid encoding a sequence of SEQ ID NO: 1 or SEQ ID NO: 11, and optionally where necessary, associated cluster genes required for conversion of the precursor polypeptide to the product. The nucleic acid may be expressed in *A. liguriae.* The *A. liguriae* may be the *A. liguriae* deposited under the Budapest Treaty deposit no: NCIMB 41362. deposited under the Budapest Treaty deposit no: NCIMB 41362. Compounds of the invention may be produced by expression of a nucleic acid, for example in the form of an expression construct encoding a precursor polypeptide carried in a recombinant expression vector, in a host cell which carries a *LanA* gene together with where necessary, associated cluster genes required for conversion of the precursor polypeptide to the product. As noted above, where the invention relates to the productions of compounds of the formula (I) in which -X1-X2- represent -Leu-Val-, the host cell may be *A. liguriae* NCIMB 41362 without any further modification.

[0124] The introduction of the expression cassette, or vector(s) into a host cell may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation". This may employ any available technique. For bacterial cells, suitable techniques may include calcium chloride transformation, polyethyleneglycol assisted transformation, electroporation, conjugation and transfection or transduction using bacteriophages.

[0125] In one aspect, the present invention provides a method of expressing nucleic acid of the invention, the method comprising providing a host cell (or other expression system) culturing the host cell, so as to express the nucleic acid of interest. The nucleic acid of interest will be in an expression cassette, such that culturing the host cell leads to the production of a product of the invention.

[0126] Preferably the nucleic acid of interest is expressed substantially only when the host cell culture reaches high cell density, more preferably at or close to the stationary phase of host cell culture. Cell cultures at or close to stationary phase may have OD650 values in the range of 1-20. Known methods of culturing cells are well known in the art, for example from Sambrook et al (1989), Ausubel et al (2002), and (in particular for Streptomyces spp.) Kieser et al (2000). The expression products of the expression systems may be collected and purified. Isolation methods may comprise capture from the fermentation medium using solvent extraction techniques, adsorption resin such as hydrophobic resins or precipitation methods such as ammonium sulfate precipitation. Purification methods may include chromatography techniques such as ion exchange, hydrophobic interaction, reverse phase, normal phase, solid phase extraction and HPLC, e.g. as described in US 5,112,806 for the isolation of mersacidin

[0127] Following culture of the cell, the compounds of the invention may be recovered from the host cell culture. The recovered compounds may be formulated in the form of a pharmaceutical composition, optionally in the form of a pharmaceutically acceptable salt.

[0128] Where host cells produce a mixture of compounds of the invention, e.g. those in which Y is -S- or -S(O)- or those in which Z is NH2 or Ala-, or mixtures of all four types, the products may be isolated using standard separation techniques such as hplc, e.g. as described in US 6,022,851 for the production of Actagardine and Ala-Actagardine.

[0129] The recovered compounds may be formulated in the form of a pharmaceutical composition, optionally in the form of a pharmaceutically acceptable salt.

Pharmaceutically acceptable salt

[0130] A "pharmaceutically acceptable salt", may be an acid addition salt in which the base retains the biological effectiveness and properties of the compound and which is physiologically acceptable. Such salts include those formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulphuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

[0131] Salts also include basic salts, such as an alkali or alkaline earth metal salt, e.g. a sodium, potassium, calcium or magnesium salt.

Pharmaceutical Compositions

[0132] The lantibiotics of the present invention may be formulated together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, diluents, fillers, buffers, preservatives, anti-oxidants, lubricants, stabilizers, solubilisers, surfactants (e.g., wetting agents), masking agents, colouring agents, flavouring agents, and sweetening agents. The formulation may further comprise other active agents, for example, other therapeutic or prophylactic agents. Thus, the present invention further provides pharmaceutical compositions, as defined above, and methods of making a pharmaceutical composition comprising admixing at least one active compound, as defined above, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, adjuvants, excipients, etc. If formulated as discrete units (e.g., tablets, etc.), each unit contains a predetermined amount (dosage) of the active compound.

[0133] The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgement, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, adjuvant, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

[0134] Compositions may be formulated for any suitable route and means of administration. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0135] For solid compositions, conventional non-toxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound as defined above may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc, an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see "Remington: The Science and Practice of Pharmacy", 20th Edition, 2000, pub. Lippincott, Williams & Wilkins. The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the subject being treated.

[0136] Dosage forms or compositions containing active ingredient in the range of 0.25 to 95% with the balance made up from non-toxic carrier may be prepared.

[0137] For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, sodium crosscarmellose, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium, carbonate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations and the like. Such compositions may contain 1%-95% active ingredient, more preferably 2-50%, most preferably 5-8%.

[0138] Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, triethanolamine sodium acetate, etc.

[0139] For topical applications, the pharmaceutically acceptable compositions may be formulated in a suitable ointment or gel containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutically acceptable compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissolved in one or more pharmaceutically acceptable carriers. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

[0140] The percentage of active compound contained in such parental or topical compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject. However, percentages of active ingredient of 0.1% to 10% w/w employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably, the composition will comprise 0.2-2% w/w of the active agent in solution.

[0141] Further teaching regarding suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington: The Science and Practice of Pharmacy", 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Administration of Compounds

[0142] Lantibiotic compounds and compositions of the invention may be administered to a subject in a method of medical treatment or prophylaxis. The subject may be a human or animal subject. The animal subject may be a mammal, or other vertebrate.

[0143] Thus there is provided a compound of the invention for use in a method of treatment or prophylaxis of a subject. There is also provided use of a compound of the invention for the manufacture of a medicament for use in a method of treatment or prophylaxis of a subject.

[0144] The method of treatment may be of a bacterial infection, including a skin, mucosal, enteric or systemic infection.

[0145] The variants and composition may be used for systemic treatment of bacteraemia (including catheter related bacteraemia), pneumonia, skin and skin structure infections (including surgical site infections), endocarditis and osteomyelitis. These and other such treatments may be directed against causative agents such as staphylococci, streptococci, enterococci. The compounds of the invention or compositions thereof may also be used for topical treatment of skin infections including acne ie. *Propionibacterium acnes.* The variants and compositions thereof may also be used in the treatment of eye infections, such as conjunctivitis, and for oral treatment for gut super-infection, such as that caused by

Clostridium difficile including multiply-resistant *C. difficile* (pseudomembranous colitis), or gut infections associated with *Helicobacter pylori.*

[0146] The variants may also be used in the treatment or prevention of infection of the skin in wounds or burns. In addition, the variants and compositions thereof may be used in prophylactic methods, such as for the clearance of the nares to prevent transmission of MRSA. This may be practiced on subjects at risk of infection (e.g. patients entering a hospital) or on health professionals or other carers at risk of being carriers of such infections. Prophylactic clearance of gut flora ahead of abdominal surgery is also contemplated.

[0147] The compounds according to the invention can be administered enterally (orally), parenterally (intramuscularly or intravenously), rectally or locally (topically). They can be administered in the form of solutions, powders (tablets, capsules including microcapsules), ointments (creams or gel), or suppositories. Possible auxiliaries for formulations of this type are the pharmaceutically customary liquid or solid fillers and extenders, solvents, emulsifiers, lubricants, flavor corrigents, colorants and/or buffer substances. As an expedient dose, 0.1-1000, preferably 0.2-100, mg/kg of body weight are administered. They are expediently administered in dose units which contain at least the efficacious daily amount of the compounds according to the invention, e.g. 30-3000, preferably 50-1000, mg.

[0148] The experimental basis of the present invention, including its best mode, will now be further described in detail, by way of example only, with reference to the accompanying drawings.

EXAMPLE 1 - Cloning of Gene Clusters

Identifying and cloning the actagardine biosynthetic gene clusters from *A. garbadinensis* and *A. liguriae.*

[0149] O/SBDIG-1 is a digoxigenin (DIG)-labelled degenerate oligonucleotide composed of 48 bases. It was designed by translating the known amino acid sequence of actagardine and considering codon usage for Actinoplanes. Southern hybridisation analysis of genomic DNA isolated from *A. garbadinensis* and digested using the restriction enzyme NcoI, identified a ~3kb fragment which hybridised to O/SBDIG-1. The *Nco*I digest of the genomic DNA was repeated and DNA fragments of ~3kb were isolated and cloned into NcoI cut pLITMUS28 (NEB). The resulting plasmids were introduced into *E. coli* DH10B cells and then analysed by Southern hybridisation using the probe O/SBDIG-1. A hybridising clone was identified and submitted for sequence analysis. Sequencing revealed that this plasmid designated pLITAG01 consists of DNA encoding the *lanA* structural gene for actagardine biosynthesis (*actA*) together with an upstream region believed to encode a portion of an ABC sugar transporter and a region downstream partially encoding lan M (*actM*).

[0150] The primers O/ACT08F and O/ACT09R were designed based upon sequence from pLITAG01. Using these primers in a PCR reaction together with DIG-labelled dNTPs (Roche) and pLITAG01 as a template, a 2296bp DIG-labelled DNA fragment was generated and designated SBDIG-2.

[0151] Two cosmid libraries were generated by cloning Sau3AI digested genomic DNA from *A. garbadinensis* ATCC 31049 and *A. liguriae* NCIMB 41362, into the cosmid SuperCos1 (Stratagene) previously digested using *BamH*I. Each cosmid library was analysed by Southern hybridisation using SBDIG-1. Twenty-five cosmids from each library believed to hybridise to SBDIG-1 were selected and re-analysed via Southern hybridisation using the probes O/SBDIG-1 and SBDIG-2. Nine cosmids derived from genomic DNA from *A. garbadinensis* and eleven cosmids derived from genomic DNA from *A. liguriae* hybridised to both probes. DNA was isolated from each cosmid and sequenced using the primers T3 and T7. The cosmids CosAL02 and CosAG14 were subsequently fully sequenced (Sequencing facility, Department of Biochemistry, University of Cambridge).

Materials and Methods

Strains

[0152] Bacterial strains used in the present invention are summarised in Table 5.

Vectors

[0153] The cosmid SuperCos1 was obtained from Stratagene.

[0154] The plasmid pLITMUS was obtained from New England Biolabs.

Primers

Primer name	SEQ. ID	Sequence 5'-3'
O/ SBDIG- 1	300	TGGGTSTGCACSCTSACSATCGARTGCGGNACSG TSATCTGCGCSTGC
O/ACT08F	301	TCCAGCACGCGCGGGG
O/ACT09R	302	GTTCGACCAGCCGCCC

Southern hybridisation

Labelling of *DNA probe*

[0155] DNA hybridisation probes were prepared using the Digoxygenin (DIG) PCR DNA labeling and detection kit supplied by Roche, according to their instructions.

Transfer of *DNA* to *nylon membrane*

[0156] The DNA of interest was initially separated by agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N, Amersham Int., UK) using a vacuum blotter (Q BIO gene). The time taken for depurination of the DNA using 0.5 M HCl was judged by the time taken for the bromophenol blue marker band to turn completely yellow (typically 15-20 min for a 0.7% agarose gel). The DNA was then systematically denatured with 1.5 M NaCl, 1.5 M NaOH and then neutralised using 0.5 M Tris, 1.5 M NaCl, pH 8.0 for a further 15-20 min each. Complete blotting of the DNA was facilitated by flooding with 20 x SSC for a minimum of 60 min. After removing the blotted membrane from the vacuum it was left to air dry at room temperature. The DNA was cross-linked by placing the membrane (DNA face down) on a UV transilluminator (UVP) and exposing it to UV at a wavelength of 365 nm for 5 min.

Colony lifts and hybridisation

[0157] Colonies to be screened by hybridisation were transferred onto a nylon membrane (Roche diagnostics). This was achieved by placing the positively charged nylon membrane over the colonies and pressing firmly for 1 min to ensure effective transfer. Reference points were marked on the membrane to indicate its orientation with respect to the colonies. Following this, the membrane was removed and prepared for hybridisation as directed in the Roche user's manual (DIG Application manual for filter hybridisation).

Hybridisation and development of membranes

[0158] DNA was hybridised with the prepared probe overnight (~16hr) at 68°C in a HB-1000 hybridisation oven (UVP). Following hybridisation the membrane was washed for 2 periods of 5 min at room temperature using 2 x salt sodium citrate (SSC) + 0.1% sodium dodecyl sulphate (SDS). These washes were followed with a second series of 2 x 15 min washes at 68°C using 1 x SSC + 0.1% SDS for the membrane hybridised in the presence of SBDIG-1 and 0.1 x SSC + 0.1% SDS for the membrane screened using SBDIG-2. Membranes were then developed as recommended in the Roche user's manual (DIG Application manual for filter hybridisation).

Software

[0159] The consensus sequences were analysed using FramePlot version 2.3.2, BioEdit sequence alignment editor, ClustalW (EMBL-EBI) and the Basic Local Alignment Search Tool (BLAST, NCBI).

Results and discussion

CosAG14

[0160] The cosmid, CosAG14, contains a 38168bp fragment of genomic DNA isolated from *A. garbadinensis.* Sequence analysis has identified DNA encoding the leader and actagardine prepeptide, this gene has been assigned the name actA. Two alanine residues lie immediately upstream of the actagardine prepeptide. These residues are believed to represent the recognition site for protease cleavage of the leader peptide from actagardine. Partial cleavage at this position resulting in the retention of an alanine is thought to result in the production of ala-actagardine routinely observed in fermentation broths of *A. garbadinensis.* Downstream of the *actA* gene lies a 3162bp region of DNA with strong sequence similarity to several IanM proteins, for example, *mrsM* (30% identity) from the mersacidin gene cluster. This putative gene has been designated *actM* and is thought to be involved in the modification of the actagardine prepeptide, catalysing dehydration and thioether formation. An open reading frame designated *actO*, that is located 11 bp downstream of *actM* encodes a 341-amino-acid protein with sequence similarity (~39% identity) to several luciferase-type monooxygenases. The role of the monooxygenase, ActO, is believed to be to catalyse the incorporation of oxygen generating actagardine from deoxy-actagardine. In reverse orientation to *actO* and located 62bp downstream is the open reading frame named *actR.* The protein product of this orf shows sequence similarity (~37% identity) to several two-component response regulators. Positioned 789bp downstream and in the same orientation to *actR* lies a putative 812 amino-acid protein that shows sequence similarity (~25% identity) to ABC transporter permeases. This putative protein designated actT is potentially reponsible for the export of the modified lantibiotic from the cell. The amino acid sequences of the second and fourth orf downstream of *actT* show similarity (~30% identity) to reponse regulator kinases and pencillin binding proteins respectively. Recent work on the nisin biosynthetic gene cluster in *Listeria monocytogenes* (Gravesen et al., 2004) has demonstrated that a histidine kinase together with a pencillin-binding protein and protein of unknown function are involved in conferring nisin resistance. The presence of analogous genes within close proximity to the actA may indicate that these genes are involved in an actagardine resistance mechanism.

