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(54) **Title**: ANTIMICROBIAL COMPOUNDS

(57) **Abstract**: The present invention relates to antimicrobial compounds and their uses, and in particular to peptide antibiotics which may be used in the treatment of Gram-negative bacterial infections.

Antimicrobial compounds

Field of the invention

The present invention relates to antimicrobial compounds and their uses, and in particular to peptide antibiotics which may be used in the treatment of Gram-negative bacterial infections, particularly those caused by multidrug-resistant (MDR) Gram-negative bacteria.

Background of the invention

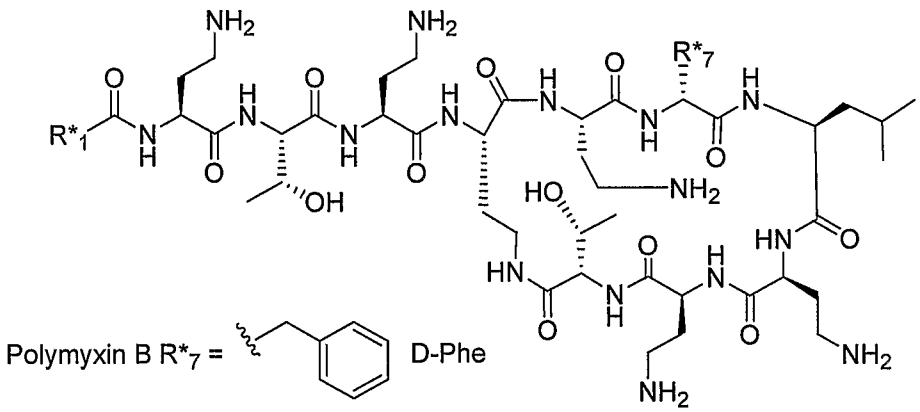
The world is facing an enormous and growing threat from the emergence of bacteria that are resistant to almost all available antibiotics. Whilst a small number of new antibiotics targeting MDR Gram-positive bacteria have been approved in the past two decades, there has been a marked decline in the discovery of novel antibiotics for the treatment of Gram-negative bacteria.

Representative genera of Gram-negative bacteria are: *Acinetobacter*; *Actinobacillus*; *Bartonella*; *Bordetella*; *Brucella*; *Burkholderia*; *Campylobacter*; *Cyanobacteria*; *Enterobacter*; *Erwinia*; *Escherichia*; *Francisella*; *Helicobacter*; *Hemophilus*; *Klebsiella*; *Legionella*; *Moraxella*; *Morganella*; *Neisseria*; *Pasteurella*; *Proteus*; *Providencia*; *Pseudomonas*; *Salmonella*; *Serratia*; *Shigella*; *Stenotrophomonas*; *Treponema*; *Vibrio*; and *Yersinia*.

While the recently approved tigecycline is active against a range of clinically important Gram-negative pathogens, including *Acinetobacter baumannii*, it is reported to not be effective against *Pseudomonas aeruginosa*. Numerous hospitals worldwide have experienced outbreaks of infections caused by *P. aeruginosa, A. baumannii* or *Klebsiella pneumoniae* that are resistant to all commercially available antibiotics, including the last-line therapies colistin (polymyxin E) and polymyxin B. The Infectious Diseases Society of America (IDSA) has placed *P. aeruginosa, A. baumannii* and *K. pneumoniae* on a 'hit list'

of the six top-priority dangerous MDR microorganisms, the so-called 'superbugs', in its recent '*Bad Bugs Need Drugs*' campaign.

Polymyxins belong to a class of peptides which was discovered more than 50 years ago. The structures of representative polymyxins are shown below:



Polymyxins are produced by nonribosomal biosynthetic enzymes from the secondary metabolic pathways of *Bacillus polymyxa*. There are two polymyxins clinically available, colistin (polymyxin E) and polymyxin B, and cross resistance exists between these two polymyxins. Despite the efficacy of polymyxins in treating certain Gram-negative bacterial infections, it has been shown that parenteral administration of colistin and polymyxin B can be potentially nephrotoxic and/or neurotoxic. Polymyxins are now being used as last-line antibiotics in patients where all other available antibiotics are inactive.

Although the incidence of resistance to polymyxins is currently relatively low, it has been demonstrated that resistance can emerge *in vitro* in *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, and polymyxin resistance in hospitalised patients has been increasingly reported. There is only one amino acid difference between colistin and polymyxin B and,

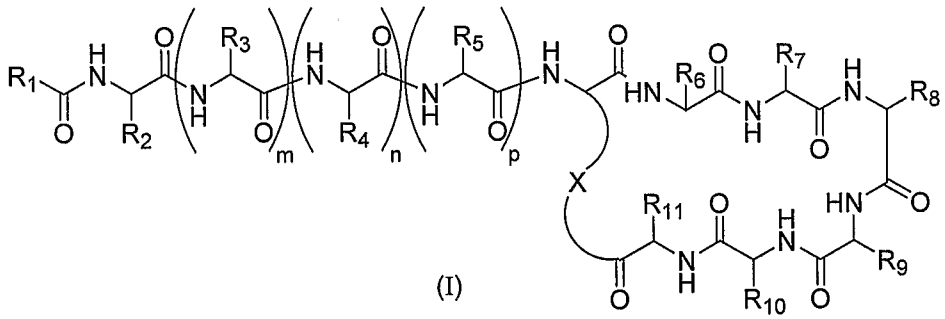
not surprisingly, cross resistance has been found to exist. In essence, resistance to polymyxins implies a total lack of antibiotics for treatment of life-threatening infections caused by these MDR Gram-negative 'superbugs'.

Accordingly there exists an urgent need to discover new antibiotics which are effective against susceptible as well as MDR, and especially polymyxin-resistant, Gram-negative bacteria.

Summary of the invention

It has now been found that certain polymyxin analogs are effective against Gram-negative bacteria, including both polymyxin-susceptible and -resistant MDR Gram-negative bacteria.

Accordingly in one aspect the present invention provides a peptide of formula (I):



(I)

wherein:

R_1 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{2-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{2-22}alkenyl;

m, n and p are each independently 0 or 1;

R_3, R_4 and R_5 where present and R_2, R_6, R_9, R_{10} and R_{11} are each independently selected from the side chain of an amino acid selected from the group consisting of α,γ-diaminobutyric acid, arginine, histidine, lysine, ornithine, glutamatic acid, glutamate, aspartic acid, aspartate, glutamine, asparagine, serine, threonine and cysteine;

X is a residue of the side chain of an amino acid selected from the group consisting of α,γ-diaminobutyric acid, arginine, histidine, lysine, ornithine, glutamatic acid, glutamate, aspartic acid, aspartate, glutamine, asparagine, serine, threonine and cysteine;

R_7 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{1-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{1-22}alkenyl;

R_8 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{1-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{1-22}alkenyl;

wherein any alkyl, alkenyl, aryl and cycloalkyl group within the peptide is optionally substituted with one or more groups selected from the group consisting of C_{1-22}alkyl, C_{2-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, hydroxy, hydroxyC_{1-22}alkyl, amino, aminoC_{1-22}alkyl, C_{1-22}alkyloxy, C_{1-22}alkylamino, (C_{1-22}alkyl)(C_{1-22}alkyl)amino, C_{1-22}alkylcarbonyloxy, C_{2-22}alkenylcarbonyloxy, C_{6-16}arylcarbonyloxy, C_{3-12}cycloalkylcarbonyloxy, C_{1-22}alkylcarbonylamino, C_{2-22}alkenylcarbonylamino, C_{6-16}arylacylamino and C_{3-12}cycloalkylcarbonylamino; and

wherein the number of carbon atoms in the R_8 group is greater than 4, or a pharmaceutically acceptable salt thereof.

It has now been found that the peptides of the present invention are active against Gram-negative bacteria and surprisingly against not only polymyxin-susceptible but also polymyxin-resistant MDR Gram-negative bacteria. It is believed that increasing the number of carbon atoms in the R_8 group to greater than 4 enhances lipophilicity at that position to an extent that may be sufficient to render the peptide effective against both polymyxin-susceptible and -resistant Gram-negative bacteria. The number of carbon atoms in the R_8 group may be greater than 5, greater than 6 or greater than 7. The number

of carbon atoms in the R_7 group may be greater than 3, greater than 4, greater than 5 or greater than 6. In some embodiments the combined number of carbon atoms in the R_7 and R_8 groups is greater than 11 and may be greater than 12, greater than 13 or greater than 14.

In another aspect the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a peptide as hereinbefore defined, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutically acceptable carrier or diluent.

In another aspect the invention provides a method of preventing or treating a bacterial infection comprising the step of administering a therapeutically effective amount of a peptide as hereinbefore described, or a pharmaceutically acceptable salt thereof, to a subject in need thereof.

In another aspect the invention provides a use of a peptide as hereinbefore described, or a pharmaceutically acceptable salt thereof, in the preparation of a medicament for the prevention or treatment of a bacterial infection.

Brief description of the Figures

Figure 1 shows static time-kill of compound **3** and colistin against a colistin-resistant MDR clinical *P. aeruginosa* isolate D.

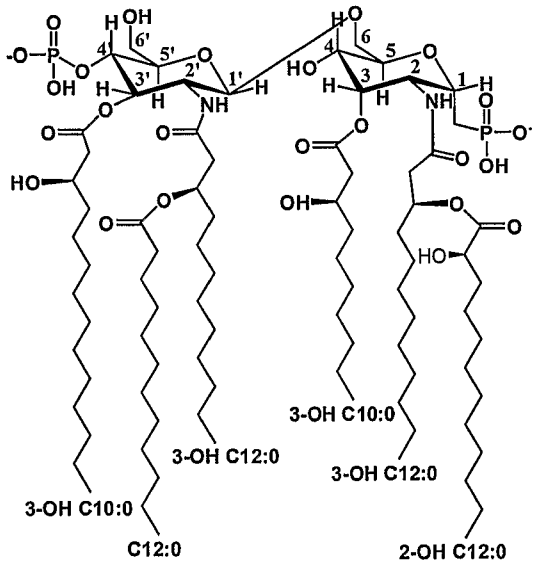
Figure 2 shows static time-kill of compound **2** and colistin against a colistin-resistant MDR clinical *P. aeruginosa* isolate D.

Figure 3 shows static time-kill of compound **3** and colistin against *P. aeruginosa* ATCC 27853.

Detailed description of the invention

The initial cellular target of polymyxins in Gram-negative bacteria is the lipopolysaccharide (LPS) component of the outer membrane (OM). It is believed that the LPS target is conserved across most, if not all, Gram-negative bacteria.

LPS is composed of three domains, a conserved inner core 2-keto-3-deoxyoctonoic acid bound to lipid A and a variable O-antigen composed of repeating units of various polysaccharides. The consensus structure of lipid A consists of a β-1'-6-linked D-glucosamine disaccharide that is phosphorylated at the 1- and 4'-positions and is shown below:

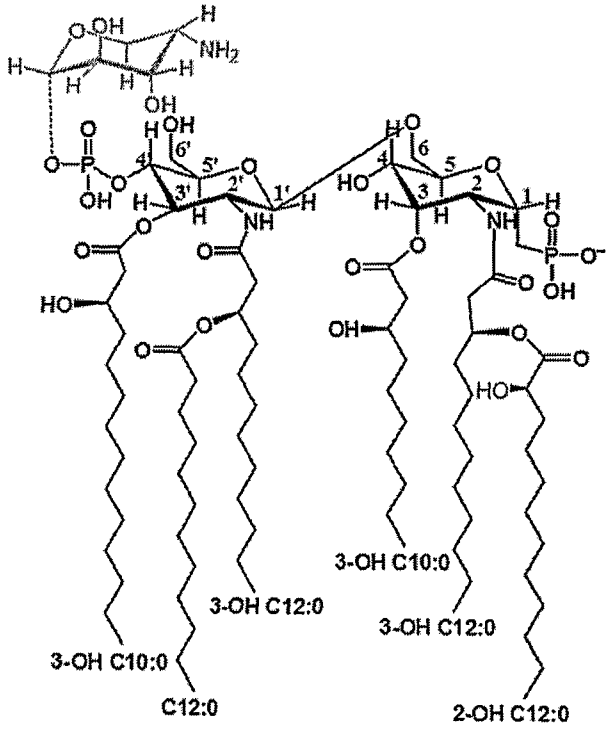


Lipid A usually contains six acyl chains. Four β-hydroxy acyl chains (usually C_{10} and C_{12} in length) are attached directly to the glucosamine sugars, while a secondary acyl chain is often attached to the β-hydroxy group on each of two of the chains. Lipid A acts as a hydrophobic anchor with the tight packing of the fatty acyl chains helping to stabilise the overall membrane structure.

It is believed that there is an initial polar interaction between the cationic polymyxin peptide (particularly the charged α,γ-diaminobutyric acid (Dab) residues) and the lipid A

component of LPS in the OM, thereby displacing divalent cations (Ca^{2+} and Mg^{2+}) from the negatively charged phosphate groups of lipid A. This initial interaction is followed by uptake across the OM and interaction with the cytoplasmic membrane. It has now been found that the binding of a naturally occurring polymyxin with lipid A may be enhanced by substitution with one or more lipophilic groups at particular locations within the polymyxin peptide structure.