CosAL02

[0161] The cosmid CosAL02 contains a 40402bp fragment of genomic DNA isolated from *Actinoplanes liguriae.* Sequence analysis has identified a *lanA* gene encoding a 64-amino-acid protein with strong sequence homology (50 identical residues) to the *actA* gene identified in the cosmid CosAG14. We have termed this species of *lanA* gene as *ligA*. The amino acid sequence of the prepeptide of this *lanA* differs from that of actagardine by two residues indicated in the alignment of the two genes shown below (SEQ. ID 119 and SEQ. ID 212):

```
AG actA MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAASSGWVCTLTIECGTVICAC 64
AL lanA MSAITVETTWKNTDLREDLTAHPAGLGFGELSFEDLREDRTIYAASSGWVCTLTIECGTLVCAC 64
*** : : :* . : ** : . * * * : . . * : * * * * * * * * * * : * * * * * * * * * * * * * * * * * * * * * * * * * * * : : * * *
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[0162] The mutations V15L and I16V would generate a protein with an identical mass to actagardine and would therefore not be distinguished by mass spectroscopy (Ic-ms) analysis. The potential product of the *lanA* gene identified in CosAL02 represents a novel lantibiotic. An open reading frame that lies 321 bp upstream of *ligA* encodes a putative 286-amino acid protein that shows sequence similarity (46% identity) to the StrR protein of *Streptomyces glaucescens.* The StrR protein is a pathway-specific DNA binding activator involved in the regulation of streptomycin gene expression. The sequence similarity (31 % identity) of the orf lying downstream of *ligA* suggests that it encodes for a 1046-amino-acid IanM polypeptide (called "ligM" below) potentially involved in modification of the ligA prepeptide. The start codon of the following downstream open reading frame, *lanT* (*ligT*), overlaps the stop codon of *ligM.* LigT is a 575 amino-acid protein with sequence similarity to several ABC-transporters. LanT proteins are responsible for the secretion of either the final mature product or the posttranslationally modified product still attached to its leader sequence. As observed in the cosmid, CosAG14, the next orf downstream of *ligT* encodes for a 347 amino-acid protein with sequence similarity (~38% identity) to luciferase-type monooxygenases. This putative monooxygenase (ligO) is believed to be involved in the incorporation of oxygen and sulfoxide bond formation. Positioned downstream of *ligO* and in reverse orientation lies a putative 217 amino-acid protein that shows sequence similarity (~37%) to several two-component response regulators. This putative regulator has been designated ligR.

Table 1- Annotation of CosAG14 (38168bp fragment isolated from A. garbadinensis. The SuperCos1 vector backbone sequence is omitted)

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	Start - end (3AA)
orf1	Hypothetical protein	482 - 42	- 1	146 (441)	MPR - RCG
orf2	Hypothetical protein	2824 - 2375	- 2	149 (450)	VSV - ERA

(continued)

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	Start - end (3AA)
orf3	ATPase AAA involved in cell division	4432 - 2876	- 2	518 (1557)	VER - TNR
orf4	Sugar hydolase	6002 - 5391	- 1	203 (612)	VGE- NYS
orf5	Endoglucanase	6484 - 5825	- 2	219 (660)	MRR - TVR
orf6	Cytosine/adenine deaminase	6627 - 7112	+3	161 (486)	MTI - PAQ
orf7	Unknown	7756 - 8997	+1	413 (1242)	VTT - YDK
orf8	Unknown	9586 - 8933	- 2	217 (654)	VGK - FRG
orf9	Pyruvate oxidase	11886 - 10108	- 3	592 (1779)	VSD - DPS
orf10	Hydrolase or acyltransferase	12066 - 12866	+3	266 (801)	VSR - SGT
orf11	Aldose epimerase	13116 - 14306	+3	396 (1191)	MTE - TAD
orf12	ABC sugar transport periplasmic component	14385 - 15521	+3	378 (1137)	MPR - AHG
orf13	ABC sugar transport permease	15514 - 16572	+1	352 (1059)	MDD - GRS
orf14	ABC transport protein. ATP-binding	16569 - 17330	+3	253 (762)	MTA - RGR
orf15	Hypothetical protein. Methyltransferase	18102 - 17335	- 3	255 (768)	MES - RKR
orf16	ABC transport permease	18962 - 18120	- 1	280 (843)	MPP - RKG
orf17	ABC transport permease	19896 - 18991	- 3	301 (906)	MSA - ESE
orf18	ABC transport substrate binding	21236 - 19899	- 1	445 (1338)	MFI - SGR
orf19	actA, structural gene	21572 - 21766	+2	64 (195)	MSA - CAC
orf20	actM,modification gene	21837 - 24998	+3	1053 (3162)	MSP - PLT
orf21	actO, monooxygenase	25009 - 26034	+1	341 (1026)	MPE - PAA
orf22	actR, response regulator	26791 - 26096	- 2	231 (696)	MRS - CLS
orf23	actT, ABC transporter associated permease	29323 - 26885	- 2	812 (2439bp)	MLA - LTR

(continued)

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	Start - end (3AA)
orf24	Hypothetical protein	29462 - 30196	+2	244 (735)	MIV - RNR
orf25	Reponse regulator kinase	30235 - 31338	+1	367 (1104)	VLR - ARA
orf26	Response regulator sensor	31335 - 31997	+3	220 (663)	MTR - AVG
orf27	Penicillin binding protein	32138 - 34486	+2	782 (2349)	MLI - PPR
orf28	Methyltransferase	35209 - 34448	- 2	253 (762)	MAP - DRR
orf29	Hydrolase	36030 - 35245	- 3	261 (786)	VPR - PPP
orf30	Response regulator	36086 - 36820	+2	244 (735)	VSP - TGS
orf31	Fructose biphosphate aldolase	36844 - 37689	+1	281 (846)	MKD - RAW
orf32	Hydrolase	37590 - 38168	+3	192 (579)	MGS - DPA

Table 2 - Annotation of CosAL02 (40402bp fragment isolated from A. liguriae. The SuperCos1 vector backbone sequence is omitted).

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	start-end (3AA)
orf1	Secretion system protein	1008 - 1	-2	335 (1008)	VRL - VDI
orf2	Response regulator	2198 - 1122	-3	358 (1077)	MSE - LFP
orf3	Hypothetical protein	3088 - 2288	-1	266 (801)	MRR - WR
orf4	Hypothetical protein	4410 - 3112	-2	432 (1299)	MRR - RTG
orf5	Response regulator ATP-binding	5205 - 4795	-2	136(411)	MWK - SAR
orf6	ABC sugar transporter	5516 - 6607	+2	363 (1092)	MFN - SAY
orf7	ABC sugar transporter ATP-binding	6673 - 8178	+1	501 (1506)	MLL - DEH
orf8	ABC transport permease	8168 - 9127	+2	319 (960)	MST - RTR
orf9	ABC transporter permease protein	9130 - 10092	+1	320 (963)	MSI - RRS
orf10	Metallopeptidase	12046 - 10586	-1	486(1461)	MRT - PGS

(continued)

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	start-end (3AA)
orf11	Putative StrR-like regulator	12460 - 13320	+1	286 (861)	MDS - DAA
orf12	ligA	13641 - 13835	+3	64 (195)	MSA - CAC
orf13	ligM	13907 - 17047	+2	1046 (3141)	MSS - THV
orf14	ligT, ABC transporter	17040 - 18767	+3	575 (1728)	MSE - LLT
orf15	ligO, Luciferase type monoxygenase	18785 - 19828	+2	347 (1044)	MLS - RRW
orf16	ligR, Response regulator	20459 - 19806	-3	217 (654)	MAD - ELA
orf17	ABC-transporter associated permease	23069 - 20625	-3	814 (2445)	MIF - LVR
orf18	ABC-transporter. ATP-binding protein	23788 - 23066	-1	240 (723)	MVS - VTS
orf19	Histidine kinase	23980 - 25068	+1	362 (1089)	VIA - AVP
orf20	Response regulator	25065 - 25721	+3	218 (657)	MTE - GPS
orf21	Putative membrane protein	26673 - 25768	-2	301 (906)	MPI - RFP
orf22	alpha-beta hydolase	26697 - 27569	+3	290 (873)	MRN - ASR
orf23	Transcriptional regulator	27574 - 28011	+1	145 (438)	VRL - RLG
orf24	Pyruvoyl-dependent arginine decarboxylase	28102 - 28629	+1	175 (528)	MAD - GMN
orf25	Putative diaminopimelate decarboxylase	30946 - 29626	-2	406 (1221)	MTL - LYA
orf26	Kinase	31860 - 30931	-2	309 (930)	VRS - PDL
orf27	Transcriptional regulator	33248 - 32145	-3	367 (1104)	VVF - ANS
orf28	Glycosyl transferase	33600 - 34553	+3	317 (954)	MPS - NAG
orf29	Glycosyl transferase	34543 - 35652	+1	369 (1110)	MPA - ARV
orf30	Dihydrolipoamide dehydrogenase	36432 - 37811	+3	459 (1380)	MGE - INF

(continued)

Gene	Description	Position (DNA)	Frame	Size AAs (bp)	start-end (3AA)
orf31	Putative membrane protein	37973 - 39019	+2	348 (1047)	MTT - TPG

EXAMPLE 2 - Expression Cassette

Generation of an Expression Cassette

[0163] This example illustrates the production of an expression cassette according to the present invention. This expression cassette, plasmid pAGvarX has been designed for the efficient generation of variant *lanA* genes of the present invention which can then be introduced into a host cell, such as a strain of *A. garbadinensis in which the wild-type actA has been removed* (*A. garbadinensis* Δ *actA*). This plasmid, a derivative of the vector pSET152 (Bierman *et al.*, 1992) will integrate into the host's chromosome via the attP attachment site. Expression of the mutated actA gene by the host organism together with the remaining wild-type genes of the actagardine biosynthetic gene cluster should generate actagardine variants.

Construction of the plasmid pAGvarX

[0164] Unless stated otherwise all quoted positions relate to SEQ ID NO:100. The scheme for the construction of plasmid pAGvarX is shown in Figure 3. The base adjacent to the orf lying upstream of the *actA* at postion 21237 to the leucine residue within the *actA* encoding region at position 21672 was amplified by PCR using the primers O/AGvar01bF and O/AGvar02bR (primer table) and pLITAG01 as a template. The primers were designed to introduce a flanking *XbaI* site at the 5' end and a *Bgl*II site via a silent mutation at the 3' leucine region encoding the *actA.* This fragment was introduced into dephosphorylated pUC19 previously digested using SmaI to yield pAGvar1.

[0165] The region of DNA spanning from the C-terminus of the actA to the adjacent downstream orf (21758-21836 inclusive) was amplified by PCR using the primers O/AGvar05F and O/AGvar06R and pLITAG01 as a template. The primers were designed to introduce a flanking *Avr*II site at the 5' position and an *Eco*RI site at the 3' end. The resulting PCR product was cloned into dephosphorylated pUC19 previously digested using *Sma*I to yield pAGvar2. The plasmids pAGvar1 and pAGvar2 were then digested using *XbaI* and the PCR fragment from pAGvar1 recovered and cloned into dephosphorylated *XbaI* digested pAGvar2, the correct orientation of the incoming fragment was determined by restriction analysis. The resulting plasmid pAGvar3 was subsequently digested using *Bgl*II and *Avr*II and ligated to the annealed oligonucleotides O/AGvar03F and O/AGvar04R generating pAGvar4. The plasmid pAGvar4 was subsequently digested using *Eco*RI and *XbaI* and the resulting ~620bp fragment including the annealed oligonucleotides introduced into pSET152 previously digested using *Eco*RI and *Xba*I yielding the vector pAGvarX.

[0166] The region of pAGvarX constructed by annealing the respective oligonucleotides, introduce a *Bsr*GI site via a silent mutation at the amino acids 6 and 7 (C and T respectively) with respect to the actagardine peptide. This site can be used in conjunction with either the upstream *Bgl*II site or downstream *Avr*II site to introduce DNA encoding targeted mutations to any of the amino acids encoded within the actA peptide.

EXAMPLE 3 - Host Cell

[0167] This example illustrates the production of a lantibiotic-producing host cell in which the *lanA* gene has been inactivated. In this example, the host cell is *A. garbadinensis* in which the *actA* gene has been deleted.

Construction of the strain A. garbadinensis Δ actA

[0168] The strain *A. garbadinensis* Δ *actA* is utilized as a host for expressing variants of the actagardine structural gene *actA.* This strain was generated from wild-type *A. garbadinensis* using the Redirect technology developed by Gust *et al.*, 2002. Firstly, the region of DNA from the cosmid CosAG14 encoding *actA* was replaced with the cassette SBdel-1. SBdel-1 consists of the apramycin resistance gene (*aac*(3)IV) and *ori*T flanked by FLP recognition target (FRT) sites and was amplified by PCR using the plasmid pIJ773 as the template together with the primers O/SB50F and O/SB51R which bind at 21536 and 21802 of SEQ ID NO:100 respectively. Following the Redirect protocol (Gust et *al.,* 2004), *actA* of CosAG14 was replaced with SBdel-1 generating the cosmid CosAG14ΔA. The central part of the SBdel-1 cassette was subsequently removed from CosAG14ΔA by FLP-mediated excision following step 7 of the Redirect protocol generating CosAG14ΔB. Removal of this region allows the generation of non-polar, unmarked in-frame deletions as well as repeated use of the same resistance marker (Gust *et al.*, 2003).

[0169] The second stage of construction was to engineer the cosmid so that it could be introduced into *A. garbadinensis* via conjugation. This began by first inserting CosAG14ΔB into the *E. coli* strain BW25113/pIJ790 by transformation. The ampicillin gene of CosAG14ΔB was then replaced with SBdel-2 following the Redirect protocol (Gust *et al.*, 2004) generating the cosmid CosAG14ΔC. The cassette SBdel-2, like SBdel-1, houses the apramycin resistance gene (*aac (3)IV*) and *ori*T flanked by FRT sites but was generated using the primers O/SB52F and O/SB53R together with the template pIJ773.

[0170] CosAG14ΔC was used to tranform electrocompetent cells of *E. coli* ET12567/pUZ8002 before being conjugated with *A. garbadinensis* following the Redirect protocol (Gust et *al.,* 2004; see also following paragraph). The resulting strain in which the *actA* gene has been removed from the chromosome of the wild-type producer is *A. garbadinensis* Δ *actA.*

[0171] In more detail, to obtain the *A. garbadinensis* Δ *actA* strain above, CosAG14ΔC was used to transform electrocompetent cells of *E. coli* ET12567/pUZ8002 before being conjugated with *A. garbadinensis.* Apramycin resistant exconjugants were obtained and sub-cultured through six successive rounds of growth in TSB without apramycin. Cells from culture 6 were plated onto medium 65 and incubated at 30˚C. After 5 days colonies were transferred and patched out over an area of approximately 1 cm^2 onto medium 65. After 3 days incubation at 30˚C the patched cells were transferred to medium 65 containing apramycin at a final concentration of 50 μg/ml. Following 72 h incubation at 30˚C, cells sensitive to apramycin were selected and the respective patches used to inoculate 50 ml flasks containing 10 ml TSB and grown at -30˚C, 250 rpm for 4 days. Genomic DNA was prepared from each culture and analysed by PCR using oligonucleotides O/AGvar01bF and O/AGvar06r. PCR products of a size consistent with the deletion of the *actA* gene were generated. In parallel, analysis of fermentation broths by hplc demonstrated that these same samples did not produce actagardine.

EXAMPLE 4 - Heterologous Expression

[0172] This example illustrates the expression of actagardine from the SEQ ID NO:100 gene cluster in a host cell which is a non-producer cell, *S. lividans.* Such host cells provide an alternative means of generating active variants of these two peptides.

[0173] The cosmids CosAG14 and CosAL02 containing the biosynthetic gene clusters encoding the production of actagardine and deoxy-actagardine B do not possess an origin of transfer (*oriT*) necessary to facilitate conjugal transfer to a heterologous host. Using Redirect technology (Gust et *al.,* 2002) an *oriT* together with a phage attachment site *att*P and integrase (*int*) can be introduced into the SuperCos1 backbone of CosAG14 and CosAL02 replacing the neomycin resistance gene, neo.

Construction of *vectors for heterologous expression.*

[0174] The cosmid pMJCOS1 (supplied by the JIC, Norwich) is a derivative of SuperCos1 (Stratagene) in which the gene encoding for neomycin resistance has been replaced by a cassette (HEapra) which includes DNA encoding an *oriT, attP,* integrase (*int*) and apramycin resistance gene (*aac(3)IV*). The cassette HEapra was isolated by digesting pMJCOS1 with SspI and recovering the DNA from an agarose gel. This cassette together with CosAG14 and CosAL02 were used to generate the cosmids CosAG14HEapra and CosAL02HEapra respectively following the Redirect protocol as described by Gust *et al.*, 2004.

[0175] The cosmid CosAG14HEapra was subsequently introduced into *S. lividans* via conjugation. Apramycin resistant exconjugants of *S. lividans*/CosAG14HEapra were isolated. Three exconjugants were used to inoculate TSB seed media. *S. lividans, A. garbadinensis* and *A. liguriae* were grown in parallel to provide controls. Following 48 h incubation the seed cultures were used to inoculate a range of four different production media namely, AAS1, GM1, GM3 and TSB. These cultures were incubated for a total of nine days at 30˚C with 1.5 ml aliquots being removed from each flask after 5, 7 and 9 days incubation. The aliquots were centrifuged at 14000 rpm (IEC micromax benchtop centrifuge) for 10 minutes and the supernatants then decanted and used undiluted for bioassays and HPLC-MS analysis.

[0176] Zones of inhibition (haloes) indicative of the presence of a biological active compound(s) were observed around all of the wells loaded with supernatants of *S. lividans* containing the cosmid CosAG14HEapra (*S. lividans*/ CosAG14HEapra) except for wells loaded with supernatant from fermentations in TSB where no haloes were generated. No biological activity was observed around wells loaded with supernatant from fermentations of *S. lividans* grown in any of the four media. Haloes were evident around all wells loaded with supernatants from cultures of *A. liguriae* and *A. garbadinensis* where growth was supported. All haloes were consistently generated from the first day of sampling on day 5 through to day 9 although a general reduction in the diameter of the haloes was evident.

[0177] HPLC-MS analysis of the supernatants from the fermentations of *S. lividans*/CosAG14HEapra confirm the presence of peaks with retention times and masses corresponding to ala(O)actagardine. These same peaks were absent from supernatants of *S. lividans* only. Table 3 summarises the HPLC-MS analyses of supernatants from fermentation of S. *lividans, S. lividans*/CosAG14HEapra, *A. garbadinensis* and *A. liguriae* following incubation for 5 days.

Table 3:

Sample	**Fermentation medium**	**Concentration of product (μg/ml)**	**Retention Time (min)**	**Molecular ion (m/z)**	**Identity**
S.lividansl CosAG14HEapra	GM1	83	6.75	981 991	Ala (0) Actagardine $(M+2H)^{+2}$ Ala (0) Actagardine $(M+H+Na)^{+2}$
S.lividansl CosAG14HEapra	GM3	33	6.75	981 991	Ala (0) Actagardine $(M+2H)^{+2}$ Ala (0) Actagardine $(M+H+Na)^{+2}$
S.lividans	GM1	Not Detected	Not Detected	Not Detected	Not Detected
S.lividans	GM3	Not Detected	Not Detected	Not Detected	Not Detected
A. garbadinensis	GM1	58	6.9	945	Actagardine $(M+2H)^{+2}$
A. garbadinensis	GM3	24	6.8	981 991	Ala (0) Actagardine $(M+2H)^{+2}$ Ala (0) Actagardine $(M+H+Na)^{+2}$
A. liguriae	GM1	Not detected	7.06	937	Deoxy-actagardineB $(M+2H)^{+2}$
A. liguriae	GM3	Not detected	7.06	937	Deoxy-actagardineB $(M+2H)^{+2}$

EXAMPLE 5 - Antibacterial Activities

MIC Determination

[0178] A selection of the variants produced as disclosed herein above were tested further for activity against a range of bacteria. Minimum inhibitory concentrations (MICs) for all organisms with the exception of *Streptococcus pneumoniae* were determined by two-fold serial antibiotic dilutions in Mueller-Hinton broth (MHB) supplemented with calcium chloride dehydrate to a final calcium concentration of 400μg/ml. Minimum inhibitory concentrations (MICs) for *S. pneumoniae* were determined by two-fold serial antibiotic dilutions in Brain Heart Infusion (BHI) broth supplemented with 400 μg/ml calcium chloride dihydrate. Antimicrobial agent stock solutions were prepared and stored according to NCCLS standard M7-A6.

[0179] Actively growing broth cultures were diluted to contain 105 to 106 CFU/ml by adjusting to an absorbance of 0.2 - 0.3 at 600nm, equivalent to the McFarland 0.5 standard. They were then diluted a further 1:100 in broth. The assays were performed in duplicate in sterile 96-well microtitre plates in a total volume of 200 μl (160 μl broth, 20 μl antibiotic, 20 μl inoculum) in a concentration range from 64 μg/ml to 0.06 μg/ml. The 12th well of the microtitre plate contained no antimicrobial agent. Vancomycin was used as a reference antibiotic for quality control. Plates were incubated aerobically, shaking, for 18 - 20 hours at 37˚C with the MIC defined as the lowest concentration of drug that produced no visible growth.

	E.faecium	*E.faecalis*	*S.aureus*	*S.aureus*	*S.epidermidis*	*S.pneumoniae*
	19579	29212	R33	SH1000	11047	R6
Actagardine	4,4	<4, <4	16, 8	8, 8	8, 8	<4, <4
Actagardine	4, 4	<4, <4	16,16	8,8	8, 8	<4, <4
Ala (0) Actagardine	8,8	4,4	8, 8	8,8	8,4	<4, <4
Ala (0) Actagardine	32, 16	8,8	<4, <4	8,8	8, 8	<4, <4
Deoxyactardine B	16,16	4,4	16,16	16,16	16,16	8,8
Deoxyactardine B	16,16	<4, <4	16,16	16,16	16,16	<4, <4

EXAMPLE 6 - NMR Analysis

NMR Studies on Actagardine and Deoxy-actagardine

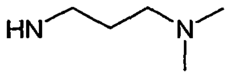
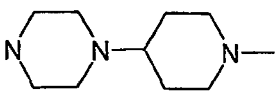
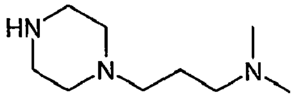
[0180] NMR spectroscopy (COSY, TOCSY, HSQC and NOESY) was successfully used to confirm the sequencing results obtained from producers of actagardine (*A. garbadinensis*) and deoxyactagardine B (*A. liguriae*). Whilst the data obtained did not permit a completely unambiguous assignment of all residues, it was consistent with the structures shown in Figure 4 and sufficient to confirm that deoxy-actagardine B from *A. liguriae* has at positions 15 and 16 the residues Leu and Val respectively.

EXAMPLE 7 - SYNTHESIS OF DERIVATIVES

[0181] The following derivatives of deoxy-actagardine B were made, in which the groups Z and the C-terminal amide were as follows:

Compound Structures

[0182]

Compound	**Z**	**C-terminal amide**
I	H	
II	H	
III	H	
IV	D-Ala	H
V	L-Ile	H
VI	L-Val	H
VII	L-Phe	H

(continued)

Compound	Z	C-terminal amide
VII	L-Lys	H
IX	L-Tryp	H

[0183] The synthesis of the compounds I - XI was as follows:

General procedure 1. Preparation of compounds I - III

[0184] To a solution of deoxy-actagardine B (20mg, 11 nmol), the appropriate amine (11nmol) and diisopropylethylamine (7.2μl, 70nmol) in dry dimethylformamide (0.8ml) were added 200μl of a solution of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop) (70mg, 134nmol) in dry dimethylformamide (1.0ml). The mixture was analysed by HPLC to follow the progress of the reaction, adding further aliquots of the Pybop solution until all the starting material had been consumed. HPLC analysis at this stage also showed variable amounts (5-20%) of the diamide. After completion of the reaction, the mixture was diluted with 30% acetonitrile in 20mM Kpi aqueous phosphate buffer, pH7 (10ml) and the monoamide was purified by preparative HPLC using the conditions described in Table 4. The appropriate fractions were concentrated to 25% of their original volume and desalted by loading on to a preconditioned C18 Bond Elut column (500mg) which was subsequently washed by sequential elution with two column volumes of 30, 40, 70 and 90% aqueous methanol. Evaporation of the appropriate fractions gave the desired products as white solids.

Compound I : Deoxy-Actagardine B N-[3-dimethylaminopropyl] monocarboxamide

[0185] Was obtained from coupling of deoxyactagardine B and 3-(dimethylamino)propylamine according to General Procedure 1. Yield 18mg, 85% yield. [$M+2H\ 2^+$] calculated 979.0, found 980.2

Compound II : Deoxy-Actagardine B N-[1-(1-methyl-4-piperidinyl)piperazine] monocarboxamide

[0186] Was obtained from the coupling of deoxyactagardine B and 4-(piperidino)piperazine according to General Procedure 1. Yield 8mg, 37% yield. [$M+2H\ 2^+$] calculated 1019.5, found 1020.0; [$M+3H\ 3^+$] calculated 680.0, found 680.0

Compound III: Deoxy-Actagardine B [1-(3-dimethylaminopropyl)piperazine] monocarboxamide

[0187] Was obtained from the coupling of Deoxy-actagardine B and 1-(3-dimethylaminopropyl)piperazine according to general procedure 1. Yield 10mg, 46% [$M+2H\ 2^+$] calculated 1013.5, found 1014.0

General procedure 2. Preparation of compounds IV-IX

[0188] A solution of the appropriate Fmoc proctected amino acid (34nmol) in dry dimethylformamide (0.4ml) was treated with a solution of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop) (11.4mg, 22nmol) and diisopropylethylamine (11μl, 68 nmol) in dry dimethylformamide (0.4ml). The mixture was then added to a solution of Deoxy-Actagardine B (2mg, 11 nmol) in dry dimethylformamide (0.5ml) The mixture was left at room temperature for 1h, after which time analytical HPLC (30-65% acetonitrile in 20mM Kpi aqueous phosphate buffer, pH7) showed complete conversion of the starting material. The reaction mixture was diluted with 40% aqueous methanol (20ml) and the mixture was passed through a C18 Bond Elute column (500mg) that had been preconditioned by washing with two column volumes of 100% methanol followed by two column volumes of water. The column was eluted sequentially with two column volumes of 40, 50, 60, 70, 80, 90 and 100% aqueous methanol. The fractions were analysed by HPLC and the fractions containing the Fmoc-protected coupling product were evaporated to dryness. The residue was taken up in dimethylformamide (1 ml) and piperidine (50μl) was added to remove the Fmoc protecting group. Progress of the reaction was monitered by HPLC and after complete consumption of the starting material the solution was diluted into 30% aqueous methanol (20ml). The mixture was then eluted through a C18 Bond Elut cartridge (500mg) as previously described and the product obtained after evaporation of the appropriate fractions was further purified by preparative HPLC using the conditions described in Table 4. The appropriate fractions were concentrated to 25% of their original volume and desalted by loading on to a preconditioned C18 Bond Elut column (500mg) which was subsequently washed by sequential elution with two column volumes of 30, 40, 70 and 90% aqueous methanol. Evaporation of the appropriate fractions gave the desired products as white solids.

Compound IV : D-Ala <0>deoxy-actagardine B

[0189] Was prepared according to general procedure 2 from Deoxy-actagardine B and Fmoc-D-alanine in 74% yield. [M+2H 2^+] calculated 972.5, found 973.0 043/188

Compound V : L-Ile(0)deoxy-actagardine B

[0190] Was prepared according to general procedure 2 from Deoxy-actagardine B and Fmoc-L-isoleucine in 27% yield. [M+2H 2^+] calculated 993.5, found 993.8

Compound VI : L-Val(0)deoxyactagardine B

[0191] Was prepared according to general procedure 2 from Deoxy-actagardine B and Fmoc-L-valine in 55% yield. [M+2H 2^+] calculated 986.5, found 985.9.

Compound VII : L-Phe(0)deoxyactagardine B

[0192] Was prepared according to general procedure 2 from Deoxy-actagardine B and Fmoc-L-phenylalanine in 22% yield. [M+2H 2^+] calculated 1010.5, found 1010.9.

Compound VIII : L-Lys(0)deoxyactagardine B

[0193] Was prepared according to general procedure 2 from Deoxy-actagardine B and Bis(Fmoc)-L-lysine in 45% yield. [M+2H 2^+] calculated 1001.0, found 1001.6

Compound IX : L-Tryp(0)deoxyactagardine B

[0194] Was prepared according to general procedure 2 from Deoxy-actagardine B and Fmoc-L-tryptophan in 55% yield. [M+2H 2^+] calculated 1030.0, found 1029.9.

EXAMPLE 8 - FURTHER ANTIBACTERIAL DATA

MIC Determination

Staphylococcus, Streptococcus, Enterococcus spp.

[0195] Minimum inhibitory concentrations (MICs) were determined and antimicrobial agent stock solutions were prepared and stored according to the NCCLS reference microdilution broth method for aerobic bacteria (M7-A6, 2003). MICs were determined by two-fold serial antibiotic dilutions in Mueller-Hinton broth (MHB) or Brain Heart Infusion (BHI) broth (*S. pneumoniae*). Actively growing broth cultures were adjusted in sterile broth or by direct colony suspension (*S. pneumoniae*) to a turbidity equivalent to the McFarland 0.5 standard (1 x 10^8 CFU/ml), then further diluted in sterile broth for a final inoculum in sterile 96-well microtitre plates of approximately 5 x 10^5 CFU/ml. The assays were performed in duplicate with *Enterococcus faecalis* ATCC 29212 included as a reference control strain and Vancomycin as a reference antibiotic for quality control. Plates were incubated aerobically, shaking, for 18 - 20 hours at 37˚C with the MIC defined as the lowest concentration of drug that produced no visible growth.

Clostridium difficile

[0196] Minimum inhibitory concentrations (MICs for *C. difficile* were determined and antimicrobial agent stock solutions were prepared and stored according to the NCCLS reference agar dilution method for anaerobic bacteria (M11-A5, 2001). Two-fold serial antibiotic dilutions were prepared in Wilkens-Chalgren agar (WCA). Test organisms were selected from 48 hour growth on Braziers (C.C.E.Y.) agar, subcultured in Schaedler broth to a density equivalent to a McFarland 0.5 standard (1 x 10^8 CFU/ml), with a final inoculum onto WCA plates of approximately 10^5 CFU/spot. *Bacteroides fragilis* ATCC 25285 was included as a reference control strain and Metronidazole was used as a reference antibiotic for quality control. All manipulations were performed in duplicate in ambient atmosphere in pre-reduced media with only brief exposure to oxygen. Plates were incubated anaerobically for 48 hours at 37˚C with the MIC defined as the concentration of drug where a marked reduction occurred in the appearance of growth on the test plate compared to growth on the anaerobic control plate.

Propionibacterium acnes

[0197] Test organisms were selected from 3-7 day growth on Wilkens-Chalgren agar (WCA) supplemented with furazolidone (1-2 μg/ml). Fresh Wilkens-Chalgren broth (WCB) was inoculated by direct colony suspension with single colonies of *P. acnes* and adjusted to a density equivalent to the McFarland 0.5 standard (1 x 10^8 CFU/ml), then further diluted in sterile WCB for a final inoculum in sterile 96-well microtitre plates of approximately 10^5 CFU/ml. Two-fold serial antibiotic dilutions were performed in sterile water with stock solutions prepared and stored according to NCCLS standards (M11-A5, 2001). The assays were performed in duplicate with Vancomycin and Clindamycin used as reference antibiotics for quality control. Plates were incubated anaerobically for 48-72 hours at 37°C with the MIC defined as the concentration of drug where a marked reduction occurred in the appearance of growth on the test plate compared to growth on the anaerobic control plate. All manipulations were performed in duplicate in ambient atmosphere in pre-reduced media with only brief exposure to oxygen.

[0198] Culture media were supplemented with calcium ions (as calcium chloride) at 50μg/ml except where higher concentrations are indicated. MIC values in μg/ml are shown in the following Tables:

Table 6: MIC values against Enterococci, Streptococci and Staphylococci

Organism	Ala(O)-deoxyactagardine-B	Deoxyactagardine-B
M. luteus 4698 +200 μg/ml Ca2+	4	8
E. faecalis 29212	16	16
E. faecalis 29212 +200 μg/ml Ca^{2+}	4	8
E. faecalis 29212 +400 μg/ml Ca^{2+}		4
E. faecium 7131121 (VRE)	>64	>64
E. faecium 7131121 (VRE) +200 μg/ml Ca^{2+}	>64	>64
E. faecium 7131121 (VRE) +400 μg/ml Ca^{2+}		32
E. faecium 19579	>64	>64
E. faecium 19579 +200 μg/ml Ca2+	>64	>64
E. faecium 19579 +400 μg/ml Ca^{2+}		16
S. aureus R33 (MRSA)	32	32
S. aureus R33 (MRSA) +200 μg/ml Ca^{2+}	16	8
S. aureus R33 (MRSA) +400 μg/ml Ca^{2+}		16
S. aureus SH1000	16	16
S. aureus SH1000 +200 μg/ml Ca^{2+}	8	8
S. aureus SH1000 +400 μg/ml Ca^{2+}		16
S. epidermidis 11047	16	32
S. epidermidis 11047 +200 μg/ml Ca^{2+}	8	16
S. epidermidis 11047 +400 μg/ml Ca^{2+}		16
S. pnuemoniae R6	16	16
S. pnuemoniae R6 +200 μg/ml Ca2+	32	6
S. pnuemoniae R6 +400 μg/ml Ca^{2+}		4
S. aureus 12232 MRSA		16
S. aureus R36 (MRSA)		16
S. aureus R34 (MRSA)		16
S. aureus R39 (MRSA)		>32
S. aureus R40 (MRSA)		>32
S. aureus W71 (MRSA)		>32
S. aureus W74 (MRSA)		>32
S. aureus W96 (MRSA)		>32
S. aureus W97 (MRSA)		>32
S. aureus W98 (MRSA)		>32
S. aureus W99 (MRSA)		>32
S. epidermidis 7755298 (MRSE)		>32
S. epidermidis 7865688 (MRSE)		>32
S. epidermidis 7753921 (MRSE)		>32

(continued)

Organism	**Ala(O)-deoxyactagardine-B**	**Deoxyactagardine-B**
S. epidermidis GRL05011 (MRSE)		>32

Table 7: MIC values against fusidic acid-resistant Staphylococcus aureus

Organism	**Deoxy-Actagardine B**
Fusidic acid-res *S. aureus* 8325-4	8, 8
Fusidic acid-res S. aureus CS1116	32, 32
Fusidic acid-res S. aureus CS957	32, 32
Fusidic acid-res S. aureus CS767	32, 32
Fusidic acid-res S. aureus CS858	32, 32
Fusidic acid-res S. aureus CS741	32, 32
Fusidic acid-res S. aureus CS1145	16, 16
Fusidic acid-res S. aureus CS872	16, 16
Fusidic acid-res S. aureus CS866	32, 32
Fusidic acid-res S. aureus CS607	64, 64
Fusidic acid-res S. aureus CS22	16, 16
Fusidic acid-res S. aureus 8325-4 +200 μg/ml Ca2+	4, 4
Fusidic acid-res S. aureus CS1116 +200 μg/ml Ca2+	16, 16
Fusidic acid-res S. aureus CS957+200 μg/ml Ca2+	16, 16
Fusidic acid-res S. aureus CS767 +200 μg/ml Ca2+	16, 16
Fusidic acid-res S. aureus CS858 +200 μg/ml Ca2+	16, 16
Fusidic acid-res S. aureus CS741+200 μg/ml Ca2+	16, 16
Fusidic acid-res S. aureus CS1145 +200 μg/ml Ca2+	8, 8
Fusidic acid-res S. aureus CS872 +200 μg/ml Ca2+	8, 8
Fusidic acid-res S. aureus CS866 200 μg/ml Ca2+	8, 8
Fusidic acid-res S. aureus CS607 +200 μg/ml Ca2+	32, 32
Fusidic acid-res S. aureus CS22 +200 μg/ml Ca2+	4, 4

Table 8: MIC values against mupirocin-resistant Staphylococcus . aureus

Organism	**Deoxy-actagardine B**
8325-4	8, 8
GISA-2	8, 8
LZ6	16, 16
LZ8	16, 16
LZ9	16, 16
LZ10	8, 8
420	4, 4
1205	16, 16
1120	16, 16
1454	16, 16
1086	8, 8

Table 9: MIC values against Propionibacterium acnes

Organism	Deoxy-Actagardine B
Propionibacterium acnes P37 (lab strain)	4, 4
P. acnes AT1	4, 4
*P. acnes*AT26	2, 2
*P. acnes*101897d	2, 2

(continued)

Organism	Deoxy-Actagardine B
P. acnes PF284 (tet res)	2, 2
P. acnes PF286 (erythro & clin res)	2, 2
P. acnes PF289 (clin and co-trimazole res)	4, 8

Table 10: MIC values against C.difficile

Organism	Ala(O)-deoxyactagardine- B	Deoxyactagardine-B
C. diffcile 37779	4	4
C. diffcile 19126	2	4
MIC$_{50}$ 10 *C.difficile* strains		2
MIC$_{90}$ 10 *C.difficile* strains		4

Table 11: MIC values against C.difficile

Organism	I	II	III	IV	V	VI	VII	VIII	IX
C. difficile 37779			>8	4	4	8	1	4	1
C. difficile 19126			>8	4	4	8	2	4	2
MIC$_{50}$ *C.difficle*	2	2					2		
MIC$_{90}$ *C.difficile*	4	4					2		

Materials & Methods

[0199] The materials and methods used in Examples 2-7 above are as follows:

Media

[0200] All buffers, solutions and media were made up using reverse osmosis (RO) water and contained per litre the following ingredients:

AAS1		*ABB13*	
Soluble starch	10 g	Soytone peptone	5 g
Glucose	10 g	Soluble starch	5 g
Peptone	5 g	$CaCO_3$	3 g
Dry corn steep liquor	1 g	MOPS	2.1 g
Yeast extract	2 g	Agar	20 g
Adjust pH to 6.0		Adjust pH to 7.0	
GM1		*BHI*	
Lablemco meat extract	4 g	Brain Heart Infusion	37 g
Peptone	4 g		
NaCl	2.5g	*GM3*	
Yeast extract	1 g	Arkasoy soyflour	20 g
Soy flour	10 g	Mannitol	20 g
Glucose	25 g	Adjust pH to 7.0	
$CaCO_3$	5 g		
Adjust pH to 7.6		*LA*	
		Luria agar	40 g
Mueller Hinton			

(continued)

Mueller Hinton broth	21 g	*LB*	
For agar plates add;		Luria broth	25 g
Agar	10 g		
SFM		*SV2*	
Soya Flour	20 g	Glucose	15 g
D-mannitol	20 g	Glycerol	15 g
Agar	16 g	Peptone	15 g
		NaCl	3 g
		$CaCO_3$	1 g
		Adjust pH to 7.0	
TAE buffer		*TSB*	
Tris	48.44 g	Tryptic soy broth	30 g
EDTA	3.72 g	Adjust pH to 7.0	
Adjust pH to 8.3			
2TY		'65'	
Tryptone	16 g	Glucose	4 g
Yeast extract	10 g	Yeast extract	4 g
NaCl	5 g	Malt extract	10 g
Adjust pH to 6.5 - 7.0		$CaCO_3$	2 g
		Agar	12 g
		Adjust pH to 7.2	

Bioassays

[0201] *Micrococcus luteus* was inoculated from frozen stock into 10ml Mueller-Hinton broth and grown overnight at 30˚C with shaking at 200 rpm. 1 ml of this culture was used to inoculate 300 ml of Mueller-Hinton agar which was then poured into petri dishes. Wells (6 mm diameter) placed equidistant apart were made using a cork-borer and subsequently loaded with 50 μl of the respective sample. The bioassay plate was placed into a laminar air flow until the loaded samples had diffused, at which point the plates were transferred to a 30˚C incubator and left overnight.

Endonuclease restriction digestions

[0202] Digestions of DNA with restriction enzymes were carried out in the supplied buffers and in accordance with the manufacturer's guidelines. Typically, for preparative digests 5 μg of DNA was digested with 12 units of enzyme for 3 h at the recommended temperature. For analytical digests, 0.5 μg of DNA was digested with 2 units of enzyme for 2-3 h again at the recommended temperature. The digested DNA was analysed by agarose gel electrophoresis.

Sub-culturing exconjugants

[0203] Agar plugs of patched exconjugants were used to inoculate 50 ml flasks containing 8 ml TSB and 2 glass beads. The cultures were incubated at 30˚C, 250 rpm for 10 days then 100 μl were removed and added to 10 ml TSB in a 50 ml flask containing 2 glass beads. The flasks were incubated for 2 days then 1 ml was removed and used to inoculate a 50 ml flask containing 10 ml TSB. Using 1 ml inoculum a total of six successive rounds of growth were carried out each incubated for 2 days at 30˚C, 250 rpm. Cells from the sixth round of sub-culturing were pelleted by centrifuging at 4000 rpm for 20 minutes (Heraeus Sepatech Megafuge) then sonicated (MSE Sanyo Soniprep 150, amplitude 10-15 microns) for 30 seconds in TSB to disrupt the mycelium. Serial dilutions (10^{-1} to 10^{-5} in TSB) of the sonicated cells were plated onto medium 65 and incubated at 30˚C.

Fermentation for heterologous expression

[0204] 50 ml conical flasks each containing 2 glass beads and either 8 ml TSB or AAS1 media supplemented with nalidixic acid and the appropriate selective antibiotic were inoculated using agar plugs or 250 µl of a -80˚C glycerol stock. Following 2 - 4 days incubation at 30˚C, 200 rpm, 1.2 ml (3%) per seed culture was used to inoculate 40 ml of the respective production media in 250 ml conical flasks containing 2 glass beads. These cultures were incubated at 30˚C, 200 rpm for 9 days.1.5 ml whole broth aliquots were removed periodically from each culture for analysis by bioassay and/or HPLC-MS analysis.

Fermentation of A. *liguriae for the isolation of deoxy-actagardine B*

[0205] 250 ml conical flasks each containing 2 glass beads and 50 ml SV2 media were inoculated with 500 µl (1%) of *A. liguriae* cells from a glycerol stock. Following 4 days incubation at 30˚C, 250 rpm, 12 ml (3%) per seed culture was used to inoculate 400 ml of GM3 in 2L conical flasks. These cultures were incubated at 30˚C, 225 rpm for nine days. The culture broth was harvested by centrifugation at 4000 rpm (Heraeus Sepatech Megafuge) for 30 minutes after which the supernatant was decanted from the pellet of cells.

Fermentation of *A. garbadinensis for the isolation* of *actagardine and Ala(O)-actagardine*

[0206] 250 ml conical flasks each containing 2 glass beads and 50 ml AAS media were inoculated with 500 µl (1%) of *A. garbadinensis* cells from a glycerol stock. Following 9 days incubation at 30˚C, 250 rpm, 12 ml (3%) per seed culture was used to inoculate 400 ml of AAS in 2L conical flasks. These cultures were incubated at 30˚C, 200 rpm for eight days. The culture broth was harvested by centrifugation at 4000 rpm (Heraeus Sepatech Megafuge) for 30 minutes after which the supernatant was decanted from the pellet of cells.

Isolation of Deoxy-actagardine B for MIC studies

[0207] Diaion HP-20 resin (50 g/L) was added and mixed with supernatant isolated from a fermentation of *A. liguriae* and left overnight at 4˚C. The suspension was aliquoted into Bond Elut columns (60 ml) and the resin washed sequentially with four bed volumes of water followed by three bed volumes of 25, 50, 75 and 100% methanol. HPLC analysis confirmed the presence of Deoxy-actagardine B in the 50, 75 and 100% methanol fractions. These fractions were combined then concentrated to approximately a quarter of the volume of the starting pool. The concentrate from 1L of broth was loaded onto two C18 Bond Elut columns (5 g) that had been pre-conditioned by washing with two column volumes of 100% methanol followed by two column volumes of water. The columns were eluted sequentially with two column volumes of 50, 60, 70, 80, 90% methanol followed by two column volumes of 100% methanol. HPLC analysis confirmed the presence of Deoxy-actagardine B in the 80, 90 and 100% methanol fractions, these fractions were pooled and concentrated to a third of the starting volume. An equal volume of 40 mM potassium phosphate pH 2.5 in 50% methanol was added and the concentrate then loaded evenly onto three pre-equilibrated SCX Bond Elut columns (1g). The SCX columns were initially washed with 40 mM potassium phosphate pH 2.5 in 50% methanol and then eluted using 1.5 column volumes of 250 mM potassium phosphate pH 7.0 in 50% methanol. The eluent was desalted by loading onto a C18 Bond Elut column (5 g) that had been pre-conditioned with two column volumes of methanol followed by two column volumes of water. The column was washed with two column volumes of 50% and then 60% methanol. Deoxy-actagardine B was eluted following the addition of two column volumes each of 70, 80, 90 and 100% methanol. Fractions containing purified Deoxy-actagardine B as confirmed by HPLC and LC-MS analyses were pooled and evaporated to dryness.

Isolation of Ala(0)-Deoxyactagardine B from fermentation of A.liguriae

[0208] Diaion HP-20 resin (50 g/L) was mixed with supernatant from a four litre fermentation of *A. liguriae* and left overnight at 4˚C. The suspension was collected into a glass sinter funnel and the resin was washed sequentially with four bed volumes of water followed by four bed volumes of 50% Methanol. Deoxy-actagardineB and Ala(0)-deoxyactagardine B were eluted from the resin by washing with five bed volumes of 100% Methanol. The 100% Methanol fraction was concentrated to a third of the original volume and was then diluted by addition of water to a final concentration of 60% Methanol. The resulting solution was loaded onto four 10g C18 Bond Elut columns prior to washing with two column volumes of 50% Methanol. Deoxy-actagardine B-related components were eluted from the column using two column volumes of Methanol/0.5% Formic Acid. The resulting eluent was concentrated by evaporation to 40 ml and Ala(0)-deoxyactagardine B was separated from Deoxy-actagardine B by preparative HPLC using the conditions described in the table below.

Column	Capitol HPLC Ltd C18 - BDS - HL5 - 26052 15cm x 20mm
Solvent A	30% ACN in 20mM Potassium Phosphate pH 5.0

(continued)

Column	Capitol HPLC Ltd C18 - BDS - HL5 - 26052 15cm x 20mm
Solvent B	65% ACN in 20mM Potassium Phosphate pH 5.0
Detection	210 nm
Flow Rate	10 ml/min
Time (T) = 0 min	100% A
T = 1 min	100% A
T = 29 min	35% B
T = 30 min	100% B
T = 33 min	100% B
T = 34 min	100% A
T = 35 min	100% A
Collection	Start 10 min; End 30 min; 0.5 or 0.25 minute fractions

[0209] Fractions containing Ala(0)-deoxy-actagardine B (as confirmed by HPLC and LC-MS analyses) were desalted using C18 Bond Elut columns as described above before being evaporated to dryness.

[0210] Ala(0)-deoxy-actagardine B was eluted from the column at Retention Time = 5.04 minutes. MS analysis confirmed a species of 972.2 m/z $(M+2H)^{+2}$.

Isolation of actagardine and Ala(O)-actagardine for MIC studies

[0211] Actagardine and Ala(0)-actagardine were purified using the method described for the purification of Deoxy-actagardine B from *A. liguriae* with the exception that preparative HPLC was required to resolve Ala(0)actagardine and Actagardine following the SCX Bond Elut step. Eluent from the SCX Bond Elut column was concentrated by rotary evaporation from 70 to 18 ml and the resulting concentrate was purified by preparative HPLC using the conditions described in Table 4 The respective fractions containing Actagardine and Ala(0)actagardine (as confirmed by HPLC and LC-MS analyses) were desalted using C18 Bond Elut columns as described previously before being evaporated to dryness.

Table 4: Preparative HPLC conditions for the separation of Actagardine and Ala(0)actagardine.

Column	Capitol HPLC Ltd C18 - BDS - HL5 - 26052 15 cm x 20 mm
Solvent A	30% Acetonitrile in 20mM Potassium Phosphate pH 7.0
Solvent B	65% Acetonitrile in 20mM Potassium Phosphate pH 7.0
Detection	268 nm
Flow Rate	10 ml/min
Time (T) = 0 min	100% A
T = 1 min	100% A
T = 19 min	25% B
T = 20 min	100% B
T = 25 min	100% B
T = 26 min	100% A
T = 30 min	100% A
Collection	Start 8 min; End 20 min; 1 minute fractions

Agarose *gel electrophoresis*

[0212] Electrophoresis of DNA was carried out as described by Sambrook et *al*., 1989. Agarose gels (0.7-1%) were prepared in TAE buffer containing a final concentration of 0.1 µg/ml ethidium bromide to allow visualisation of the DNA by UV light. 0.1 volumes of 10 x agarose gel loading solution was mixed with the samples. Samples were loaded onto the gel alongside a 100bp, 1 kb, or lambda DNA-*Hind*III digest ladders (NEB) and run at 1-5 V/cm. The gel was visualised at λ = 300 nm and photographed using a UVP video camera.

Recovery of *DNA from agarose gels*

[0213] DNA was excised from agarose gels and recovered using a Qiaquick gel extraction kit (Qiagen) and eluted in either sterile reverse osmosis purified water, Tris-HCl (10 mM, pH 8.5) or TE buffer.

End-filling

[0214] Filling the recessed 3' termini created by digestion of DNA with restriction enzymes was done using *E. coli* DNA polymerase Klenow fragment. In a typical reaction 1 unit of enzyme was added per µg DNA along with 250 µM each dNTP. The reaction was incubated at 25°C for 15-30 min and stopped by adding EDTA to a final concentration of 10 mM.

Phosphorylation of DNA

[0215] PCR products were treated with T4 polynucleotide kinase at 37°C for 30 min, following the method described by Sambrook et *al*., 1989. The enzyme was inactivated by incubating at 65°C for 20 min.

Dephosphorylation of linearised vectors

[0216] To avoid self-ligation of linearised vectors, 5'-phosphate groups were removed using shrimp alkaline phosphatase (SAP) following the manufacturer's guidelines. In a typical reaction 1 unit of SAP was added to the restriction mixture for the last hour of the DNA restriction reaction. The enzyme was inactivated by incubating at 65°C for 20 min.

Ligations

[0217] DNA ligations were performed as described by Sambrook *et al.,* (1989) using 1 unit (U) of T4 DNA ligase in a total volume of 15 µl and incubating for 12-16 h at 16°C.

Maintenance of bacterial cultures

[0218] Viable cells were stored as glycerol suspensions by freezing 0.5 ml of the respective culture at -80°C with glycerol at a final concentration of 10%. Single colonies of *A. garbadinensis* and *A. liguriae* were obtained by streaking 50 µl from a fermentation broth or glycerol stock onto either medium 65 or ABB13 plates.

Polymerase chain reaction

[0219] Polymerase chain reactions (PCRs) were performed on a Stratagene Robocycler Gradient96. In a typical reaction 100-200 ng template DNA was mixed with 20 pmol of each oligonucleotide primer and dNTP's at 250 µM each. Thermophilic DNA polymerase buffer as supplied by the manufacturer and DMSO made up 10% (v/v) each of a final volume of 50 or 100 µl reaction mixture. A typical reaction began with an initial cycle of 1 min denaturation (94°C), 1 min, Y°C (annealing) and 30 seconds - 3 min extension (72°C), at which point 5 units of thermophilic DNA polymerase was added. This was followed by 30 cycles of 94°C for 1 min, Y°C (annealing) for 1 min and 72°C for Xmin and a final cycle of 72°C for 2X min. The extension time X, was 1 min per kb of product when *Taq* polymerase was used and 2 min per kb of product when *Pfu* polymerase was used. The annealing temperature Y was 55°C and 49°C in the generation of pAGvar1 and pAGvar2 respectively. The conditions used for the generation of SBdel-1 and SBdel-2 were as described in the Redirect protocol (Gust et *al*., 2004).

Primers

[0220]

Primer name	SEQ ID NO:	Sequence 5'-3'
O/ AGvar01bF	303	TTCTAGACGTTGTTCTCCCATTTTCAC
O/ AGvar02bR	304	AAGATCTTCGAAGGTGAGCTCGCCGAA
O/ AGvar03F	305	GATCTTCGCGAGGACCGCACCATCTACGCCGCCAGCAGCGGCTGGGTGTGTACACTGACGATCGAGTGCGGCACCGTGATCTGCGCCTGCTGAC
O/ AGvar04R	306	CTAGGTCAGCAGGCGCAGATCACGGTGCCGCACTCGATCGTCAGTGTACACACCCAGCCGCTGCTGGCGGCGTAGATGGTGCGGTCCTCGCGAA
O/ AGvar05F	307	GCCTGCTGACCTAGGTCGACGATCGT
O/ AGvar06r	308	TGAATTCGGCTGCTCCCCGCGCGAAAT
O/SB50F	309	ATTCGCCCGGGAAGTCCACCGAAAGGAAGACACACCATGATTCCGGGGATCCGTCGACC
O/SB51R	310	GGGCGATGCCCGCCCCGGGCCGGAAACGATCGTCGATCATGTAGGCTGGAGCTGCTTC
O/SB52F	311	AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGATTCCGGGGATCCGTCGACC
O/SB53R	312	GCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCATGTAGGCTGGAGCTGCTTC

Preparation of plasmid DNA

[0221] Plasmid DNA was prepared on a small scale (less than 20 µg preparation) by inoculating 3 ml of sterile 2TY or LB containing the appropriate antibiotic with single colonies picked from 2TY (or LA) agar plates. The cultures were incubated overnight (12-16 h) at 37°C and 250 rpm. The cells were collected by centrifugation at 12,000 xg for 1 min and plasmid DNA obtained using Wizard (Promega) Miniprep kits according to the manufacturer's guidelines. In the case of larger preparations of up to 100 µg of plasmid DNA, 30 ml of 2TY cultures were grown and plasmid DNA extracted using a Qiagen Midi-prep kit, following the manufacturer's instructions. All plasmid preparations were checked by a combination of restriction analysis and/or sequence analysis.

Preparation of cosmid DNA

[0222] Cosmid DNA was prepared by inoculating 50 ml of sterile 2TY or LB containing the appropriate antibiotic with single colonies picked from 2TY (or LA) agar plates. The cultures were incubated overnight (12-16 h) at 37°C and 250 rpm. The cells were collected by centrifugation at 4,000rpm (Heraeus sepatech Megafuge 2.0R) for 20 min and Cosmid DNA isolated using a Qiagen Midi-prep kit according to the manufacturer's guidelines.

Preparation and transformation of electrocompetent E. coli cells.

[0223] Electrocompetent *E. coli* DH10B were prepared by the method of Dower *et al.*. (1988). Aliquots (60 µl) of competent cells were thawed on ice and 1.8 µl of ligation mixture or plasmid DNA added. The mixture was placed into an electroporation cuvette (Sigma 0.1 cm) and transferred to the electroporator (Stratagene electroporator-1000). A potential difference of 1.8 kV/mm (25 µF, 200 Ω) was applied and 0.5 ml of 2TY or LB medium subsequently added. The cells were then incubated at 37°C for 45-60 min to allow expression of the antibiotic resistance genes, prior to plating on the appropriate selective medium.

Preparation of genomic DNA

[0224] Genomic DNA templates were prepared using the procedure described by Kieser *et al.* (2000).

Conjugation procedure for Actinoplanes sp.

[0225] Intergeneric conjugation between *E. coli* and *Actinoplanes* sp. was performed following the procedure described by Heinzelmann *et al.* (2003), except, the strain *E. coli* ET12567/pUB8002 (Kieser *et al.*, 2000) was used in place of the strain *E. coli* ET12567/pUB307 (Flett *et al.*, 1997). Exconjugants were transferred and patched out over an area approximately 1 cm^2 onto medium 65 or ABB13 containing 50 μg/ml nalidixic acid and the relevant selective antibiotic. These plates were incubated at 30°C for 4-7 days prior to being used as inoculum for broth cultures.

Conjugation procedure for Streptomyces sp.

[0226] Intergeneric conjugation between *E. coli* and *Streptomyces* sp. was performed following the procedure described by Kieser et *al.*, 2000. Exconjugants were transferred and patched out over an area approximately 1 cm^2 onto SFM containing 50 μg/ml nalidixic acid and the relevant selective antibiotic. These plates were incubated at 30°C for 4-7 days prior to being used as inoculum for broth cultures.

Table 5- Bacterial Strains

Name	**Description/Use**
Actinoplanes garbadinensis ATCC31049	Isolation of the biosynthetic gene cluster for the production of actagardine.
Actinoplanes garbadinensis Δ	*Actinoplanes garbadinensis* ATCC31049 in which the actA gene has been removed.
actA	Expression of variant actA genes
Actinoplanes liguriae NCIMB 41362	Isolation of the biosynthetic gene cluster for the production of deoxy-actagardine B. Expression of variant *ligA* genes.
Escherichia coli XL1-Blue MR	Generation of a cosmid library.
Escherichia coli DH10B	Routine cloning.
Escherichia coli ET12567	Isolation of non-methylated DNA.
Escherichia coli ET12567/pUZ8002	Intergenic transfer of DNA via conjugation.
Escherichia coli BW25115/pIJ790	Strain containing the lambda red recombination plasmid pIJ790. Facilitates the targetted recombination of a cassette flanked by FLP recognition sites.
Escherichia coli DH5α/BT340	Strain containing the plasmid BT340 facilitating FLP-mediated excision of disruption cassettes.
Micrococcus luteus ATCC4698	Bioassay test organism.
Streptomyces lividans 1326	Host organism for the heterologous expression.
Streptomyces coelicolor B757	Host organism for the heterologous expression
Streptomyces cinnamoneus DSM 40005	Host organism for the heterologous expression

Antibiotics

[0227] Antibiotic stock solutions were prepared in water (unless stated otherwise) and filter sterilised by passing through a 0.22 μm Millipore filter. Solutions dissolved in ethanol were not sterilised (Sambrook *et al.,* 1989). All antibiotics were stored at -20°C. In media where apramycin was used, $MgCl_2$ was added to a final concentration of 10 mM (from a stock of 2.5 M).

	Stock solution	Working concentration
Ampicillin (amp)	100 mg/ml	100 μg/ml
Apramycin (apra)	100 mg/ml	50 μg/ml
Carbenicillin (car)	100 mg/ml	100 μg/ml
Chloramphenicol (cm)	25 mg/ml in ethanol	25 μg/ml

(continued)

	Stock solution	Working concentration
Kanamycin (kan)	50 mg/ml	50 μg/ml
Nalidixic acid (na)	25 mg/ml	25 μg/ml

Cassettes

[0228]

Name	Size (bp)	Source	Description/Use
SBdel-1	1462	PCR using the primers O/SB50F and O/SB51R and pIJ773 as a template.	Contains an origin of transfer (*oriT*) and apramycin resistance gene flanked by FLP recognition target sites. The 5' and 3' regions are homologous to DNA flanking the *actA* gene from *A. garbadinensis.*
SBdel-2	1462	PCR using the primers O/SB52F and O/SB53R and pIJ773 as a template.	Contains an origin of transfer (*oriT*) and apramycin resistance gene flanked by FLP recognition target sites. The 5' and 3' regions are homologous to DNA flanking the ampicillin resistance gene from SuperCos1.
HEapra	5247	pMJCOS1	SspI fragment isolated from pMJCOS1. Cassette consists of an apramycin resistance gene, origin of transfer (*oriT*), attachment site (*attP*) and øC31 integrase.

Vectors

[0229]

Name	Size (kb)	Resistance marker	Source	Description/Use
pAGvar1	3.1	amp	This study.	449bp PCR fragment generated using the primers O/AGvar01 bF and O/AGvar02bR and template pLITAG01 cloned into pUC19 previously digested using *Sma*I.
pAGvar2	2.8	amp	This study.	91bp PCR fragment generated using the primers O/AGvar05F and O/AGvar06R and template pLITAG01 cloned into pUC19 previously digested using *Sma*I.
pAGvar3	3.2	amp	This study.	*Xba*I fragment (-450bp) cloned into pAGvar2 previously digested using *Xba*I.

(continued)

Name	Size (kb)	Resistance marker	Source	Description/Use
pAGvar4	3.3	amp	This study.	Annealed oligonucleotides O/AGvar03F, O/AGvar04R ligated to pAGvar3 previously digested using *Bgl*II and *Avr*II.
pAGvarX	6.3	apra	This study.	*Xba*I - *Eco*RI fragment (~650bp) from pAGvar4 ligated to pSET152 previously digested using *Eco*RI/*Xba*I. Variant actagardine genes can be assembled and introduced into the hosts chromosome via the attachment site *attP*.
CosAL02	47.2	amp and neo.	This study.	40402 bp *Sau*3AI DNA fragment from *A*. *liguriae* cloned into SuperCos1 previously digested using *Bam*HI.
CosAL02HEapra	49.1	amp and apra.	This study.	CosAL02 in which the gene encoding neomycin has been replaced with the HEapra cassette.
CosAG14	45	amp and neo.	This study.	38168 bp Sau3AI DNA fragment from *A*. *garbadinensis* cloned into SuperCos1 previously digested using *Bam*HI.
CosAG14ΔA	46.3	amp, neo and ampra.	This study.	CosAG14 in which the *actA* gene has been replaced by the cassette SBdel-1.
CosAG14ΔB	44.9	amp and neo.	This study.	CosAG14ΔA in which the cassette SBdel-1 has been removed by FLP-recombinase leaving an 81bp scar.
CosAG14ΔC	45.5	neo and apra.	This study	CosAG14ΔB in which the ampicillin resistance gene has been replaced with the cassette SBdel-2.
CosAG14HEapra	46.9	amp and apra.	This study.	CosAG14 in which the gene encoding neomycin has been replaced with the HEapra cassette.
pIJ773	4.3	amp and apra.	John Innes Centre (JIC), Norwich.	Redirect template (Gust et *al*., 2003) used to generate the cassettes SBdel-1 and SBdel- 2.

(continued)

Name	Size (kb)	Resistance marker	Source	Description/Use
pLITAG01	6.1	amp.	This study.	3263bp Ncol fragment isolated from *A. garbadinensis* (19955-23217 CosAG14rc) cloned into pLITMUS28 previously digested using *Nco*I.
pLITMUS28	2.8	amp.	New England Biolabs (NEB).	Routine cloning
pMJCOS1	9.8	amp and apra.	JIC, Norwich.	SuperCos1 in which the gene encoding neomycin has been replaced by an *Ssp*I fragment consisting of an apramycin resistance gene, *oriT*, *attP* and øC31 integrase. Source of HEapra cassette.
pSET152	5.7	apra.	NRRL B14792	Conjugative plasmid which can facilitate introduction of DNA into the host's chromosome via the *attP* site.
SuperCos1	7.9	amp and neo.	Stratagene.	T3 and T7 promoter regions flanking a unique cloning site.
pUC19	2.7	amp.	NEB.	Routine cloning

High performance liquid chromatography

[0230] HPLC analyses were performed using a Hewlett Packard 1050 series HPLC system with the parameters as described below:

Column: Zorbax SB-C18, 4.6x150 mm, 5μ
Mobile Phase A: 30% Acetonitrile in 20 mM potassium phosphate buffer pH 7.0
Mobile Phase B: 65% Acetonitrile in 20 mM potassium phosphate buffer pH 7.0
Flow rate: 1ml/min
Gradient: Time 0 min 100% A 0% B
Time 10 min 0% A 100% B
Time 11 min 0% A 100% B
Time 11.2 min 100% A 0% B
Cycle time 15 min
Injection volume: 10 μl
Detection: 210 nm

High performance liquid chromatography -mass *spectrometry (HPLC-MS)*

[0231] HPLC-MS analyses were performed on a Hewlett Packard 1050 series HPLC system linked to a Micromass Platform LC (operated with MassLynx version 3.5 software) with the following parameters:

Column: Agilent Zorbax SB-C18 150 x 4.6mm 5μ

Flow rate: 1ml/min
Mobile phase: A 10% acetonitrile, 0.1% formic acid 90% water.
B 90% acetonitrile, 0.1% formic acid, 90% water.
Linear gradient A to B over 10 minutes, hold 1 min, B-A
Wavelength: 200 - 400nm
Injection volume: 10μl
Post column split: 1:10
Mass spectrometer: Micromass Platform LC
Mode: Electrospray positive
Nitrogen flow: 380 l/hr
Capillary voltage: 40V
Skimmer lens offset: 5V

Deposit

[0232] NCIMB 41362 was deposited under the Budapest Treaty on 7 December 2005 at NCIMB Ltd, Aberdeen, AB21 9YA, Scotland, UK, by Novacta Biosystems Limited.

Sequence Listing

[0233]

SEQ ID NO:1 Actagardine B:
SSGWVCTLTIECGTLVCAC

SEQ ID NO:2 Actagardine B variant VV:
SSGWVCTLTIECGTVVCAC

SEQ ID NO:3 Actagardine B variant LI:
SSGWVCTLTIECGTLICAC

SEQ ID NO:4 Actagardine:
SSGWVCTLTIECGTVICAC

SEQ ID NO:5 - SEQ ID NO:10

SEQ ID NO:11 Ala-Actagardine B:
ASSGWVCTLTIECGTLVCAC

SEQ ID NO:12 Ala-Actagardine B variant VV:
ASSGWVCTLTIECGTVVCAC

SEQ ID NO:13 Ala-Actagardine B variant LI:
ASSGWVCTLTIECGTLICAC

SEQ ID NO:14 Ala-Actagardine:
ASSGWVCTLTIECGTVICAC

SEQ ID NO:15 - SEQ ID NO:21

SEQ ID NO:22 pre-pro-Actagardine B variant VV:
MSAITVETTWKNTDLREDLTAHPAGLGFGELSFEDLREDRTIYAASSGWVCTLTIECGTVVCAC

SEQ ID NO:23 pre-pro-Actagardine B variant LI:
MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAASSGWVCTLTIECGTLICAC

SEQ ID NO:24 - SEQ ID NO:99

SEQ ID NO:100 CosAG14 (38168bp)

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GGCTACCAGGCCGGCTCGGTGATGAAGATGTTCACCATCGTCGCCGCGCTGGAGCAGGGCTACCCGCTCGCCTACA
CGATCAGGACGCAGAGCCGGTACCGCTCCCGCTACATCATCGAGAGCAGCAACGACGCGGCCTGCCCCGGAACGCA
CTTCTGGTGTCCCAGCAACGCCGGTGGCGGCGGCGAGGGTGTCTTCAACATGTGGACCGGCCTGGGCAGGTCGATC
```