As noted above, it is believed that a critical first step in the action of polymyxins on Gram-negative bacteria is the polar interaction between the positively charged Dab residues of polymyxins and the negatively charged phosphate groups on lipid A. It has been discovered that many of the bacterial mechanisms of resistance to polymyxins are based on modifications to lipid A which reduce or abolish this initial polar interaction. In *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *K. pneumoniae* and *P. aeruginosa*, modification of one or both of the phosphates of lipid A with moieties, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn), reduces the net negative charge of lipid A thereby reducing the initial interaction of a polymyxin with lipid A leading to polymyxin resistance. One example of such a modification is shown below:



In addition, in *K. pneumoniae* the presence of a capsule can also cause polymyxin resistance.

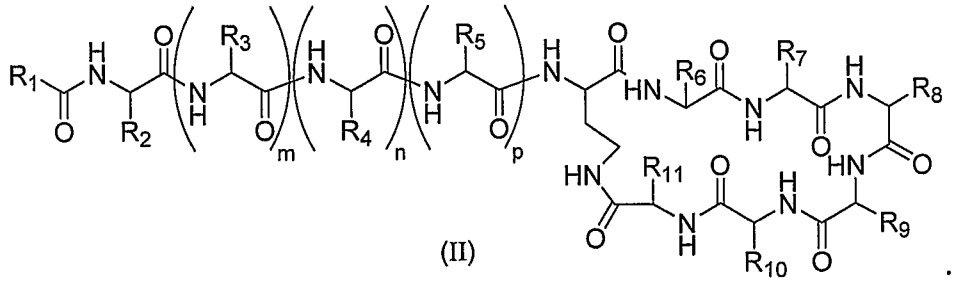
The issue of polymyxin resistance is complicated by the recent discovery from population analysis profiles of 42 *P. aeruginosa*, 46 *A. baumannii* and 30 *K. pneumoniae* polymyxin-susceptible (Minimum Inhibitory Concentrations (MICs, the lowest concentration of an antibiotic that inhibits visible growth of a microorganism) ≤ 2 mg/L) MDR clinical strains from various regions in the world that more than 50% of the strains contained a small sub-population that was able to grow in the presence of 10 mg/L, or higher, of colistin - a phenomenon known as heteroresistance which potentially explains how apparently susceptible Gram-negative bacteria can rapidly develop polymyxin resistance. Consistent with such findings of heteroresistance to colistin, recent studies have revealed very high ratios of Minimum Bactericidal Concentration (MBC, the lowest concentration of an antibiotic that results in the killing of more than 99.9% of the bacteria being tested) to MIC in MDR strains of all three species (MBC/MIC ≥ 32). In other words, whilst visible bacterial growth was inhibited at MICs of colistin, 100% of bacterial cells were not killed. It has also been recently demonstrated in an *in vitro* pharmacokinetic/pharmacodynamic

(PK/PD) model which mimics clinical dosing regimens that polymyxin-resistant sub-populations have the potential to rapidly amplify upon exposure to colistin.

It has now been surprisingly found that the peptides of the present invention are not only effective against polymyxin-susceptible but also polymyxin-resistant MDR Gram-negative bacteria. Without wishing to be bound by theory it is believed that sufficient binding between a polymyxin analog and lipid A may be achieved by overcoming, or at least ameliorating, the adverse polar interactions between a modified moiety on the phosphate group of lipid A and the positively charged residues on polymyxins through enhancement of the interactions between the lipophilic groups on lipid A and certain residues on the polymyxin analog.

It is believed that, for the polymyxin analogs in this invention, the lipophilic groups R_1, R_7 and R_8 interact with the acyl chains in lipid A, whereas the X, R_2, R_6, R_9, R_{10} and R_{11} groups and the R_3, R_4 and R_5 where present, which are generally hydrophilic, interact with the polar, charged portions of lipid A. If lipid A has undergone glycosylation or modification with phosphoethanolamine as part of the resistance mechanism in the bacteria, it is believed that the lipophilic groups and the generally hydrophilic groups remain able to maintain these same interactions. Accordingly, the peptides of the present invention are effective against not only polymyxin-susceptible but also polymyxin-resistant MDR Gram-negative bacteria.

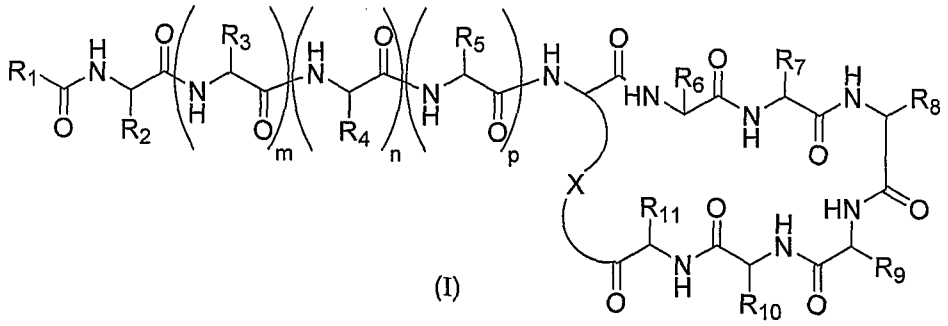
In some embodiments, the side chain residue X is the residue of the side chain of α,γ-diaminobutyric acid (Dab) and the peptide is of formula (II):



(II)

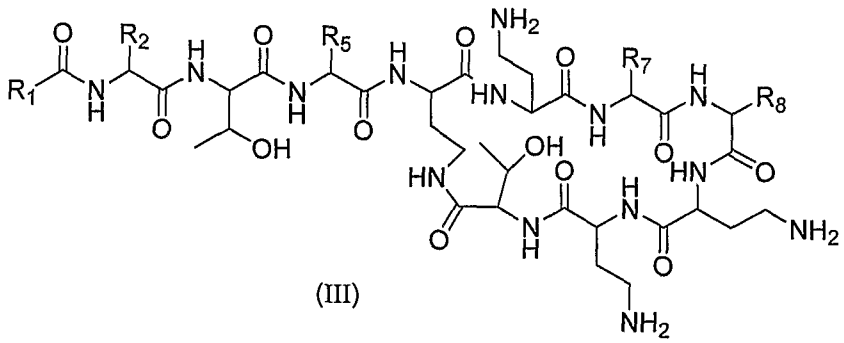
In some embodiments, the linear peptide portion of the molecule comprises three amino acids, as do the naturally occurring polymyxin structures. In these cases, m and p may be equal to 1 and n may be equal to 0.

In some embodiments the X, R_3, R_6, R_9, R_{10} and R_{11} groups in the peptide of formula (I):



(I)

are the same as the corresponding groups that are common to the naturally occurring polymyxin structures, m and p equals 1 and n equals 0, and the peptide may accordingly be depicted as formula (III):



(III)

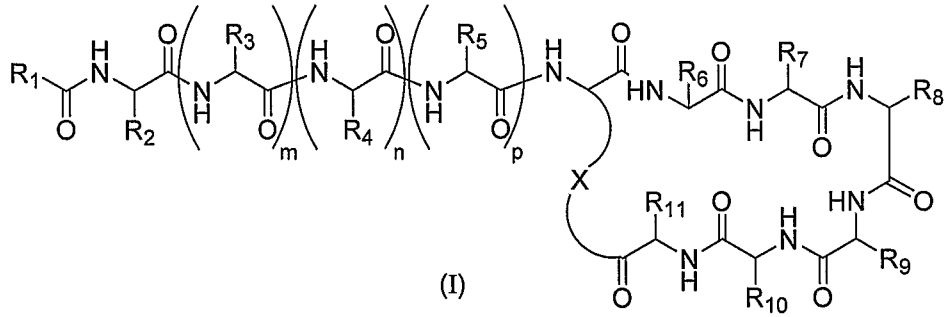
wherein R_1 and R_8 are lipophilic groups as hereinbefore defined;

R_2 is the side chain of α,γ-diaminobutyric acid or threonine;

R_5 is the side chain of α,γ-diaminobutyric acid, serine or threonine; and

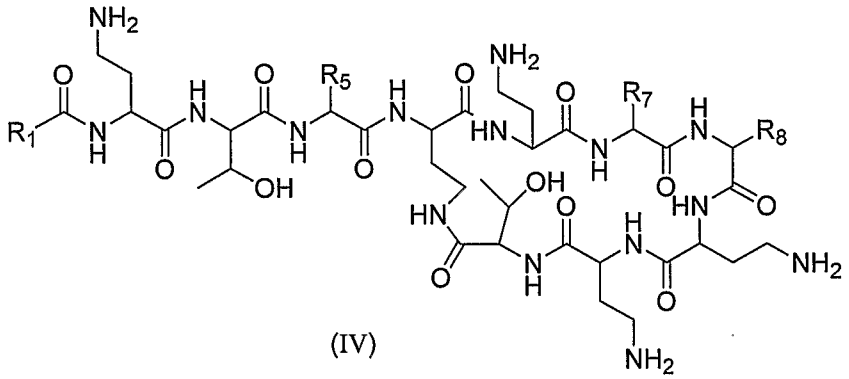
R_7 is the side chain of leucine or phenylalanine.

In some embodiments the X, R_2, R_3, R_6, R_9, R_{10} and R_{11} groups in the peptide of formula (I):



(I)

are the same as the corresponding groups that are common to the naturally occurring polymyxin structures, m and p equals 1 and n equals 0, and the peptide may accordingly be depicted as formula (IV):



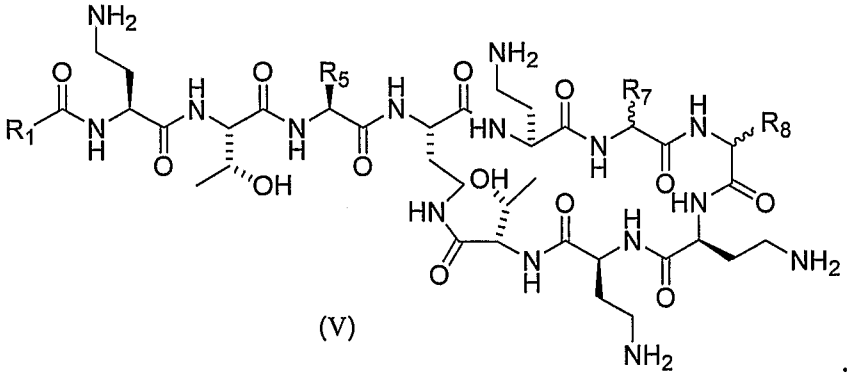
(IV)

wherein R_1 and R_8 are lipophilic groups as hereinbefore defined;

R_5 is the side chain of α,γ-diaminobutyric acid or serine; and

R_7 is the side chain of leucine or phenylalanine.

Each amino acid residue within the peptide may be in the L- or D-form. In some embodiments the absolute stereochemistry at each of the stereocentres in the compound of formula (V) shown below is the same as the corresponding stereochemistry in the naturally occurring polymyxin structures:



(V)

In the compound of formula (V) (as well as compounds of formulae (I) to (IV)) shown above, the absolute stereochemistry of the stereocentres adjacent to the R_7 and R_8 groups respectively have not been defined. It will be understood that where the amino acid(s) providing the R_7 and/or R_8 group is/are used as a mixture of enantiomers, such as a racemate, the peptide so formed that includes the R_7 and R_8 groups will, in the absence of purification of an intermediate peptide, generally be prepared as a mixture of diastereomers. In some embodiments, the peptide may be used as a mixture of diastereomers, such as in the treatment of a bacterial infection. In some embodiments the peptide may be used as a single diastereomer, which may be isolated from a mixture of diastereomers or may be prepared using an enantiopure, or enantioenriched, amino acid(s) that provides the R_7 and/or R_8 group. It is believed that preferred peptides are those in which the stereocentre adjacent to the R_7 group has the stereochemistry of D-phenylalanine - having *R* stereochemistry and/or where the amino acid is in the D-form. It is believed that preferred peptides are those in which the stereocentre adjacent to the R_8 group has the stereochemistry of naturally occurring amino acids such as L-leucine - having *S* stereochemistry and/or where the amino acid is in the L-form. Where the peptide is used as a single diastereomer, it is typically preferable that the peptide is made using an enantiopure, or enantioenriched, amino acid containing the R_7 and/or R_8 group rather than being purified following synthesis using a racemic mixture of the amino acid.

Preferred examples of lipophilic groups are as follows:
R_1 is selected from the group consisting of C_{3-11}alkyl, phenyl, bi-phenyl, cyclohexanylbutyl, trans-4-propylcyclohexanyl, cyclododecanyl and cyclopentylphenyl;
R_7 is the side chain of leucine or phenylalanine; and/or
R_8 is selected from the group consisting of C_{5-10}alkyl, phenyl, bi-phenyl, cyclohexylbutyl, trans-4-propylcyclohexanyl, cyclododecanyl and cyclopentylphenyl.

As used herein, the term "alkyl", used either alone or in compound words denotes straight chain or branched alkyl. Preferably the alkyl group is a straight chain alkyl group. Prefixes such as "C_{3-22}" are used to denote the number of carbon atoms within the alkyl group (from

3 to 22 in this case). Examples of straight chain and branched alkyl include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec*-butyl, *t*-butyl, *n*-pentyl, hexyl, heptyl, 5-methylheptyl, 5-methylhexyl, octyl, nonyl, decyl, undecyl, dodecyl and docosyl (C_{22}).