AACACGTACTTCGTGCCGCTGGAGGAACGCGTCGGCGCGGAGAAGGTGGTCAGCGCCGCGAAACGCTTCGGCATCC
AGTTCCGGGAGCCGGACGACGCACTGCTGGCCGAACCGGGCAACGCACACCAGTGGGGCGCCTTCACCCTGGGCGT
CTCGGCCACCACCCCGCTGGACATGGCCAACGCCTACGCCACCCTGGCCGCCGACGGGATGTACTGCCCGCCGACC
CCGATCGAGCGGATCGCCACCCGTGACGGCGACCAGCTGGACGTCGGCCGGTCCCCGTGCGTACGGGCGACCGCCA
AGGACGTCGCCCGCGCCGCCCTCGACGCGGCCCGCTGCCCGGTGGGCGACTCCGCGCAGCTCGGCCGGTGCGGGGG
AAGCACCGCCGGCATCACCCGGTCGGTGGTCGGGCATCCGGTGTTCGGCAAGACCGGCACCACCGACCGCGACCGG
ACCGCCTCGCTGATCGCCGGCACCACCGCGCTGGTGGTCGCCGGTTACCTGGTCAACCCCGACTACCAGAACCACC
GCGACCGGCTCGACCACGACCAGGTCAACCCGGCCGTCTACCGCACGCTCGCCGACTACATGGAGGGCAGGCCACG
GGAGTCGTTCAAGCGGCCGTCGAGCGGGCGGATCGCGTTCGGTGACCAGCGCTCGATCCCGGACGTCGAGTGCGAC
CCGATGCCCCGCGCCCGCGACCGTCTGGAGGATGCCGGCTTCGACGTCTGGCGAGGCCAGGAAGTGGAGTCGGACT
GTCCCGCGGGCACCGCGGCCGGCACCGAGCCGAGCGGCCGGACCGTCAAGAACGGCGTGGTGGTGATCCAGGTGAG
CAAGGGCCGCCGGGGCGCGTCACCGCCGATCTTCCCGCCGATCGGGCCACCCCGCTGACCGGCTGACGGCGTAGAC
GTCGCCGGCGTTCGCCTCGTGCGGCAGCCCGGCCAGGTGCGCGAGCATCTCGTCGCGGGCGGCCCACCGGCCGAAG
GTGCGGGCCATGGTCTCGCCCAGGGAGACGTCGAAGCCGTCGAAGCCCAGCTCGCCGGCACGGTCGAGGCAGCGCC
GGGCCACGTCCCGGGCGATCGTCGTGAACTCGAACGACAACGCCCGGACACCGCGGCTCAGACCGGCGAGCACCGC
GTCCTCGTACCCTTCGACGTCGATCTTGATGAAGGCCGGCAGGCCGAATCGCTGGACGAGCGTGTCCAGGGTGACC
GCAGGGACGGTGATCTGCTGGTCCCAGACCTCGTTCTCCCAGCCGCGCGAGCCGGTCGCCGCGGTCAGGAAGCGCA
CCGAGTTCGTGGAGACCGTCGGGTTCGCCGAGTTGATGTAGAGCGGCACGCTCCCGCCGGCGGGCCCGCACGCGGC
CTCGACGAGCGCCACCCGGTCGTCGTGGGCGTAGAGCGCGCGCAGCGCCCGCATGCACAGCGGCTGCGGTTCGACG
GCGACCACGCGGGCGCCGAGCCGCCGGAAGCAGGCGACCCGGTCACCGACGTGCGCGCCGATGTCGAAGACGACGT
CACCCGGCCGGACGAAACGCCGGTACATCGCGTCCATCGCGGCCTCCCGGCCGGCGTCACCGTAGTAGTAACGCAG
CGACCGGGTCAGCGGCGCCATGGCCGGGTCGACTCTCAGTTTGCGCAGGTCATCGGTCACGGCGGGGGCATTCCCC
CGATCAGGCGATCCATGCATCGCCGGCGCGTGCGCAGGTAGGCGGCGATCGCCCGGGCGGAACGCACGCCCGAGGT
GGTCATGACGTTGTGCGCGGCGGCCGGGACGGTCACCGACACGCCGCCGGTCATCGCCGCCACCTCGGCACGCCAG
CGCAGCGGCGCCACCGGATCAAGCGACCCGCCGAGCACCAGCGGGGGTACGGGCAACCGGCGCAGATCCTCCTCGA
TCGCGTTGCGTACCGAGTGGCCGACCGTGGCCAGAACCCGCCACGGCTTGGCGTCGGCGATGTCGCGGACCAGCAC
CGGCGCCTGCCACGGCGCCTCGATCCACAGGTCCACCGCCCACCGGCCGATCAGCGCCCGCCGGGACCGGGCCGCC
GGGTCGGTGGTCGGGCTGGCCAGCACGACGGCGGCGACCAGGTCCGGCCGCAGCACCGCGAGACGGGCCGCCACCT
CGGCGCCGAACGAGTGCCCGGCGATGCAGGCCCGGGGCACGCCGAGCACGTCCAGCCAGGCCGCGAGGTGTTCGGC
GTGCCGGCCCACGTCGTACGCGCGGCGCGGCTTGTCGCTGAACCCGAACCCGGGAAGGTCCGGAACGAGGACCGGA
TGGCGGCCCGCCAGCGCGTGGGCGGTGGGCATCAGGTAGCGGTGCGAGACGGCGAGCCCGTGTACCAGCACCACCG
GAAGGCCGCCGGCGTGGCACGCGTGCCGGTCGTGCGTACGCACTCCACCGACCGTCACCAGGCGGCTGTCGAATCG
GGGCACCGCGGAGATGTACCCCGTCCTTCCGCAATCTTTCCCGCGCGGTTAGTTTCGAGTTGTGTCGCCGCCCTTC
CGGCTCGACGAGGCGGTCGCCGACGTCTACGGCGACCTGCGCCTGGCGCCCGTGCTGCGGCGGCTGCTGCGGCACA
GCGGCCGCCTGACCGGCTCGGTGGCCGGCAGCGTGTCGCTGATCGACGCGGACCGCGGCTGCTACGTCAAGGCCGC
CGAGTACGGCGCGAACTGCCAGCTCGGACGCTCTTTCCCGCTCGACGAGGGCGCCACCGGCCGCGCCTTCGGCAGC
CGCCGGCCCGTGGTGATCCCGGACTACGGTCAGCTGCGGGCCGGTCATCTCGCGGCGGCGCATCCGGCACGGAAGG
GCCCGGCCGTCGCTGTGCCGATCTGGTGGCGCGGCGACGTGATCGCGGTGAACGTCGCCTTCGCGCCGGCCTTCTC
CCTGGGTGGTGTCGACGAACTGGAGGCGCTGACCCAGTCCGCGGCCGCCGCGATCGTCCGCAGCCGGGGTGTGCGG
GTCCGCGCCGACCCGCCGTACGCGGCTCCGGCCGCACCGTTCACCCCGCGCGAGGCCGAGGTCCTCGATCTGCTCC
GGCAGGGTCTGACCGACCGCGAGATGGCCCGCCGGCTCGGCCTGTCCGCGAAGACCGTGGAGAAGCACGTCGGCGC
GATCAGGCGCAAGACCGGGACCTCCAACCGTACGGCGGCGGTCGTCACGGCCCTGGACAACGACTGGGTGGGGAAT
CTTCCCCATACCGCGGAGCACACCACCGGGTCTTGACTGGCGGCATGCCCGTCGTCTCCATGAAGGACATCCTCGA
CCGTGCGCTGGCCGAGCGGTACGGTGTGGCCGCCTTCAACATCGTCAACGACCTGACCGTCGAGGCCGTGCTCGCC
GCCGCGGCGGAGGAACGCGCCCCGGTCATCCTGCAGACCTCGGTCAAGACGGTCCGGATGTACGGCCGCCCCCGGC
TGTACGAGATCGTCCACGCCTTCGCCCACGACGCGCCCGTCCCGGTGACCCTGCACCTGGACCACTGCCCCGAGCG
GTCGGTCATCTCCGACTGCCTCGCCGGCGGCTGGAACTCCGTGCTCTTCGACGCGCACGAGCTCGACGTGGCCGAC
AACCTGCGCCAGACCACCGAGGTGGTGGCCGAGGCCCGTCGCGCCGGCGCCCACGTCGAGGGCGAGATCGAGGGCA
TCCAGGGTGTCGAGGACGACGTCGGCAACGACTACGCCCCGATGGTGCAGAGCCTGGAGGTGGCGGTCGACTTCAT
CAAACGCACCGGCGTCGACTGTTTCGCGCCGGCCATCGGCAACGCGCACGGCCAGTACAAGCAGGCGCCCGTGCTC
AACACCCGCCGGGTCAGCGACCTCGTTGCGGCCACCGGCATCCCGATGGCCCTGCACGGCGGCACCGGCCTCTCCG
ACGAGCAGTTCACCGACCTCATCGCCCGTGGCTGCGCGAAGGTCAACATCTCCACGGCGCTCAAGGAGTCGTTCAT
GAAATCCGGCCTGGAGTTCCTGCGCGAGGCCGATGAGCGCGGCAAATGGGATCCGCCGTCGCTGTTCCGGCATCAG
CGGGCGGCGGTCGTAGAGATGGCCCGGCAGCACATCCGGCTCTTCGGCGGATCGGGGCGCGCGTGGTGAACGCCCT
GGTCTTCGACTGCGACGGCGTGCTGGCCGACACCGAACGGCACGGCCACCTGCCCGCGTTCAACGCCACGTTCGAG
CAGTTCGGGCTGCCCGTGCGGTGGAGCGAGGAGGAATACGGCGAGAAGCTGCGCATCGGCGGCGGCAAGGAGCGGA
TGGCGTCGCTGTTCGCCGATCCCGCCTTCGCCGCGGCGGCCGGCGACACCGACCGTACGGAACTGCTGCGAACCTG
GCACCGCGCCAAGACCGCGGCTTTCACGAAGCTGGTCGCCGAGGGCCGGATTCCGGCCCGTCCGGGCACAGCCCGG
ATCATCAGCGAGGCACTCCGGGCAGGATGGACGGTCGCGGTCGCTTCCACGTCGGCCGAGGATTCGGTACGCGCAG
TGCTCGTCAACGCCGTGGGAGCGACGACTGCCGAGCGGATCCCGGTGTTCGCCGGAGACGTCGTGCCCGCGAAGAA
ACCCGACCCGGCGATC