As used herein, the term "cycloalkyl", used either alone or in compound words denotes a cyclic alkyl group. Prefixes such as "C_{3-12}" are used to denote the number of carbon atoms within the cyclic portion of the alkyl group (from 3 to 12 in this case). Examples of cyclic alkyl include mono- or polycyclic alkyl groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl and cyclododecyl.

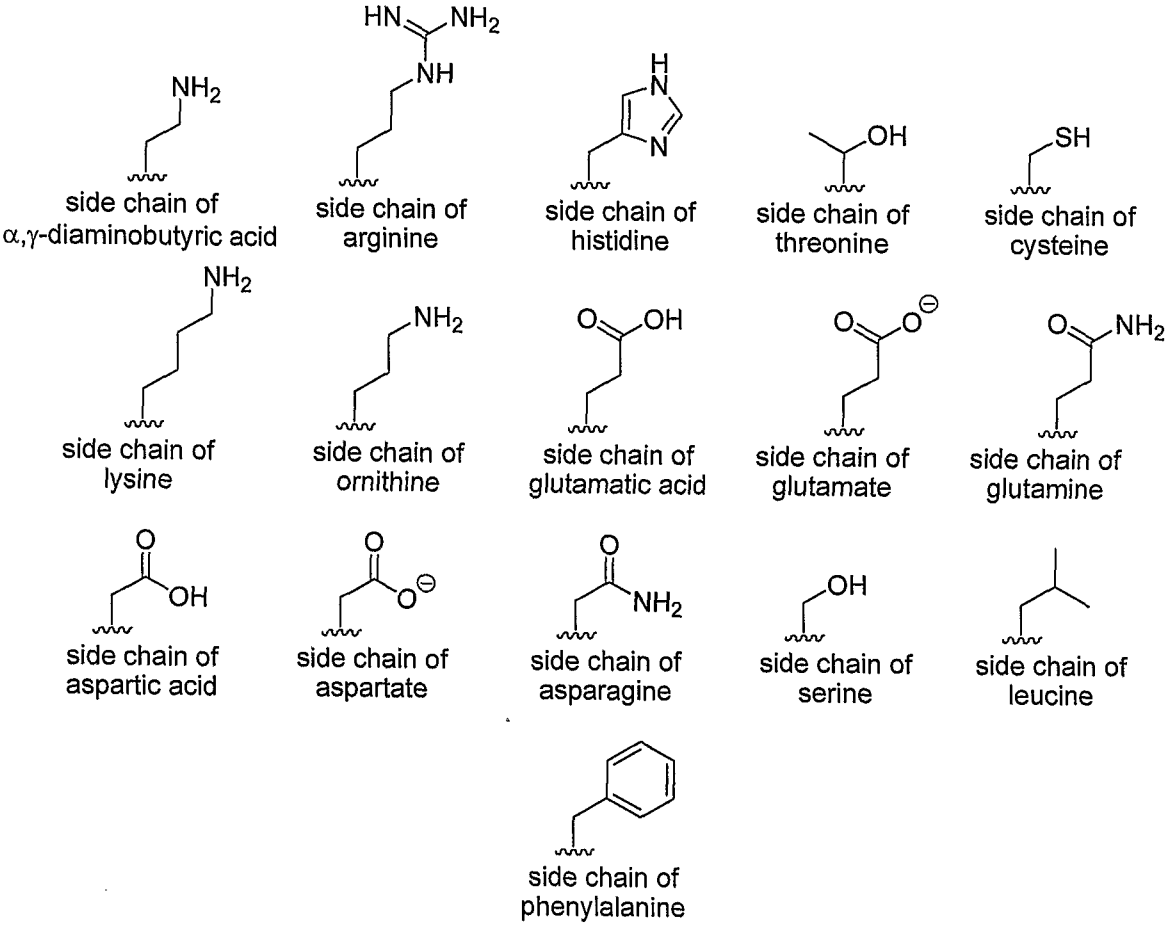
As used herein, the term "alkenyl", used either alone or in compound words denotes straight chain or branched hydrocarbon residues containing at least one carbon to carbon double bond including ethylenically mono-, di- or polyunsaturated alkyl groups as previously defined. Preferably the alkenyl group is a straight chain alkenyl group Prefixes such as "C_{3-22}" are used to denote the number of carbon atoms within the alkenyl group (from 3 to 22 in this case). Examples of alkenyl include vinyl, allyl, 1-methylvinyl, butenyl, iso-butenyl, 3-methyl-2-butenyl, 1-pentenyl, 1-hexenyl, 3-hexenyl, 1-heptenyl, 3-heptenyl, 1-octenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-butadienyl, 1,4-pentadienyl, 1,3-hexadienyl, 1,4-hexadienyl and 5-docosenyl (C_{22}).

As used herein, the term "aryl" denotes any of single, polynuclear, conjugated and fused residues of aromatic hydrocarbon ring systems. Prefixes such as "C_{6-16}" are used to denote the number of carbon atoms within the cyclic portion of the aryl group (from 6 to 16 in this case). Examples of aryl include phenyl, naphthyl and tetrahydronaphthyl.

As used herein, the term “lipophilic” refers to a property of a chemical compound, or part thereof, wherein the chemical compound or part thereof more readily associates with members of a large and diverse group of oils, fats and fat like substances (that occur, for example, in living organisms) than with water. As referred to herein, "lipophilic groups"

are groups which, of themselves, display a preference to be solubilised by lipidic solvents rather than aqueous solvents.

As used herein, reference to an amino acid "side chain" takes its standard meaning in the art. Examples of side chains of amino acids are shown below:

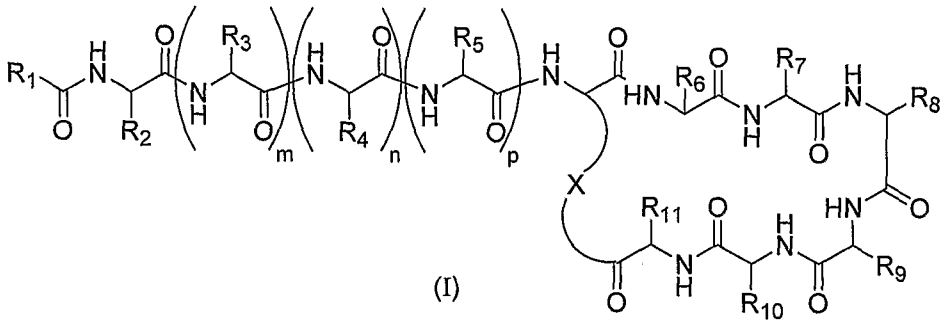


In addition to the negatively charged side chains shown above, it will be appreciated that a number of the side chains may also be protonated and so become positively charged, such as the side chain of lysine. The present invention contemplates within its scope these protonated side chains as well.

As discussed above, it is understood that the peptides of the present invention may exist in one or more stereoisomeric forms (eg enantiomers, diastereomers). The present invention includes within its scope all of these stereoisomeric forms either isolated (in for example enantiomeric isolation), or in combination (including racemic mixtures and diastereomic

mixtures). The present invention contemplates the use of amino acids in both L and D forms, including the use of amino acids independently selected from L and D forms. For example, where the peptide comprises two Dab residues, each Dab residue may have the same, or opposite, absolute stereochemistry.

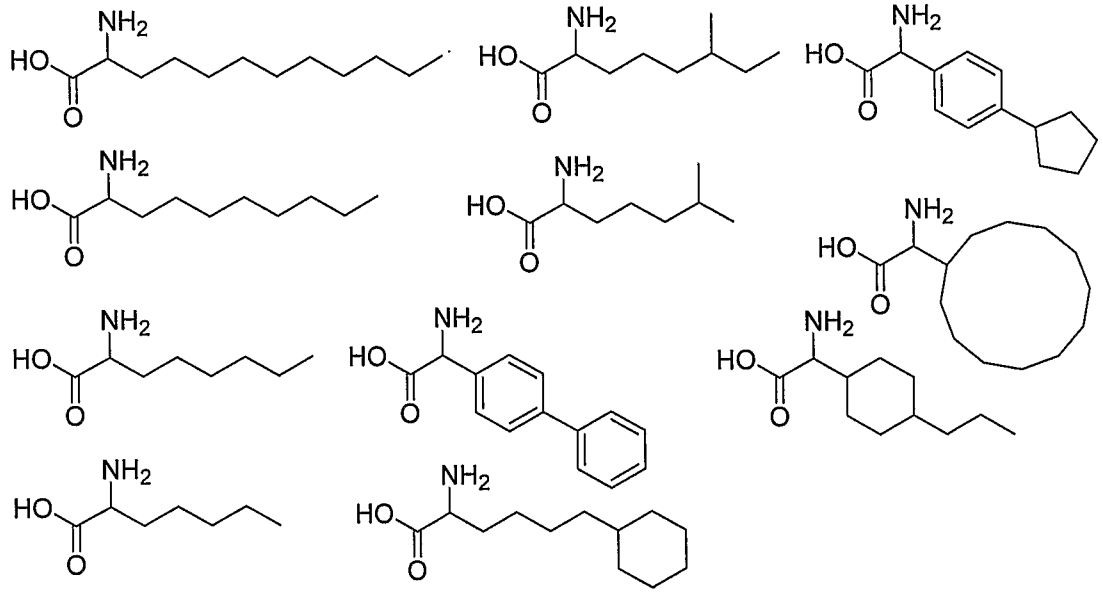
It is understood that the R_7 and R_8 groups in the peptide of formula (I):



(I)

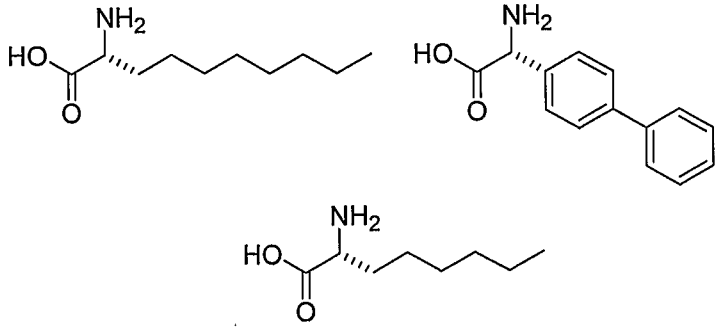
are appended to a 2-aminoethanoic acid subunit and accordingly are amenable to incorporation into the peptide backbone through standard peptide chemistry. The actual synthesis of the R_7 and R_8 appended 2-aminoethanoic acid reagents may be accomplished using known techniques - for example: Wimmer, N., *et al.* (2002) Syntheses of polycationic dendrimers on lipophilic peptide core for complexation and transport of oligonucleotides, *Bioorg. Med. Chem. Lett.* **12,** 2635-2637; and Gibbons, W. A., *et al.* (1990) Synthesis, Resolution and Structural Elucidation of Lipidic Amino Acids and Their Homo- and Hetero-Oligomers, *Liebigs Ann. Chem.* 1175. The entire contents of these documents are incorporated herein by reference.

Accordingly, lipidic amino acids such as:

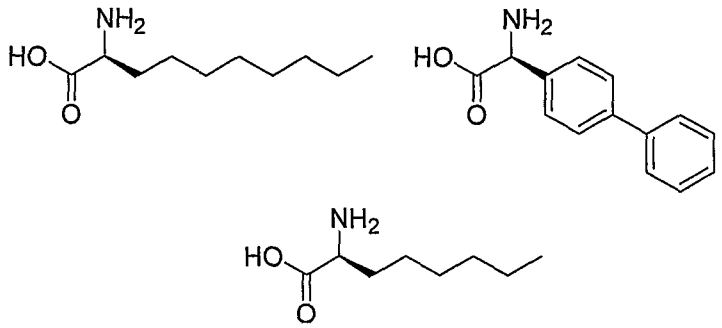


may be prepared for use in the synthesis of the polymyxin peptide analogs of the present invention.

The R_7 and R_8 appended amino acids (the "lipidic" amino acids) may be prepared as racemates or, alternatively, the amino acids or synthetic intermediates may be resolved to provide enantiopure, or at least enantioenriched, products. The resolution may take place chemically or enzymatically for example. The amino acids bearing the R_7 and R_8 side chains may be synthesised in enantiopure, or at least enantioenriched, form through stereoselective synthesis and/or purification techniques such as recrystallisation. A number of amino acids bearing lipophilic groups defined by R_7 and/or R_8 in enantiopure, or enantioenriched, form are commercially available. Examples of R_7 appended amino acids that may be used in enantiopure, or at least enantioenriched, form are shown below:



Examples of R_8 appended amino acids that may be used in enantiopure, or at least enantioenriched, form are shown below:



In general, techniques for preparing the peptides of the invention are well known in the art for example see:

a) Alewood, P.; Alewood, D.; Miranda, L.; Love, S.; Meutermans, W.; Wilson, D., "Rapid *in situ* neutralisation protocols for Boc and Fmoc solid-phase chemistries" *Meth. Enzymol.* 1997, 289, 14-28;

b) Merrifield, *J. Am. Chem. Soc.* 1964, 85, 2149;

c) Bodanzsky, "Principles of Peptide Synthesis", 2nd Ed., Springer-Verlag (1993); and

d) Houghten, *Proc. Natl. Acad. Sci. USA*, 1985, 82, 5131.