SEQ ID NO:101   orf1 Hypothetical protein 146aa

MPRRSTRCSPACRPGAADQPDPGRACRPDCGRADRSGSGRACRPDCGRAD
RSGADRPSAAALVSGMPRRTGVGGAPGRLIEARAVLPELIGAARDRRASC
VEQRVRSLTACRACACRRAAAPAAPRTPPRPSAAPAPGSDAGGRCG

SEQ ID NO:102 orf2 Hypothetical protein 149aa

VSVSLLLVPLAMAGAAAVQAAAGRMDDGRLICQVQTRMRDVTLLDAALRD
TGATVTAAGDTISATWTQSAATFTRGADGIWAAHVTGVDQPGAVELMTTV
DTAYGRRVQQAVLERLRAQAPEAGLRLESESVGQDASVRLVFEVERERA

SEQ ID NO:103 orf3 ATPase AAA involved in cell division 518aa

VERSFAETFAQLLKARFPVLHLETYEEQRALHHLAGIAGDADLVRVPRAV
WTWSLTAGLVQPNGEARSGAQRATDALRAVQRIDEPAVFVFRDLHPLFAQ
SPEVVRLVRDIAQAFRAGRSPRTLVLLSPVLDLPVELSKDVTIVDFPLPG
QLELRALLDAMVRGNTASGRLRVELDEQSRERFVTAAAGLTMQEAENAYA
RAMVNDAVLDLADLEIVHEEKRQTVRKSGVLEFMAAGTVLDDVGGLENLK
AWLVKRNGSWLDEAAGYGLPAPRGVLITGVPGCGKSLTAKAVATAWNLPL
LRFDIGRVFSGLVGSSEHNMRTALRTAEAVAPCVLWVDEIEKGFAGGTGG
DSGTGARVFGTFLTWMQEKRTPVFVIATANDFDGLPPELLRKGRFDETFF
VDLPSRSERVAVWRVHLGRALRHRRAAGELRVDAELLTELAGLTEGYSGA
EIEQAVIAGLFDAFSERRPLRRDDLVRAVMSIVPLSVTQAERVDALRGWA
RNRAVSATGTDDWDLTNR

SEQ ID NO:104 orf4 Sugar hydrolase 203aa

VGELDQQRIHPHNNIWGSGAGTQTIWARSGTNWGVVANHPRTSGVKSYPN
TGKTLNRTLSSLNSLTSSFNVSVPSSGDYSTTYDIWANNHAYEVMIWTNQ
QGAVGPIAEQYDANGAVPNVRNLSVGGHTWNVYRGSNGANAVFSFIRTNT
NSGTVDILAILNWLRTNGWWGDVTVGEAQFGFEISGTAGQSNFTVNNFSL
NYS

SEQ ID NO:105 orf5 Endoglucanase 219aa

MRRRTLALSLAASAALIAGAGVVTALPASAAAGCRVAYTVSSQWPGGFGA
NVTITNLGDPLTNWTLVWSYSGGQTVTQAWNTSLTQSGSQVTARNAGYNG
SVGTNATVSFGFNGSGAATPAPGTFTLNGTACTGSAGPTSPSSQPPTNGV
PSDAVWVDSGQWANWTNNGYILTTTSGARAPAPRPSGRAAAPTGASSRIT
RAPAGSSPTPTPERPSTVR

SEQ ID NO:106 orf6 Cytosine/adenine deaminase 161aa

MTITETDLAHLRRCVDLAREALDDGDEPFGSVLVSADGKVLFEDRNRVRH
GDATQHPEFAISRWAAEHLTPRERASATVYTSGEHCPMCSASHGWVRLGR
IVYAASSAQLTAWYKEWGIPAGPVAPLPITTVVPGAVVEGPVPAFEAELR
ELHRARFTPAQ

SEQ ID NO:107 orf7 Unknown 413aa

VTTPLVAQAGDSTTGLTGLGLVEDAHQIAEAIRGNSWVDGVLGGVGASLD
GLALAIDPLGTLAAWGVAWLIEHVQPLQDALDWLAGDVDEIAAQAATWRN
VAAFTDSAQQDYADRLRTEVAGWFGASGDAYRAHASEHLAALKGISTAAG
GISSAVEGAGLLVSLVRGIVRDLIAQFVATLAVRLPQWLAAEGLTLGLAT
PVVASQVAALVARGVNKIQHFIRALLNSLRRLMPMIDRLGEVLERLRMLT
DRLARSSPSTRPEPTPGPATHAGTENASGNKPEGDLEPNEPRPAEADARD
STPQAFVDEVVSNPRSVAGHSAQSIADQFNAAGYSAVVEQSTRSGTSGNA
IQVRIHGHPDITNIQVHPGGGRHTPEGSPYWKISTNTVGKIWIIPENFRG
ADELRGNVVRYDK

SEQ ID NO:108 ***orf8 Unknown 217aa***

VGKPCPDLVEVLSREIQAGNQGASASEVVEIFDLELVGIFRGAVTQKLPG
IEVLRKHLHLKQGGVQVRSVVVRAEDPAGIVVVGDLCARIDNRDVGLAQG
DAYWERRDDTVDRLDQIRADSPCEFDDMVRLGAGVHGDGQRRVRQRFADV

ANLFGVQRAIINESRIRTKIDPDDVDAQGDERGSFPRGSHPIHFDDLICH
SAPHSHAVHRRPGNFRG

SEQ ID NO:109 ***orf9 Pyruvate oxidase 592aa***

VSDVVVERLTAWDVERVFGYSGDGIDGVIGALRRAGRPTFVQARHEEGAA
FMAVGHAKYTGGAGVCLATQGPGAVHLLNGLYDAKLDSKPVVAIVGQQVS
TVLGSAYQQEIDLVRLFGDVCAQFVQAAHTSEQVPMLLDRAFRTALATRS
PTCVVLPHDVQTAPAPDPQAHEHGVLATSAGLRPARVVPRPEDLREAAEV
LRSGERVAIMVGQGAYGAEREIVELAQRLGAGVTTSLLGKPVLDENLPFH
TGVMGHLGTTASAELMRHCDTLLLIGTNDPWTEFYPRPGQARAVQIDVDG
RRLGVRYPVEVPLLGDAVETLRELLGLLPSRASGAWGARVEEWVQRWRLI
SAARAAAPAEPVNPQHVIRSLSDHLPADAQVAVDVGSVVYWYARHLRLPR
GVPAHLSSTLASMGCGLPYGLAAKLAAPQRPVVVLAGDGAMQMAGMAELI
TVAARWRDWADPRFVVCVLNNRDLAEVSWEQRETEGEPRFVTSQELPDVP
YARYAELLGLRGVRITDPSDLTGAWEAALSADRPTLIEAVVDPAIPLLPP
GQPYEKVQAMYAGLAAEKGDQARRAEAHLRRERADEGFDDPS

SEQ ID NO:110 ***orf10 Hydrolase or acyltransferase 266aa***

VSRDKTYRPVRPDGIRTTFHDGVLGRMRIRCLGEPRPGVPEIVMIQGMTV
SDYLLPGLGALSAWTRVHLVELPGGSGSGRPPHDLTVEEYARAAADWLCA
QRLGRIVLAGHSSGTQVAAETALLCPDEVAGVVLAGPAIDPVARGGLRVF
ARWWIDRRGDPKSLDEVHKPEREQVGFRRLFQVLRAHLRHDLEKPVVGLC
VPVLVIRGSEDRLGTARWARRLADLAAVGGRYVEVPGTHSFCWRYPQAWS
APIREFAGWSVSVSGT

SEQ ID NO:111 ***orf11 Aldose epimerase 396aa***

MTEADHRPTVLRRILRLCAVLLMVLGVGLVGAPTSHAGGKPTISKEAFGS
VGGKAVDRYTLTNGRLQVRILTYGGILQTITFPDHRGRRANVTLGFRTLD
EYVTTKNPAYFGAIIGRYGNRIADGRFTLDGTTYQLATNNDPNHLHGGVV
GFDKRVWDATPIRDGDSVGLRLTYTSPHGEENYPGTLRVTMTYTVTRQMG
IRMDYRATTDRPTIVNLTNHAYWNLGGEGTGTIDDHLLKLNANRYTPVDA
TLIPTGAIDAVAGTPMDFRRPTPIGARNRDPFQQLVYGRGYDHNWVLNRE
DGQFRRLEFAARAVDPDSGRQLTIYTTEPGIQFYGGNFLDGTLYGTSGRA
YRQGDGFALETQHFPDSPNHANFPSTVLRPGQTYNSTTIYQFGTAD

SEQ ID NO:112 ***orf12 ABC sugar transport periplamsic component 378aa***

MPRIHPKVEEAVSTLDLNRTTRRRLLSGTGLFSASLAAGALLSACSDQND
GQNQTEGAGNFPDTPEWRFTFVNHVTTNPFFTPTQYGMEDAATLLGIAKP
QWTGSQNSIVAEMVNATNTAVSAKVDGIAIAVVDKDAFRGPVDQALNAGI
PVVSYNADGARGAPGTNRLAYIGQGLYESGYALGQRALQVLDSGEVAAFI
ATPGALNIQPRIDGAQQAFKDSGKPITFTAVATNADVTRGLSIIDAYAQG
HANLAGMLAVDAGSTSSVGQTVKKYNMRGKGLKVAGGFDLIPETLTGIQE
GSLDYTIDQQPYLQGFLPVLALYFYKVSGGLIAPSETNTGLLFVTKDNVA
PYQSTKSRYEGSTTDKVLVPRSGPIAHG

SEQ ID NO:113 orf13 ABC sugar transport permease 352aa

MDDRISPAPAQAPSLEVEQRRGRWQPVTAAGRKVLDAFLRRREASVLLVA
IGLMIYFRASSPVFLSRDNLVNIAQATAPVAIIAVGIVLLLVSGEIDLSV
GIVAALAPFLFHFGINFYSLPVVPAFVVALAIAAGIGLVNGLIVTQLHVP
SFVTTLGTFFAVQGILLITSHAYPVPIPDAAKGTFQTWLGAGPWASITWA
LIIVAIFHTVLTLTRWGLHTISVGGNPVGATEAGIRASRIKIGNFVITST
LGGLVGIMEAFRINTIDPNIGGGTTLTFYAISAAVIGGTALAGGSGTIVG
AFLGALVLAELQNGFNLIGYSANTIFLILGLAILVSMIANQYLSRLRRAG
RS

SEQ ID NO:114 orf14 ABC transport protein ATP-binding 253aa

MTAETVSDALRVQNIAKRFGALTALQDVTLRVAEGEVLGLIGDNGAGKST
LIKIICGYHRPDAGRIFVGGEEVTLRSVDHARSVGIDAVYQDLALVNELS

VYHNMFLNRELVRWPLLNNRAMRRRAEEHLRDMGVNLPDVGVEVAKLSGG
QRQAIAVARCVYSDARILLLDEPLAAMGAKEGTMILDLIRDLKARGNVSI
IIIAHNYAQVLDVCDRVNLLQHGRITFDKRSADTSLAELTELVVAEYRTG
RGR

SEQ ID NO:115 orf15 Hypothetical protein. Methyltransferase 255aa

MESGASVPQSARIWNYWLGGTDNLPVDRAAGDEYRAVFPGIDEIARESRR
YLSRAVRYLAGEAGVRQFLDVGAGLPTVDNTHQIAQRVAPDARVLYVDKD
PYAVEHGRELLAGSSDVYLEGDLQKPADILAVAARELDMGRPVALILNGV
LGHIPSTAEVRDIVRQLMAGLPPGSYLSINDGVRVAGEEALNQAQDAYNS
SGAVPYLMHTPDEIAGFFEGLDLVPPGVVPSPQWHPDPGDETTGASQYSG
VGRKR

SEQ ID NO:116 orf16 ABC transport permease 280aa

MPPRTSRGPHPWVLWTALAAAAVIFVYPFVWLVSASLKPRPDVFDNRLLP
AEWAPGNYTAIWDAAPVLTWMFNSVVVALAAAAAVTISSAVVAFGFAYFR
FPGRNVLFALVVGTMMLPGAVTMIPTYLIWNELGLAATQVPLWAGNLFGS
AFYIFLIRQFFLGVPRELFEAARVDGAGYWRLFWRIAVPLCRPALIVAFV
FELRASWSDLLKPLIYLRDPALFTMPRGMKAILDQFGQAGEARWEIVLAG
AVITTVPMIIAFFLCQRYFVEGVATQARKG

SEQ ID NO:117 orf14 ABC transport permease 301aa

MSAAVRRRETLAAFGFLSPWLIGFTVFMAGPMVASLVLSFTDYDVLTSTD
FVGGENYRQMLADPRVRTSIANTLIYTALHVPVTMIVSLALAMLLARVGR
RSAGFFRTIFYLPTITPKVAVGVLFLLLFNGQVGIVNEALGTVGIDGPNW
TVDGPWIKPGLVLIGAWSLGSTVIIYLAALQNVPRDLYEAAEMDGASAWA
RFRAVTVPMISGALFFTLIINTIASLQTFDEVYTAFYGSANQQTYGNDAA
LFYVVYLFQQAFQFLHMGYASALAWLLFLIIVIITVVQVRLSRRFVYYES
E

SEQ ID NO:118 ***orf18 ABC transport substrate binding 445aa***

MFIRSIRFVVGGALVLTLAAGCGGVGGSDSDSDSNAAVTLTMMGFGTGDE
IAKTRFDAANAVIAPSLAKASEGSFDAQAFLSAVASRTPPDLVYMERRLL
GTYAAKKALTPLGDCVEREKIDMSQFREAAVTEATLNGQLYGLPDFYNNR
VLMLNDAAFAEVNLDPAGFDTGDWQALSTATARLTRMSGGKLQRIGFDPK
LPEFLPVWARANGAALVSDDGRTAQLNNPKVVEALEYAVGLINAQGGWSN
FKSFRDSWDFFGAKNQFASNQLGAFPMEDFYLNVLADNSPKVKVTVAPFR
GVDGQPIDWITGNAWAIPANSAHPGQACKWIKTMTASETWIAAARARAEL
RKKENKPFAGVYTGNKKADEVIFRDVVKPDANVQIVLQTQESGFSEPALA
AGEEFKAAWQNAVNRVLEGKQKPAQAMAEAQQQAQAALDKANSGR

SEQ ID NO:119 ***orf19 actA lanA structural gene 64aa***

MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAASSGWV
CTLTIECGTVICAC

SEQ ID NO:120 ***orf20 actM lanM modification gene 1053aa***

MSPVPSLNSTSVRDSAYLHERTVTGEDQPAPAAQARIASWRDSAFLDDRV
LDIRLRQWGIDRATFGRLLTDDDFTVPGRLLAWADELATVLATDTTPVTG
LELSTKLWSQGFDRLLFAGLLHPFLAHYEQRLHERVPRPIAGSLRRPLLE
SLANRLLAVAARTLLLELNVARVHGRLTGDTPQQRYDDYDRRLLTDPAYL
AALFEEYPVLGRCLVECGRRWVDHAAELFNRLHDDEPELRAAGLLPPSAE
ALRSVRLDLGDPHNGGRSVVQLTFDDGTDLVYKPRPVGSERAYAETMAAL
ARHGLPVPVTAPRVLDRGGHGWCEFVRPAPCADAAELSRFYRRAGSVLAA
MLLLGGVDMHMENVIAAGSSFTPIDLETVLQSGELGDGATDAYGRALDLL
NRSVLAIGILPARAFGGRQRKSVDVSALGGGEPQTAPRPVPRIVDAYTDT
ARLEAVEATMAGAQNRPSLPGAEVRPWEHTADVVAGFTDAYDIMLAHRAD
FDRLLRGFHDVEVRYLPRPTRRYSIFLTESYHPDYLRDASDRDRLLDKLW
TAADARPELIPIIESEKRQLLAGDIPCFRSVAGSRQIRTASGPLHPEFFT
APAVTVLTRRLGEFGPVHRAAQVRIIRDSMATMPGPRPAAQPSPDRAAGP

RPRVTGADPATLADRIARRLADEAILGDRDVSWIGVSIEGVAQETYSYKP
MATGLYDGVAGLALTFAYAARTLGDDRYLDLAHRAARPVAGYLRYLAEHR
IVETVGAYSGTAGLLYALDHVAHATGDDSYLDAVSEAVPWLRECATREEC
PDLIAGLAGCALISLDLHGRHRIDGLREVAAICAERLAALAVDVDGAAGW
PATPDGPLLGGFSHGAAGIAWPLHRLATELGDPSLRELARRAVQFDRDLY
VPAAGAWRDLRPEMAGTDSYPALWCHGAAGIGLSRLLIAQHDQDTRLAAE
ATAALDLVGAHGFGHNHSICHGDFGALALFDLAARTGFDPGRHDAAAAAV
TADIAANGARCGLVGDIHMPGLMLGAAGICLSLLRIAHPAQVPAVAWLQP
PLT

SEQ ID NO:121 ***orf21 actO monooxygenase 341aa***

MPEEIVLSVLDQVPVFRDGSPAEAVRDAVALARSAEQYGYHRFWIAEHHG
SAANACAAPEVVTAAVAAATSRIRVGSGGVLLPHYSPLKVAETFRVLAAL
YPGRIDLGFGRAPGGPPAMAELLNPYAVRTDEAFLEQIGRLLGFLGDTRT
VSRVSVTPQVEEPPVPWMLGAGTGSARMAGMLGLPFCFAQFIATEECPEA
IEAYRDAFRPSPWLERPQPMLALRVLCADSDAEAEELATCFWMSCTTGWR
AQVQLTDDYRGGAPNLDDARRYRLTAEDLALRESRPFLQISGTPAAVGKE
IRRLQAVYGVSEVVLTTNCPGLPARRRSYELLAGEFASPAA

SEQ ID NO:122 *orf22 actR response regulator 231aa*

MRSAARGGPIVTDVLVVDGEALVSIGIKMILESTGGFAVATTDRENLRSA
VEQHRPAVVLLDGHSAQSDGLEVLDQLRALSSPPAIAMLTTLAPPELVLD
SLRGGACGFLLRDSQPEQLVAAVRALAEGSIVLAPEASSVVVRAGSRGSA
AGAGSPACERVKQLSDREQSILRLLGAGLTNAEISRQLFLSAATVKEHVS
VILSKLGVANRVQAAVLAYASGLSSDDVCLS

SEQ ID NO:123 *orf23 actT ABC transporter associated permease 812aa*

MIFALAWSQLRAYPARLFAIVAAVMLATGFLAATATFAATSGEGLRRTAA
APLTTADIVLDADDTVRDPAWYEAAAAVPGVRSVDAQYARTVSVFGGSRR
GSANVQSIAATPQVRWFTLDRGAWPTGPGQLVADQRTLDDLGIDVGATLT
VRHGEAAPQPVTVTGAADLGFRPLTGSDFRFYADASFFAGDVPPAALLTV
ADDASLTGTVDALRRSMGPGISATDASAAADQAAARFAGGNGQLVVIMLA
FAAVALLAAVLVIANTFHVVIVQRIRQIALLRLVGGHRAQVSRVVLAEAA
IAGTAGGLVGAAAGVGLGYLGADLLDISGGGLRVNPFALAGCVLAGVLAT
LVAAWAPARRATRIAPVRALQAADEPPAGTVRGGRRLVVGTVVTVVGAAA
LAVAAIGASLPLALLGGLLLAAGLLAALPRLIALSLAPAARLLERFGVAA
GLAGTSLSQNARRTASAAMAVVVGAALITCLAVAATSGRATVNADLEARY
PVAAGLRTDGEPISGATAGAFAAVPQLSASGTVGTVAARFPDGGKATPRL
LAAPGDELAARVAPELAGEPVVLVPATYLAELGLPDSAPIVVEVGERRVN
LIARASRLADTTGQLLGVVSARTLAANRIEAVPTTVWGVADPGFDREALS
AAVGAVAARDALVQVGGGVTEGGDIANVLSILLGLSLAMLAVTVVIALLG
IANLLGLSVVERVREMALLRALGTRRARLRAMLAVEAVVITLLGTVAGLV
VGVPVGLAAVAAAVGRTADPVIRLPWGQLAAVLVVAVLTGVVASLAPARR
AARVAPAEGLTR

SEQ ID NO:124 *orf24 Hypothetical protein 244aa*

MIVWPERRAASRSPPSSARPVAMSSAPVGSSAKTTDGLASSARATATRCC
WPPDSSDGRCPSRSVICSESVIFRSSPGSTRRPASRSGSTMFCSAVSVGS
RLNAWKTKPIRSRRRSVRARSSRPANDRPARLTVPAVGVSNPASRCISVV
FPEPDGPMIAVNWPAEKPADTPSTAVTAAGPVPYVLLTSRQLTISACVVC
VVTAPTLEIRADRHIPPRDPGRAVDRYLRMTRRYRPRAPCSRNR

SEQ ID NO:125 *orf25 Response regulator kinase 367aa*

VLRIDALVAAAVVIGCLLLGLAGLSEWYWSAAVAVPLLLRRSAPRCFLAL
VAGVSGLHLLASHSFMFPGDLVALVAVHAAAAHAPGRARHAGLLLGAAGA
LVVAAQALQDQRLGSALPAVLIVASTMAAWSIGLMQRQQRSAVLDAEHRR
RLAEQDSAMRAQLAVHEERTRISQEMHDIIAHSLASIIAQAEGGRVAARA
DARIAGPVFDRIAGLGRQALTDVKRLLTVVDHDDEWHDDGLERLPVLLAG
VTEAGLDVTVDSSGAPQPLAAGMDLAVYRVIQESLTNVLKHAPARRACLR

MRWTPALLTVTVSSPLPGGRGAGLVEGRGLSGIRQRCSLFNGDCTVTATT
ELTVTTTWPLTPEGARA

SEQ ID NO:126 orf26 Response regulator sensor 220aa

MTRPPIAVLIADDQELVRTGFAMVVDAAPDMRVVAIAASGAEAIELAAEH
RPDVILMDIRMPGTDGITATSAILAAGGERPPKIIALTTYDSSDYATRIL
TAGASGYLLKDATAEGLTAAIRSAYHGGSVIAPTTTRNLVAARAEPPPPA
RDPAPLDTFTARERDVFDLIVAGANNAEIAARLHLAEVTVKTHVGRVLAK
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SEQ ID NO:127 orf27 Penicillin binding protein 782aa

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SEQ ID NO:128 orf28 Methyltransferase 253aa

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SEQ ID NO:129 orf29 Hydrolase 261aa

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SEQ ID NO:130 orf30 Response regulator 244aa

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SEQ ID NO:131 orf31 Fructose biphosphate aldolase 281aa

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SEQ ID NO:132 orf32 Hydrolase 192aa

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CTGTACCGGTTGGTCGGCGCCGCGCGTCGCGGGGATC

SEQ ID NO:201 orf1 Secretion system protein 334aa

VRLRIQRRLADELGPTLYTTGQSEDLDARVRDALHDLLAREETPLSGADR
ARITREVTDEVLGHGPIESLLRDPSVTEVMVNGPYSVYVERFGRLEKVAA
EFDDEQHLRRIIDRICSRVGRRVDEASPTVDARLPDGSRVNAVVPPIALD
GSTLTIRKFAAVPLTVGDLLQYGTLTRQAAEFLDACVRGRRTILVSGGTG
SGKTTILNVLSSFVPADERIVTIEDAAELQLVQDHVVRLESRPPNSEGRG
TITTRDLVRNALRMRPDRIVVGEVRDAAALDMLQAMNTGHDGSLTTLHAN
TPRDALSRLETMVLMAGMDLPIRAIRDQISSAVD

SEQ ID NO:202 orf2 Response regulator 358aa

MSELESYLPRYPETLLVVIGAVVDLDEALMFTAYQRLQRPVIGVVLIRHA
IEAGVVLRCLQSGIRDLVAEEDEEGIRSATVRSVDLSKALSTTMRGTGDN
APYASVVTVFAGKGGCGRSVVAVNLAVALAGAGRRVLLMDLDLQFGDVAI
MMKLSPERNIAGGLQMAGRLDEPGLRSIVTTYRQSLDALLAPASPAEGEQ
VRREFVVELLDVARPLYDFIVVDTPSVVTDQVLAALDMSDWFIPIVTPDL
PALKSVRLTAEMFDLLDYPKDKRLLVFNRAGAQVGLSPSEVEVAAGMPFA
VQVPASRDVTVSVNHGEPIAVTDPLHPVSRAIRELGDRIAGVGTTPQRRR
GFLGRLFP

SEQ ID NO:203 orf3 Hypothetical 266aa

MRRRILILLAALVLAGISGAAVLSYARSADRRALSGREGTWILVAGQHIP
SGTSGAEIRARGLTERLLVPTVTVPAGALTTWDSALDPLRLGGDLQPRQL
LMRTLFVPASPAPSHTGRIPVPRRSLAVSVALNVAPQVAGNITPGDQVAV
YYTYQAKVLMAEDHETIPMTELLLPKARVITIGEAEPGQAPVTPSPSPTP
GPSASAGDSTAAVKEIQRYVVTLAVGDTDALRLVHAAQSGSLYLALLGPN
ATASTGPAIDTSWVVR

SEQ ID NO:204 orf4 Hypothetical protein 432aa

MRRLIHALFPPRGEHGVITALVAVLAGAGVLLGMAALVIDIGALYAEREQ
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SDVQFCITTVSAAGVTTADAGCPSSWNTPVTCPAPPSASGPYRYVEVRTS
TRNSDNTSVVPPLFGRGLAGSAYHGAKMGACGRVAWGAPAVTDVLALGVS
RCDFLRLTGDYTRFFAPPPPTGPNPQTGVHPLLGLSDPTAGYIPISDGIL
ATSCPADAGETVAGYTWLGQPDGPPALPGLLPVSAPDASCELTGIPATDG
PADSWVGGFTIGPGNASAATACLDRLNVLITSGQPVLVPIFDRQVAVIGT
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YGYFTRALVTDHVPTRFATSRYYGAMVIGRTG

SEQ ID NO:205 orf5 REsponse regulator ATP-binding 136aa

MWKATERQGPVVLLVEDDEDLRELAAQMLEMRGFVVLVAKDPVSAIMTCR
VHSGAIDVLLTDLGLPGVSGGELARSASEVRPGMKIVYVSGVPEEIAIKK
GLIRAGSPFVAKPYTADRLAGMLRTVLAQGAESSAR

SEQ ID NO:206 orf6 ABC sugar transporter 363aa

MFNYVSFVRPKTFAATFAAAALLLGSGACAKSEDSGDTVAAGPAPSAAQV
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EAAKLGITNLKTSNANSQFNKQIADVEQMIDAGVQLLVIAPLNSDGWDSV
FAKATAKHIPIITIDRKINATACKDYLTFIGSDFAEQGKRAADALAKSLG
NKGEVAILLGAPGNNVTTLRTSGFKDEIAKVAPDIKITFEQTGNFSREDG
QKVAEQLLQSKPNINGIYGENDEMALGAITALKGAGKKAGDVKIVSIDGT
KGAVQGIVDGWVSAVIESNPRFGPLAFDTATKFFGGEPVGQDIVIQDRAY
DESNAKTDIGSAY

SEQ ID NO:207 orf7 ABC sugar transporter ATP-binding 501aa

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GREPRNRFGLLDEARMVAEATEILAGYGVRTDVRRRLGTLALGAQQMVAL
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ELYRVCDAVTILRDGKLVHTGRMADLDRRTLVSLMLGREFGADFTSFSES
PQSTPEGEPVLRVSGLTSRPRLDDISFDVRPGEVVGLGGLLGAGRSETIK
AIGGAYPIDSGVIEVGGVRLGRPSTVRAVRAGVATQPEDRKAEGIVPGLS
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GGNQQKVLLARLLATGPKVLLLDEPTRGIDVGAKAEVQALIDELAKEGLG
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SEQ ID NO:208 orf8 ABC transport permease 319aa

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LVSLLAGVAVGLINGVLVAKAGLQPIVATLALFVGGRGLAVVISGGQLKD
VRNADLLYLGSGDLLGVPVLVWIAALLVLVVAFVVRRTVFGRRLLAVGGN
RPAAELAGLPVKRVLIGVYVFCAVLASIAGLLSVARIQSSDASAVGLLIE
LSAITAVVVGGTPLTGGRVRVLGTVAGALLMQLVVATMIKHDLPPSTTEM
VQAVIILVAVYVARERRTR

SEQ ID NO:209 orf9 ABC transporter permease protein 320aa

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LLCGAIGVLNGILISRTGMAPFIVTLAALLGARGLMRSISDEGSTTYLVR
SDVFHELGTGSLLGVGLPVWLAAVLVGAGILVLNRTRFGHAVHAIGGSED
AAALMGLPVRRIKVWVYLLSGLLAGLAGAINAAKLGSGVTVLGSGMELDA
IAAVVIGGTLLTGGSGSIAGTVAGVLLLGVIQNLINQVGNVNSNWQQVIS
GGFLAAVVVAQTTLVRARRS

SEQ ID NO:210 orf10 metallopeptidase 486aa

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RVSVDGQPARAARDGDELTVIPRRPLPRGRLFRVTVANFTAAPAALVATP
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GTLLPAIAGEREQLAWMEKQAGPYPFEDYGSLVIDDDLGYALETQTLSLY
GAALFTGPETTRGPSMTHELAHQWFGDSVSPFSWSDVWLNEGHATWYEML
WSEETGGFPQYTGLADREAFFKAVYAAGDIFRARYGPVAAPLDAATTWDV
FNPNVYAGGALVLYALRQKIGAATFQRVERAWLTTYRGRSASTGDFITLA
SRVARQDLRPFLTSWLLGVTTPPMPNHPDWVVTPGS

SEQ ID NO:211 orf11 putative StrR-like regulator 286aa

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SDRMIAASSGLGARTVGGLRRRRAASGESPAGLRSRAGRDSRVRPAGSTA
GRLKAVDYLQDRPDASLREIARHAGVSPSTARDVRDRLHRGEDPIPATQR
AAARPGNDSPPLRSLVQGLASDPSLRFSESGRDLLRWLIAHAVQDGEWKG
LVDTIPAHSAQALAKIARHCSREWREFADILEKDAA

SEQ ID NO:212 orf12 lanA 64aa

MSAITVETTWKNTDLREDLTAHPAGLGFGELSFEDLREDRTIYAASSGWV
CTLTIECGTLVCAC

SEQ ID NO:213 orf13 lanM 1046aa

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GFSHGSAGIAWALHELAAEFGDRDLRELADRAVEFDRRLYVPAAGAWRDL
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SEQ ID NO:214 orf14 lanT 575aa

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IIDPVLLGVSLAPVLCGAAGVRLVGRRLRPLSSAVQESIGALTASTTRAL
GAIRTIRVAGATERETALIVAEADRARAAGVRLALVAAQAGPIVRLALQG
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LGALTRIEEILALPDEDSALGVRARTPATVRHDPVLLEFDHVSFRYPTGG
EILRDVSFRVPAGSTTALVGPSGAGKSTILALIARLYEVHGGRILLHGRD
IRDYPLAELRAALGYVEQEAPVLAGTVRDNLTLAAPDVAEHAIRHVTASV
NLDDLLARDPAGLDAPVGDGGVLFSGGERQRLAVARTLLAPGELLLFDEP
TAHLDARNEQALQHGLTAHAAGRTLVVVAHRLATVAHADQILVIDDGRSV
AAGRHEELLVRDPTYREFATRQLLT

SEQ ID NO:215 orf15 lanO (Luciferase type monooxygenase) 347aa

MLSVLDQVPVFRGDDPAEAVREAVGLARAAESLGYHRFWIAEHHGSAANA
CAAPEIVAAAVAGATERIRVGTGGVLLPYYSPLKVAEAFRVLAALYPGRI
DLGFGRGRGGPAVMAELLNPYAIATEEAYAEQVGRLLAFLGDARTVSRVS
VTPAVQDPPLPWLLGSGVGSARLAGMLGVPFCFAQFIATEECPEAIAAYQ
ESFRSSPWLDEPQAMLALRVLAAGTAEDAEELATGFWMSCTTGWRAQVRP
DDDYRGGVPNLADAQRYTLTEEDLAMRASRPYLQISGTAETVGEEIRRLR
KVYDVAEVMLTTNCPGAAAPAPVLRAAGRRARADRAGVTRVRPARRW

SEQ ID NO:216 orf16 lanR (Repsonse regulator) 217aa

MADVLVVAEEALVSIGIKMILETMGGFSVVAADRDSALAAVSEHRPAVVL
LDAHATLPESVPLLTRLRDLESGPALAVLATLAASSTVLESLRGGACGFL
LKDSQPEQLVAAVRALASGVTVLAPEASSIMLGAACRGTPAAENAVDEVK
QLSDREQAVLGLIGQGLTNAEIAGRLFISDSTVKEYVSVILRKLGVANRV
QAAVLAYAAGLTTDELA

SEQ ID NO:217 orf17 ABC-transporter associated permease 814aa

MIFTLAWSQVRSHPGRLLAIVAAVVLATGFLAATATFASTSDEGLRLTAA
APLTTADIVLDADDTVHDPQWYQAAAGVRGVRSVDPQYARTVSVFGGDRR
GSANVQSIPATASVRWFTVDEGTWPSAAGQVVADRRTLTDLGVGVGAHLD
FRQGTAAPVPVTVVGSADLGFRPLTGSDYRFYAAASFFAGDTPPAALLTV
TDRDRLAETVDAVGRALPPGATATDASAAADAAAGRFAGGNTQLVVLMLA
FAAVALLASILVIANTFQVIVSQRVRQVALLRLVGGHRRQVSRVVLAEAA
IAGSIGAVIGAVAGVGLGYLGAGLLDINGGGLAVNPIVLALCVLTGVGAT
VVAAWAPARRATRVPPVRALQEVPDALPAQVRGGRRLVAGLILIGLAVGV
LGLAAIGTSLPLALVGGVLLAAGLLTALPLGIALLLPPAARGLERFGVAA
SLAGSNLRQNARRTASATMAVVVGAALITGLAVASASGRATVEADLEARY
PVAVSVHTDGAAIDDRTVRALSGITGLTTATVATSAATFPAAGKPTPARI
AALPTDVAGRLAPELSASTGDPVLLVPASYLTARGLTDGAPLTVTAGGRD
LRFTARGSRLADTTGQLLGVTTGDVLTAAGVRTVPTTVWGTAPGGFDRET
LAADVNAVAARDAGVEVGGGVTEGGDIMNVLSILLGLSLGMLAVTVVIAL
LGIANLLGLSVIERTREMALLRALGTRRSRLRAMVAVEAVTITLVGTVAG
IVIGVPVGLVGVIAAVGRQAEPVIMLAWPQLGLVLVAAAVTGVLASLAPA
RRATRIAPAEGLVR

SEQ ID NO:218 orf18 ABC-transporter ATP-binding protein 240aa

MVSCRNLRKRYGTGDAAVVAVDGVSTSFAAGEFTAIMGPSGSGKTTLMHL
LAGLDTPTEGEVSLAGTALAGLDDRALTDLRRDRVGFIFQAFNLLPTLTA
EQNIVLPLRLAGRPVDRDRLQRIAASLQIGDRLGHRPAELSGGQQQRVAV

ARALLTEPSVVFADEPTGALDIATGRALLAGLQNAARQASQTIIMVTHDA
AAATYADRVLIMADGRLWDELRAPTLESIMSVMASVTVTS

SEQ ID NO:219 orf19 Histidine kinase 362aa

VIAGAAVMVCLLLGLAGLDEWYWSAALCVPLVIRRSAPVVFLALVAVLSG
IHMIYSGSFAFPGDLVDLVAVHAVAGYGPARVRHLGLLLGVAGSLVVTAR
ALHDGLPSSATLPAALIVAATLAAWSTGLMQRRQRADVIEADHRRRLAEQ
DSAMRARLAAIEERTRISQEMHDIIAHSLASVIAQAEGGRVAARADAVVA
GPLFDRIAQIGREALNDVKRLLNSIDGDTPDDFAQGLPDLPGLLAGVSAA
GLDVTFEVAGPEQPLASGMDLAVYRVIQESLTNVLKHATQRQARLSLVWT
PAWLEVSVTSPLTFAGALREGRGLSGIRQRCSLFNGDCEIVAGQTFSVIT
RWPLARPEVAVP

SEQ ID NO:220 orf20 Repsonse regulator 218aa

MTEPQIDVVIADDQDLVRTGFALVVDSAPDMRVVATAADGAEVVRLAAEF
RPDVVLMDIRMPRVDGITAARAILEGNAQPPKIVALTTYDNDEYASRILA
AGASGYLLKDTTAEGLTAAIRTVHRGGSVLAPSTTHRLVTAHRQHPARPS
ALLDSFTTREREVFDLIVAGASNAEIADRLNLAEVTIKTHVGRVLAKIGV
RDRVNVVIWAYRNGAGPS

SEQ ID NO:221 orf21 Putative membrane protein 301aa

MPILWTAVLAGAVILGHRLVPNAVGNAGSLIEAFLPWFGLAVPVLLLLAL
MRRSLTGLAAVLLPLGAWLIHFGGYVVDRDTGTPDLIVVQHNVSDENPDP
AGTARALLAAHPDLVGLEEVLPEAVAAYRGVLDAELPFHTVQGTVALWSR
YPLTGAEAIDIRPHDLGEDWNRGLRAVARTPGGDTAVYVAHLPSVRVTAA
GLTSARRDESARKLGALLAADPVPRLVVIGDLNTSVDDRGLRPIRQVMID
SPADFAFTWPARTPVARIDQVLARSMTVTRLTALPRTGSDHLPLAAEIRF
P

SEQ ID NO:222 orf22 alpha-beta hydrolase 290aa

MRNNETVRIPVATGGAVTATLFAPESARAVLVVHPATATPQGFYASFATY
LAENGIATVTYDYRGTGRSGSPRDHRDLGMRDWIGADAPAVAAWAADRFP
GLPRLAAGHSLGGHVIALGAAGPDLAASVIVASHIAALRTIPSRLERFRV
RIMLHILGPALGRLLGYVPARSLGLGEDLPAAAMLEWGGWARRDNYFFDD
PSMRAAERAATLTGPVLAVGTTDDPWSTPRQMDALTVHLTSASVERRTYS
PAAAGVPVIGHHGLFRRAVRDTVWPELLAWLHAHSEKASR

SEQ ID NO:223 orf23 Transcriptional regulator 145aa

VRLPRLIFLLFNADRAVRRWIDARSGDTGIGASGAGVLFYLAGHENALIG
DVTAALGASPSGMSGLVNRLERGGCLTRSQDPADARAVRLALTPRGHQVV
IHARGLVDDLNEQLTAGFDDAEIAVVQRWLEHVTRVSVQREERLG

SEQ ID NO:224 orf24 Pyruvoyl-dependent arginine decarboxylase 175aa

MADSVVFDQIPVVRAIGRGTTSLSAFHDALVTMECGFYNLVRLSSVIPPG
TAVDPSGKAPVPVGAWGDKLYCVYAEQHASQPGEEAWAGIGWVQRRDGQG
GLFVEHEGTSESFVREAIKASLRDLVKGHEDDFDGPDFVVHGVVSDGEPV
CAMVLAPYETAPWRGVRATDPPGMN

SEQ ID NO:225 orf25 Putative diaminopimelate decarboxylase 406aa

MTLSEILPSLGASLRPRLDPANWPLTARWTEPGDLTVGGVPVTRIAAAHG
TPVHVIDETDVHSRCAEYVAAFGPGAVCCSAKGGLLRGAARWIARDGLGC
YCRSAAELRTALDAGIRPESLALFGSGKSVADLEAALSCGAAVVIGSASE
AEVVAARSRPGQRVLLRVRPGSAQRGYGVRLNSSAALAAVATVTRSRRLV
LAGLDCSLGHRLNRFGTYESCLREAIGFVARLRTTVPVLNLGGGHAADLP