Of particular relevance to polymyxin peptide synthesis are: Kline, T., Holub, D., Therrien, J. *et al.* (2001), Synthesis and characterization of the colistin peptide polymyxin E1 and related antimicrobial peptides, *J. Pept. Res.* **57**, 175-187; and Vaara, M., Fox, J., Loidl, G., Siikanen, O. *et al.* (2008), Novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents, *Antimicrob. Agents Chemother.*, **52**(9), 3229–3236. The entire contents of these documents are incorporated herein by reference.

Known solid or solution phase techniques may be used in the synthesis of the peptides of the present invention, such as coupling of the N- or C-terminus to a solid support (typically a resin) followed by step-wise synthesis of the linear peptide. An orthogonal protecting group strategy may be used to facilitate selective deprotection and cyclization to form the cyclic heptapeptide core of the peptide. Protecting group chemistries for the protection of amino acid residues, including side chains, are well known in the art and may be found for example in: Theodora W. Greene and Peter G. M. Wuts, *Protecting Groups in Organic*

Synthesis, (Third Edition, John Wiley & Sons, Inc, 1999) – the entire contents of which is incorporated herein by reference.

As a general strategy, the synthesis of the peptides of the present invention may be performed in four stages. In the first stage, amino acids may be protected for incorporation into the peptide (such as the protection of biphenylglycine as Fmoc-biphenylglycine). Second, a partially protected linear peptide which selectively exposes only the functional groups required for cyclization may be synthesised using solid phase techniques. Third the cyclization reaction may be performed in solution to produce the protected cyclic lipopeptide. Fourth the remaining side chain protecting groups may be deprotected to furnish the peptide.

Where the compounds of the present invention require purification, chromatographic techniques such as high-performance liquid chromatography (HPLC) and reversed-phase HPLC may be used. The peptides may be characterised by mass spectrometry and/or other appropriate methods.

Where the peptide comprises one or more functional groups that may be protonated or deprotonated (for example at physiological pH) the peptide may be prepared and/or isolated as a pharmaceutically acceptable salt. It will be appreciated that the peptide may be zwitterionic at a given pH. As used herein the expression "pharmaceutically acceptable salt" refers to the salt of a given compound, wherein the salt is suitable for administration as a pharmaceutical. For example, such salts may be formed by the reaction of an acid or a base with an amine or a carboxylic acid group respectively.

Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids. Examples of inorganic acids include hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like. Examples of organic acids include 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.

Pharmaceutically acceptable base addition salts may be prepared from inorganic and organic bases. Corresponding counterions derived from inorganic bases include the sodium, potassium, lithium, ammonium, calcium and magnesium salts. Organic bases include primary, secondary and tertiary amines, substituted amines including naturally-occurring substituted amines, and cyclic amines, including isopropylamine, trimethyl amine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethylaminoethanol, tromethamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, N-alkylglucamines, theobromine, purines, piperazine, piperidine, and N-ethylpiperidine.

Acid/base addition salts tend to be more soluble in aqueous solvents than the corresponding free acid/base forms.

The compounds of the invention may be in crystalline form or as solvates (e.g. hydrates) and it is intended that both forms are within the scope of the present invention. The term "solvate" is a complex of variable stoichiometry formed by a solute (in this invention, a peptide of the invention) and a solvent. Such solvents should not interfere with the biological activity of the solute. Solvents may be, by way of example, water, ethanol or acetic acid. Methods of solvation are generally known within the art.

The compounds of the invention may be in the form of a "pro-drug". The term "pro-drug" is used in its broadest sense and encompasses those derivatives that are converted *in vivo* to the peptides of the invention. Such derivatives would readily occur to those skilled in the art and include, for example, compounds where a free hydroxy group is converted into an ester derivative or a ring nitrogen atom is converted to an N-oxide. Examples of ester derivatives include alkyl esters (for example acetates, lactates and glutamines), phosphate esters and those formed from amino acids (for example valine). Any compound that is a prodrug of a peptide of the invention is within the scope and spirit of the invention.

Conventional procedures for the preparation of suitable prodrugs according to the invention are described in text books, such as "Design of Prodrugs" Ed. H. Bundgaard, Elsevier, 1985 – the entire contents of which is incorporated herein by reference.

The present invention also provides a pharmaceutical composition comprising a therapeutically effective amount of a peptide as hereinbefore defined, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutically acceptable carrier or diluent.

The term "composition" is intended to include the formulation of an active ingredient with encapsulating material as carrier, to give a capsule in which the active ingredient (with or without other carrier) is surrounded by carriers.

While the peptide as hereinbefore described, or pharmaceutically acceptable salt thereof, may be the sole active ingredient administered to the subject, the administration of other active ingredient(s) with the compound is within the scope of the invention. For example, the compound could be administered with one or more therapeutic agents in combination. The combination may allow for separate, sequential or simultaneous administration of the peptide as hereinbefore described with the other active ingredient(s). The combination may be provided in the form of a pharmaceutical composition.

As will be readily appreciated by those skilled in the art, the route of administration and the nature of the pharmaceutically acceptable carrier will depend on the nature of the condition and the mammal to be treated. It is believed that the choice of a particular carrier or delivery system, and route of administration could be readily determined by a person skilled in the art. In the preparation of any formulation containing the peptide actives care should be taken to ensure that the activity of the peptide is not destroyed in the process and that the peptide is able to reach its site of action without being destroyed. In some circumstances it may be necessary to protect the peptide by means known in the art, such as, for example, micro encapsulation. Similarly the route of administration chosen should be such that the peptide reaches its site of action.

Those skilled in the art may readily determine appropriate formulations for the peptides of the present invention using conventional approaches. Identification of preferred pH ranges and suitable excipients, for example antioxidants, is routine in the art. Buffer systems are routinely used to provide pH values of a desired range and include carboxylic acid buffers for example acetate, citrate, lactate and succinate. A variety of antioxidants are available for such formulations including phenolic compounds such as BHT or vitamin E, reducing agents such as methionine or sulphite, and metal chelators such as EDTA.

The peptide as hereinbefore described, or pharmaceutically acceptable salt thereof, may be prepared in parenteral dosage forms, including those suitable for intravenous, intrathecal, and intracerebral or epidural delivery. The pharmaceutical forms suitable for injectable use include sterile injectable solutions or dispersions, and sterile powders for the extemporaneous preparation of sterile injectable solutions. They should be stable under the conditions of manufacture and storage and may be preserved against reduction or oxidation and the contaminating action of microorganisms such as bacteria or fungi.

The solvent or dispersion medium for the injectable solution or dispersion may contain any of the conventional solvent or carrier systems for peptide actives, and may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about where necessary by the inclusion of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include agents to adjust osmolarity, for example, sugars or sodium chloride. Preferably, the formulation for injection will be isotonic with blood. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Pharmaceutical forms suitable for injectable use may be delivered by any

appropriate route including intravenous, intramuscular, intracerebral, intrathecal, epidural injection or infusion.

Sterile injectable solutions are prepared by incorporating the active peptide in the required amount in the appropriate solvent with various of the other ingredients such as those enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, preferred methods of preparation are vacuum drying or freeze-drying of a previously sterile-filtered solution of the active ingredient plus any additional desired ingredients.

Other pharmaceutical forms include oral and enteral formulations of the present invention, in which the active peptide may be formulated with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active peptide may be incorporated with excipients and used in the form of ingestible tablets, buccal or sublingual tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. It will be appreciated that some of these oral formulation types, such as buccal and sublingual tablets, have the potential to avoid liver metabolism. However the peptides of the present invention may also be delivered to the stomach where liver metabolism is likely to be involved. The amount of active peptide in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain,

in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active peptide, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active peptide(s) may be incorporated into sustained-release preparations and formulations, including those that allow specific delivery of the active peptide to specific regions of the gut.

Liquid formulations may also be administered enterally via a stomach or oesophageal tube.

Enteral formulations may be prepared in the form of suppositories by mixing with appropriate bases, such as emulsifying bases or water-soluble bases. It is also possible, but not necessary, for the peptides of the present invention to be administered topically, intranasally, intravaginally, intraocularly and the like.

The present invention also extends to any other forms suitable for administration, for example topical application such as creams, lotions and gels, or compositions suitable for inhalation or intranasal delivery, for example solutions, dry powders, suspensions or emulsions.

The peptides of the present invention may be administered by inhalation in the form of an aerosol spray from a pressurised dispenser or container, which contains a propellant such as carbon dioxide gas, dichlorodifluoromethane, nitrogen, propane or other suitable gas or combination of gases. The peptides may also be administered using a nebuliser.

Pharmaceutically acceptable vehicles and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active

substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate the compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutically acceptable vehicle. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding active materials for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

As mentioned above the principal active ingredient may be compounded for convenient and effective administration in therapeutically effective amounts with a suitable pharmaceutically acceptable vehicle in dosage unit form. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.25 μg to about 2000 mg. Expressed in proportions, the active compound may be present in from about 0.25 μg to about 2000 mg/mL of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The term "therapeutically effective amount" refers to that amount which is sufficient to effect treatment, as defined below, when administered to an animal, preferably a mammal, more preferably a human in need of such treatment. The therapeutically effective amount will vary depending on the subject and nature of bacterial infection being treated, the severity of the infection and the manner of administration, and may be determined routinely by one of ordinary skill in the art.

The terms "treatment" and "treating" as used herein cover any treatment of a condition or disease in an animal, preferably a mammal, more preferably a human, and includes: (i) inhibiting the bacterial infection, i.e. arresting its proliferation; (ii) relieving the infection, i.e. causing a reduction in the severity of the infection; or (iii) relieving the conditions caused by the infection, i.e. symptoms of the infection. The terms "prevention" and preventing" as used herein cover the prevention or prophylaxis of a condition or disease in an animal, preferably a mammal, more preferably a human and includes preventing the bacterial infection from occurring in a subject which may be predisposed to infection but has not yet been diagnosed as being infected.

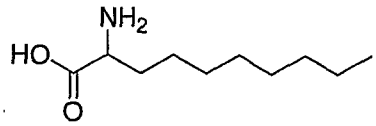
In some embodiments the bacterial infection is a Gram-negative bacterial infection. The bacterial infection may be caused by one or more species selected from one or more of the genera *Acinetobacter*; *Actinobacillus*; *Bartonella*; *Bordetella*; *Brucella*; *Burkholderia*; *Campylobacter*; *Cyanobacteria*; *Enterobacter*; *Erwinia*; *Escherichia*; *Francisella*; *Helicobacter*; *Hemophilus*; *Klebsiella*; *Legionella*; *Moraxella*; *Morganella*; *Neisseria*; *Pasteurella*; *Proteus*; *Providencia*; *Pseudomonas*; *Salmonella*; *Serratia*; *Shigella*; *Stenotrophomonas*; *Treponema*; *Vibrio*; and *Yersinia*. Specific examples of species are *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella enterica*.

The invention will now be described with reference to the following non-limiting examples:

Example 1: Synthesis of compounds of the invention

(a)

2-Aminodecanoic acid:



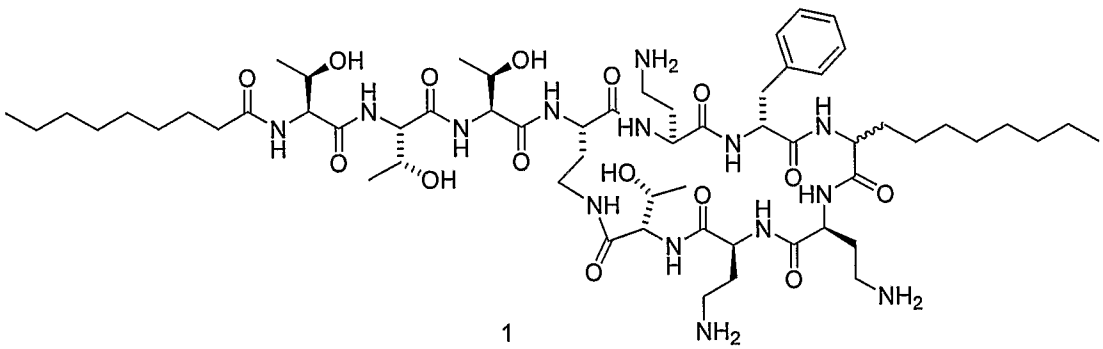
is prepared according to the procedure described in Gibbons, W. A., *et al.* (1990) Synthesis, Resolution and Structural Elucidation of Lipidic Amino Acids and Their Homo- and Hetero-Oligomers, *Liebigs Ann. Chem.* 1175.

L-2-aminodecanoic acid is available commercially from a number of sources including: www.chemicalbook.com; www.anaspec.com; www.chemexper.com; and www.icis.com.

L-2-aminooctanoic acid is available commercially from a number of sources including: www.chemicalbook.com.

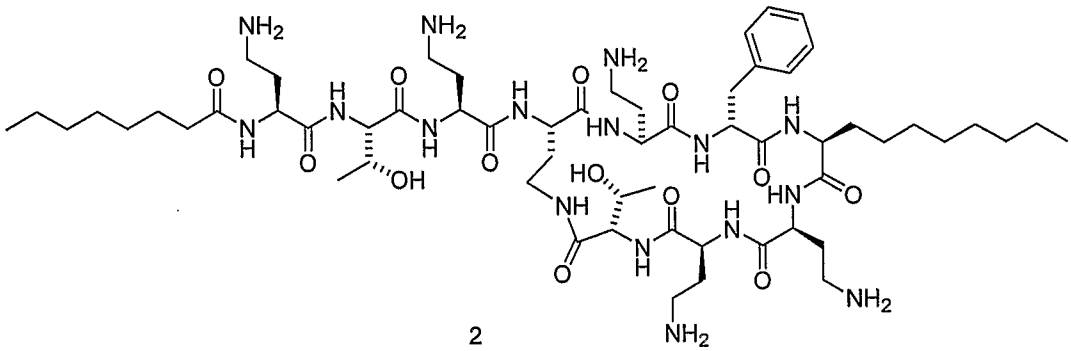
(b)

Representative syntheses used to make compounds of the invention including compounds **1** (mixture of diastereomers):



1

and **2**:



2

by Fmoc-based automated solid phase peptide synthesis are detailed below:

(i) Peptide was synthesized by conventional solid phase chemistry, by a standard Fmoc protection strategy. The Dab γ-amino groups and threonine hydroxyl groups were

protected by benzyloxycarbonyl (Z). The γ-amino group of the Dab residue involved in cyclization was protected by t-butoxycarbonyl (tBoc), which is an acid labile group that can be removed at the cleavage step.

Synthesis was performed in an automated synthesizer using acid labile Wang resin. The synthesis used *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU) or *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) as activators. Fmoc deprotection was carried out using 20% piperidine in DMF. The N^{α} acylation was performed for 30 min using a four-fold molar excess of nonanoic acid, a four-fold molar excess of the activator HCTU or TBTU, and an eightfold molar excess of *N*-methyl morpholine.

Cleavage of the peptide from the resin and deprotection of the Dab(Boc) residue was achieved by treating the resin with 95% TFA:5% water mixture. The partially protected linear peptide was isolated by treatment with hexane.

The cyclization mixture (consisting of benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBop), *N*-hydroxybenzotriazole (HOBt), and *N*-methyl morpholine (NMM)) at the molar excess of 2-, 2- and 4-fold respectively was added to the peptide (dissolved in DMF) and allowed to react for 2 h. The cyclized, protected peptide was precipitated by the addition of cold diethyl ether. The residual protecting groups were removed by subjecting the peptide (dissolved in acetic acid-methanol-water [5:4:1]) to an atmosphere of hydrogen in the presence of a palladium charcoal catalyst. The deprotected and cyclized peptide was purified by reversed phase chromatography using conventional gradients of acetonitrile:water:trifluoroacetic acid. Compound **1** was dried by lyophilization. The purity was >95% as estimated by reversed-phase HPLC (MALDI-TOF: *m/z* 1262.3 (100%, M^+), 1284.5 (16%, $M+Na^+$)).

(ii) Peptide was synthesized by conventional solid phase chemistry, by a standard Fmoc protection strategy. The Dab γ-amino groups were protected by *t*-butoxycarbonyl (tBoc), and threonine was protected as the t-butyl ether (tBu). The γ-amino group of the Dab

residue involved in cyclization was protected by ivDde, which is a hydrazine labile group that can be removed prior to the cleavage step.

Synthesis was performed in an automated synthesizer using super acid labile chlorotrityl resin. The synthesis used *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU). Fmoc deprotection was carried out using 20% piperidine in DMF. The *N*α acylation was performed for 30 min using a four-fold molar excess of octanoic acid, a four-fold molar excess of the activator HCTU, and an eightfold molar excess of *N*-diisopropylethylamine.

Cleavage of the peptide from the resin was achieved by treating the resin with 2% TFA in dichloromethane or hexafluoroisopropanol or acetic acid in trifluoroethanol. The partially protected linear peptide was isolated by treatment with hexane.

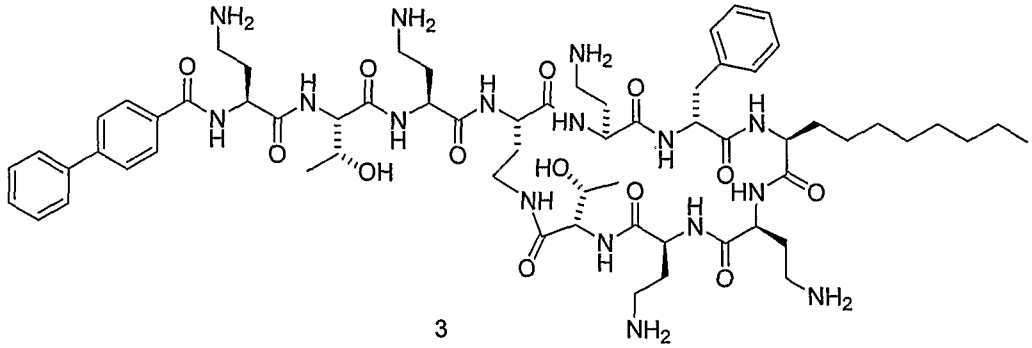
The cyclization mixture (consisting of diphenylphosphorylazide, and diisopropylethylamine at the molar excess of 2, and 4-fold respectively) was added to the peptide (dissolved in DMF) and allowed to react for 2 h. The DMF was removed and the cyclized, protected peptide was precipitated by the addition of cold diethyl ether. The residual protecting groups were removed by treating the peptide with 95% TFA:5% water.

The deprotected and cyclized peptide was purified by reversed-phase chromatography using conventional gradients of acetonitrile:water:trifluoroacetic acid. Compound **2** was dried by lyophilization. The purity was >95% as estimated by reversed-phase HPLC (MALDI-TOF: *m/z* 1245.7 (55%, M+), 1267.7 (100%, $M+Na^+$)).

(iii) Peptide was synthesized by conventional solid phase chemistry, by a standard Fmoc protection strategy. The Dab γ-amino groups were protected by *t*-butoxycarbonyl (tBoc), and threonine was protected as the t-butyl ether (tBu). The γ-amino group of the Dab residue involved in cyclization was protected by Mtt, which is a super acid labile group that can be removed in the cleavage step by treatment the resin with 1-2% TFA in

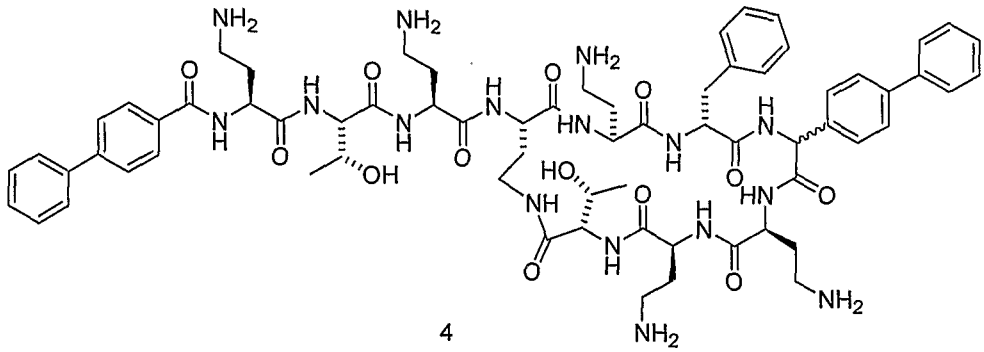
dichloromethane or hexafluoroisopropanol or acetic acid in trifluoroethanol. The remainder of the synthesis proceeds as above for (i) or (ii).

It will be understood that these representative syntheses may be applied to the synthesis of a range of compounds described herein. For example, the representative syntheses may be applied to the synthesis of the following compounds **3** to **5**:



3

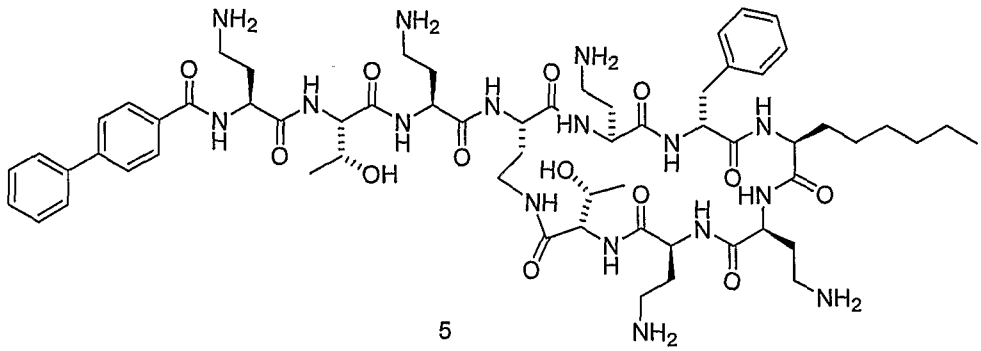
MALDI-TOF: *m/z* 1321.6 (100%, $M+Na^+$);



4

(mixture of diastereomers)

MALDI-TOF: *m/z* 1340.4 (29%, M^+), 1362.9 (100%, $M+Na^+$), 1378.9 (41%);



5

MALDI-TOF: *m/z* 1272.7 (100%, M^+), 1295.6 (48%, $M+Na^+$), 1311.4 (18%).

Example 2: Measurements of minimum inhibitory concentrations (MICs)

Compounds were subjected to antibacterial activity measurements. MICs were determined by broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB, magnesium concentration 10 to 12.5 mg/L, and calcium concentration 20 to 25 mg/L) (Oxoid Australia, Thebarton, SA, Australia) according to the Clinical and Laboratory Standards Institute protocol (Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement M100-S18.* Wayne, PA, 2008). The strains examined were: *P. aeruginosa* (ATCC 27853 and 9 multidrug-resistant strains, including 7 polymyxin-resistant strains (strains A-G)); *A. baumannii* (ATCC 19606 and 6 multidrug-resistant strains, including 5 polymyxin-resistant strains (strains J-N)) and *K. pneumoniae* (ATCC 13883 and 5 multidrug-resistant strains, including 4 polymyxin-resistant strains (strains P, R-T)).

As the initial step to evaluate their antibacterial activities, MICs were measured against *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. The results for compounds **2 – 5** are shown in **Table 1**. Viable counting was conducted at the end of the MIC experiments for the wells of MIC, 2× MIC and 4× MIC. Importantly, these compounds usually killed all bacterial cells (detected by viable counting with 50 μL broth culture) either at the MIC or 2-4× MIC (i.e. MBC) in the MIC experiments, which is in sharp contrast to polymyxins usually requiring 8-32× MIC to achieve complete bacterial kill. As referred to herein, the expressions "100% bacterial killing", "complete bacterial kill" and variants thereof, refer to the killing of substantially all of the bacterial cells present as measured by viable counting.

The MIC for compound **1** was 5.2-10.3 mg/L against a number of polymyxin-susceptible (*P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606 and *K. pneumoniae* B5055Δ*lpxM*) and polymyxin-resistant (*P. aeruginosa*, *A. baumannii* and *K. pneumoniae*) bacterial strains. Counting of viable bacteria after 24 h incubation revealed that compound **1** achieved 100% bacterial killing at 10.3 mg/L (the MBC) against both polymyxin-susceptible and polymyxin-resistant *A. baumannii*. In contrast, colistin was inactive (even at 128 mg/L) against the resistant strain, and required at least 16 mg/L to achieve complete

killing of the susceptible strain. Likewise, bacterial counting indicated substantial killing of *K. pneumoniae* and *P. aeruginosa* by compound **1**.

In addition, the MICs of compound **3** were not affected by calcium concentrations in CAMHB (100 mg/L *versus* 20 mg/L) against both colistin-resistant and colistin-susceptible *P. aeruginosa* strains (2 strains for each type were examined). On the other hand, the MIC for colistin against *P. aeruginosa* ATCC 27853 increased from 1 mg/L (in the presence of 20 mg/L calcium) to 4 mg/L with 100 mg/L calcium. This indicates that much stronger interactions exist between the compounds of the present invention and lipid A, compared to the interaction between colistin and lipid A. This conclusion is consistent with the findings shown in Example 4 below.

Table 1: MICs (mg/L) for compounds and colistin (colistin resistance: colistin MIC > 2 mg/L); ND = not determined; * = lab-derived strain that has no LPS.