VGIFAARLRAVAQVTSEGYGIEPPEVHVSPGRALLGRAGITVHRVVAAGD
GVIELDGDVPDCLPGADCAGLHTAALIGRASPAPGRSITVRCGDATVAVA
ELPGDMAAGDLVALSGTGAYHQRRDVYVGRPAVVAVCGGRARTLLPRETI
DRILYA

SEQ ID NO:226 orf26 Kinase 309aa

VRSKREHSADIRRGRRAVLVVNTRSRRGRLLYEEARRRLVEAGFELLGTY
ALEQSGGLDGLISEALRKEPDLLIAGGGDGTISTAGRMLAHRDVALGVLP
LGTTNNFARTVRIEPDLEAAIATLVDGKVIDVDLGVAGDVPFTNHVGIGL
SGEVMISAPPRLKRAVGRLAYPMTALGLLARHRPVRAVIRAEGRELRFHT
HQVYVANGGFHAGRPITADAHADDRLLVAYPVGGASRRELLRETARNAAT
GHRRTLHERPFIAVRELWLETDRPVAVEVDGEPRGTTPMRIAIDPNALRI
MAPADSPDL

SEQ ID NO:227 orf27 Transcriptional regulator 367aa

VVFGSLLLVGGGGGAIGLNATVAAATSSVGQESLLGSAKPAEEKKNANLD
GAKNLLLVGIDQRPTQTNGEPLRSDSIILLHINKDHSSGYMISLPRDSYV
YIPAYDNGKQKWAGGKTKINAAFAFGTRGLKGNEALQHGFELLTMTVKEL
TGITPDAGAIIDFQGFRDVVNVLGKVCMYVDTTTKSIHLGKDQNGKTAKP
FVINPDGTLKSKISGVTPNTYTKGDHCFTPGQALDFVRQRDLLADNSLDY
GRQRHQQQFFKAIINQALKDGLDSPTKLPKLLSAFGKAMTVDDGGIDLAD
WALAMRSLKPDKLLTIKTNAGKLNSENVPGSGSVELLSDDSMDLLKSIKK
DQIDTFLLSHPAFIANS

SEQ ID NO:228 orf28 Glycosyl transferase 317aa

MPSEPDVSVVIPTCNRPELAVRAVRSALGQTHRNLEVIVVVDGPDEATVT
ALGEVGDPRLSVIVLPERGKAPNARNTGARAARGRWTAMLDDDDEWLPTK
IERQLETAAAATVERPVVACRMISRTPRADTIMPRRLPEPGEPISEYLLV
RRGLFYGDGFVQTSCIMAPTELWRKVPFTVGLRRAQELDWTLRAMREPGT
ALIYAEEPLVLWHQDENRDRISLQNPWREQLEWLRGNRELFTPRAYAAFT
LSVLSSMAAPTRDTGLFRELLAEARTHGDPGTVDYLTHMQIWALPPSVRH
RLRDVVVGRGKTSSNAG

SEQ ID NO:229 orf29 Glycosyl transferase 369aa

MPAERRVAIWRSSMLPGSETFVRNQADALTRWTPAYVGAVRHESVLSRPD
DVIAFPGGKGFLRLRLTGASPQLQKTISAVRPNLVHAHFGGDGWLVSHSA
QQLGVPLAVTVHGHDVTRQPSSPGAKGVRYRRNLQTVFTRASLVIAVSEV
IRGQAIRWGADPAKVKVHYTGIAVPPEQPEEVPKRWDVVFIGRFVAKKGV
DDLLTALAAVESRPRALLIGDGELMTAMRARAEQLGVDVTFAGSRTPEQV
RRHLLESRLLACPSKTAPDGDTEGLPTTILEAAALGLPVVATRHSGIPEA
VIDGETGLLSPEADPAALAVSLTRLLGDEDLQRRLGARARRHVTAHFDLV
EQTRRLEDLYDEVVAGARV

SEQ ID NO:230 orf30 Dihydrolipoamide dehydrogenase 459aa

MGEHFDLVVLGAGPGGYVAAIRGAQLGLTTAIVEDKYWGGVCLNVGCIPS
KALLRNAELAHIFHHQAQTFGIEGKVTFDFAVAHQRSRSVADGRVKGVHF
LMKKNGITEIQGRGEFTDAHTLRVGDRTVTFDNCILATGASTRMIPGTSV
SKRVVTYEEQILDPDLPDSIVIVGAGAIGVEFAYVLRNYGVDVTIVEFLD
RMLPLEDEEVSKELLRQYRKLGVDVRVGTRVEGIEEGADSVRVTVSKNGK
TEVLEADKVMQAIGFKPNVEGYGLETTGVTVSDRGAVEIDDFCRTNVPGI
YAIGDVTAKLMLAHAAEAMGIVAAETIAGAETMALDYRMIPRATFCQPQV
ASFGWTEAQAREQGFDVKVAKFPFTANGKAHGLGDATGFVKILSDAKYGE
LLGAHLIGPDVTELLPELTLAQQWDLTVHEVGRNVHAHPTLAEAVKEAIH
GLAGHMINF

SEQ ID NO:231 orf31 Putative membrane protein 348aa

MTTPPRRSGTLSLVTLTVEPPIAPAPPAAPGRSRRRRLGYLAFVLVAVVA
VVTLRDRLPDPGEFLDALRAADWRWAALAVGAGVLSQIAYAEQQRRLLAA
FGVRVPARRAIAMTYVRSALSMALPAGSAASAAYAFQVYRRHGATAAISA
TATLISTVVTVMSLGLLYAATWSLTATVVAGLAVLLLWIYRTVRGPVPAR
AGVPRRLRVAPIARLLQRPAVAQALRGARSVPARTWLTVTLAGVINWLLD
MACLLLAADALHAGLGWSRLALIYLAVQVVRQIPLTPGGIGLIETSMLAG
LIAAGAPQVTAAGIVLIYRLISFWLILPSGLAAHLTLRRGTVPPVTPG

SEQ ID NO:232-SEQ ID NO : 299

SEQ ID NO:300 O/SBDIG-1
TGGGTSTGCACSCTSACSATCGARTGCGGNACSGTSATCTGCGCSTGC

SEQ ID NO:301 O/ACT08F
TCCAGCACGCGCGGGG

SEQ ID NO:302 O/ACT09R
GTTCGACCAGCCGCCC

SEQ ID NO:303 O/AGvar01bF
TTCTAGACGTTGTTCTCCCATTTTCAC

SEQ ID NO:304 O/AGvar02bR
AAGATCTTCGAAGGTGAGCTCGCCGAA

SEQ ID NO:305 O/AGvar03F

GATCTTCGCGAGGACCGCACCATCTACGCCGCCAGCAGCGGCTGGGTGTGTACACTGACGATCGAGTGC
GGCACCGTGATCTGCGCCTGCTGAC

SEQ ID NO:306 O/AGvar04R

CTAGGTCAGCAGGCGCAGATCACGGTGCCGCACTCGATCGTCAGTGTACACACCCAGCCGCTGCTGGCG
GCGTAGATGGTGCGGTCCTCGCGAA

SEQ ID NO:307 O/AGvar05R
GCCTGCTGACCTAGGTCGACGATCGT

SEQ ID NO:308 O/AGvar06r
TGAATTCGGCTGCTCCCCGCGCGAAAT

SEQ ID NO:309 OlSB50F
ATTCGCCCGGGAAGTCCACCGAAAGGAAGACACACCATGATTCCGGGGATCCGTCGACC

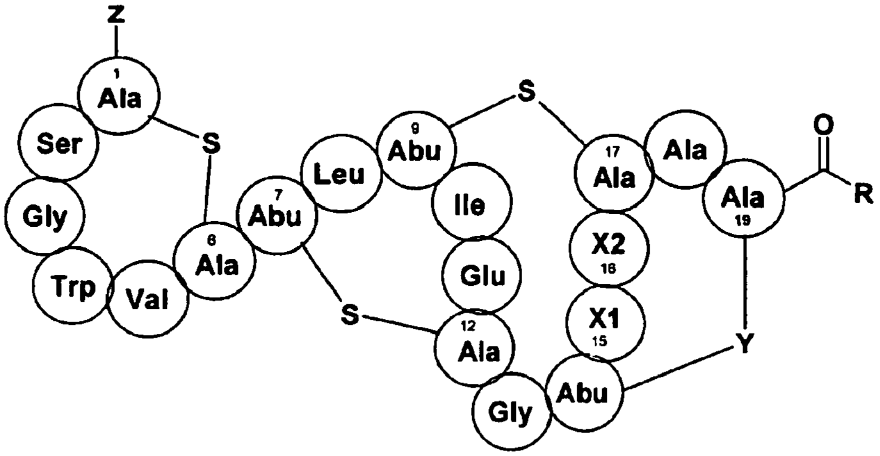
SEQ ID NO:310 OlSB51R
TGGGTSTGCACSCTSACSATCGARTGCGGNACSGTSATCTGCGCSTGC

SEQ ID NO:311 OlSB52F
AAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGATTCCGGGGATCCGTCGACC

SEQ ID NO:312 OlSB53R
GCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCATGTAGGCTGGAGCTGCTTC

Claims

1. A compound of the formula:



wherein:

-X1-X2- represents -Leu-Val-;
-Y- is -S-;
Z is either an amino acid or $-NH_2$ wherein the latter represents the N-terminus of the Ala at position 1;
R represents -OH or$-NR^1R^2$, wherein R^1 and R^2 independently represent:

(i) hydrogen;
(ii) a group of formula $-(CH_2)_n-NR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or C_{1-4}alkyl, or R^3 and R^4 taken together represents a group $-(CH_2)_3-$, $-(CH_2)_4-$, $(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2$ or $-(CH_2)_5-$; or
R^1 and R^2 taken together with the adjacent nitrogen atom represent a piperazine moiety which may be substituted at position 4 with a substituent selected from:

(a) C_{1-4}alkyl;
(b) C_{5-7}cycloalkyl;
(c) pyridyl,
(d) $-(CH_2)_p-NR^5R^6$ in which p represents an integer from 1 to 8 and R^5 and R^6 independently represent hydrogen or C_{1-4}alkyl;
(e) piperidinyl;
(f) substitute piperidinyl, wherein the substituted piperindinyl bears a N-substituent which is C_{1-4}alkyl;
(g) benzyl; and
(h) substituted benzyl, wherein the phenyl moiety bears 1 or 2 substituents selected from chloro, bromo, nitro, C_{1-4}alkyl and C_{1-4}alkoxy,

or a pharmaceutically acceptable salt thereof.

2. The compound according to claim 1, wherein Z is an amino acid.

3. The compound according to claim 2, wherein the amino acid is Ala.

4. The compound according to claim 1, wherein Z is $-NH_{2.}$

5. The compound according to any one of claims 1 to 4, wherein R is OH.

6. The compound according to any one of claims 1 to 4, wherein R^1 and R^2 independently represent:

(i) hydrogen;
(ii) a group of formula $-(CH_2)_n-NR^3R^4$, in which n represents an integer from 2 to 8 and R^3 and R^4 independently represent hydrogen or C_{1-4}alkyl.

7. The compound of claim 1, wherein the compound is selected from the group consisting of:

deoxyactagardine B *N*-[3-dimethylaminopropyl]monocarboxamide;
deoxyactagardine B *N*-[1-(1-methyl-4-piperidinyl)piperazine]monocarboxamide;
deoxyactagardine B [1-(3-dimethylaminopropyl)piperazine]monocarboxamide;
deoxyactagardine B;
D-Ala(0)deoxyactagardine B;
L-Ile(0)deoxyactagardine B;
L-Val(0)deoxyactagardine B;
L-Phe(0)deoxyactagardine B;
L-Lys(0)deoxyactagardine B; and
L-Trp(0)deoxyactagardine B.

8. A pharmaceutical composition comprising a compound as defined in any one of claims 1 to 7 together with a pharmaceutically acceptable carrier.

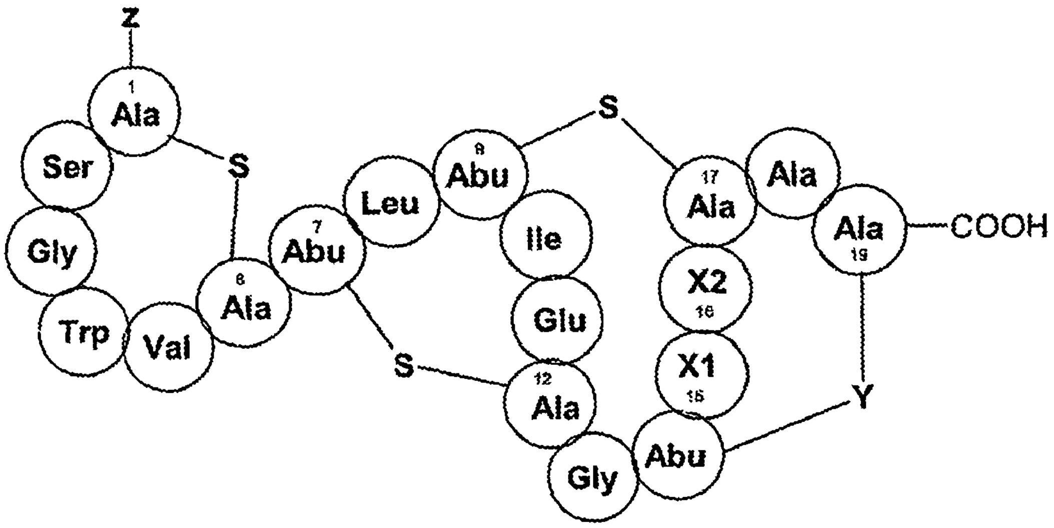
9. The pharmaceutical composition according to claim 8, for oral administration.

10. A compound as defined in any one of claims 1 to 7 for use in a method of treatment.

11. A compound as defined in any one of claims 1 to 7 for use in a method of treatment of a bacterial infection.

12. Use of a compound as defined in any one of claims 1 to 7 in the preparation of a medicament for use in the treatment of a bacterial infection.

13. A method of preparing a compound of formula:



wherein:

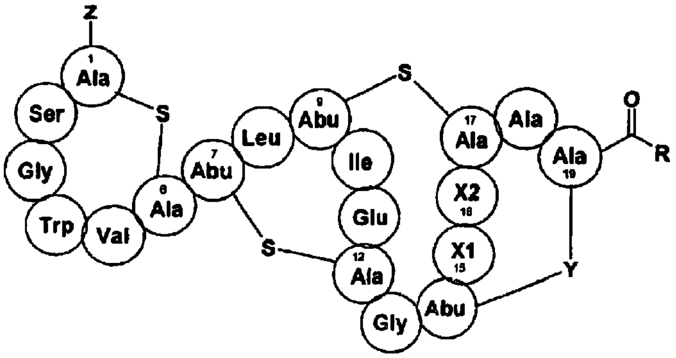
-X1-X2- represents -Leu-Val-;
-Y- is -S-;
Z is Ala or -NH_2 wherein the latter represent the N-terminus of the Ala at position 1, the method comprising expressing a nucleic acid encoding a sequence of SEQ ID NO: 1 or SEQ ID NO: 11, and optionally where necessary, associated cluster genes required for conversion of the precursor polypeptide to the product.

14. A method according to claim 13, wherein the nucleic acid is expressed in A. *liguriae.*

15. A method according to claim 14 wherein the *A. liguriae* is deposited under the Budapest Treaty deposit no: NCIMB 41362.

Patentansprüche

1. Verbindung der folgenden Formel:



worin:

-X1-X2- für -Leu-Val- steht;
-Y- -S- ist;
Z entweder eine Aminosäure oder -NH_2 ist, wobei Letzteres den N-Terminus des Ala an Position 1 darstellt;
R für -OH oder -NR^1R^2 steht, worin R^1 und R^2 jeweils unabhängig voneinander Folgendes darstellen:

(i) Wasserstoff:

(ii) eine Gruppe der Formel $-(CH_2)_n-NR^3R^4$, worin n für eine ganze Zahl von 2 bis 8 steht und R^3 und R^4 jeweils unabhängig voneinander für Wasserstoff oder C_{1-4}-Alkyl stehen oder R^3 und R^4 zusammengenommen für eine Gruppe $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_2-O-(CH_2)_2-$, $-(CH_2)_2-S-(CH_2)_2-$ oder $-(CH_2)_5-$ stehen, oder R^1 und R^2 zusammen mit dem benachbarten Stickstoffatom eine Piperazingruppierung darstellen, die an Position 4 gegebenenfalls mit einem aus der aus

(a) C_{1-4}-Alkyl,
(b) C_{5-7}-Cycloalkyl,
(c) Pyridyl,
(d) $-(CH_2)_p-NR^5R^6$, worin p für eine ganze Zahl von 1 bis 8 steht und R^5 und R^6 jeweils unabhängig voneinander für Wasserstoff oder C_{1-4}-Alkyl stehen,
(e) Piperidinyl,
(f) substituiertem Piperidinyl, worin das substituierte Piperidinyl einen N-Substituenten aufweist, der C_{1-4}-Alkyl ist,
(g) Benzyl und
(h) substituiertem Benzyl, worin die Phenylgruppierung 1 oder 2 aus Chlor, Brom, Nitro, C_{1-4}-Alkyl und C_{1-4}-Alkoxy ausgewählte Substituenten aufweist,

bestehenden Gruppe ausgewählten Substituenten substituiert ist,
oder ein pharmazeutisch annehmbares Salz davon.

2. Verbindung nach Anspruch 1, worin Z eine Aminosäure ist.

3. Verbindung nach Anspruch 2, worin die Aminosäure Ala ist.

4. Verbindung nach Anspruch 1, worin Z $-NH_2$ ist.

5. Verbindung nach einem der Ansprüche 1 bis 4, worin R OH ist.

6. Verbindung nach einem der Ansprüche 1 bis 4, worin R^1 und R^2 jeweils unabhängig voneinander für Folgendes stehen:

(i) Wasserstoff,
(ii) eine Gruppe der Formel $-(CH_2)_nNR^3R^4$, worin n für eine ganze Zahl von 2 bis 8 steht und R^3 und R^4 jeweils unabhängig voneinander für Wasserstoff oder C_{1-4}-Alkyl stehen.

7. Verbindung nach Anspruch 1, worin die Verbindung aus der aus

Desoxyactagardin-B-N-[3-dimethylaminopropyl]monocarboxamid,
Desoxyactagardin-B-N-[1-(1-methyl-4-piperidinyl)piperazin]monocarboxamid,
Desoxyactagardin-B-N-[1-(3-dimethylaminopropyl)piperazin]monocarboxamid,
Desoxyactagardin B,
D-Ala(0)desoxyactagardin B,
L-Ile(0)desoxyactagardin B,
L-Val(0)desoxyactagardin B,
L-Phe(0)desoxyactagardin B,
L-Lys(0)desoxyactagardin B und
L-Trp(0)desoxyactagardin B
bestehenden Gruppe ausgewählt ist.

8. Pharmazeutische Zusammensetzung, umfassend eine Verbindung nach einem der Ansprüche 1 bis 7 zusammen mit einem pharmazeutisch annehmbaren Träger.

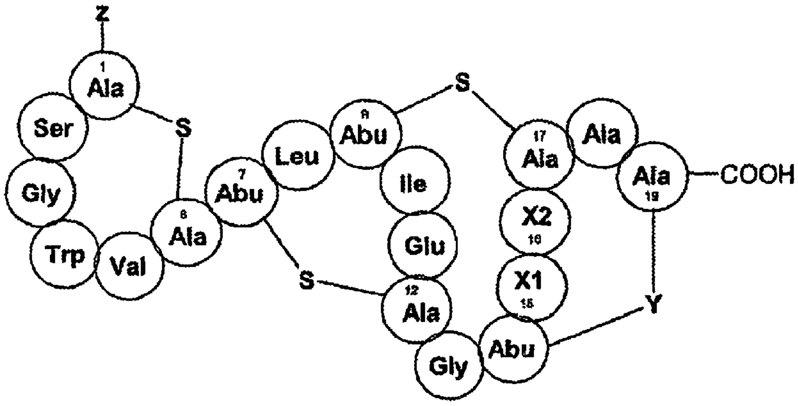
9. Pharmazeutische Zusammensetzung nach Anspruch 8 zur oralen Verabreichung.

10. Verbindung nach einem der Ansprüche 1 bis 7 zur Verwendung in einem Behandlungsverfahren.

11. Verbindung nach einem der Ansprüche 1 bis 7 zur Verwendung in einem Verfahren zur Behandlung einer Bakterieninfektion.

12. Verwendung einer Verbindung nach einem der Ansprüche 1 bis 7 bei der Herstellung eines Medikaments zur Verwendung bei der Behandlung einer Bakterieninfektion.

13. Verfahren zur Herstellung einer Verbindung der folgenden Formel:



worin:

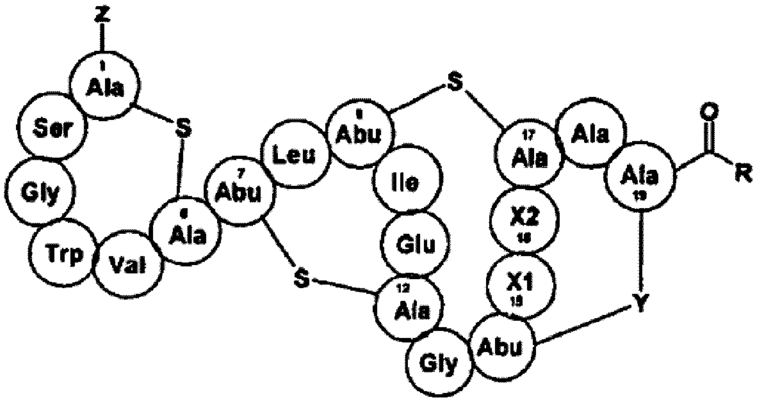
-X1-X2- für -Leu-Val- steht;
-Y- -S- ist;
Z Ala oder -NH_2 ist, worin Letzteres den N-Terminus des Ala an Position 1 darstellt, wobei das Verfahren das Exprimieren einer Nucleinsäure umfasst, die für eine Sequenz nach Seq.-ID Nr. 1 oder Seq.-ID Nr. 11 und gegebenen- und erforderlichenfalls assoziierte Clustergene, die zur Überführung des Vorläuferpolypeptids in das Produkt notwendig sind, kodiert.