Strain	**Test molecule**				
	Colistin	**2**	**3**	**4**	**5**
P. aeruginosa					
ATCC 27853	1	4	4	2	4
A	>128	8	2	2	2
B	>128	8	8	8	ND
C	>128	8	8	32	ND
D	>128	4	4	8	ND
E	>128	4	2	4	ND
F	16	4	4	4	ND
G	8	4	4	4	ND
H	1	4	4	2	ND
I	2	4	4	4	ND
A. baumannii					
ATCC 19606	1	4	2	2	4
J*	128	64	32	32	16
K	16	ND	ND	8	4
L	16	2	4	2	ND
M	8	4	4	2	ND
N	16	2	4	2	ND
O	0.5	2	2	4	ND
K. pneumoniae					
ATCC 13883	1	2	2	16	8
P	>128	128	64	>32	128
Q	1	4	8	2	ND
R	32	16	16	8	ND
S	128	64	64	>32	ND
T	32	16	16	32	ND

Example 3: Static time-kill studies

The time-kill kinetics of a colistin-resistant MDR clinical *P. aeruginosa* isolate by compounds **2** and **3** and colistin (sulfate) was examined. The antibiotic was added to a logarithmic-phase broth culture of approximately 10^6 CFU/mL to yield concentrations of 0, 0.5, 1 and 4×MIC of the isolate (32 mg/L for colistin). Viable counting was performed on samples collected at 0, 0.5, 1, 2, 3, 4 and 24 h after antibiotic addition. After appropriate dilutions with saline, samples of bacterial cell suspension (50 μL) were spirally plated on nutrient agar plates (Medium Preparation Unit, University of Melbourne) using a Whitley automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted by a ProtoCOL automated colony counter (Synbiosis, Cambridge, UK) after 24 h incubation of subcultures at 35 °C. The lower limit of counting was 20 CFU/mL.

The antibacterial killing kinetics of compounds **3** and **2** a gainst two colistin-resistant (colistin MICs ≥ 128 mg/L) MDR clinical isolates (shown in Figures 1 and 2, respectively) of *P. aeruginosa* and ATCC 27853 reference strain (shown in Figure 3) of *P. aeruginosa* indicate that compounds **2** and **3** achieved 4 - 6 $\log_{10}$ kill within 2 h.

Example 4: Displacement of dansyl-polymyxin bound to lipopolysaccharide (LPS) from *P. aeruginosa*, *Escherichia coli*, *K. pneumoniae* and *Salmonella enterica* by the compounds of the invention, colistin and polymyxin B

Following the procedures of: Moore RA, Bates NC, Hancock RE, Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob. Agents Chemother.* (1986) **29**, 496-500.

Dansyl-polymyxin B (identified by the following structure, where * represents the location of cyclisation: octanoyl-Lys(dansyl)-Thr-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*) was synthesized according to one or more of the representative syntheses described in Example 1 using dansyl-appended lysine. Suspensions of purified LPS (3 mg/L) from *P. aeruginosa* (Sigma-Aldrich, cat # L9143), *E. coli* (Sigma-Aldrich, cat # L2630), *K.*

pneumoniae (Sigma-Aldrich, cat # L4268) and *S. enterica* (Sigma-Aldrich, cat # L6511) were prepared in 5 mM HEPES buffer (pH 7.2). LPS was added into a quartz cuvette containing 1 mL of the same buffer, after which time aliquots of dansyl-polymyxin B solution were titrated at 2 min intervals until fluorescence intensity reached a plateau. Fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (Varian, Mulgrave, Victoria Australia) set at an excitation wavelength of 340 nm. Slit widths were set to 5 and 10 nm for the excitation and emission monochromators, respectively. Emission spectra were collected from a wavelength of 400 - 650 nm (the emission maxima for bound dansyl-polymyxin B was observed at approximately 485 nm).

Fluorescence enhancement was determined from the integrated area under the emission spectra. The K_d of the LPS-dansyl-polymyxin complex was determined by non-linear regression analysis of the binding isotherms. The background corrected dansyl-polymyxin binding fluorescence data were fitted to a one-site binding model (Eq1):

$$\Delta F=\Delta F_{max}*[L]/(K_d+[L]) \qquad \text{(Eq1)}$$

where ΔF represents the specific fluorescence enhancement upon the addition of dansyl-polymyxin B to a fixed concentration of LPS, ΔF_{max} is the maximum specific fluorescence enhancement at saturation, K_d represents the dissociation constant for dansyl-polymyxin B binding to LPS obtained from the concentration of dansyl-polymyxin B equivalent to half F_{max} determined from the data fit, and [L] represents the free concentration of dansyl-polymyxin B.

Dansyl-polymyxin B was added to a quartz cuvette containing LPS at a concentration necessary to obtain 90 to 100% of the maximum fluorescence when bound. Displacement of dansyl-polymyxin B by compounds **2**, **3** and **5**, polymyxin B and colistin, tested separately, was measured as the corresponding decrease in fluorescence upon the progressive titration of aliquots of stock solutions of the compound to be tested at 2-minute intervals, until no further decrease in fluorescence was observed.

Fluorescence readings were corrected for dilution before plotting the fraction of dansyl-polymyxin B bound as a function of displacing test compound concentration. The concentration of test compound required to displace 50 % of the bound dansyl-polymyxin B (I_{50}) was determined by fitting the fluorescence data to a sigmoidal dose-response model which assumed a single class of binding sites (Eq 2):

$$\%\text{Initial Fluorescence} = F_{min} + (F_{max}-F_{min})/(1+10^{((\log I50 - \log[L])*\text{HillSlope})}) \quad \text{(Eq 2)}$$

where I_{50} is the midpoint of competition and is defined as the concentration of displacer at which the maximum fluorescence intensity (F_{max}) of the dansyl-polymyxin B saturated complex is reduced to 50 % of the initial value, F_{min} represents the maximum dansyl-polymyxin B displacement produced by the competitor and is defined by the bottom plateau of the displacement curve; [L] represents the concentration of the competitor. The inhibition constant (K_i) was determined from the following equation (Eq 3):

$$K_i=[I_{50}]/(1+[\text{dansyl-polymyxin B}]_{free}/K_{d\ \text{dansyl-polymyxin B}}) \quad \text{(Eq 3)}$$

$K_{d\ \text{dansyl-polymyxin B}}$ represents the dissociation constant for the LPS-dansyl-polymyxin B complex. All data modeling operations were performed with GraphPad Prism V4.0 software (GraphPad software, San Diego, CA, USA).

The displacement of dansyl-polymyxin B bound to LPS from the various Gram-negative species (including *P. aeruginosa*) indicated that compounds **2, 3** and **5** had significantly greater binding affinity, compared to colistin (up to 27.1-fold) (**Table 2**).

Table 2: Displacement of dansyl-polymyxin B bound to LPS from *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *S. enterica* by compounds of the invention, colistin and polymyxin B. Binding affinity (μM) expressed as K_d^* or $K_i^\#$.

LPS of *P. aeruginosa*					
Dansyl-polymyxin B*	Polymyxin B#	Colistin#	Compound **2**#	Compound **3**#	Compound **5**#
1.2 ± 0.2	3.5 ± 0.2	3.8 ± 0.1	0.2 ± 0.07	0.14 ± 0.09	0.4 ± 0.1
LPS of *E. coli*					
Dansyl-polymyxin B*	Polymyxin B#	Colistin#	Compound **2**#	Compound **3**#	Compound **5**#
0.5 ± 0.1	0.5 ± 0.08	0.8 ± 0.1	0.015 ± 0.006	0.05 ± 0.008	0.07 ± 0.006
LPS of *K. pneumoniae* ATCC 13883					
Dansyl-polymyxin B*	Polymyxin B#	Colistin#	Compound **2**#	Compound **3**#	Compound **5**#
0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.05	0.016 ± 0.004	0.04 ± 0.05	0.06 ± 0.01
LPS of *K. pneumoniae* ATCC 13883 Col10 (colistin-resistant derivative of wild-type ATCC 13883)					
Dansyl-polymyxin B*	Polymyxin B#	Colistin#	Compound **2**#	Compound **3**#	Compound **5**#
1.5 ± 0.1	2.6 ± 0.1	2.9 ± 0.1	0.12 ± 0.05	0.7 ± 0.06	0.8 ± 0.1
LPS of *S. enterica*					
Dansyl-Polymyxin B*	Polymyxin B#	Colistin#	Compound **2**#	Compound **3**#	Compound **5**#
0.6 ± 0.09	0.4 ± 0.1	0.6 ± 0.08	0.02 ± 0.006	0.05 ± 0.01	0.06 ± 0.02

Example 5: Tolerability

Hemolysis was assessed using a modified method (Worlitzsch D, Kaygin H, Steinhuber A et al. Effects of amoxicillin, gentamicin, and moxifloxacin on the hemolytic activity of *Staphylococcus aureus in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* (2001) **45,** 196-202) with healthy human red blood cells (Australian Red Cross). Tested concentrations of compound **2**, compound **3**, colistin and polymyxin B were 0.5 to 128 mg/L. A 2% solution of Triton X-100 was employed as a positive control and red blood cells in isotonic phosphate buffer - saline was a negative control. Hemolysis was calculated as: % Hemolysis = (Abs_{450nm} sample – Abs_{450nm} blank) / (Abs_{450nm} Triton-X100 – Abs_{450nm} of negative control) × 100.

No hemolysis of human red blood cells was observed after exposure to compound **2**, compound **3**, colistin or polymyxin B at concentrations up to 128 mg/L.

In addition, no adverse effect was observed after administration of compounds **2** and **3** in rats (a single intravenous dose of 1 mg/kg) and mice (a single subcutaneous dose of 40 mg/kg). The results of these preliminary animal studies indicate that compounds **2** and **3** have similar safety profiles to colistin and polymyxin B.

Example 6: *In vivo* efficacy of compound 3 and colistin in neutropenic mouse lung infection model

This animal study was approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Six-week old, specific-pathogen-free, female Swiss mice (22 - 26 g) were purchased from Monash Animal Services (Clayton, Victoria, Australia). Mice were fed normal mouse chow and water *ad libitum* and were housed in M.I.C.E.® cages (Australian Animal Care Systems Pty Ltd, Keilor, Victoria, Australia) at room temperature (20 – 23 °C). The animals were maintained in accordance with the criteria of the Australian code of practice for the care and use of animals for scientific purposes.

Mice were rendered neutropenic by intraperitoneal administration of two doses of cyclophosphamide (Endoxan®, Baxter Healthcare Pty Ltd, NSW, Australia) 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to experimental infection (Dudhani RV, Turnidge JD, Coulthard K *et al.* Elucidation of the pharmacokinetic/pharmacodynamic determinant of colistin activity against *Pseudomonas aeruginosa* in murine thigh and lung infection models. *Antimicrob. Agents Chemother.* (2010) **54**, 1117-24). Before introducing the inoculum into lungs, mice were anesthetized very lightly with isoflurane by inhalation. Lung infection was produced by introducing intranasally, via a 29-gauge needle, 50 μL of a suspension of bacterial cells (~10^8 CFU/mL) in early logarithmic phase. The bacterial suspension was delivered in ~10 μL volumes which, when placed directly in the nares of the anesthetized mouse, was inhaled spontaneously. This procedure was repeated until animals received the entire 50 μL of bacterial suspension. Thereafter, animals were held in a vertical position with head up for 1 min. Administration of colistin (a single

subcutaneous dose of 40 mg/kg) or compound **3** (a single subcutaneous dose of 40 mg/kg) occurred 2 h after inoculation by which time infection was reproducibly established and bacterial load in the lung was determined as time zero. At 24 h after colistin or compound **3** administration, lungs were collected aseptically and homogenized in 2 mL of normal saline in a polystyrene round-bottom tube (Becton Dickinson, NJ, USA). The homogenate was mixed with a further 2 mL of sterile saline and filtered through a sterile filter bag (280 μm, Bagpage®, Interscience, France). The filtrate was serially diluted with saline and 50 μL aliquots were plated on nutrient agar plates using a WASP2® spiral plater (Don Whitley Scientific Ltd, England). Following incubation at 37 °C for 24 h, colonies were counted using a Synbiosis protoCOL® colony counter (Don Whitley Scientific Ltd, England). CFU were enumerated for each lung and expressed as $\log_{10}$ CFU per lung. The lower limit of counting was 220 CFU/lung (equivalent to one colony per plate).

A proof-of-concept study using a neutropenic mouse lung infection model demonstrated significantly better ($p < 0.045$) *in vivo* efficacy of compound **3** against a polymyxin-resistant clinical isolate of *P. aeruginosa* compared with colistin (polymyxin E) (Table 3).

Table 3: *In vivo* efficacy of compound **3** and colistin against a polymyxin-resistant clinical isolate of *P. aeruginosa*

Time (h)	**Bacterial burden in the lung (Log_{10} CFU/lung)**		
	Control	**Compound 3 (40 mg/kg)**	**Colistin (40 mg/kg)**
0	5.42 ± 0.54		
24	7.39 ± 0.17	4.75 ± 0.80*	6.71 ± 0.46

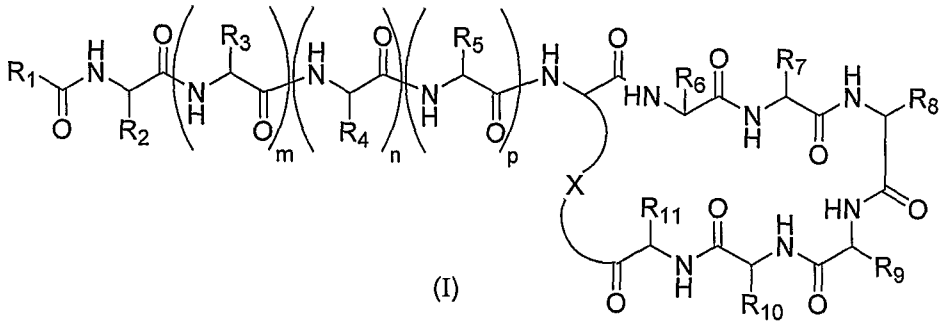
* $p < 0.045$ compared with colistin.