14. Verfahren nach Anspruch 13, worin die Nucleinsäure in *A. liguriae* exprimiert ist.

15. Verfahren nach Anspruch 14, worin die *A. liguriae* gemäß dem Budapester Vertrag unter der Hinterlegungsnummer NCIMB 41362 hinterlegt sind.

Revendications

1. Composé de formule:



dans laquelle:

-X1-X2- représente -Leu-Val-;
-Y- est -S-;
Z est soit un acide aminé soit $-NH_2$, ce dernier représentant l'extrémité N de l'Ala en position 1;
R représente -OH ou $-NR^1R^2$, où R^1 et R^2 représentent indépendamment:

(i) l'hydrogène;
(ii) un groupe de formule $-(CH_2)_n-NR^3R^4$ dans laquelle n représente un entier de 2 à 8 et R^3 et R^4 représentent indépendamment l'hydrogène ou un alkyle en C_1 à C_4, ou bien R^3 et R^4, pris ensemble, représentent un groupe - $(CH_2)_3$-, $-(CH_2)_4$-, $-(CH_2)_2-O-(CH2)_2$-, $-(CH_2)_2-S-(CH_2)_2$ ou $-(CH_2)_5$-; ou
R^1 et R^2, pris ensemble avec l'atome d'azote adjacent, représentent un fragment pipérazine qui peut être substitué en position 4 avec un substituant choisi parmi:

(a) alkyle en C_1 à C_4;
(b) cycloalkyle en C_5 à C_7;
(c) pyridyle;
(d) $-(CH_2)_p-NR^5R^6$ où p représente un entier de 1 à 8 et R^5 et R^6 représentent indépendamment l'hydrogène ou un alkyle en C_1 à C_4;
(e) pipéridinyle;
(f) pipéridinyle substitué, lequel pipéridinyle substitué porte un N-substituant qui est un alkyle en C_1 à C_4;
(g) benzyle; et
(h) benzyle substitué, dans lequel le fragment phényle porte 1 ou 2 substituants choisis parmi chloro, bromo, nitro, alkyle en C_1 à C_4 et alcoxy en C_1 à C_4,

ou un sel pharmaceutiquement acceptable de celui-ci.

2. Composé selon la revendication 1, dans lequel Z est un acide aminé.

3. Composé selon la revendication 2, dans lequel l'acide aminé est Ala.

4. composé selon la revendication 1, dans lequel Z est $-NH_2$.

5. composé selon l'une quelconque des revendications 1 à 4, dans lequel R est OH.

6. Composé selon l'une quelconque des revendications 1 à 4, dans lequel R^1 et R^2 représentent indépendamment :

(i) l'hydrogène;
(ii) un groupe de formule $-(CH_2)_n-NR^3R^4$ dans laquelle n représente un entier de 2 à 8 et R^3 et R^4 représentent indépendamment l'hydrogène ou un alkyle en C_1 à C_4.

7. Composé selon la revendication 1, lequel composé est choisi dans le groupe constitué par les suivants:

N-[3-diméthylaminopropyl]monocarboxamide de
désoxyactagardine B;
N-[1-(2-méthyl-4-pipéridinyl)pipérazine]monocarboxamide de désoxyactagardine B;
[1-(3-diméthylaminopropyl)pipérazine]monocarboxamide de désoxyactagardine B;
D-Ala(0)désoxyactagardine B;
L-Ile(0)ésoxyactagardine B;
L-Val(0)désoxyactagardine B;
L-Phe(0)désoxyactagardine B;
L-Lys(0)désoxyactagardine B; et
L-Trp(0)désoxyactagardine B.

8. Composition pharmaceutique comprenant un composé tel que défini dans l'une quelconque des revendications 1 à 7 conjointement avec un véhicule pharmaceutiquement acceptable.

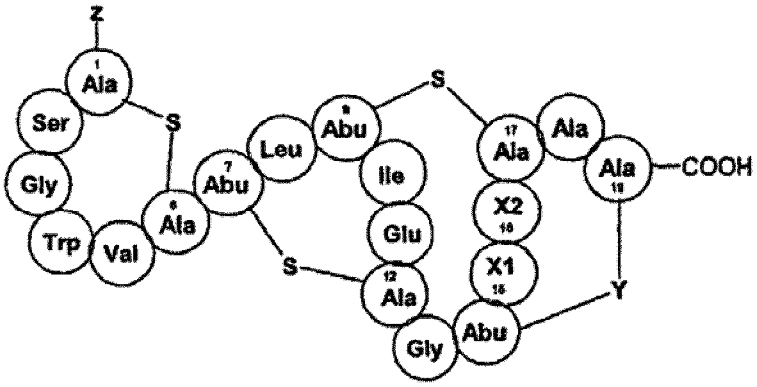
9. Composition pharmaceutique selon la revendication 8, pour une administration par voie orale.

10. Composé tel que défini dans l'une quelconque des revendications 1 à 7, pour une utilisation dans une méthode de traitement.

11. Composé tel que défini dans l'une quelconque des revendications 1 à 7, pour une utilisation dans une méthode de traitement d'une infection bactérienne.

12. Utilisation d'un composé tel que défini dans l'une quelconque des revendications 1 à 7 dans la préparation d'un médicament pour une utilisation dans le traitement d'une infection bactérienne.

13. Procédé de préparation d'un composé de formule:



dans laquelle:

-X1-X2- représente -Leu-Val-;
-Y- est -S-;
Z est Ala ou $-NH_2$, ce dernier représentant l'extrémité N de l'Ala en position 1,
lequel procédé comprend l'expression d'un acide nucléique codant une séquence de la SEQ ID NO: 1 ou de la SEQ ID NO: 11, et éventuellement, lorsque c'est nécessaire, des gènes en groupe associés requis pour la conversion du polypeptide précurseur en le produit.

14. Procédé selon la revendication 13, dans lequel l'acide nucléique est exprimé dans A. *liguriae.*

15. Procédé selon la revendication 14, dans lequel l'A. *liguriae* est déposé conformément au Traité de Budapest sous le numéro de dépôt NCIMB 41362.

FIG. 1 Cosmid CosAG14 from *Actinoplanes Garbadinensis*

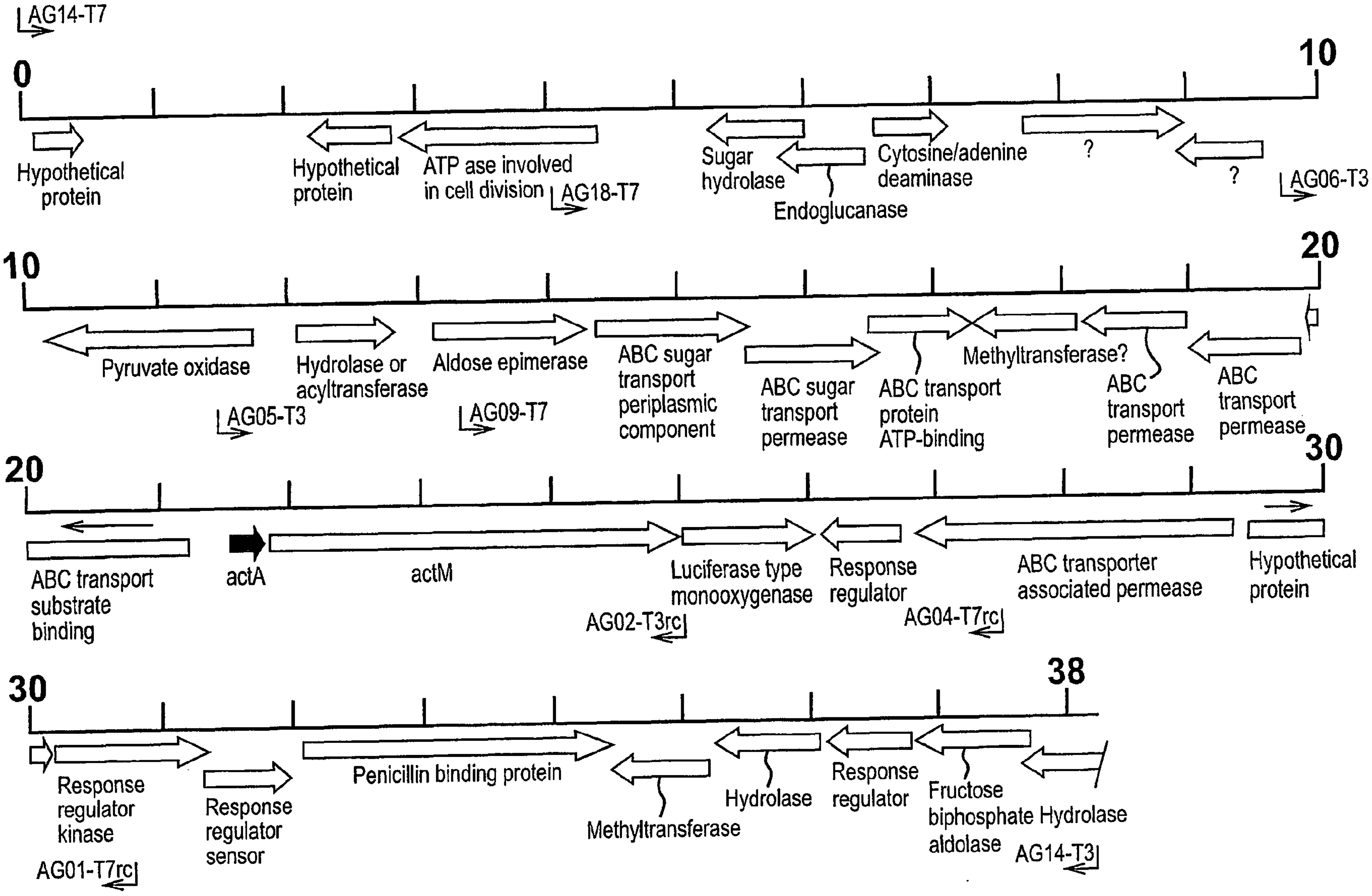


FIG. 2 AL02 from *Actinoplanes liguriae*

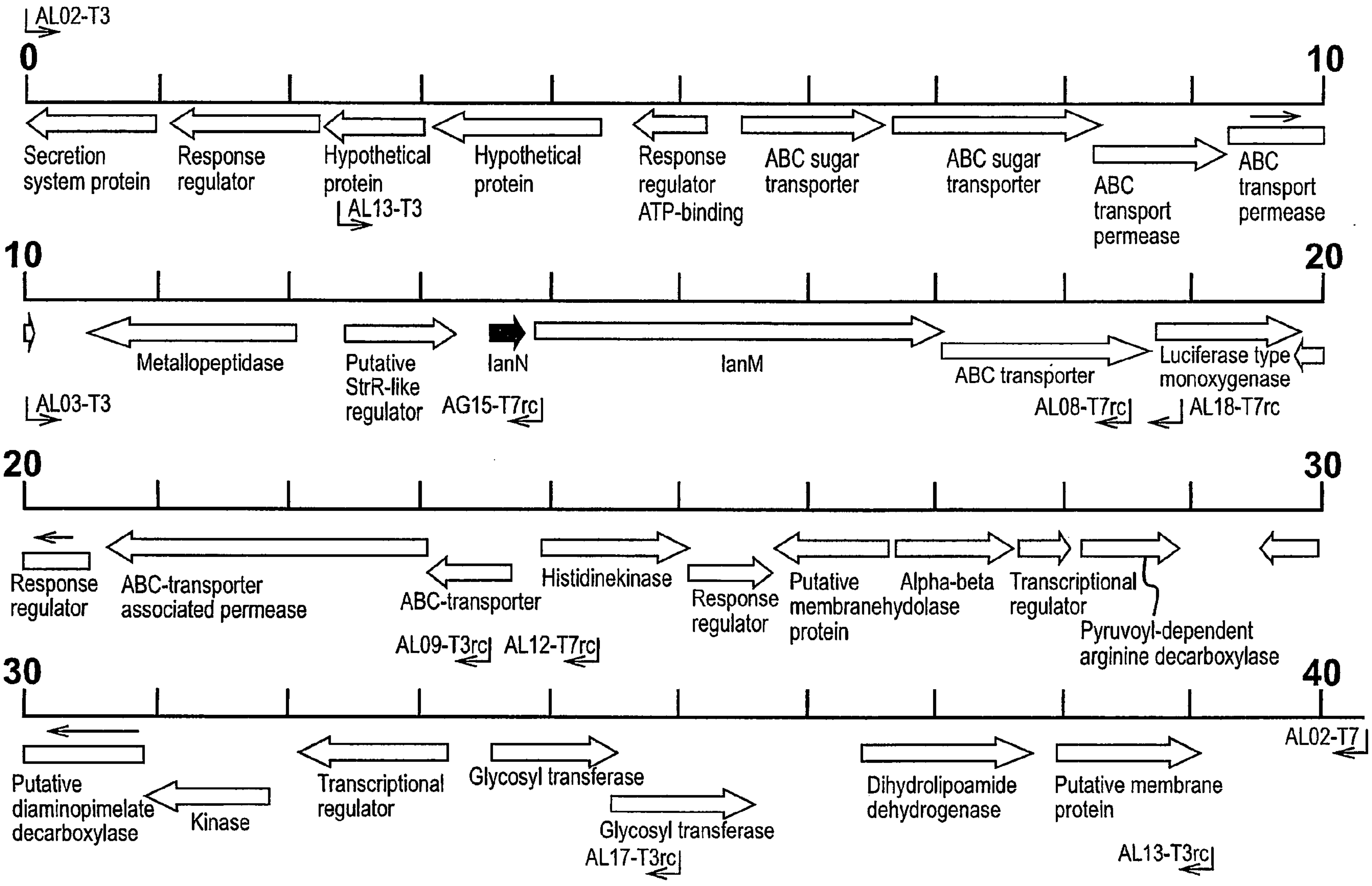
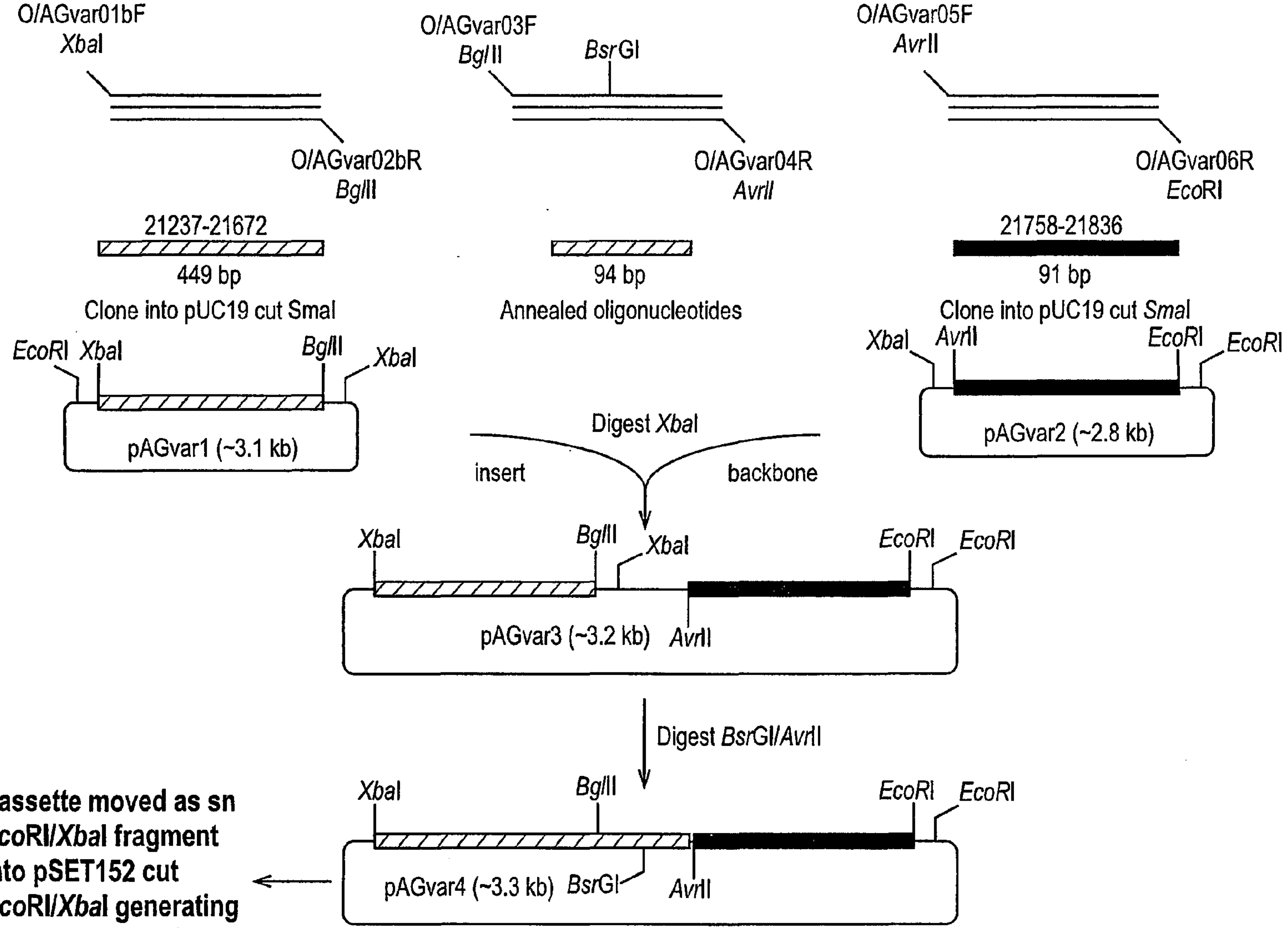
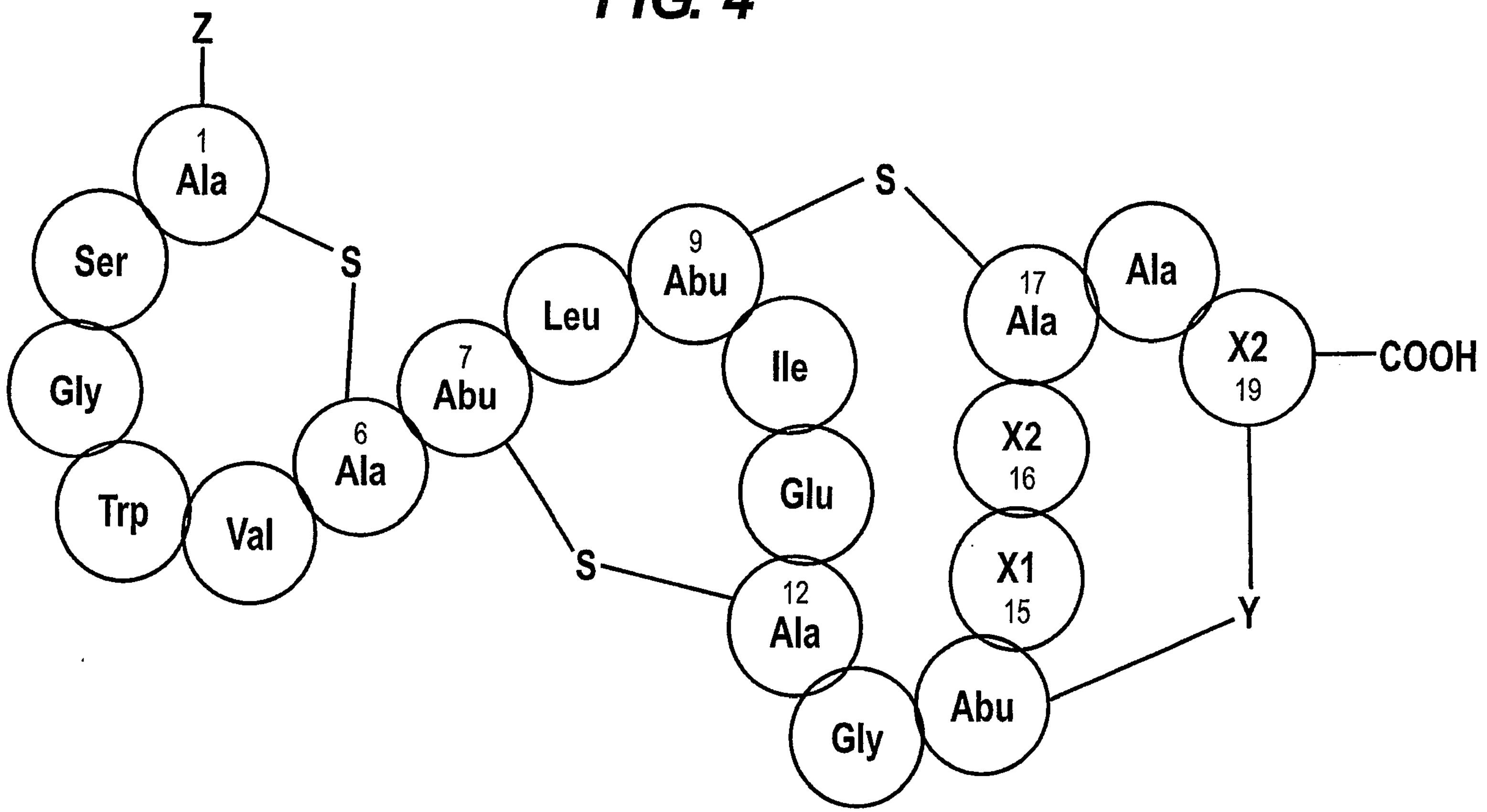


FIG. 3 Strategy for generating pAGvarX



Cassette moved as sn *EcoRI/Xba*I fragment into pSET152 cut *EcoRI/Xba*I generating pAGvarX (~6.3kb).

FIG. 4


Z
1
Ala
Ser
Gly
Trp
Val
S
6
Ala
7
Abu
Leu
9
Abu
S
Ile
Glu
12
Ala
Gly
S
17
Ala
X2
16
X1
15
Abu
Ala
X2
19
COOH
Y

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