Throughout this specification and claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers or steps but not the exclusion of any other integer or group of integers.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Claims:

1. Peptide of formula (I):



(I)

wherein:

R_1 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{2-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{2-22}alkenyl;

m, n and p are each independently 0 or 1;

R_3, R_4 and R_5 where present and R_2, R_6, R_9, R_{10} and R_{11} are each independently selected from the side chain of an amino acid selected from the group consisting of α,γ-diaminobutyric acid, arginine, histidine, lysine, ornithine, glutamatic acid, glutamate, aspartic acid, aspartate, glutamine, asparagine, serine, threonine and cysteine;

X is a residue of the side chain of an amino acid selected from the group consisting of α,γ-diaminobutyric acid, arginine, histidine, lysine, ornithine, glutamatic acid, glutamate, aspartic acid, aspartate, glutamine, asparagine, serine, threonine and cysteine;

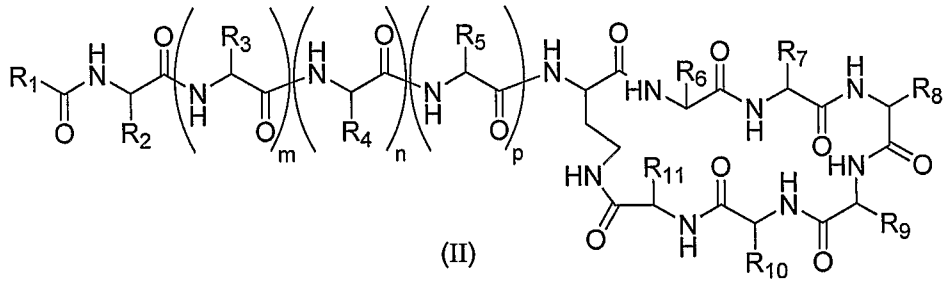
R_7 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{1-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{1-22}alkenyl;

R_8 is a lipophilic group selected from the group consisting of C_{3-22}alkyl, C_{3-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, C_{6-16}arylC_{1-22}alkyl, C_{6-16}arylC_{1-22}alkenyl, C_{3-12}cycloalkylC_{1-22}alkyl and C_{3-12}cycloalkylC_{1-22}alkenyl;

wherein any alkyl, alkenyl, aryl and cycloalkyl group within the peptide is optionally substituted with one or more groups selected from the group consisting of C_{1-22}alkyl, C_{2-22}alkenyl, C_{6-16}aryl, C_{3-12}cycloalkyl, hydroxy, hydroxyC_{1-22}alkyl, amino, aminoC_{1-22}alkyl, C_{1-22}alkyloxy, C_{1-22}alkylamino, (C_{1-22}alkyl)(C_{1-22}alkyl)amino, C_{1-22}alkylcarbonyloxy, C_{2-22}alkenylcarbonyloxy, C_{6-16}arylcarbonyloxy, C_{3-12}cycloalkylcarbonyloxy, C_{1-22}alkylcarbonylamino, C_{2-22}alkenylcarbonylamino, C_{6-16}arylacylamino and C_{3-12}cycloalkylcarbonylamino; and

wherein the number of carbon atoms in the R_8 group is greater than 4,
or a pharmaceutically acceptable salt thereof.

2. Peptide according to claim 1 of formula (II):



(II)

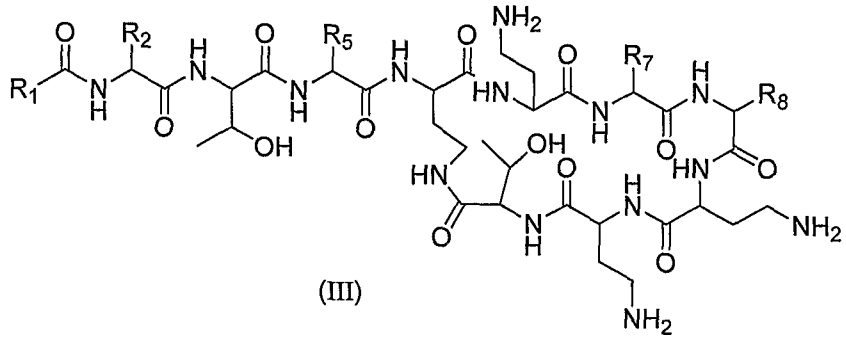
wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, m, n and p are as defined in claim 1, or a pharmaceutically acceptable salt thereof.

3. Peptide according to claim 1 or claim 2 where the number of carbon atoms in the R_8 group is greater than 5, greater than 6 or greater than 7.

4. Peptide according to any one of claims 1 to 3 wherein the number of carbon atoms in R_7 is greater than 3, greater than 4, greater than 5 or greater than 6.

5. Peptide according to any one of claims 1 to 4 wherein m=1, n=0 and p=1, or a pharmaceutically acceptable salt thereof.

6. Peptide according to any one of claims 1 to 5 of formula (III):



(III)

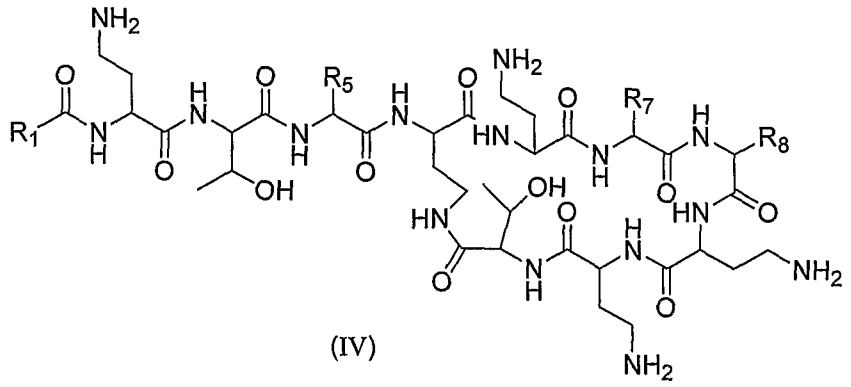
wherein R_1 and R_8 are as defined in claim 1;

R_2 is the side chain of α,γ-diaminobutyric acid or threonine;

R_5 is the side chain of α,γ-diaminobutyric acid, serine or threonine; and

R_7 is the side chain of leucine or phenylalanine.

7. Peptide according to any one of claims 1 to 6 of formula (IV):



(IV)

wherein:

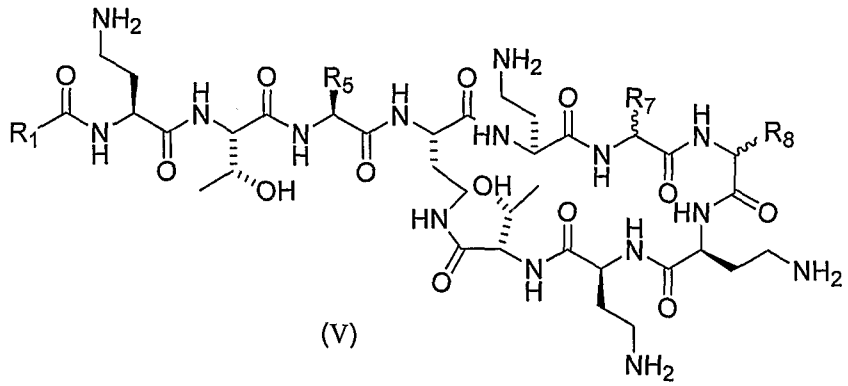
R_1 and R_8 are as defined in claim 1;

R_5 is the side chain of α,γ-diaminobutyric acid or serine; and

R_7 is the side chain of leucine or phenylalanine,

or a pharmaceutically acceptable salt thereof.

8. Peptide according to claim 7 of formula:



(V)

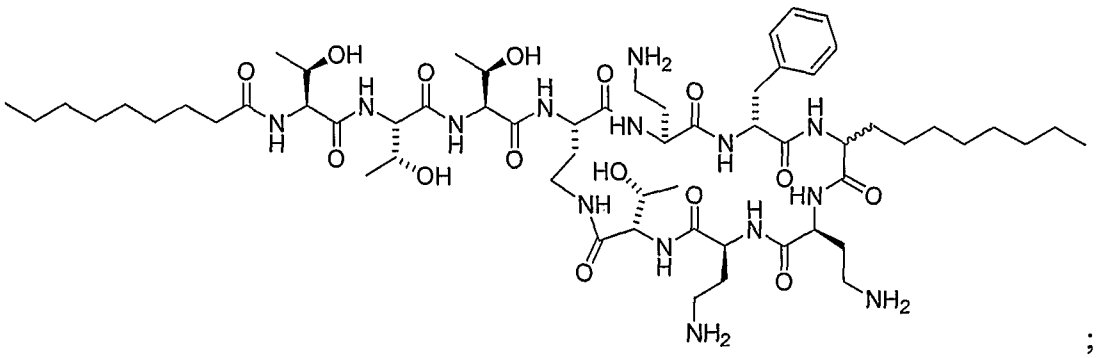
wherein R_1, R_5, R_7, and R_8 are as defined in claim 7,
or a pharmaceutically acceptable salt thereof.

9. Peptide according to any one of claims 1 to 8 wherein:

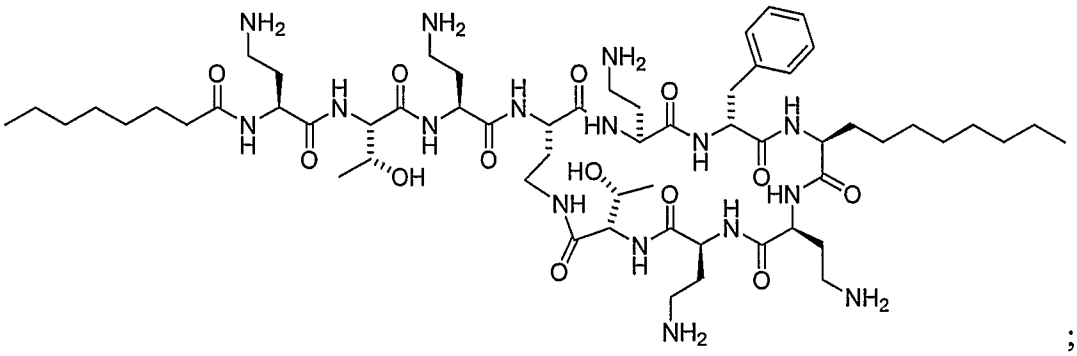
R_1 is selected from the group consisting of C_{3-11}alkyl, phenyl, bi-phenyl, cyclohexanylbutyl, trans-4-propylcyclohexanyl, cyclododecanyl and cyclopentylphenyl; and

R_8 is selected from the group consisting of C_{5-10}alkyl, phenyl, bi-phenyl, cyclohexylbutyl, trans-4-propylcyclohexanyl, cyclododecanyl and cyclopentylphenyl,
or a pharmaceutically acceptable salt thereof.

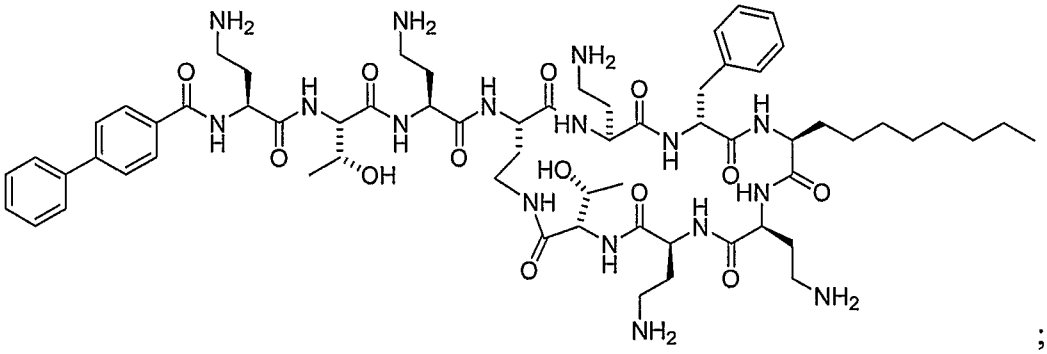
10. Peptide according to any one of claims 1 to 9 of formula:



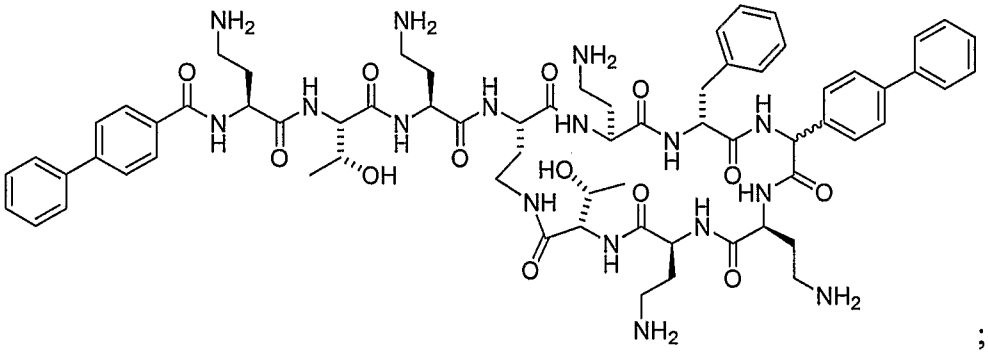
;



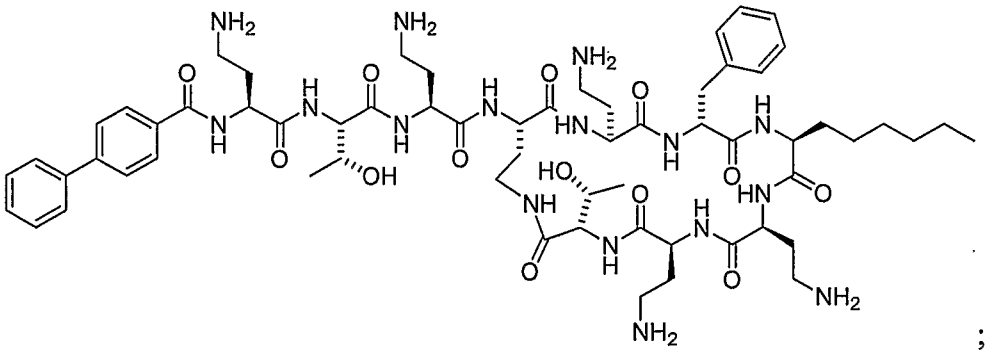
;



;



; or



;

or a pharmaceutically acceptable salt thereof.

11. Peptide according to any one of claims 1 to 9 wherein the combined number of carbon atoms in the R_7 and R_8 groups is greater than 11.

12. Peptide according to any one of claims 1 to 11, or a pharmaceutically acceptable salt thereof, for use in the prevention or treatment of a bacterial infection.

13. Pharmaceutical composition comprising a therapeutically effective amount of a peptide according to any one of claims 1 to 11, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutically acceptable carrier or diluent.

14. Method of preventing or treating a bacterial infection comprising the step of administering a therapeutically effective amount of a peptide according to any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof, to a subject in need thereof.

15. Use of a peptide according to any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof, in the preparation of a medicament for the prevention or treatment of a bacterial infection.

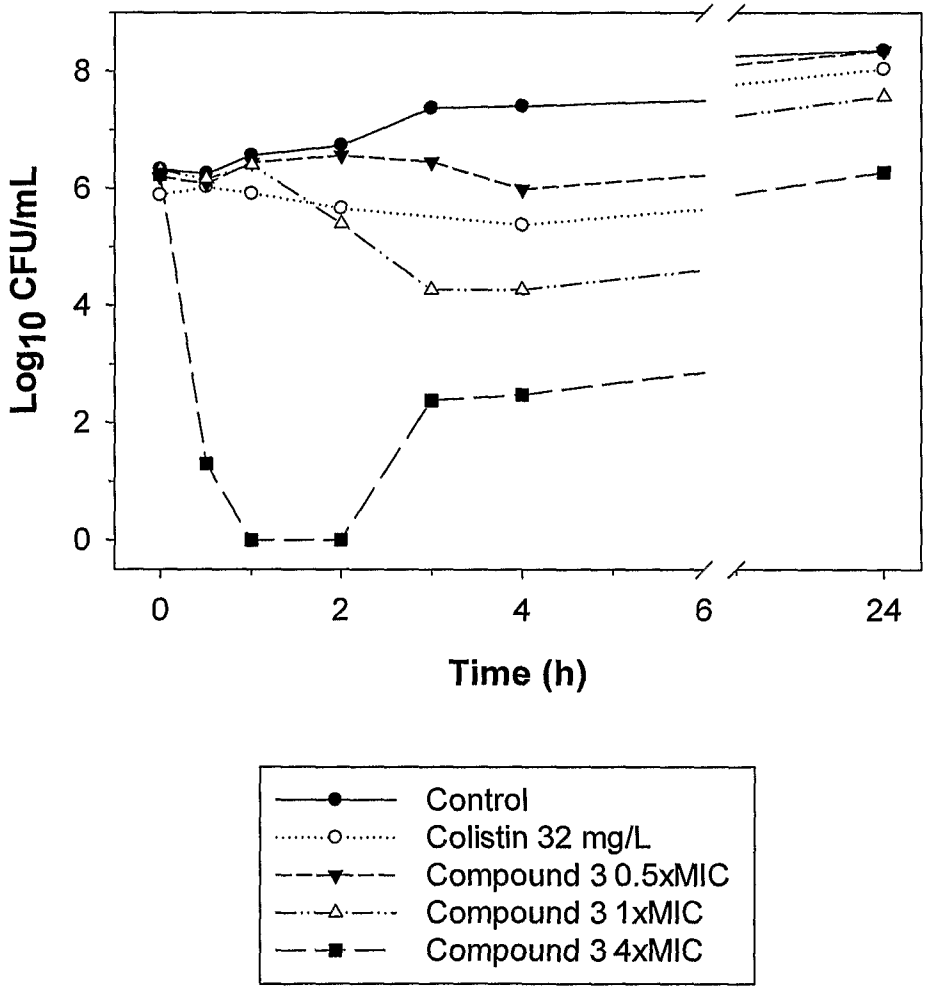

Log10 CFU/mL
8
6
4
2
0
0
2
4
6
24
Time (h)
Control
Colistin 32 mg/L
Compound 3 0.5xMIC
Compound 3 1xMIC
Compound 3 4xMIC

Figure 1

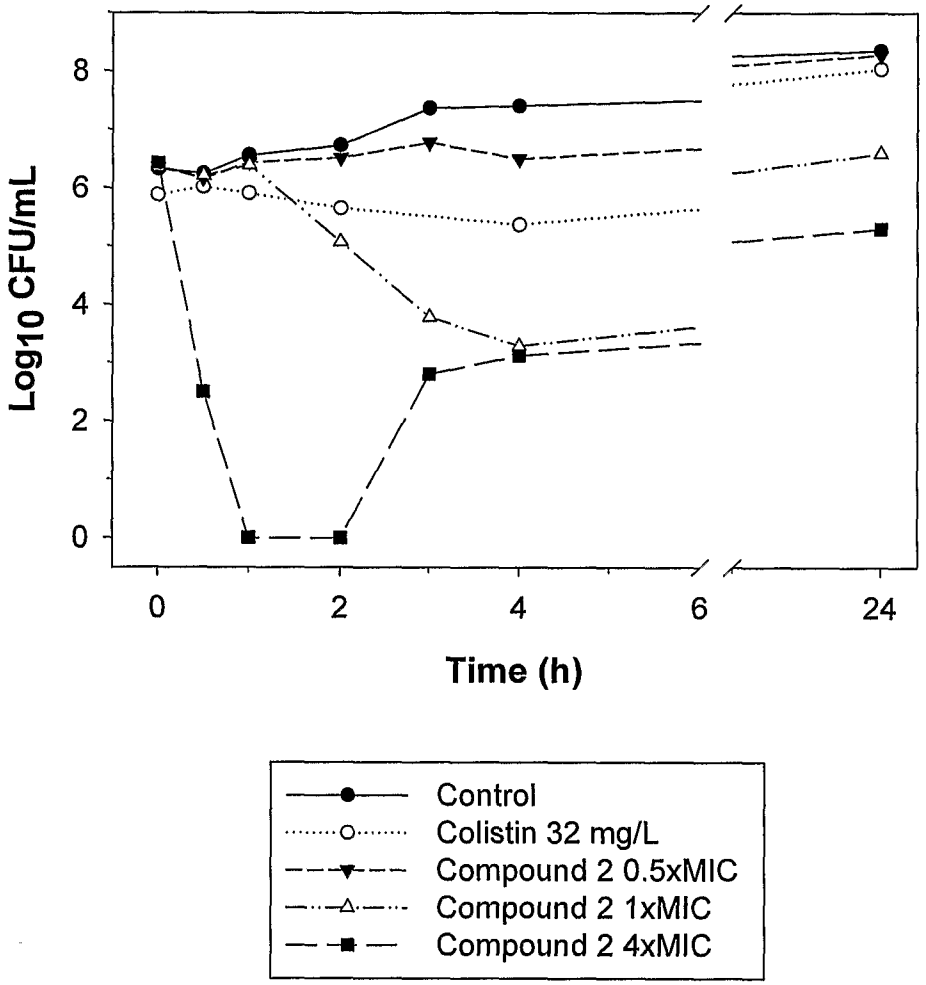

Log10 CFU/mL
8
6
4
2
0
0
2
4
6
24
Time (h)
Control
Colistin 32 mg/L
Compound 2 0.5xMIC
Compound 2 1xMIC
Compound 2 4xMIC

Figure 2

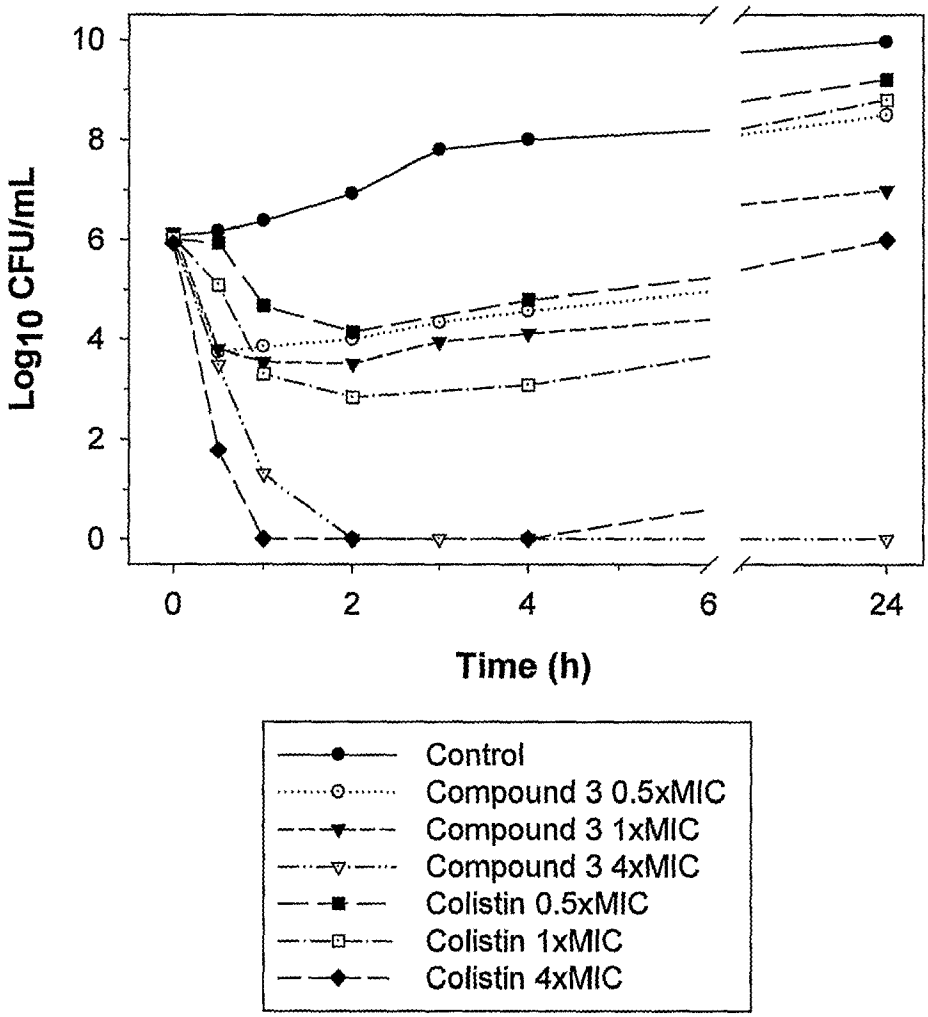

10
8
6
4
2
0
Log10 CFU/mL
0
2
4
6
24
Time (h)
Control
Compound 3 0.5xMIC
Compound 3 1xMIC
Compound 3 4xMIC
Colistin 0.5xMIC
Colistin 1xMIC
Colistin 4xMIC

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/000568

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 7/62 (2006.01) *A61K 38/12* (2006.01) *A61P 31/04* (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS: substructure search based on formula (I).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/083317 A2 (BIOSOURCE PHARM, INC.) 10 August 2006 See Formula 7; Table 2, Octapeptin C; page 56, paragraph [0151] – page 67, paragraph [0191].	1-5, 12-15
X	KANAZAWA, K. *et al.* "Contribution of Each Amino Acid Residue in Polymyxin B_3 to Antimicrobial and Lipopolysaccharide Binding Activity", Chem. Pharm. Bull. (2009), 57(3), 240-244 (published online January 9, 2009) See Compound 11; pages 241-242, section entitled Bacteria and Susceptibility Test.	1-8, 11-13
A	TSUBERY, H. *et al.* "N-terminal modifications of Polymyxin B nonapeptide and their effect on antibacterial activity", Peptides (2001), 22, 1675-1681 See whole document.	1-15

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 June 2010	11 JUN 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **RACHEL NORFOR** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6225 6127

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2010/000568

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2006083317	AU	2005326770	BR	PI0512941	CA	2571944
		CN	101010336	EP	1761554	KR	20070047770
		MX	PA06015239	NO	20070563	NZ	552730
		RU	2007103811	US	2006004185	US	2008207874
		ZA	200610818				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX