

POLYMYXIN DERIVATIVES AND USES THEREOF

TECHNICAL FIELD

The present invention relates to polymyxin derivatives and to uses thereof in the treatment of infections caused by Gram-negative bacteria. The polymyxin derivatives may have antibacterial effects or may sensitize bacteria to enhance the effects of other antibacterial agents.

BACKGROUND

Sepsis kills more than 215,000 Americans each year. It is estimated that 750,000 Americans are infected with severe sepsis and 29% of them die from it each year. Sepsis deaths make 9% of all death cases in the U.S. Sepsis kills as many Americans as myocardial infections, even more than traffic accidents.

Two to three million Americans acquire a hospital infection each year and 10% of these infections progress to sepsis. More than 90,000 of these patients die from sepsis infected in hospitals.

Severe sepsis and septic shock (severe sepsis combined with low blood pressure) took up to 135,000 lives each year in the intensive care units (ICU) in the European Union according to the OECD Health Report of 2000. In Britain, 5,000 out of 100,000 patients who acquired a hospital infection die from sepsis every year in acute care hospitals belonging to the NHS organisation.

The death toll has increased year after year due to the fact that the number of patients predisposed to sepsis, such as the elderly, premature neonates, and cancer patients, has increased, not least because many serious illnesses are more treatable than before. Also the use of invasive medical devices and aggressive procedures has increased.

Gram-negative bacteria cause more than 40% of all septicemic infections and many of the Gram-negative bacteria are extremely multiresistant. Gram-negative bacteria provide a harder challenge in therapy than Gram-positives, as they possess a unique structure, the outer membrane, as their outermost structure. Lipopolysaccharide molecules located on the outer membrane inhibit the diffusion of many antibacterial agents deeper into the cell, where their ultimate targets are located. More than 95% of the novel antibacterial agents isolated from nature or chemically synthesized in 1972-1991 lacked activity against Gram-negatives (Vaara 1993).

Polymyxins are a group of closely related antibiotic substances produced by strains of *Paenibacillus polymyxa* and related organisms. These cationic drugs are relatively simple peptides with molecular weights of about 1000. Polymyxins, such as polymyxin B, are decapeptide antibiotics, i.e. they are made of ten (10) aminoacyl residues. They are bactericidal and especially effective against Gram-negative bacteria such as *Escherichia coli* and other species of *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter baumannii*, and others. However, polymyxins have severe adverse effects, including nephrotoxicity and neurotoxicity. These drugs thus have limited use as therapeutic agents because of high systemic toxicity.

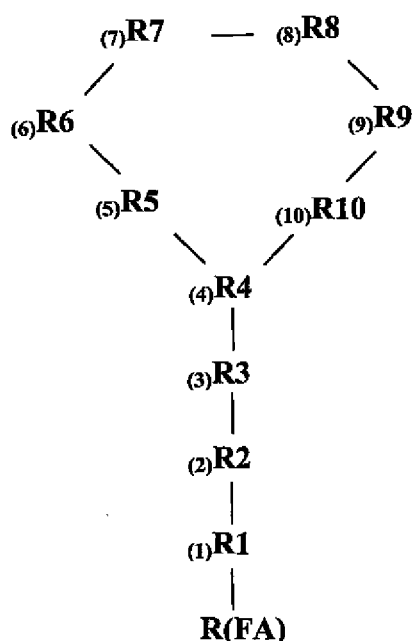
Polymyxins have been used in the therapy of serious infections caused by those bacteria, but because of the toxicity, their use was largely abandoned in the 70's when newer, better tolerated antibiotics were developed. The recent emergence of multiresistant strains of Gram-negative bacteria has necessitated the therapeutic use of polymyxins as the last resort, in spite of their toxicity, and as many of the less toxic antibiotics have already lost their effectiveness against particular strains of the said bacteria, the use of polymyxins has again increased.

Accordingly, polymyxins have now been recalled to the therapeutic arsenal, although, due to their toxicity, on a very limited scale. Their systemic (i.e. non-topical) use is, however, largely restricted to the therapy of life-threatening infections caused by multiply resistant strains of *Ps. aeruginosa* and *A. baumannii* as well as by carbapenem-resistant enteric bacteria.

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Polymyxins consist of a cyclic heptapeptide part and a linear part consisting of a tripeptide portion and a hydrophobic fatty acid tail linked to the α -amino group of the N-terminal amino acid residue of the tripeptide and may be represented by the general formula:

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wherein R1-R3 represent the tripeptide side chain portion; R4-R10 the heptapeptide ring portion and R(FA) represents the hydrophobic fatty acid tail linked to the α -amino group of the N-terminal amino acid residue of the tripeptide.

The polymyxin group includes the following polymyxins: A1, A2, B1-B6, C, D1, D2, E1, E2, F, K1, K2, M, P1, P2, S, and T (Storm *et al.* 1977; Srinivasa and Ramachandran 1979). All polymyxins are polycationic and possess five (5) positive charges, with the exception of polymyxin D, F, and S which possess four (4) positive charges. It should be noted that modified polymyxins that lack the fatty acid part R(FA) but carry R1-R10 have one additional positive charge when compared to the natural polymyxins they derived from, due to the free α -amino group in the N-terminus of the derivative. Accordingly, for example, such a derivative of polymyxin B or polymyxin E carries six (6) positive charges in total.

The clinically used polymyxin B and polymyxin E differ from each other only in the residue R6, which is D-phenylalanyl residue in polymyxin B and D-leucyl residue in polymyxin E.

Also circulin A and B are classified as polymyxins (Storm *et al.* 1977). They differ from other polymyxins only in carrying isoleucyl residue in the position R7 whereas other polymyxins have either threonyl or leucyl residue in the said position. For an overview of the structures of some polymyxins, see

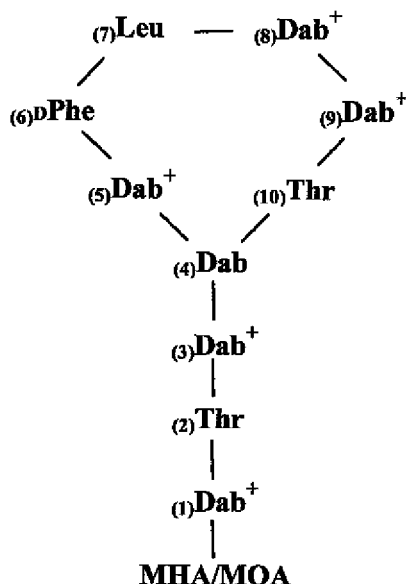
5 Table 1.

Table 1

The structure of selected polymyxins and octapeptin as well as selected derivatives thereof

Compound	R(FA)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Polymyxin B	MO(H)A-	Dab-	Thr-	Dab-	*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr
Colistin (polymyxin E)	MO(H)A-	Dab-	Thr-	Dab-	*Dab-	Dab-	D Leu-	Leu-	Dab	Dab	*Thr
Colistin sulphomethate	MO(H)A-	sm-Dab-	Thr-	sm-Dab-	*Dab-	Sm-Dab-	D Leu-	Leu-	sm--Dab-	sm--Dab-	*Thr
Polymyxin A	MO(H)A-	Dab-	Thr-	D Dab-	*Dab-	Dab-	D Leu-	Thr-	Dab	Dab	*Thr
Polymyxin M	MOA	Dab-	Thr-	Dab-	*Dab-	Dab-	D Leu-	Thr-	Dab	Dab	*Thr
Polymyxin D	MO(H)A-	Dab-	Thr-	D Ser-	*Dab-	Dab-	D Leu-	Thr-	Dab	Dab	*Thr
Circulin A	MOA	Dab-	Thr-	Dab-	*Dab-	Dab-	D Leu-	Ile-	Dab	Dab	*Thr
Octapeptin A	OHMDA	—	—	Dab-	*Dab-	Dab-	D Leu-	Leu-	Dab	Dab	*Thr
Deacylcolistin (DAC)		Dab-	Thr-	Dab-	*Dab-	Dab-	D Leu-	Leu-	Dab	Dab	*Thr
Polymyxin E nonapeptide (PMEN)			Thr-	Dab-	*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr
Deacylpolymyxin B (DAPB)		Dab-	Thr-	Dab-	*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr
Polymyxin B nonapeptide (PMBN)			Thr-	Dab-	*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr
Polymyxin B octapeptide (PMBO)				Dab-	*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr
Polymyxin B heptapeptide (PMHP)					*Dab-	Dab-	D Phe-	Leu-	Dab	Dab	*Thr

Polymyxin B is represented by the following formula:



Commercially available polymyxin B is a mixture, where R-FA is
 5 predominantly 6-methyloctanoyl (6-MOA, in polymyxin B1) but may also be a
 related fatty acyl such as 6-methylheptanoyl (6-MHA, in polymyxin B2), oc-
 tanoyl (in polymyxin B3), or heptanoyl (polymyxin B4) (Sakura *et al.* 2004). All
 these variants are equally potent against Gram-negatives such as *E. coli* (Sa-
 kura *et al.* 2004). Quite analogously, in polymyxin E1 (colistin A) and in circulin
 10 A the R-FA is 6-MOA and in polymyxin E2 (colistin B) and in circulin B the R-
 FA is 6-MHA. Numerous researchers have attached various hydrophobic moie-
 ties including various fatty acyl residues to the N-terminus of polymyxin deriva-
 tives and analogues and have shown that the resulting derivatives have potent
 antibacterial activity (Chihara *et al.* 1973, Sakura *et al.* 2004 and in US patent
 15 publication 2006004185. Even the derivative that carries the bulky hydrophobic
 9-fluorenylmethoxycarbonyl residue as the R-FA is almost as potent as po-
 lymyxin B in inhibiting the growth of *E. coli* and other Gram-negative bacteria
 (Tsubery *et al.* 2001).

For biological activity the heptapeptide ring structure is essential
 20 (Storm *et al.* 1997). A derivative with an octapeptide ring is significantly less
 active as an antibiotic.

Multiple modifications of polymyxins and multiple polymyxin-like synthetic molecules have been made, and with certain limits they have preserved their biological activity. The modifications comprise but are not limited to those in the side chain, as well as molecules in which an inherent hydrophobic amino acid residue (such as DPhe or Leu) has been replaced with another hydrophobic amino acid residue or in which the cationic Dab has been replaced with another cationic amino acyl residue, such as Lys, Arg, or ornithine residue (Storm *et al.* 1997, Tsubery *et al.* 2000a, Tsubery *et al.* 2002, US patent publication 2004082505, Sakura *et al.* 2004, US patent publication 2006004185).

Other modifications that result in microbiologically at least partially active compounds comprise but are not limited to alkanoyl esters where the OH-groups of the threonyl residues form esters with alkanoyls such as propionyl and butyryl (US Patent 3,450,687).

Octapeptins are otherwise identical to polymyxin E (colistin) but have a covalent bond instead of the residues R1-R2 (Table 1). In this invention, the R positions are numbered according to those in the natural polymyxins and thus the only amino acyl residue in the side chain of octapeptins is defined as R3. Accordingly, octapeptins are octapeptides whereas all natural polymyxins are decapeptides, and they possess only four (4) positive charges. The R-FA residues among various octapeptins (A1, A2, A3, B1, B2, B3, C1) include the following: 3-OH-8-methyldecanoic acid, 3-OH-8-methylnonanoic acid, and β -OH-6-methyloctanoic acid. Derivatives that possess a fatty acyl residue with 6 to 18 carbon atoms have a potent antibacterial activity against *E. coli* (Storm *et al.* 1977).

The first target of polymyxins in Gram-negative bacteria is their outer membrane (OM) that is an effective permeability barrier against many noxious agents including large (Mw more than 700 d) antibiotics as well as hydrophobic antibiotics. By binding to the lipopolysaccharide (LPS) molecules exposed on the outer surface of the OM, polymyxins damage the structure and function of the OM and, as a result, permeabilize (i.e. make permeable) the OM to polymyxin itself, as well as to many other noxious agents (Nikaido and Vaara 1985, Vaara 1992, Nikaido 2003). The final and lethal target (the bactericidal target) of polymyxins is believed to be the cytoplasmic membrane (the inner membrane) of bacteria.

Numerous efforts have been made to reduce the toxicity of polymyxins. The treatment of polymyxin E (colistin) with formaldehyde and sodium bisulfite yields colistin sulphomethate, in which the free amino groups of the five diaminobutyric acid residues have partially been substituted by sulphomethyl groups (Table 1). The preparations consist of undefined mixtures of the mono-, di-, tri-, tetra-, and penta-substituted compounds. The sulphomethylated preparations, when freshly dissolved in water, initially lack both the antibacterial activity and toxicity of the parent molecule, but when the compounds start decomposing in the solution, in the blood or in the tissues to yield less substituted derivatives and free colistin, both the antibacterial activity and the toxicity are partially brought back. Furthermore, the degree of initial sulphomethylation apparently varies between the commercially available pharmaceutical preparations. Many other ways to block all the free amino groups have been published. Examples comprise but are not limited to the formation of unstable Schiff bases with amino acids (Storm *et al.* 1977).

Polymyxin E nonapeptide (PMEN, colistin nonapeptide, Table 1), obtained by treating polymyxin E enzymatically and lacking the R-FA and R1, was shown in 1973 to be less toxic than the parent compound in acute toxicity assay (immediate death presumably due to direct neuromuscular blockade) in mice (Chihara *et al.* 1973). However, it also lacked the antibacterial activity, as measured as its ability to inhibit bacterial growth (Chirara *et al.* 1973).

Vaara and Vaara, on the other hand, showed, that polymyxin B nonapeptide (PMBN, Table 1) retains the ability to permeabilize the OM of Gram-negative bacteria (Vaara and Vaara 1983a,b,c; US Patent 4,510,132; Vaara 1992). Accordingly, even though it lacks the direct antibacterial activity (i.e. the ability to inhibit bacterial growth), it is able to sensitize (i.e. make sensitive or, as also termed, make susceptible) the bacteria to many antibacterial agents such as hydrophobic antibiotics as well as large antibiotics and some other noxious agents.

PMBN also sensitizes bacteria to the bactericidal activity of the human complement system, present in fresh human serum as a first-line defence system against invaders (Vaara and Vaara 1983a, Vaara *et al.* 1984, Vaara 1992). Furthermore, it sensitizes the bacteria to the joint bactericidal activity of serum complement and human polymorphonuclear white cells (Rose *et al.* 1999).

PMBN resembles PMEN in being less toxic in the acute toxicity assay in mice than unmodified polymyxins. In further toxicological assays, several criteria proved PMBN to be less toxic than its parent compound, but this polymyxin derivative was still judged to be too nephrotoxic for clinical use
5 (Vaara 1992).

PMBN carries five (5) positive charges. Subsequent studies revealed, quite expectedly, that PMEN, also carrying five (5) positive charges as well as deacylpolymyxin B and deacylpolymyxin E, both carrying six (6) positive charges are potent agents to sensitize bacteria to other antibiotics (Viljanen *et al.* 1991, Vaara 1992). In addition, it has been shown that a structurally further reduced derivative polymyxin B octapeptide (PMBO) retains a very effective permeabilizing activity while polymyxin B heptapeptide (PMBH) is less active (Kimura *et al.* 1992). PMBN, PMEN and PMBO have five (5) positive charges while PMBH has only four (4) positive charges. This difference may
10 explain the weaker activity of PMBH.
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The group of Ofek, Tsubery and Friedkin recently described polymyxin-like peptides that were linked to chemotactic peptides, such as fMLF, that attract polymorphonuclear leucocytes (US patent publication 2004082505, Tsubery *et al.* 2005). They described peptides fMLF-PMBN, MLF-PMBN,
20 fMLF-PMEN, fMLF-PMBO and MLF-PMBO, all carrying four (4) positive charges, that sensitize Gram-negative bacteria to antibiotics, even though no comparative studies with increasing concentrations of the compounds were published (Tsubery *et al.* 2005).

In order to study the structures and functional properties of polymyxins, a few works have disclosed, among other compounds, polymyxin derivatives having less than four (4) positive charges.
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Teuber (1970) has described the treatment of polymyxin B with acetic anhydride that yields a preparation containing polymyxin B as well as its mono-, di-, tri-, tetra-, and penta-N-acetylated forms. Teuber also separated
30 each group and nonquantitatively reported using an agar diffusion assay that penta-acetylated and tetra-acetylated forms lacked the ability to halt the growth of *Salmonella typhimurium*, whereas di- and monoacetylated forms did have such ability. Triacetylated form had some ability.

Srinivasa and Ramachandran (1978) isolated partially formylated
35 polymyxin B derivatives and showed that a diformyl derivative as well as a tri-formyl derivative inhibited the growth of *Pseudomonas aeruginosa*. They did

not disclose the compounds' ability to sensitize bacteria to antibiotics. Furthermore, in 1980 they showed that the free amino groups of trimethylpolymyxin B in residues R1 and R3, as well as the free amino groups of diformylpolymyxin B in residues R1, R3, and R5 are essential while the free amino groups in R8 and R9 are not essential for the growth inhibition (Srinivasa and Ramachandran, 1980a).

A shortened polymyxin B derivative octanoyl polymyxin B heptapeptide has been disclosed by Sakura *et al.* (2004). The attachment of the octanoyl residue to the N-terminus of the residue R4 of the polymyxin B heptapeptide results in a compound having only three (3) positive charges. Sakura *et al.* found that octanoyl polymyxin B heptapeptide inhibits the growth of bacteria only at a very high concentration (128 µg/ml), whereas the other derivatives such as octanoyl polymyxin B octapeptide and octanoyl polymyxin B nonapeptide, both having four charges (4) were very potent agents to inhibit bacterial growth.

US patent publication 2006004185 recently disclosed certain polymyxin derivatives and intermediates that can be used to synthesize new peptide antibiotics. The antibacterial compounds described possessed four (4) or five (5) positive charges.

There is still an urgent need for effective treatments for bacterial infections, in particular for the infections caused by multiresistant Gram-negative bacteria.

SUMMARY

The present invention relates to a polymyxin derivative wherein the total number of positive charges at physiological pH is at least two but no more than three, with the proviso that R8 and R9 are not both formylated when R(FA)-R1-R2-R3 constitutes the native polymyxin B side chain; and R4 is not directly linked to octanoyl residue when R4-R10 constitutes a native polymyxin B ring structure. More specifically, the present invention relates to a derivative, wherein R1-R10 is selected from the group consisting of SEQ ID NO:s 9-26, preferably SEQ ID NO:s 9-20.

The invention also relates to a combination product comprising two or more of the derivatives according to the present invention, and to a pharmaceutical composition comprising such derivative(s) or a combination thereof and pharmaceutically acceptable carriers and excipients.

Furthermore, the present invention relates to a method for treating, alleviating or ameliorating an infection in an individual caused by a Gram-negative bacterium, comprising administering a therapeutically effective amount of a derivative or a combination according to the present invention to
5 said individual, wherein said bacterium may be selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

In another embodiment, the present invention relates to a method
10 for sensitizing Gram-negative bacteria to an antibacterial agent, comprising administering, simultaneously or sequentially in any order, a therapeutically effective amount of said antibacterial agent and a derivative according to the present invention, wherein said antibacterial agent may be selected from the group consisting of clarithromycin, azithromycin, erythromycin and other mac-
15 rolides, ketolides, clindamycin and other lincosamines, streptogramins, rifampin, rifabutin, rifalazile and other rifamycins, fusidic acid, mupirocin, oxazolidinones, vancomycin, dalbavancin, telavancin, oritavancin and other glycopeptide antibiotics, fluoroquinolones, bacitracin, tetracycline derivatives, beta-lactam antibiotics, novobiocin, pleuromutilins, folate synthesis inhibitors, de-
20 formylase inhibitors, and bacterial efflux pump inhibitors.

Also provided are methods for developing novel antibiotics; for reducing the toxicity of natural polymyxins, octapeptins and their derivatives; for improving the pharmacokinetic properties of natural polymyxins, octapeptins and their derivatives; and for sensitizing clinically important Gram-negative
25 bacteria to a host defence mechanism complement present in the serum.

The present invention also provides uses of a polymyxin derivative according to the present invention in the manufacture of medicament for treating infections caused by Gram-negative bacteria, such e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter
30 freundii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*; for the manufacture of a medicament for sensitizing Gram-negative bacteria against antibacterial agents; and for sensitizing Gram-negative bacteria to a host defence mechanism complement present in the serum.

Finally, the present invention relates to a process for preparing a po-
35 lymyxin derivative according to the present invention, comprising modifying a

natural or synthetic polymyxin or octapeptin compound or a derivative thereof having 4 to 6 positively charged residues by replacing 1 to 4 of said residues by neutral residues or a covalent bond, or by converting 1 to 4 of said residues into neutral residues in order to obtain a polymyxin derivative of formula (I)
5 having 2 or 3 positively charged residues.

Definitions

"Physiological pH" as used herein refers to a pH value of more than 7,0 and below 7,6, such as a pH value in the range of from 7.1 to 7.5, for example in the range of from 7.2 to 7.4.

10 "Positive charge" as used herein denote positive charges at the above-defined physiological pH.

"Cationic" molecule as used herein refers to a molecule that contains one or more positive charges.

15 "Amino acid residue" as used herein refers to any natural, non-natural or modified amino acid residue, either in L- or D-configuration.

"Equivalent residues" as used herein, is intended to include obvious modifications to e.g., amino acids, resulting in non-natural amino acids or derivatives thereof, but retaining the structural and/or functional capacity of the replaced residue.

20 "Natural polymyxin(s)" as used herein, refers to polymyxins and circulins.

"Polymyxin derivative" refers, for the purpose of this invention, to synthetic or semisynthetic derivatives of natural polymyxins or octapeptins, which have a cyclic heptapeptide (or heptapeptide ring) portion R4-R10 and a side chain linked to the N-terminal aminoacyl residue R4. The side chain may consist of an R(FA)-triaminoacyl(R1-R3), an R(FA)-diaminoacyl(R2-R3), an R(FA)-monoamino-acyl(R3), or of R(FA) alone.

"Compounds" as used herein include all stereochemical isomers of said compound.

30 "Sensitizing activity" or "ability to sensitize" as used herein is intended to include any ability to increase the sensitivity, make sensitive or make susceptible a bacterium to an antibacterial agent.

Abbreviations

Fatty acids: FA, fatty acyl residue; 6-MOA and MOA, 6-methyloctanoyl residue; 6-MHA and MHA, 6-methylheptanoyl residue; MO(H)A, the mixture
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of 6-methyloctanoyl, 6-methylheptanoyl and related fatty acyl residues occurring in polymyxin B; OHMDA, 3-OH-8-methyldecanoic acid;

Amino acids: Dab, α,γ -diamino-n-butyryl residue; fDab, N- γ -formyl-diamino-n-butyryl residue; acDab, N- γ -acetyldiamino-n-butyryl residue; Abu, α -aminobutyryl residue; Thr, threonyl residue; Ser, serinyl residue; Phe, phenylalanyl residue; Leu, leucyl residue; Ile, isoleucyl residue; Ala, alanyl residue; sm-Dab, γ -sulphomethylated α,γ -diamino-n-butyryl residue. One-letter codes for modified amino acyl residues: X, Dab; Z, Abu; B, N- γ -fDab; J, N- γ -acDab.

Peptides: DAPB, deacylpolymyxin B; DAC, deacylcolistin; PMBN, polymyxin B nonapeptide; PMEN, polymyxin E nonapeptide; PMBO, polymyxin B octapeptide; PMHP, polymyxin B heptapeptide.

Other: cy, cyclo (to denote the cyclic part of the peptide, enclosed within brackets); f, formyl; ac, acetyl; LPS, lipopolysaccharide; OM, outer membrane; CFU, colony forming unit. The symbol * is used herein to mark the residues between which the heptapeptide ring portion of the compound is closed leaving the remaining part of the molecule as a side chain.

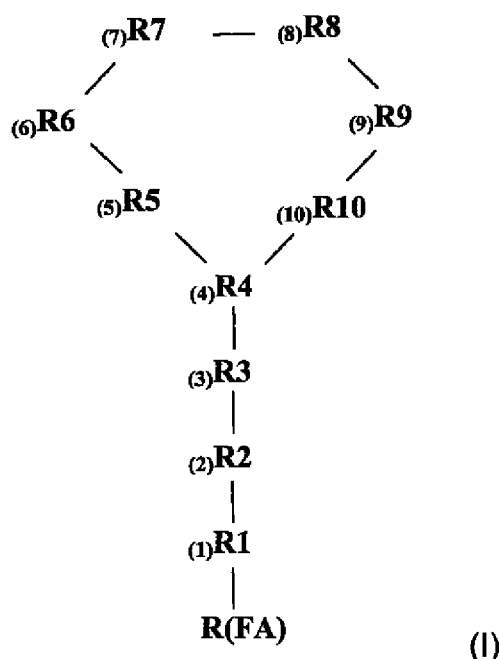
DETAILED DESCRIPTION OF THE INVENTION

It has now been found that polymyxin derivatives containing at least two (2) but no more than three (3) positive charges still possess antibacterial activity against Gram-negative bacteria, and/or possess the ability to sensitize Gram-negative bacteria to antibacterial agents, such as antibiotics, semisynthetic antibiotics, chemotherapeutic agents and host defence factors, such as complement.

This reduction of positive charges may improve the pharmacological properties of the derivatives according to the present invention when compared to natural polymyxins and their known derivatives. More specifically, it may reduce the toxicity, including nephrotoxicity, of the compounds, and/or reduce the histamine liberation from the host tissue, exerted by the compounds, and/or result in more suitable pharmacokinetic properties such as longer serum half life or lower susceptibility to the inactivation by polyanionic tissue and pus constituents, as compared to clinically used polymyxins and their previously described and characterized derivatives, such as polymyxin B nonapeptide.

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The present invention thus relates to a polymyxin derivative which may be represented by the general formula I:



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wherein R1, R2 and R3 may be absent; and

wherein the total number of free, unsubstituted cationic charges in the compound is at least two (2) and does not exceed three (3); or a pharmaceutically acceptable salt thereof.

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In natural polymyxins and octapeptins, R(FA) is 6-methyloctanoic acid (6-MOA), 6-methylheptanoic acid (6-MHA), octanoic acid, heptanoic acid, nonanoic acid, 3-OH-6-methyloctanoic acid, 3-OH-8-methyldecanoic acid, 3-OH-8-methylnonanoic acid, 3-OH-8-decanoic acid, and 3-OH-6-methyloctanoic acid. Examples of known derivatives that have antibacterial activity include those wherein R(FA) is γ -phenylbutyric acid, isovaleric acid, 9-fluorenylmethoxycarbonic acid, a series of C:9 to C:14 unbranched fatty acids as well as iso C:9 and iso C:10 fatty acids.

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In a derivative according to the present invention, R(FA) may be any hydrophobic fatty acid residue, and is preferably selected from the group consisting of octanoyl, decanoyl and 6-MHA residues.

A person skilled in the art may readily recognize equivalents of these preferred hydrophobic R(FA) residues, which may be selected from the group consisting of e.g. acyl residues such as an optionally substituted alkyl

residue, an optionally substituted isoalkyl residue, an optionally substituted cycloalkyl residue, an optionally substituted alkenyl residue, an optionally substituted cycloalkenyl residue, an optionally substituted aryl residue, an optionally substituted heteroaryl residue, an optionally substituted heterocyclic residue, wherein said residues preferably have more than five (5) carbon atoms and wherein the substitutions may also include those optionally designed between the acyl residue and the N-terminus of the peptide. R(FA) may also be a stretch of a hydrophobic oligopeptide. Examples of possible R(FA) residues include (but are not limited to) octanoyl, nonanoyl, isononanoyl, decanoyl, isodecanoyl, undecanoyl, dodecanoyl, tetradecanoyl, cyclohexyl, cycloheptanoyl, cyclooctanoyl, cyclononanoyl, cycloisononanoyl, cyclodecanoyl, cycloisodecanoyl, cycloundecanoyl, cyclododecanoyl, cyclotetradecanoyl, hexyl, heptanoyl, and 9-fluorenylmethoxycarbonyl residues.

In natural polymyxins and octapeptins, R1 is Dab or absent (i.e. replaced by a covalent bond). Examples of known derivatives that have antibacterial activity include those wherein R1 is Ala or a covalent bond.

In a derivative according to the present invention R1, if present, may be any amino acid residue, provided that the total number of positive charges in said derivative does not exceed three and that the total number of positive charges in the side chain portion does not exceed two, and is preferably Abu, if present.

In natural polymyxins and octapeptins, R2 is Thr or absent (i.e. replaced by a covalent bond). Examples of known derivatives that have antibacterial activity include those wherein R2 is O-acetyl-Thr, O-propionyl-Thr, O-butyryl-Thr or a covalent bond.

In a derivative according to the present invention, R2, if present, may be any amino acid residue, provided that the total number of positive charges in said derivative does not exceed three and that the total number of positive charges in the side chain portion does not exceed two, and is preferably selected from the group consisting of Thr, DThr, and DAla, if present. A person skilled in the art may also recognize an equivalent residue of Thr to be Ser.

In natural polymyxins and octapeptins, R3 is Dab, DDab or DSer. Examples of numerous known synthetic derivatives that have antibacterial activity include those wherein R3 is Lys or 2-amino-4-guanidino butyric acid.

In a derivative according to the present invention, R3, if present, may be any amino acid residue, provided that the total number of positive charges in said derivative does not exceed three and that the total number of positive charges in the chain portion does not exceed two, and is preferably
5 selected from the group consisting of Thr, DThr, Ser, DSer, DAla, Dab and Abu, if present.

A person skilled in the art may readily recognize equivalent residues of these preferred residues R1, R2 and R3, and may select such from a group consisting of e.g. a covalent bond, alanine, 2-aminoadipic acid, α -n-butyric
10 acid, N-(4-aminobutyl)glycine, α -aminobutyric acid, γ -aminobutyric acid, α -amino-caproic acid, aminocyclopropanecarboxylate, aminoisobutyric acid, aminonorbornylcarboxylate, α -amino-n-valeric acid, arginine, N $_{\omega}$ -methyl-arginine, asparagine, α -methylasspartate, aspartic acid, N-benzylglycine, N-(2-carbamylethyl)glycine, N-(carbamylethyl)glycine, 1-carboxy-1(2,2-diphenyl
15 ethylamino)cyclopropane, cysteine, N $_{\alpha}$ -methyldiamino-n-butyric acid, N $_{\gamma}$ -acetyldiamino-n-butyric acid, N $_{\gamma}$ -formyldiamino-n-butyric acid, N $_{\gamma}$ -methyl-diamino-n-butyric acid, N-(N-2,2-diphenylethyl)carbamyln-methyl-glycine, N-(N-3,3-diphenylpropyl) carbamyln-methyl(1)glycine, N-(3,3-diphenylpropyl) glycine, glutamic acid, glutamine, glycine, t-butylglycine, 2-amino-4-guanidinobutyric
20 acid, N-(3-guanidinopropyl)glycine, histidine, homophenylalanine, isodesmosine, isoleucine, leucine, norleucine, hydroxylysine, N $_{\alpha}$ -methyllysine, lysine, N $_{\alpha}$ -methylhydroxylysine, N $_{\alpha}$ -methyllysine, N $_{\epsilon}$ -acetylhydroxylysine, N $_{\epsilon}$ -acetyl-lysine, N $_{\epsilon}$ -formylhydroxylysine, N $_{\epsilon}$ -formyllysine, N $_{\epsilon}$ -methylhydroxylysine, N $_{\epsilon}$ -methyllysine, methionine, α -methyl- γ -aminobutyrate, α -methyl-aminoiso-
25 butyrate, α -methylcyclohexylalanine, α -naphthylalanine, norleucine, norvaline, α -methylornithine, N $_{\alpha}$ -methylornithine, N $_{\delta}$ -acetylornithine, N $_{\delta}$ -formyl-ornithine, N $_{\delta}$ -methylornithine, ornithine, penicilamine, phenylalanine, hydroxyproline, proline, N $_{\alpha}$ -methyldiamino-n-propionic acid, N $_{\beta}$ -acetyldiamino-n-propionic acid, N $_{\beta}$ -formyldiamino-n-propionic acid, N $_{\beta}$ -methyldiamino-n-propionic acid, phos-
30 phoserine, serine, phosphothreonine, threonine, tryptophan, tyrosine, nor-valine, and valine.

In natural polymyxins and octapeptins, R4 is Dab. Examples of syn-
thetic derivatives that have antibacterial activity include those wherein R4 is
Lys.

35 In a derivative according to the present invention R4 is an amino acid residue comprising a functional side chain able to cyclicize the molecule,

and may be selected from the group of equivalent residues consisting of Lys, hydroxylysine, ornithine, Glu, Asp, Dab, diaminopropionic acid, Thr, Ser and Cys, preferably Dab.

In natural polymyxins and octapeptins, R5, R8 and R9 are Dab. Examples of synthetic derivatives that have antibacterial activity include those wherein R5, R8, and R9 may be Lys or 2-amino-4-guanidino butyric acid.

In a derivative according to the present invention R5, R8 and R9 may be a positively charged or a neutral amino acid residue, preferably Dab or Abu, provided that the total number of positive charges in said derivative does not exceed three.

A person skilled in the art, may readily recognize equivalent residues of these preferred residues, and may select such from a group consisting of e.g. diaminobutyric acid, diaminopropionic acid, lysine, hydroxylysine, ornithine, 2-amino-4-guanidinobutyric acid, glycine, alanine, valine, leucine, isoleucine, phenylalanine, D-phenylalanine, methionine, threonine, serine, α -amino-n-butyric acid, α -amino-n-valeric acid, α -amino-caproic acid, N $_{\epsilon}$ -formyllysine, N $_{\epsilon}$ -acetyllysine, N $_{\epsilon}$ -methyllysine, N $_{\epsilon}$ -formylhydroxylysine, N $_{\epsilon}$ -acetylhydroxylysine, N $_{\epsilon}$ -methylhydroxylysine, L-N $_{\alpha}$ -methylhydroxylysine, N $_{\gamma}$ -formyldiamino-n-butyric acid, N $_{\gamma}$ -acetyldiamino-n-butyric acid, N $_{\gamma}$ -methyldiamino-n-butyric acid, N $_{\beta}$ -formyldiamino-n-propionic acid, D-N $_{\beta}$ -formyldiamino-n-propionic acid, N $_{\beta}$ -acetyldiamino-n-propionic acid, N $_{\beta}$ -methyldiamino-n-propionic acid, N $_{\delta}$ -formylornithine, N $_{\delta}$ -acetylornithine and N $_{\delta}$ -methylornithine.

In natural polymyxins and octapeptins, R6 is DPhe or DLeu and R7 is Leu, Ile, Phe or Thr. Synthetic derivatives that have antibacterial activity include those wherein R6 is DTrp and wherein R7 is Ala.

In a derivative according to the present invention, R6 is an optionally substituted hydrophobic amino acid residue, preferably DPhe or DLeu, and R7 is an optionally substituted hydrophobic residue, preferably Leu, Thr or Ile.

A person skilled in the art may readily recognize equivalent residues of these preferred hydrophobic residues, and may select such from a group consisting of e.g. phenylalanine, α -amino-n-butyric acid, tryptophane, leucine, methionine, valine, norvaline, norleucine, isoleucine and tyrosine. A person skilled in the art may also recognize the equivalent residue of threonine to be serine.

In natural polymyxins and octapeptins, R10 is Thr and Leu. Examples of known derivatives that have antibacterial activity include those wherein R10 is O-acetyl-Thr, O-propionyl-Thr or O-butyryl-Thr.

In a derivative according to the present invention, R10 is Leu or any
 5 non-hydrophobic amino acid residue, provided that the total number of positive charges in said derivative does not exceed three. Preferably R10 is Thr or Leu.

A person skilled in the art may also recognize the equivalent residue of threonine to be serine.

10 More specifically, preferred residues are chosen in such a manner that R8 and R9 are not both formylated when R(FA)-R1-R2-R3 constitutes the native polymyxin B sidechain; and R4 is not directly linked to octanoyl residue when R4-R10 constitutes a native polymyxin B ring structure.

The specific positions of the at the most three (3) positive charges
 15 referred to herein above can be located in the heptapeptide ring portion and/or in the side chain, if present. When three (3) positive charges are present in the derivatives according to the invention, said three (3) positive charges can be located in the heptapeptide ring portion; or two (2) positive charges can be located in heptapeptide ring portion while the remaining one positive charge is
 20 located in the side chain; or one (1) positive charge can be located in the heptapeptide ring portion while the remaining two (2) positive charges are located in the side chain. Preferably at least two (2) positive charges are located in the heptapeptide ring portion.

In one embodiment, derivatives according to the present invention
 25 can be selected from the group of derivatives wherein R1-R10 is selected from the group consisting of Thr-DSer-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 10; Thr-DThr-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. SEQ ID NO. 11; Thr-DSer-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. SEQ ID NO. 12; Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 13; Abu-Thr-
 30 Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 14; Thr-Dab-cy[Dab-Dab-DPhe-Leu-Abu-Dab-Thr-], i.e. SEQ ID NO. 15; Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Leu-], i.e. SEQ ID NO. 16; Thr-DAla-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. SEQ ID NO. 17; Thr-Dab-cy[Dab-Dab-DPhe-Leu-Dab-Abu-Thr-], i.e. SEQ ID NO. 18; Thr-Abu-cy[Dab-Dab-DLeu-Leu-Dab-
 35 Dab-Thr-], i.e. SEQ ID NO. 19; DAla-DAla-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 20; cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID

NO. 9; Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 21; Thr-Dab-cy[Dab-Abu-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 22; Dab-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Abu-Abu-Thr-], i.e. SEQ ID NO. 23; Thr-Abu-cy[Dab-Lys-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 24; Thr-Abu-cy[Dab-Abu-DPhe-Leu-Dab-Dab-Thr-], i.e. SEQ ID NO. 25; and Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Abu-Thr-], i.e. SEQ ID NO. 26.

In other embodiments, derivatives according to the present invention can be selected from the group consisting of: OA-Thr-DSer-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 10; DA-Thr-DSer-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 10; OA-Thr-DThr-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 11; OA-Thr-DSer-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 12; DA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 13; OA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 13; MHA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. MHA-SEQ ID NO. 13; MHA-Abu-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. MHA-SEQ ID NO. 14; OA-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Abu-Dab-Thr-], i.e. OA-SEQ ID NO. 15; OA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Leu-], i.e. OA-SEQ ID NO. 16; OA-Thr-DAla-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 17; OA-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Dab-Abu-Thr-], i.e. OA-SEQ ID NO. 18; OA-Thr-Abu-cy[Dab-Dab-DLeu-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 19; OA-DAla-DAla-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 20; DA-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 9; OA-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 21; OA-Thr-Dab-cy[Dab-Abu-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 22; MHA-Dab-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Abu-Abu-Thr-], i.e. MHA-SEQ ID NO. 23; OA-Thr-Abu-cy[Dab-Lys-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 24; OA-Thr-Abu-cy[Dab-Abu-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 25; and OA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Abu-Thr-], i.e. OA-SEQ ID NO. 26.

Preferably, derivatives according to the present invention are selected from the group consisting of: OA-Thr-DSer-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 10; DA-Thr-DSer-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 10; OA-Thr-DThr-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 11; OA-Thr-DSer-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 12; DA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 13; OA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-

Dab-Thr-], i.e. OA-SEQ ID NO. 13; MHA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. MHA-SEQ ID NO. 13; MHA-Abu-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. MHA-SEQ ID NO. 14; OA-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Abu-Dab-Thr-], i.e. OA-SEQ ID NO. 15; OA-Thr-Abu-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Leu-], i.e. OA-SEQ ID NO. 16; OA-Thr-DAla-cy[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 17; OA-Thr-Dab-cy[Dab-Dab-DPhe-Leu-Dab-Abu-Thr-], i.e. OA-SEQ ID NO. 18; OA-Thr-Abu-cy[Dab-Dab-DLeu-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 19; OA-DAla-DAla-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. OA-SEQ ID NO. 20; and DA-cy[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-], i.e. DA-SEQ ID NO. 9.

As shown in the example section herein, the compounds according to the present invention carrying only three (3) positive charges can be very potent agents to inhibit the growth of Gram-negative bacteria or to sensitize them to antibacterial agents, and even derivatives carrying only two (2) positive charges can have the same effect, although at a more moderate level.

For direct antibacterial activity at least two (2) and more preferably three (3) positive charges are located in the heptapeptide ring portion, and for sensitizing activity at least one (1) and more preferably two (2) or three (3) positive charges are located in the heptapeptide ring part. Furthermore, the presence of two hydroxyl groups in the side chain part significantly enhances the direct antibacterial activity.

The works of Teuber (1970), Srinivasa and Ramachandran (1980a), and Sakura *et al.* (2004) disclose, among other polymyxin derivatives, derivatives having only two (2) or three (3) positive charges. However, the antibacterial activity of the disclosed derivatives is very weak and clinically irrelevant (Sakura *et al.* 1980), attributed to amino acid residues in full contradiction to the findings of the present invention (Srinivasa and Ramachandran 1980a) or attributed to no specific amino acid residues at all, due to incomplete purification (Teuber 1970). Furthermore, none of the works cited above describe, suggest or motivate to study the ability of such derivatives to sensitize Gram-negative bacteria to antibacterial agents. As the examples of the present invention clearly show, one cannot predict the ability of a polymyxin derivative to sensitize Gram-negative bacteria to antibacterial agents on the basis of its ability to inhibit the growth of the same. For example, whereas a clinically irrelevant concentration of octanoyl polymyxin B heptapeptide of as high as 128 µg/ml is needed to inhibit the growth of *E. coli* (Sakura *et al.* 2004), an amount as low

as 4 µg is enough for a moderate sensitization of the bacterium to rifampin, as shown in the example section herein.

The acetylated polymyxin derivatives described by Teuber (1970) are mixtures of differently acetylated derivatives. Acetic anhydride can react with any of the five free amino groups of the polymyxin B molecule to yield a monoacetylated polymyxin. Therefore a monoacetylated polymyxin according to Teuber is a mixture of five monoacetylated polymyxin derivatives, each acetylated at a different amino group. A diacetylated polymyxin according to Teuber is a mixture of ten differently diacetylated derivatives and triacetylated polymyxin is also a mixture of ten differently triacetylated derivatives. Teuber did not attempt to isolate these derivatives from the mixtures. The problem with such modified polymyxins is that the partial modification may result in reduced specificity. Therefore, some of the amino groups that are important for the antibacterial activity are partially substituted (and hence inactivated) whereas some of the non-important amino groups remain partially unsubstituted. Furthermore, the degree of substitution may lead to lot-to-lot variations.

The polymyxin derivatives according to the present invention, on the other hand, are isolated and structurally clearly defined and identified compounds.

Srinivasa and Ramachandran (1980a) proposed that the free side chain amino groups of trimethyl-polymyxin B in residues R1 and R3, as well as the free amino groups of diformylpolymyxin B in residues R1, R3, and R5 are essential while the free amino groups in R8 and R9 are not essential for the growth inhibition of *Pseudomonas aeruginosa*. As a contrast to their conclusions, the compounds in the present invention include those that lack the free amino groups in R1 and R3, carry them in R5, R8 and R9, and yet are potent agents against *Ps. aeruginosa* as well as other Gram-negative bacteria.

A shortened polymyxin B derivative octanoyl polymyxin B heptapeptide has been disclosed by Sakura *et al.* (2004). The attachment of the octanoyl residue to the N-terminus of the residue R4 of the polymyxin B heptapeptide results in a compound having only three (3) positive charges. Sakura *et al.* found that octanoyl polymyxin B heptapeptide inhibits the growth of bacteria only at a very high (and clinically irrelevant) concentration (128 µg/ml), whereas the other derivatives such as octanoyl polymyxin B octapeptide and octanoyl polymyxin B nonapeptide, both having four charges (4) are very potent agents to inhibit bacterial growth. Sakura *et al.* did not disclose or suggest

the ability of octanoyl polymyxin B heptapeptide to sensitize bacteria to antibacterial agents, nor teach that a longer fatty acid tail increases its antibacterial activity, as shown in the example section herein.

5 The present invention in one aspect provides new polymyxin derivatives having two (2) or three (3) positive charges only and still being capable of inhibiting the growth of one or more Gram-negative bacterial species and or sensitizing one or more Gram-negative bacterial species to an antibiotic or antibacterial agent.

10 The susceptibility of bacteria to an antibacterial agent may be determined by two microbiological methods. A rapid but crude procedure uses commercially available filter paper disks that have been impregnated with a specific quantity of the antibacterial agent. These disks are placed on the surface of agar plates that have been inoculated with a suspension of the organism being tested, and the plates are observed for zones of growth inhibition. A
15 more accurate technique, the broth dilution susceptibility test, involves preparing test tubes containing serial dilutions of the drug in liquid culture media, then inoculating the organism being tested into the tubes. The lowest concentration of drug that inhibits growth of the bacteria after a suitable period of incubation is reported as the minimum inhibitory concentration (MIC).

20 Derivatives according to the present invention may inhibit the growth of or sensitize to antibacterial agents clinically important Gram-negative bacteria such as those belonging to the genus of *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bordetella*, *Branhamella*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Francisella*, *Fusobacterium*, *Haemophilus*, *Helicobacter*, *Klebsiella*, *Legionella*, *Moraxella*, *Pasteurella*, *Plesiomonas*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia* species. The bacteria may be, for example, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, other species of *Enterobacter*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, other species of *Pseudomonas*, *Acinetobacter baumannii*, as well as many other species of non-fermentative Gram-
25 negative bacteria. The bacteria also include *Helicobacter pylori*, as well as other clinically important Gram-negative bacteria.

30 The bacterial infections to be treated include, for example, bacteremia, septicemia, skin and soft tissue infection, pneumonia, meningitis, infections in the pelveoperitoneal region, foreign body infection, fever in hematological patient, infection associated with an intravenous line or other catheter,

canyl and/or device, infection in gastrointestinal tract, in the eye, or in the ear, superficial skin infection, and colonization of gastrointestinal tract, mucous membranes and/or skin by potentially noxious bacteria.

5 The bacterial infectious diseases include (but are not limited to) severe hospital-acquired infections, infections of the immunocompromised patients, infections of the organ transplant patients, infections at the intensive care units (ICU), severe infections of burn wounds, severe community-acquired infections, infections of cystic fibrosis patients, as well as infections caused by multi-resistant Gram-negative bacteria.

10 The present invention is also directed to combinations of two or more derivatives according to the present invention for combination treatment. The combinations may include derivatives having different spectra of antibacterial activity or a capability to sensitize different species or strains of Gram-negative bacteria to antibacterial agents.

15 Another aspect of the present invention is directed to pharmaceutical compositions comprising polymyxin derivatives according to the present invention, their salt forms, selected combinations thereof, and optionally an antibacterial agent formulated together with one or more pharmaceutically acceptable carriers and excipients. They facilitate processing of the active compounds into preparations which can be used pharmaceutically and include e.g.
20 diluting, filling, buffering, thickening, wetting, dispersing, solubilizing, suspending, emulsifying, binding, stabilizing, disintegrating, encapsulating, coating, embedding, lubricating, colouring, and flavouring agents as well as absorbents, absorption enhancers, humefactants, preservatives and the like, well-known to
25 a person skilled in the art.

 Pharmaceutical compositions include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to treat, prevent, alleviate or ameliorate symptoms of pathology or prolong the survival of the subject being treated at a reasonable benefit to risk ratio applicable to any medical treatment. Determination
30 of a therapeutically effective amount is well within the capability of those skilled in the art of medicine.

Compositions may be produced by processes well known in the art, e.g. by means of conventional mixing, dissolving, encapsulating, entrapping, lyophilizing, emulsifying and granulating processes. The proper formulation is dependent upon the route of administration chosen, and the pharmaceutical composition can be formulated for immediate release or slow release (e.g. in order to prolong the therapeutic effect and/or improve tolerability). Furthermore, the formulations may conveniently be presented in unit dosage form by methods known in the art of pharmacy.

Pharmaceutical compositions according to the present invention include (but are not limited to) those intended for intravenous, intramuscular, oral, or topical administration as well as those being administered as a suppository or as an inhalable aerosol. The compositions include intravenous, intramuscular, intraperitoneal, subcutaneous, intramedullary, intrathecal, intraventricular, intranasal, or intraocular injections, inhalable aerosols as well as those intended for rectal, oral, intravaginal, transmucosal or transdermal delivery.

For parenteral administration (e.g. by bolus injection, fast running infusions, or slow infusions), the compounds according to this invention as well as the combinations described above may be formulated as their suitable salt or ester forms in sterile aqueous solutions, preferably physiologically compatible fluids such as saline, 5% dextrose, Ringer's solution, and Hank's solution. The formulation may also include organic solvents such as propylene glycol, polyethylene glycol, propylene glycol or related compounds as well as preservatives and surfactants.

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

In addition, the pharmaceutical compositions for parental administration may be suspensions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Suitable lipophilic vehicles and solvents include fatty oils such as

natural and/or synthetic fatty acids esters, such as ethyl oleate and triglycerides, or liposomes. The suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran.

5 The parenteral compositions can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use.

10 For oral administration, solid form preparations include e.g. powders, tablets, pills, dragees, lozenges, capsules, cachets, and microgranular preparations. Pharmaceutical preparations can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. A solid carrier/excipient can be one or more substances which may also
15 act as diluents, solubilizers, lubricants, suspending agents, binders, preservatives, flavouring agents, wetting agents, tablet disintegrating agents, or an encapsulating material. Suitable carriers include, but are not limited to, magnesium carbonate, magnesium stearate, talc, dextrose, lactose, pectin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low
20 melting wax, cocoa butter, and the like.

 Liquid preparations suitable for oral administration include e.g. aqueous solutions, syrups, elixirs, aqueous suspensions, emulsions and gels. Aqueous solutions can be prepared by dissolving the active component in water and adding suitable stabilizing and thickening agents as well as colorants
25 and flavours. Aqueous suspensions can be prepared by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well known suspending agents. Emulsions may be prepared in solutions in aqueous propylene glycol solutions or may contain emulsifying agents such
30 as lecithin, sorbitan monooleate or acacia.

 The compounds according to the invention or combinations described above may also be formulated for topical administration. The active compounds are admixed under sterile conditions with pharmaceutically acceptable carriers/excipients, including any needed buffering agents and preservatives. Ointments, creams and lotions may, for example, be formulated
35 with an aqueous or oily base with the addition of suitable emulsifying, dispers-

ing, suspending, thickening, stabilizing, or coloring agents. Commonly used excipients include animal and vegetable fats and oils, waxes, paraffins, starch, cellulose derivatives, tragacanth, and polyethylene glycol.

Other topical formulations include, but are not limited to, ear-drops,
5 eye-drops and transdermal patches.

For transdermal as well as transmucosal administration, penetrants generally known in the art may be used in the formulation.

For administration by inhalation, the compounds according to this invention and the combinations described above are delivered in the form of an
10 aerosol spray presentation from a ventilator, pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g. gelatin for use in
15 an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds according to this invention and the combinations described above may also be formulated in rectal compositions such as retention enemas or suppositories, using conventional suppository bases such as
20 cocoa butter, other glycerides, polyethylene glycol, or a suppository wax.

The present invention also relates to a method for using the present polymyxin derivatives or a combination of such derivatives as a part of the clinical treatment of (or a preventive prophylactic regimen for) human or animal subjects suffering of an infectious disease, and comprises administering to
25 said subject an therapeutically effective dose of at least one derivative according to the present invention, optionally in combination with an antibacterial agent.

The present invention also relates to a method of sensitizing Gram-negative bacteria to an antibacterial agent, wherein the derivative according to
30 the present invention is administered simultaneously, or sequentially in any order, with a therapeutically effective amount of said antibacterial agent.

The derivative of the present invention and the antibacterial agent may be administered together as one formulation or by different routes. For example, the polymyxin derivative may be administered intravenously while the
35 antibacterial agent is administered intramuscularly, intravenously, subcutaneously, orally or intraperitoneally. Alternatively, the derivative may be adminis-

tered intramuscularly or intraperitoneally while the antibacterial agent is administered intravenously, intramuscularly or intraperitoneally, or the derivative may be administered in an aerosolized or nebulized form while the antibacterial agent is administered, e.g., intravenously. The derivative and the antibacterial agents may be administered simultaneously or sequentially, as long as they are given in a manner sufficient to allow both to achieve effective concentrations at the site of infection.

"Therapeutic effectiveness" is based on a successful clinical outcome, and does not require that a derivative according to the present invention, optionally in combination with an antibacterial agent, kills 100% of the bacteria involved in an infection. Successful treatment depends on achieving a level of antibacterial activity at the site of infection, sufficient to inhibit the bacteria in a manner that tips the balance in favor of the host. When host defenses are maximally effective, the antibacterial effect required may be modest. Reducing organism load by even one log (a factor of 10) may permit the host's own defenses to control the infection. In addition, augmenting an early bactericidal/bacteriostatic effect can be more important than long-term bactericidal/bacteriostatic effect. These early events are a significant and critical part of therapeutic success, because they allow time for host defense mechanisms to activate. Increasing the bactericidal rate may be particularly important for infections such as meningitis, bone or joint infections.

The therapeutic effectiveness of an antibacterial agent depends on the susceptibility of the bacterial species to said antibacterial agent at the clinically relevant concentration of the derivative according to this invention. The effect of compounds according to the present invention to improve the therapeutic effectiveness of antibacterial agents in vivo may be demonstrated in in vivo animal models, such as mouse peritonitis or rabbit bacteremia assays, and may be predicted on the basis of a variety of in vitro tests, including (1) determinations of the minimum inhibitory concentration (MIC) of an antibacterial agent required to inhibit growth of a Gram-negative bacterium for 24 hours, (2) determinations of the effect of an antibacterial agent on the kinetic growth curve of a Gram-negative bacterium, and (3) checkerboard assays of the MIC of serial dilutions of antibacterial agent alone or in combination with serial dilutions of compound(s). Exemplary models or tests are well known in the art.

Using in vitro determinations of MIC at 24 hours, a derivative according to the present invention may be shown to reduce the MIC of the anti-

bacterial agent. With this result, it is expected that concurrent administration of the compound in vivo will increase susceptibility of a Gram-negative bacterium to the antibacterial agent. A compound according to the present invention may also be shown to reduce the MIC of an antibacterial agent from the range in which the organism is considered clinically resistant to a range in which the organism is considered clinically susceptible. With this result, it is expected that concurrent administration in vivo of the one or more compound(s) according to the present invention with the antibacterial agent will reverse resistance and effectively convert the antibiotic-resistant organism into an antibiotic-susceptible organism.

By measuring the effect of antibacterial agents on the in vitro growth curves of Gram-negative bacteria, in the presence or absence of a compound according to the present invention, the compound may be shown to enhance the early antibacterial effect of antibacterial agents within a period of preferably less than 24 hours. Enhancement of early bactericidal/growth inhibitory effects is important in determining therapeutic outcome.

A polymyxin derivative according to the present invention and an antibacterial agent may also have synergistic or potentiating effects beyond the individual effects of each agent alone or the additive effects of the agents together. In a checkerboard assay, the combination of a compound according to the present invention with antibacterial agents may result in a "synergistic" fractional inhibitory concentration index (FIC). The checkerboard method is based on additivity, which assumes that the result observed with multiple drugs is the sum of the separate effects of the drugs being tested; according to this system a FIC of less than 0.5 is scored as synergy, 1 is scored as additive, and greater than 1 but less than 2 is scored as indifferent.

Antibacterial agents suitable for use in combination with derivatives according to the present invention, include e.g. macrolides, such as clarithromycin, azithromycin, and erythromycin, ketolides, lincosamines, such as clindamycin, streptogramins, rifamycins, such as rifampin, rifabutin and rifalazile, fusidic acid, mupirocin, oxazolidinones, glycopeptide antibiotics, such as vancomycin, dalbavancin, telavancin and oritavancin, fluoroquinolones, tetracycline derivatives, hydrophobic derivatives of penicillins, cephalosporins, monobactams, carbapenems, penems and other betalactam antibiotics, novobiocin, pleuromutilins, folate synthesis inhibitors, deformylase inhibitors, and bacterial efflux pump inhibitors. A person skilled in the art of treating Gram-negative in-

fections may easily recognize additional, clinically relevant antibacterial agents that may be useful. Preferably said antibacterial agents are selected from a group of hydrophobic or moderately hydrophobic antibacterial agents against which the outer membrane of Gram-negative bacteria acts as an effective permeability barrier.

The invention also includes the use of the present compounds or combinations thereof to sensitize clinically important bacteria listed herein to the host defence mechanism complement (present in the fresh human and animal serum) by subjecting said bacteria to the action of such compounds during a clinical infection or a suspected infection. The host defence can be exerted, e.g., by the combined action of complement and polymorphonuclear leucocytes.

Those skilled in the art of medicine can readily optimize effective dosages and administration regimens for the compounds according to the present invention as well as for the antibiotics in concurrent administration, taking into account factors well known in the art including type of subject being dosed, age, weight, sex and medical condition of the subject, the route of administration, the renal and hepatic function of the subject, the desired effect, the particular compound according to the present invention employed and the tolerance of the subject to it. Dosages of all antimicrobial agents should be adjusted in patients with renal impairment or hepatic insufficiency, due to the reduced metabolism and/or excretion of the drugs in patients with these conditions. Doses in children should also be reduced, generally according to body weight.

The total daily dose of a derivative according to the present invention administered to a human or an animal can vary, for example, in amounts from 0.1 to 100 mg per kg body weight, preferably from 0.25 to 25 mg per kg body weight.

It will also be recognised by one skilled in the art that the optimal course of treatment, i.e., the number of doses given per day for a defined number of days, will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques.

There is also provided a method for assaying a compound according to the present invention, said compound being a derivative of a natural po-

lymyxin or octapeptin, wherein said derivative has a only 2-3 positive charges, in contrast to the naturally occurring compound from which it is derived, for antibacterial activity against a harmful Gram-negative bacterium and/or for the ability to sensitize it to antibacterial agents and/or the complement present in the serum, said method comprising the step of contacting the bacterium with said derivative of a natural polymyxin or octapeptin, and identifying derivatives possessing antibacterial activity and/or sensitizing activity towards said bacterium.

There is also provided a method to screen polymyxin and octapeptin derivatives with reduced binding to renal tissue or its constituents in or from test animals or from human origin measuring their reduced ability to competitively block the binding of aminoglycosides to the same, or block the binding of other substances known to bind to the same.

In a further aspect there is provided a method for developing novel antibiotics comprising the steps of providing a natural polymyxin or octapeptin compound, or a derivative thereof, having a total of 4 or 5 positive charges, or a total of 6 positive charges, as in deacylpolymyxins, substituting from 1 to 4 residues carrying one or more positive charges with a residue not having a positive charge, or with a covalent bond, thereby generating a polymyxin derivative having 2 or 3 positive charges, assaying said derivative compound for antibacterial activity against Gram-negative bacteria and/or for the ability to sensitize Gram-negative bacteria to an antibacterial agent, and selecting compounds having antibacterial activity against Gram-negative bacteria, or the ability to sensitize Gram-negative bacteria to an antibacterial agent.

There is also provided in accordance with the present invention a semisynthetic polymyxin derivative obtainable by treating chemically or enzymatically naturally-occurring polymyxins or octapeptins, respectively, or those variants thereof which are manufactured by genetically modified organisms. Chemical treatments include, but are not limited to, those with acetanhydride, formic acid, hydrazine, and oxalic acid. Enzymatic treatments include, but are not limited to, with enzymes such as polymyxin deacylase, ficin, papain, bromelain, subtilopeptidases, subtilisin, colistin hydrolase, and Nagarse.

Preferred compounds according to one embodiment are less cationic than natural polymyxins or octapeptins, have two (2) or three (3) positive charges only, and are:

(a) able to inhibit the growth of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa* or *Acinetobacter baumannii* and/or to sensitize any of them to antibiotics, and/or

5 (b) less toxic than clinically used polymyxins, as evidenced in in vivo animal model, and/or

(c) less nephrotoxic than clinically used polymyxins, as evidenced in an animal model and/or in an in vitro test that measures affinity of the compounds to kidney structures, and/or

10 (d) able to cause less histamine liberation from the tissues than clinically used polymyxins when administered topically or when inhaled as an aerosol, and/or

(e) pharmacokinetically more favorable, such as having a longer serum half life and/or by being less inactivated by polyanionic tissue and pus constituents than clinically used polymyxins.

15 Methods for synthesising compounds according to the present invention include but are not limited to the following described below. For a specific compound to be synthesised, an expert in the art is able to choose the appropriate method.

20 1. Semisynthetic derivatives of polymyxins and octapeptins that carry an unchanged heptapeptide part and a modified acyl-aminoacyl side chain can be made by the procedures described as follows:

Protection of the free amino groups in the starting material (polymyxin or octapeptin, or modifications thereof) by methods known to those skilled in the art. The protection can be achieved by the use of residues such as t-butoxycarbonyl (tBoc), fluorenylmethoxycarbonyl (Fmoc), benzyloxycarbonyl (CBZ, Z), allyloxycarbonyl (ALOC), 3-pyridyl-N-oxide-methoxycarbonyl (as described in patent publication GB 1323962), by using Schiff bases such as benzaldehyde by the method described in Japanese Patent publication 30 7115630/1971 or the like which can be removed by conventional conditions compatible with the nature of the product.

In conditions where the poor water solubility occasionally poses a problem in the sub-sequent steps, the protection can be made by using negatively-charged blocking groups such as a sulfonic acid derivative of Fmoc or a carboxylic acid derivative of Fmoc, the method being described in US patent 35 publication 2006004185. The water solubility can also be enhanced by linking

a suitable, removable, negatively charged, very hydrophilic blocking group to the OH-group of threonine.

Thereafter, the compound is subjected to an enzymatic treatment with enzymes such as polymyxin deacylase, polymyxin hydrolase, papain, ficin, bromelain, subtilopeptidase, Nagarse or other enzymes that remove a terminal part of the side chain or even the entire side chain of polymyxin or octapeptin compounds. This treatment can optionally be followed by the Edman degradation procedure. The resultant compound lacks the entire side chain and consists of the cyclic heptapeptide part only, but has a free N-terminal alpha amino group.

Alternatively, polymyxins and octapeptins that have amino groups protected by benzyloxycarbonyl or t-butyloxycarbonyl can be treated by oxalic acid or formic acid to yield protected deacyl derivatives, the method being described by Kurihara *et al.* (1974). The procedure is followed by further enzyme treatment as above and/or by Edman degradation to yield a heptapeptide.

Thereafter, a suitable residue is linked to the free alpha-amino position of the heptapeptide ring portion. The residue might contain an acyl or related residue as well as optionally amino acid residues, preferably up to three residues. For instance, one semisynthetic compound with an acyl group and two amino acid residues can be prepared by adding to the above-described heptapeptide a synthetic N-(acyl)-threonyl-Dthreonyl residue. This can be achieved by conventional general techniques known to those familiar with the art of organic chemistry, these techniques including the use of N-hydroxy-succinimide-linked residues as described in US 2006004185. In this particular synthesis the procedure may involve the use of 2-N-(n-octanoyl)-alanyl-aminobutyl-N-hydroxysuccinimide.

2. Acylated polymyxin nonapeptides carrying three (3) free amino groups. Polymyxin D possesses only four (4) positive charges. It can be treated with papain or ficin by the method described by Chihara *et al.* (1973) and by Vaara and Vaara (1983a,b) to yield the corresponding nonapeptide. The nonapeptide can then be acylated by acylisothiocyanate (by the method well-known to a person skilled in the art and described in US 2006004185, by acyl chloride (by the method well-known to a person skilled in the art and described in Chihara *et al.* 1974), or by using residues linked to N-hydroxy-succinimide (by the method well-known to a person skilled in the art and

described in US 2006004185. The acylated polymyxin D nonapeptide carries only three (3) free amino groups, all in the heptapeptide ring portion.

Alternatively, the free amino groups of polymyxin D can be protected by the means described above. This is followed by an enzymatic treatment and an optional Edman degradation step, to yield a nonapeptide, which can then be acylated by acylisothiocyanate (by the method well-known to a person skilled in the art and described in US 2006004185, by acyl chloride (by the method well-known to a person skilled in the art and described in Chihara *et al.* 1974), or by using residues linked to N-hydroxysuccinimide (by the method well-known to a person skilled in the art and described in US 2006004185, Finally, the protective groups are removed.

In an analogous manner, acylated polymyxin S nonapeptide and acylated polymyxin F nonapeptide can be made. Both carry only three (3) free amino groups.

3. Acylated polymyxin and octapeptin heptapeptides. Heptapeptides can be made by Nagarse treatment of the natural compounds, as described by Kimura *et al.* 1992. Alternatively, they can be made by treatments with other enzymes such as polymyxin acylase, polymyxin hydrolase, ficin, papain, bromelain, and subtilopectidase, followed by optional Edman degradation steps. They can also be made by deacylating the natural compounds by hydrazine or by acids such as formic acid and oxalic acid, followed by Edman degradation steps. The heptapeptide can then be acylated for instance by using the acyl chloride technique well-known to a person skilled in the art and described in Chihara *et al.* (1974). The acylated polymyxin heptapeptide carries only three (3) free amino groups.

4. Totally synthetic polymyxin and octapeptin derivatives can be made by the very conventional methods known for those skilled in the art. Such methods include the liquid-phase synthesis procedures as well as the solid-phase synthesis procedures described for instance by Sakura *et al.* (2004), Tsubery *et al.* (2000a, 2000b, 2002, 2005), and Ofek *et al.* (2004). The methods include e.g. the use of protecting agents such as Fmoc, tBoc, and CBZ at strategic positions, as well as the cyclisation step where DPPA (diphenyl phosphorazidate) or a mixture of benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBop), N-hydroxybenzotriazole (HoBt), and N-methylmorpholine (NMM) is used. Fmoc derivatives of many non-trivial as well as D-amino acids are commercially available.

5. Exemplary reactions related to conversion of the free amino groups to generate compounds according to the present invention having 2 or 3 positive charges may include (but are not limited to) the following reactions:

5 A) reaction of free amine group of the compound with a conjugation moiety comprising a reactive epoxide group, thereby generating a β -hydroxy-amine linkage;

B) reaction of free amine group of the compound with a conjugation moiety comprising a reactive sulphonyl halide, thereby generating a sulfonamide linkage;

10 C) reaction of free amine group of the compound with a conjugation moiety comprising a reactive carboxyl acid, thereby generating an amine linkage;

15 D) reaction of free amine group of the compound with a conjugation moiety comprising a reactive aldehyde group (under reducing conditions), thereby generating an amine linkage;

E) reaction of free amine group of the compound with a conjugation moiety comprising a reactive ketone group (under reducing conditions), thereby generating an amine linkage;

20 F) reaction of free amine group of the compound with a conjugation moiety comprising a reactive isocyanate group, thereby generating a urea linkage.

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EXAMPLES

The following examples illustrate certain embodiments of the present invention and should not be construed as limiting the scope of the invention.

5 Example 1.

Peptide synthesis

Polymyxin derivatives ("NAB peptides" or "NAB compounds") were synthesized by conventional solid phase chemistry, using the standard Fmoc protection strategy. The amino acid at the C-terminus is commercially available
10 as pre-attached to the solid phase and when cleaved off the resin with acid, yields a C-terminal carboxylic acid.

The strategy in the protection was to use three levels of orthogonal protection, temporary Fmoc protection for the alpha amino functions, groups which are removed during the acid cleavage stage, and semi-permanent protection to cover reactive side chain functions while the cyclisation reaction
15 takes place. After cleavage of the peptide from the resin, the C-terminal carboxylic acid is reacted with an amino function on the side chain of one of the amino acids to form a cyclic peptide. After the cyclisation step, the semi-permanent protection groups are removed to yield NAB peptide.

20 Accordingly, the alpha amino function of the amino acid was protected by fluorenyl-methoxycarbonyl (Fmoc) and Fmoc was removed by 20% piperidine in DMF at every cycle. The amino acid that is involved with cyclisation, e.g. diaminobutyric acid, was protected by t-butoxycarbonyl (tBoc), an acid labile group which was removed at the cleavage step. All the other amino
25 acids which have functional side chain groups were protected by a group that is stable to the acid cleavage stage, i.e. benzyloxycarbonyl (Z). Amino acids phenylalanine and leucine naturally needed no side chain protection. The amino terminus was not protected; this enabled direct reaction in the acylation procedure.

30 The synthesis steps were performed in a commercial automatized synthesizer that employed 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) as activator.

6-methylheptanoic acid (6-MHA) was from Ultra Scientific Inc, North Kingstown, RI, USA (product number, FLBA 002). Other fatty acids were from
35 a standard supplier.

The acylation was performed by using a four-fold molar excess of each amino acid or the fatty acid, four-fold molar excess of the activator HCTU (see above), and an eight-fold molar excess of N-methyl morpholine. The reaction time was 30 min.

5 The amino acids were purchased already protected from a standard supplier. The peptide was removed from the resin by reaction with a solution of 95% trifluoroacetic acid and 5% water for 2 hours at room temperature, to yield the partially protected product. The resulting peptide was precipitated with diethyl ether.

10 The cyclisation mixture used was benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop), N-hydroxybenzotriazole (HoBt), and N-methyl morpholine (NMM) at the molar excess of 2, 2, and 4, respectively. The peptide was dissolved in dimethylformamide, the cyclisation mix was added and allowed to react for 2 hours. The cyclised, protected peptide was precipitated by the addition of cold diethyl ether. Any residual PyBop was removed by washing the peptide with water.

15 The remaining side chain protection groups (Z) were removed by catalytic dehydrogenation. The peptide was dissolved in acetic acid-methanol-water (5:4:1), under an atmosphere of hydrogen and in the presence of a palladium charcoal catalyst.

20 The peptide was purified by reverse phase chromatography using conventional gradients of acetonitrile:water:trifluoroacetic acid. The product was dried by lyophilisation.

25 The yield was 20-40 mg representing approx. 20%-40% of the theoretical, calculated from the molar amount (approx. 100 micromoles) of the first amino acyl residue bound to the resin.

30 The purity, as estimated by reversed phase HPLC was more than 95%. For acylated peptides, the Edman degradation product did not reveal any amino acid residue, indicating that the α -amino group of the N-terminal amino acid residue was blocked, as expected, due to the successful N-acylation. Within experimental error, the masses obtained were those expected from the theoretical values.

Example 2.**Direct antibacterial activity of the compounds against *Escherichia coli***

Peptides synthesized in Example 1, all carrying at least two (2) but not more than three (3) positive charges, were studied for their ability to inhibit the growth of *E. coli*. This was tested employing LB agar (LB Agar Lennox, Difco, BD, Sparks, MD, U.S.A) plates. The indicator organism *E. coli* IH3080 (K1:O18) was an encapsulated strain originally isolated from a neonate suffering from meningitis (Vaara *et al.* 1984) and obtained from National Public Health Institute, Helsinki, Finland.

From an overnight-grown culture of IH3080 on LB agar, a suspension of approx. 10^8 cells/ml was prepared in 0.9% NaCl. Aliquots of this suspension were then pipetted on the agar plates and the plates were gently shaken to spread the suspension evenly on the entire surface of the plate. Thereafter, the unabsorbed part of the suspension was removed by using a Pasteur pipette. After the surface had dried, small wells (diameter, 2 mm) were drilled on the plates (five wells per plate) by using a sterile sharp-edged narrow metal tube, single-use pipette tip, and vacuum suction. Samples (4 μ l and 10 μ l) of the peptide solution in 0.9% NaCl (at concentrations of 1 μ g/ml and 0.1 μ g/ml) were then pipetted to the wells and the sample fluids were allowed to absorb. Controls included 0.9% NaCl solution without the compound to be tested. The plates were then incubated for 18 h at 37°C whereafter the diameters of growth inhibition zones around each well were measured; the diameter of the well itself was not reduced. Finally, the diameters were converted to surface areas of growth inhibition (in square mm).

Table 2 shows the antibacterial activity of the derivatives against *E. coli* IH3080 as compared with that of an equal amount of polymyxin B as well as of some polymyxin derivatives not related to the present invention. NAB734, NAB737, NAB739 and NAB740 were the most antibacterial compounds and were even more antibacterial than polymyxin B against *E. coli* IH3080. A well containing 4 μ g of NAB739 produced a growth inhibition area as wide as 133 square mm. In all these four NAB compounds, the side chain consists of two amino acyl residues that carry hydroxyl groups.

Unlike NAB739, NAB7061 was not antibacterial at 4 μ g. However, it displayed notable antibacterial activity at 10 μ g. NAB7061 differs from NAB739 only by carrying Abu (instead of DSer) in R3. Prolonging the length of the fatty acyl part from C8 in NAB7061 to C10 in NAB7062 resulted in notably en-

hanced antibacterial activity manifesting at 4 μ g. In addition, three other peptides (NAB738, NAB716 and NAB719) displayed notable antibacterial activity, albeit clearly weaker than NAB739 and the other most antibacterial compounds.

5 A common property of the compounds directly antibacterial to *E. coli* was the presence of three positive charges out of which either all the three or at least two were located in suitable positions in the cyclic part. In the latter case the relative positions of said charges significantly affected the potency of the antibacterial activity against *E.coli*.

10 Furthermore, as shown in the Table 2, also the structure and length of the side chain has significant influence on the potency of the antibacterial activity. The presence of a side chain consisting of at least two amino acyl residues appears to be important for the compounds antibacterial against *E. coli*, since the compounds lacking either R2 (NAB713) or both R2 and R3 (octanoyl
15 PBHP) also lacked the direct antibacterial activity in the conditions used in the assay. However, it can be anticipated, that the lack of those residues can be compensated by using instead of octanoyl residue, a more extended residue as the R(FA).

Table 2

Structure of the compounds and their antibacterial activity* against *Escherichia coli* IH3080

	Structure**				Positive charges in		Antibacterial activity at	
	FA-part	Peptide sequence***						
		side chain	cyclic part	SEQ ID No.	cyclic part	total	4 µg	10 µg
Reference compounds								
Polymyxin B	MO (H) A	XTX	cy [XXFLXXT]	1	3	5	79	133
Colistin (polymyxin E)	MO (H) A	XTX	cy [XXLLXXT]	2	3	5	ND	ND
Deacylpolymyxin B	-	+XTX	cy [XXFLXXT]	3	3	6	57	113
Deacylcolistin	-	+XTX	cy [XXLLXXT]	4	3	6	79	95
Polymyxin B nonapeptide	-	+TX	cy [XXFLXXT]	5	3	5	0	0
Polymyxin B heptapeptide	-	- +cy	[XXFLXXT]	6	4	4	0	0
NAB704	-	+TZ	cy [XXFLXXT]	7	3	4	0	0
NAB705	-	+ZTZ	cy [XXFLXXT]	8	3	4	0	0
Octanoyl PMBH	OA	-	cy [XXFLXXT]	9	3	3	0	0
Cpds of the present invention								
NAB 739	OA	TS	cy [XXFLXXT]	10	3	3	133	177
NAB 740	DA	TS	cy [XXFLXXT]	10	3	3	95	133
NAB 737	OA	TT	cy [XXFTXXT]	11	3	3	133	201
NAB 734	OA	TS	cy [XXFTXXT]	12	3	3	113	177
NAB 7062	DA	TZ	cy [XXFLXXT]	13	3	3	50	99
NAB 7061	OA	TZ	cy [XXFLXXT]	13	3	3	0	50
NAB 706	MHA	TZ	cy [XXFLXXT]	13	3	3	5	7
NAB 707	MHA	ZTZ	cy [XXFLXXT]	14	3	3	5	7
NAB 716	OA	TX	cy [XXFLXZT]	15	2	3	13	64
NAB 719	OA	TZ	cy [XXFLXXL]	16	3	3	7	50
NAB 738	OA	TA	cy [XXFTXXT]	17	3	3	0	64
NAB 717	OA	TX	cy [XXFLXZT]	18	2	3	0	0
NAB 718	OA	TZ	cy [XXLLXXT]	19	3	3	0	0
NAB 733	OA	AA	cy [XXFLXXT]	20	3	3	0	0
NAB 736	DA	-	cy [XXFLXXT]	9	3	3	0	0
NAB 713	OA	Z	cy [XXFLXXT]	21	3	3	0	0
NAB 715	OA	TX	cy [XZFLXXT]	22	2	3	0	0
NAB 721	MHA	XTX	cy [XXFLZZT]	23	1	3	0	0
NAB 731	OA	TZ	cy [XXFLXXT]	24	3	3	0	0
NAB 710	OA	TZ	cy [XZFLXXT]	25	2	2	0	7
NAB 709	OA	TZ	cy [XXFLXZT]	26	2	2	0	0
NAB 708	OA	TZ	cy [XXFLXZT]	27	2	2	0	0
NAB 725	OA	XTX	cy [XZFLXZT]	28	1	3	0	0
NAB 726	OA	XTX	cy [XZFLXZT]	29	1	3	0	0
NAB 722	MHA	XTX	cy [XZFLZZT]	30	0	2	0	0
NAB 735	OA	XXX	cy [XZFLZZT]	31	0	3	0	0
NAB 701	-	+TX	cy [XXFLZZT]	32	1	3	0	0
NAB 702	-	+TX	cy [XXFLBBT]	33	1	3	0	0
NAB 703	-	+TX	cy [XXFLJJT]	34	1	3	0	0

* Antibacterial activity measured as the growth inhibition (in square millimeters) around a well containing 4 or 10 microgram of a compound on LB plates

** One-letter codes for amino acyl residues: A, Ala; F, Phe; K, Lys; L, Leu; S, Ser; T, Thr; X Dab; Z, Abu; B, N- γ -formyl-Dab; J, N- γ -acetyl-Dab. Underlined letters indicate residues that are in D-configuration. Bold letters indicate residues that carry a positive charge. Bold + indicates the positive charge of the α -amino group in the free N-terminus of the peptide. Abbreviation: cy, cyclo.

*** In the sequence listing X, Z, B, J and amino acids in D-configuration are denoted Xaa, and defined as modified residues (MOD_RES).

Example 3.

10 Direct antibacterial activity of selected NAB compounds against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

Direct antibacterial activity of twelve NAB compounds against *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 27853 was tested by using the susceptibility determination method described in Example 2. The results are shown in Table 3. Five compounds (NAB7062, NAB734, NAB737, NAB739, and NAB740) had notable activity against *A. baumannii*. In Example 2, the same compounds were shown to be very potent against *E. coli*. The antibacterial activity of NAB739 and NAB740 were as strong as or even stronger than that of polymyxin B.

20 Against *P. aeruginosa*, the most active NAB compounds were NAB739, NAB740 as well as NAB736, which is quite inactive against *E. coli* and *Acinetobacter baumannii*. NAB740 was the most active compound and its activity was as strong as that of polymyxin B. All the three NAB compounds lack positive charges in the side chain and were still active against *P. aeruginosa*. This finding is against the conclusion of Srinivasa and Ramachandran (1980a) that the free amino groups in R1 and R3 are essential for the growth inhibition of *P. aeruginosa*.

25 Surprisingly, NAB736 is rather effective against *P. aeruginosa* whereas octanoyl PMBH is much less effective. Accordingly, lengthening the R(FA) part from C8 to C10 has a marked effect on the activity.

Table 3

Antibacterial activity* of twelve (12) novel compounds against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

	<i>A. baumannii</i> ATCC 19606		<i>Ps. aeruginosa</i> ATCC 27853	
	4 µg	10 µg	4 µg	10 µg
NAB 7061	0	0	0	0
NAB 7062	13	38	0	0
NAB 716	0	0	0	0
NAB 717	0	0	0	0
NAB 718	0	0	0	0
NAB 719	0	0	0	0
NAB 736	0	0	38	79
NAB 734	38	95	13	50
NAB 737	38	79	0	28
NAB 738	0	0	0	0
NAB 739	113	177	38	79
NAB 740	133	201	64	133
Polymyxin B	113	154	95	133
PMBN	0	0	113	177
Octanoyl PMBH	nd	nd	0	13

5 * Antibacterial activity measured as the growth inhibition (in square millimeters) around a well containing 4 or 10 µg of the compound on LB plates

Example 4.**Direct antibacterial activity of NAB734 against selected Gram-negative bacteria**

10 The susceptibility of eleven Gram-negative bacterial strains (nine different species) to NAB734 and polymyxin B was compared by using the susceptibility determination method described in Example 2. The strains included those belonging to the species of *Serratia marcescens* and *Proteus mirabilis*, both species generally known to be resistant to polymyxin. Further-
 15 more, the susceptibility determination was also performed by using the Gram-positive bacterium, *Staphylococcus aureus*, also generally known to be polymyxin-resistant. Ten of the strains originated from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.), and one from CCUG (Culture Collection of University of Gothenburg, Sweden). The source of *E. coli* IH3080 has
 20 been described in Example 2. Polymyxin B sulfate was from Sigma-Aldrich (St. Louis, MO, USA).

The results in Table 4 show that NAB734 can be generally regarded to be approximately as potent as polymyxin B against *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Citrobacter freundii*. It

appears to be somewhat less potent than polymyxin B against *Acinetobacter baumannii* and clearly less potent than polymyxin B against *Pseudomonas aeruginosa*. Representatives of known polymyxin-resistant bacterial species were resistant to NAB734 also. This suggests that NAB734 has a very specific
 5 antibacterial action and that its mode of action is quite similar to that of polymyxin B.

Table 4

Antibacterial activity* of NAB 734 against selected Gram-negative bacteria and *Staphylococcus aureus*

Strain	NAB 734		Polymyxin B sulfate	
	4 µg	10 µg	4 µg	10 µg
<i>E. coli</i> ATCC25922	133	177	95	133
<i>E. coli</i> IH3080	113	154	95	133
<i>K. pneumoniae</i> ATCC13883	64	113	79	113
<i>K. pneumoniae</i> CCUG45421	64	104	95	133
<i>K. oxytoca</i> ATCC13182	95	143	79	104
<i>E. cloacae</i> ATCC23355	133	177	95	143
<i>C. freundii</i> ATCC8090	133	177	95	154
<i>A. baumannii</i> ATCC19606	57	79	113	154
<i>P. aeruginosa</i> ATCC27853	13	50	95	133
<i>S. marcescens</i> ATCC8100	0	0	0	0
<i>P. mirabilis</i> ATCC29906	0	0	0	0
10 <i>S. aureus</i> ATCC 25923	0	0	0	0

* Antibacterial activity measured as the growth inhibition (in square millimeters) around wells containing 4 or 10 µg of the compound on LB plates

Example 5.

**The ability of the NAB compounds to sensitize *E. coli* IH3080 to a model
 15 antibiotic rifampin**

Novel NAB peptides according to the present invention and all carrying at least two (2) but not more than three (3) positive charges, were also studied for their ability to sensitize *E. coli* IH3080 to rifampin. This was tested in parallel with the susceptibility determinations described in Example 2 and by
 20 employing LB plates that contain increasing concentrations (0.1 µg/ml, 0.3 µg/ml, 1 µg/ml) of rifampin (Sigma-Aldrich, St. Louis, MO, U.S.A).

Table 5 shows the activity of the NAB compounds (4 µg) against *E. coli* IH3080 in the presence of rifampin (0.1 and 1 µg /ml) as compared with the activity of an equal amount of previously described substances known to
 25 sensitize Gram-negative bacteria to antibacterial agents, i.e. polymyxin B hep-

tapeptide, deacylpolymyxin B, deacylcolistin, polymyxin B nonapeptide, as well as with polymyxin B. The compounds also included octanoyl PMBH, an agent that has not previously been reported to be able to sensitize bacteria to antibiotics.

5 Several NAB compounds sensitized *E. coli* IH3080 to the antibacterial effect of as low a concentration of rifampin as 0.1 µg/ml. In the absence of the compounds tested, a hundred-fold concentration (10 µg/ml) of rifampin was needed for the inhibitory effect. Several compounds that lacked a notable direct antibacterial activity at 4 µg were able to sensitize the target bacterium to
10 rifampin. Such compounds included NAB7061, NAB717, NAB718, and NAB733.

 Furthermore, most of the NAB compounds that had direct antibacterial activity, i.e. antibacterial activity in the absence of rifampin (see Example 2), inhibited the target bacterium even more effectively in the presence of
15 rifampin. The ability of the most active compounds NAB734, NAB737, NAB738, and NAB739 was clearly even better than that of PMBN, the well-known effective permeabilizer of the OM.

 The most active NAB compounds carry three (3) positive charges and have at least two (2) positive charges suitably positioned in the cyclic part,
20 the relative positioning of which affecting the potency of the sensitizing activity. It should indeed be noted, that NAB716, that carries only two positive charges in the cyclic part and has the third positive charge in the form of a Dab residue in R3, is notably able to sensitize *E. coli* to rifampin.

 Amongst the series of compounds all having octanoyl residue as
25 R(FA), octanoyl PMHP, that lacks both R2 and R3, possessed the weakest sensitizing activity, NAB713, that lacks R2, possessed a somewhat better activity, and NAB7061, that possesses both R2 and R3 had a remarkable activity. This indicates that the presence of R2 and R3 is advantageous. However, their absence can be at least partially compensated by lengthening the R(FA) part
30 as in NAB736. It carries decanoyl residue as the R(FA) part, lacks both R2 and R3, and is quite active as a sensitizer.

 NAB compounds that carry both of their two (2) positive charges in the cyclic part were less active than the structurally otherwise analogous NAB compounds that carry all of their three (3) positive charges in the cyclic part, or,
35 for one compound (NAB708), inactive in the study conditions employed.

NAB compounds that carry two (2) positive charges in the side chain and one (1) positive charge in the cyclic part have very modest, if any activity, in the study conditions employed. NAB735, that carries all of its three (3) positive charges in the side chain is inactive in the study conditions employed. Again the relative positions of the said charges in the cyclic part affected the potency of the sensitizing activity.

Table 5

Antibacterial activity of the compounds (4 µg) against *E. coli* IH3080 in the presence of rifampin*

	Antibacterial activity in the presence of rifampin concn (µg/ml) of		
	0	0.1	1.0
<i>Reference and other compounds</i>			
Polymyxin B	79	95	104
Colistin (polymyxin E)	ND	ND	ND
Deacylpolymyxin B	57	79	127
Deacylcolistin	79	87	127
Polymyxin B nonapeptide	0	20	113
Polymyxin B heptapeptide	0	0	38
NAB 704	0	0	24
NAB 705	0	0	5
Octanoyl PMBH	0	0	38
<i>Novel compounds of the present invention</i>			
NAB 739	133	177	201
NAB 740	95	95	95
NAB 737	133	177	201
NAB 734	113	154	201
NAB 7062	50	104	104
NAB 7061	0	113	155
NAB 706	5	79	133
NAB 707	5	87	113
NAB 716	13	133	165
NAB 719	7	79	95
NAB 738	0	133	177
NAB 717	0	71	95
NAB 718	0	13	133
NAB 733	0	95	113
NAB 736	0	113	133
NAB 713	0	20	38
NAB 715	0	0	33
NAB 721	0	13	28
NAB 731	0	0	5 (22**)
NAB 710	0	7	13
NAB 709	0	0	13
NAB 708	0	0	0
NAB 725	0	0	0
NAB 726	0	0	0
NAB 722	0	0	0
NAB 735	0	0	0
NAB 701	0	0	0
NAB 702	0	0	0
NAB 703	0	0	0

* Antibacterial activity measured as the growth inhibition (in square millimeters) around a well containing 4 µg of a compound on plates with no rifampin or rifampin (0.1 or 1.0 µg/ml)

** The value in parentheses was obtained using a well containing 10 µg of the compound

Example 6.**The ability of the NAB compounds to sensitize *Acinetobacter baumannii* and *Pseudomonas aeruginosa* to a model antibiotic rifampin**

NAB peptides related to present invention were also studied for their ability to sensitize *A. baumannii* and *P. aeruginosa* to rifampin (Table 6). This was tested in parallel with the susceptibility determinations described in Example 3 and by employing LB plates that contain increasing concentrations (0.1 µg/ml, 0.3 µg/ml, 1 µg/ml) of rifampin.

Several NAB compounds possessed a very marked ability to sensitize *A. baumannii* to rifampin. The ability of the most active compounds NAB734, NAB737, and NAB739 was clearly even better than that of PMBN, the well-known effective permeabilizer of the OM. NAB739 inhibited the growth of *P. aeruginosa* somewhat better in the presence of rifampin than in its absence.

Table 6

Antibacterial activity* of twelve (12) novel compounds (4 µg) against *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in the presence of rifampin (0.1 or 0.3 µg/ml)

	<i>A. baumannii</i> ATCC 19606			<i>Ps. aeruginosa</i> ATCC 27853		
	0	0.1	0.3	0	0.1	0.3
NAB 7061	0	28	50	0	0	0
NAB 7062	13	50	95	0	0	7
NAB 716	0	0	64	0	0	0
NAB 717	0	0	50	0	0	0
NAB 718	0	0	38	0	0	0
NAB 719	0	154	133	0	0	7
NAB 736	0	95	154	38	38	38
NAB 734	38	201	283	13	20	20
NAB 737	38	201	283	0	20	20
NAB 738	0	95	154	0	13	13
NAB 739	113	177	314	38	64	64
NAB 740	133	154	154	64	64	64
PMBN	0	133	154	113	113	154
Octanoyl PMBH	nd	nd	nd	0	13	13

* Antibacterial activity measured as the growth inhibition (in square millimeters) around a well containing 4 µg of a compound on plates with no rifampin (control) or with rifampin (0.1 or 0.3 µg/ml)

Example 7.**NAB7061 sensitizes *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* to a broad range of antibacterial agents**

5 The minimum inhibitory concentrations (MIC) of a representative set of clinically used antimicrobial agents were determined for two strains of *E. coli* (ATCC25922 and IH3080), *K. pneumoniae* ATCC13883, and *E. cloacae* ATCC23355 by using Mueller-Hinton agar medium (product no LabO39; LabM Ltd., Bury, Lancs, U.K.) in the presence of NAB7061 (4 µg/ml) as well as in its absence. MICs were determined by using E-strips (Biodisk Ltd., Solna, Swe-

10 den) according to the manufacturer's instructions. The NAB7061 concentration used did not itself inhibit the growth of the target bacteria. The MIC of NAB7061 for *E. coli* IH3080 and *K. pneumoniae* ATCC13883 was >16 µg/ml, for *E. coli* ATCC25922 16 µg/ml, and for *E. cloacae* ATCC23355 8 µg/ml.

The results are shown in Table 7. NAB7061 at a concentration of

15 4 µg/ml was able to sensitize the tested strains to rifampin by a factor ranging from 170 to 1500. Sensitization factor is defined as the ratio of the MIC of an antibiotic in the absence of NAB7061 to that in the presence of 4 µg/ml of NAB7061. Extremely high sensitization factors were observed also to clarithromycin (63-380), mupirocin (24-512), azithromycin (31-94), erythromycin (21-

20 48), and for some of the strains, to fusidic acid, quinupristin-dalfopristin, clindamycin, linezolid, and vancomycin. All these antibacterial agents are notably hydrophobic or large (vancomycin) and are known to be excluded by the intact OM of Gram-negative bacteria but penetrate the damaged OM. No significant sensitization (sensitization factor <2, tested by using *E. coli* ATCC25922) was

25 found to piperacillin, ceftazidime, cefotaxime, levofloxacin, ciprofloxacin, meropenem, and tobramycin, all agents that are hydrophilic or relatively hydrophilic and against which the intact OM is not an effective permeability barrier.

Table 7

Sensitization factors* to selected antibacterial agents at NAB 7061 concentration of 4 µg/ml

	<i>E. coli</i> ATCC 25922	<i>E. coli</i> IH 3080	<i>K. pneum.</i> ATCC 13883	<i>E. cloacae</i> ATCC 23355
Rifampin**	250 - 750	170 - 350	250 - 500	750 - 1500
Clarithromycin***	170 - 380	190 - 260	63	85 - 380
Mupirocin***	64 - 170	64 - 85	24 - 32	250 - 512
Azithromycin***	24 - 64	31 - 94	31 - 43	32
Erythromycin***	32 - 48	32 - 42	24 - 48	21 - 43
Fusidic acid	> 43	> 43	> 4	> 8
Quinupristin-dalfopr.	> 21	> 21	1	> 5
Clindamycin	6	12	43	32
Linezolid	> 11	> 8	> 4	> 8
Vancomycin	5	2.5	1	32
Polymyxin B	5	2	1	4
Trimetoprim	4	3	2	3
Moxifloxacin	2.5	4	1.4	4

*Sensitization factor is the ratio of the MIC of the antibiotic in the absence of NAB 7061 to that in the presence of 4 µg/ml of NAB 7061

** Results from five independent determinations

*** Results from two independent determinations

10 Example 8.

Susceptibility of 33 different strains of Gram-negative bacteria to rifampin and clarithromycin in the presence of NAB7061 (4 µg/ml)

The minimum inhibitory concentrations (MIC) of rifampin and clarithromycin for a representative set of different strains of clinically relevant Gram-negative bacteria were determined by the E-test method as in Example 7 and by using Mueller-Hinton agar with or without NAB7061 (4 µg/ml). This concentration of NAB7061 did not itself inhibit the growth of the target bacteria. The strains originated from ATCC (11 strains), CCUG (11 strains), and NCTC (The National Collection of Type Cultures, Colindale, U.K.; 2 strains). Eight strains (the F-strains) were purchased from Mobidiag Ltd., Helsinki, Finland. The source of *E. coli* IH3080 has been given in Example 2. Sensitization factor was defined as in Example 7.

The results are shown in Table 8. For all strains (17) belonging to the group consisting of *E. coli*, *K. oxytoca*, *E. cloacae*, and *C. freundii*, the MIC of rifampin was as low ≤ 0.125 µg/ml in the presence of NAB7061 (4 µg/ml) and the sensitization factor varied from 85 to 2000. Very similar results were obtained with clarithromycin. For fifteen out of seventeen strains belonging to

- the group consisting of *E. coli*, *K. oxytoca*, *E. cloacae*, and *C. freundii*, the MIC of clarithromycin was as low as ≤ 0.25 $\mu\text{g/ml}$ in the presence of NAB7061 (4 $\mu\text{g/ml}$) and for all the 17 strains the sensitization factor varied from 90 to 1000. Strains of *K. pneumoniae* remained somewhat more resistant to both
- 5 antibiotics, and the sensitization factors varied between 10 and 500. For the three strains of *A. baumannii*, the sensitization factors varied between 24 and 125, and the resulting MIC values were quite low (to rifampin ≤ 0.125 $\mu\text{g/ml}$ and to clarithromycin ≤ 0.5 $\mu\text{g/ml}$).

Table 8

The ability of NAB 7061 to sensitize Gram-negative bacteria to model antibiotics (rifampin and clarithromycin)

Bacterial strain	MIC (µg/ml) of rifampin in the presence of 4 µg/ml of NAB 7061*	Sensitization factor**	MIC (µg/ml) of clarithromycin in the presence of 4 µg/ml of NAB 7061	Sensitization factor***
<i>E. coli</i> ATCC25922****	0,016 - 0,047	250-750	0,094 - 0,125	170-400
<i>E. coli</i> IH3080****	0,023 - 0,047	170-350	0,047 - 0,064	190-260
<i>E. coli</i> CCUG41421	0,032 - 0,064	125	0,125	400
<i>E. coli</i> CCUG41422	0,094 - 0,125	170 - 256	0,25	100
<i>E. coli</i> CCUG41424	0,064 - 0,094	85 - 94	0,125	130
<i>E. coli</i> CCUG41425	0,016 - 0,023	200 - 260	0,047	340
<i>E. coli</i> CCUG41427	0,032 - 0,064	100 - 250	0,064	250
<i>E. coli</i> CCUG41429	0,032	125 - 250	0,064	250
<i>E. coli</i> CCUG41432	0,032	180 - 250	0,064	90
<i>E. coli</i> NCTC13351	0,032 - 0,047	340 - 500	0,032	750
<i>E. coli</i> NCTC13353	0,064	125 - 250	1	260
<i>K. pneumoniae</i> ATCC13883****	0,064 - 0,125	250 - 500	0,19	60
<i>K. pneumoniae</i> CCUG45421	2 - 3	10 - 20	24	10
<i>K. pneumoniae</i> F145	0,19 - 0,25	170	0,25	170
<i>K. pneumoniae</i> F144	0,19	>170	0,75	64
<i>K. pneumoniae</i> F136	0,75	>43	2	24
<i>K. oxytoca</i> ATCC13182	0,032 - 0,047	680 - 750	0,25	260
<i>K. oxytoca</i> CCUG51683	0,012 - 0,023	700 - 2000	0,19	250
<i>E. cloacae</i> ATCC23355****	0,008 - 0,016	750-1500	0,25 - 0,75	90-400
<i>E. cloacae</i> CCUG52947	0,032 - 0,047	500 - 1000	0,38	350
<i>E. cloacae</i> F230	0,016 - 0,032	1500	0,047	1000
<i>E. cloacae</i> F232	0,023	1400	0,094	500
<i>C. freundii</i> ATCC8090	0,023 - 0,032	500 - 1000	0,125	250
<i>Ac. baumannii</i> ATCC19606	0,094 - 0,125	24 - 32	0,5	50
<i>Ac. baumannii</i> F263*****	0,032 - 0,19	21 - 125	0,38	40
<i>Ac. baumannii</i> F264	0,125	32	0,25	100
<i>S. maltophilia</i> ATCC17444	2	4 - 8	64	4
<i>S. marcescens</i> ATCC8100	16	<2	96	<2
<i>P. mirabilis</i> ATCC29906	1 - 6	<2	24	<2
<i>P. vulgaris</i> ATCC13315	1 - 1,5	<2	24	<2
<i>P. aeruginosa</i> ATCC27853	12 - 16	2	32	<2
<i>P. aeruginosa</i> CCUG51971	>32	<2	64	<2
<i>P. aeruginosa</i> F58	>32	<2	256	<2

5

* Results from two independent determinations

** Sensitization factor is the ratio of rifampin MIC in the absence of NAB 7061 to that in the presence of 4 µg/ml of NAB 7061

*** Sensitization factor is the ratio of clarithromycin MIC in the absence of NAB 7061 to that in the presence of 4 µg/ml of NAB 7061

10

**** Results from five (rifampin) and two (clarithromycin) independent determinations

***** Results from three independent determinations (rifampin)

Example 9.**NAB7061 sensitizes carbapenem-resistant strains of *Acinetobacter* to carbapenems**

The minimum inhibitory concentrations (MIC) of two carbapenems, imipenem and meropenem, for three strains of *A. baumannii* were determined by the E-test method as in Example 7 and by using Mueller-Hinton agar with or without NAB7061 (4 µg/ml). This concentration of NAB7061 did not itself inhibit the growth of the target bacteria. Sensitization factor was defined as in Example 7. The results are shown in Table 9. NAB7061 sensitized both carbapenem-resistant strains (F263, F264) to both carbapenems by a factor ≥ 4 .

Table 9

Susceptibility of imipenem and meropenem against *Acinetobacter baumannii* strains in the absence of NAB 7061 and in the presence of NAB 7061 (4 µg/ml)

Strain	MIC (µg/ml) of imipenem		MIC (µg/ml) of meropenem	
	at the indicated concn (µg/ml)		at the indicated concn (µg/ml)	
	of NAB 7061		of NAB 7061	
	0	4	0	4
<i>A. baumannii</i> ATCC19606	0.38	0.38	1.5	0.75
<i>A. baumannii</i> F263	>32	8	>32	6
<i>A. baumannii</i> F264	24	6	32	4

Example 10.**NAB7061 sensitizes *E. coli* to the complement in fresh normal serum**

The ability of NAB7061 to sensitize encapsulated, smooth strain of *E. coli* to the bactericidal action of normal guinea pig serum (GPS) was studied by the method described by Vaara *et al.* (1984). *E. coli* IH3080 (018,K1) was grown in LB broth (LB broth Lennox, Difco, BD, Sparks, MD, U.S.A) at 37°C in a rotary shaker into early logarithmic growth phase, washed with PBS (phosphate-buffered saline, 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ x2H₂O and 0.2 g of KH₂PO₄ per liter) and resuspended in PBS, to approx. 10⁹ cells/ml). GPS was used as complement source. It was stored at -70°C before use. To inactivate the complement, serum was incubated at 56°C for 30 min.

The experimental procedure was as follows. 10% GPS in PBS was inoculated with approx. 500 CFU (colony forming units) of bacteria per ml and pipetted in 0.2 ml aliquots into wells of microtiter plates. The wells already contained increasing amounts of NAB7061 in 0.020 ml of 0.9% NaCl. The plate

was incubated at 37°C for 2 h whereafter each well was emptied onto LB plates. The plates were incubated overnight at 37°C and the developed colonies were counted.

The results are shown in Table 10. NAB7061 itself did not significantly reduce CFU count in the absence of GPS or in the presence of heat-inactivated 10% GPS. However, as low a concentration of NAB7061 as 2 µg/ml was sufficient to reduce CFU count by a factor of approx. 100 in the presence 10% fresh GPS. Accordingly, NAB7061 acts synergistically with the bactericidal complement machinery present in fresh serum, as does PMBN, the agent well known to have this property.

Table 10

The synergistic bactericidal activity of NAB7061 and 10% guinea pig serum (GPS) against *E. coli* IH3080 (O18:K1)*

	Concentration of NAB7061 (µg/ml)			
	0	1	2	4
none (PBS)	100	97	97	79
10% GPS	270	230	2	0
10% GPS, heat inactivated	500	500	500	250

* measured as % survival after 2-hour treatment at 37°C

Example 11.

Reduced affinity of NAB7061 to the brush-border membrane (BBM) of the renal cortex

The binding of the compounds according to this invention to isolated brush-border membrane (BBM) from the renal cortex can be measured indirectly by measuring their ability to inhibit the binding of radiolabelled gentamycin to BBM. Accordingly, the compounds according to this invention that have less affinity to BBM than for example polymyxin B inhibit the binding of radiolabelled gentamycin to a lesser degree than does polymyxin B.

BBM was isolated from the renal cortex of male albino rats by using the Mg²⁺/EGTA precipitation technique as described by Nagai *et al.* (2006). The binding of gentamycin was measured, according to the method described by Nagai *et al.* (2006) by incubating BBM vesicles (20 µl) in 10 mM HEPES (pH 7.5) with 100 mM mannitol in the presence of 20 µM [³H] gentamycin (Amersham Biosciences Inc., Buckinghamshire, U.K.) with or without the compound to be tested or a positive control. After an incubation of 60 min at 4°C, 1 ml of

ice-cold buffer described above was added, and the mixture was filtered through a Millipore filter (0.45 μm ; HAWP). The filter was washed with the buffer and the radioactivity remaining in the filter was measured by using a liquid scintillation counter. The IC_{50} values were determined as in Nagai *et al.* (2006) using the Hill equation.

The IC_{50} values (μM) for the NAB compound studied and the controls were the following: 187.3 ± 24.3 for NAB7061 (average of two independent experiments, each with three parallel determinations), 39.3 ± 5.5 for polymyxin B (average of two independent experiments, each with three parallel determinations), and 90.2 ± 9.7 for unlabelled gentamycin (three parallel determinations). Accordingly, the affinity of NAB7061 to BBM is only approximately half of the affinity of gentamycin to BBM and approximately one fifth of the affinity of polymyxin B to BBM.

Example 12.

15 The activity of NAB7061 in experimental *E. coli* peritonitis model in mice

A suspension of *E. coli* IH3080 (K1:O18) in saline (0.9% NaCl) was prepared from an overnight culture on a blood agar plate (Statens Serum Institut, Copenhagen, Denmark). All mice (female NMR1 from Harlan Scandinavia, Allerød, Denmark; weight, 25-30 g) were inoculated intraperitoneally with 0.5 ml of the suspension containing 0.96×10^6 CFU per ml in the lateral lower quadrant of the abdomen. At 1h, the CFU count was determined from three mice and the remaining mice (four mice per group) were treated with a subcutaneous injection of 0.2 ml of erythromycin solution in saline (corresponding to 5 mg/kg body weight) or NAB7061 solution in saline (corresponding to 5 mg/kg body weight), or both erythromycin and NAB7061 (corresponding to 5 mg/kg body weight of both drugs; given at two separate sites). Control group received two 0.2 ml injections of saline. At 4.5 h postinfection, all mice were anaesthetized with CO_2 and sacrificed. Sterile saline (2 ml) was injected intraperitoneally and the abdomen was gently massaged before it was opened and the fluid sampled. Appropriate dilutions of the fluid were plated on blood agar plates, the plates were incubated overnight, and the colonies were counted.

At 1 h postinfection, the CFU count was $0.74 (\pm 0.7) \times 10^6$ per ml. At 4.5 h postinfection (corresponding to 3.5 h after the treatment), the CFU counts (per ml) were $11.1 (\pm 6.2) \times 10^6$ (control group), $8.9 (\pm 6.4) \times 10^6$ (erythromycin group), $1.1 (\pm 0.6) \times 10^6$ (NAB 7061 group), and $2.1 (\pm 1.2) \times 10^6$ (NAB plus

erythromycin group). Accordingly, in the absence of NAB 7061, the bacterial count increased by a factor of 15 (saline group) or by a factor of 12 (erythromycin group), while in the presence of NAB 7061, the corresponding factors ranged from 1.5 to 3.

5 Example 13.

Toxicity studies on NAB7061

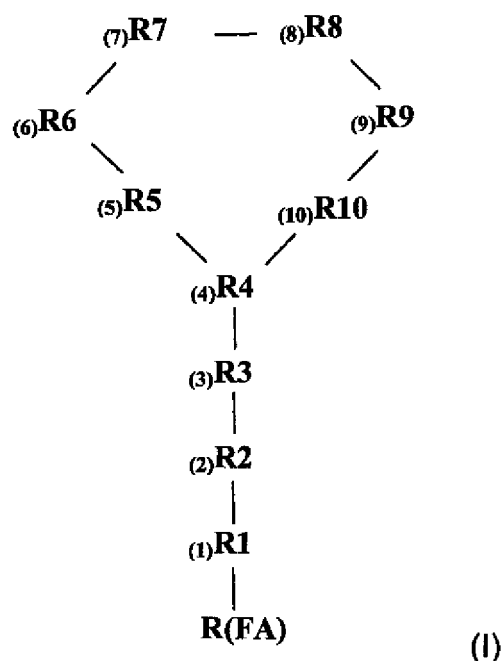
Toxicity in young rats (weighing approx. 150 g in the beginning of the study) was determined by administering doses (1, 2, 4, 8, 16, and 32 mg/kg per day) of NAB7061 as well as the control compound polymyxin B intravenously twice (2) a day for two weeks. A group of ten (10) rats was studied for each dosing regimen. Clinical observations were made daily, the body weight measured twice weekly, and the food consumption twice weekly. In the end of week 2, all animals were sacrificed.

The control compound, polymyxin B negatively influenced the body weight gain at as low a dose as 1 mg/kg per day whereas the lowest dose of NAB7061 that had this effect was 8 mg/kg. Polymyxin B caused mortality at 32 mg/kg per day (100% mortality), whereas all rats receiving NAB7061 remained alive throughout the study. In the end of the study, blood urea nitrogen (BUN) was 15% higher in the group receiving polymyxin 16 mg/kg per day than in the control or low dose polymyxin group (1 mg/kg per day). In the group that received NAB7061 at the dose of 16 mg/kg per day, no such a rise was found, and in the group that received NAB7061 at the dose of 32 mg/kg per day, the rise was 7%.

Histopathology of kidneys was performed for each animals and histopathology of all tissues was performed in three high-dose groups receiving NAB7061. No NAB7061-related pathological findings were found in the general pathology and histopathology of any organs or in the histopathology of kidneys in any animals that received NAB7061.

CLAIMS

1. A polymyxin derivative of the general formula (I),



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wherein R1, R2 and R3 are optional; **characterized in that** R1, R2, R3, R5, R8 and R9 are cationic or neutral amino acid residues selected so that the total number of positive charges at physiological pH is at least two but no more than three;

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with the proviso that R8 and R9 are not both formylated when R(FA)-R1-R2-R3 constitutes the native polymyxin B side chain; and R4 is not directly linked to octanoyl residue when R4-R10 constitutes a native polymyxin B ring structure;

or a pharmaceutically acceptable salt thereof.

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2. The derivative according to claim 1, wherein said positive charges are selected from the group consisting of free, unsubstituted amino groups and other cationic groups.

3. The derivative according to any one of claims 1-2, wherein R1-R10 is selected from the group consisting of SEQ ID NO: 9-26.

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4. The derivative according to any one of claims 1-3, wherein R(FA) is selected from the group consisting of OA, DA and MHA.

5. The derivative according to any one of claims 1-4, wherein the number of said positive charges is three.

6. The derivative according to claim 5, wherein R1-R10 is selected from the group consisting of SEQ ID NO: 9-20.

7. The derivative according to claim 6, selected from the group consisting of OA-SEQ ID NO. 10, DA-SEQ ID NO. 10, OA-SEQ ID NO. 11, OA-SEQ ID NO. 12, DA-SEQ ID NO. 13, OA-SEQ ID NO. 13, MHA-SEQ ID NO. 13, MHA-SEQ ID NO. 14, OA-SEQ ID NO. 15, OA-SEQ ID NO. 16, OA-SEQ ID NO. 17, OA-SEQ ID NO. 18, OA-SEQ ID NO. 19, OA-SEQ ID NO. 20, and DA-SEQ ID NO. 9.

8. A combination product comprising two or more of the derivatives according to any of claims 1 to 7.

9. A pharmaceutical composition comprising at least one derivative according to any one of claims 1 to 7, and at least one pharmaceutically acceptable carrier and/or excipient.

10. The pharmaceutical composition according to claim 9, further comprising an antibacterial agent.

11. A method for treating, alleviating or ameliorating an infection in a subject caused by a Gram-negative bacterium, comprising administering a therapeutically effective amount of a derivative according to any one of claims 1 to 7 or a combination according to claim 8.

12. The method according to claim 11, wherein said bacterium is selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

13. A method for sensitizing Gram-negative bacteria to an antibacterial agent, comprising administering, simultaneously or sequentially in any order, a therapeutically effective amount of said antibacterial agent and a derivative according to any one of claims 1 to 7 or a combination according to claim 8.

14. The method according to claim 13, wherein said antibacterial agent is selected from the group consisting of clarithromycin, azithromycin, erythromycin and other macrolides, ketolides, clindamycin and other lincosamines, streptogramins, rifampin, rifabutin, rifalazile and other rifamycins, fusidic acid, mupirocin, oxazolidinones, vancomycin, dalbavancin, telavancin, oritavancin and other glycopeptide antibiotics, fluoroquinolones, bacitracin, tetracycline derivatives, betalactam antibiotics, no-

vobiocin, pleuromutilins, folate synthesis inhibitors, deformylase inhibitors, and bacterial efflux pump inhibitors.

15 15. The method according to claim 14, wherein said anti-bacterial agent is selected from the group consisting of: clarithromycin, azithromycin, erythromycin, clindamycin, the streptogramin combination
5 quinupristin-dalfopristin, rifampin, fusidic acid, mupirocin, the oxazolidinone linezolid, vancomycin, the fluoroquinolone moxifloxacin, and the folate synthesis inhibitor trimetoprim.

10 16. The method according to claim 13, wherein said bacterium is selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

15 17. A method for developing novel antibiotics comprising the steps of

 a) providing a natural polymyxin or octapeptin compound, or a derivative thereof, having a total of 4 to 6 positive charges;

 b) substituting from 1 to 4 residues carrying one or more positive charges with a residue not having a positive charge, or with a covalent bond, thereby generating a derivative of a polymyxin compound having 2 or
20 3 positive charges;

 c) assaying said derivative compound for antibacterial activity against Gram-negative bacteria; or the ability to sensitize Gram-negative bacteria to an antibiotic; and

 d) selecting compounds having antibacterial activity against
25 Gram-negative bacteria, or the ability to sensitize Gram-negative bacteria to an antibacterial agent.

18. A method for reducing the toxicity of natural polymyxins, octapeptins and their derivatives during application thereof in the treatment of infections in an individual, said method comprising the steps of:

30 a) providing a natural polymyxin or octapeptin compound, or a derivative thereof, having a total of 4 to 6 positive charges;

 b) substituting from 1 to 4 residues carrying one or more positive charges with a residue not having a positive charge, or with a covalent bond, thereby generating a derivative having 2 or 3 positive charges; and

35 c) administering said derivative to said individual.

19. The method according to claim 18, wherein said toxicity is nephrotoxicity.

20. A method for improving the pharmacokinetic properties, of natural polymyxins, octapeptins and their derivatives, said method comprising the steps of:

a) providing a natural polymyxin or octapeptin compound, or a derivative thereof, having a total of 4 to 6 positive charges;

b) substituting from 1 to 4 residues carrying one or more positive charges with a residue not having a positive charge, or with a covalent bond, thereby generating a derivative having 2 or 3 positive charges;

wherein said improved pharmacokinetic properties consist of prolonged serum half life; or lower susceptibility to the inactivation by polyanionic tissue and pus constituents, as compared to the starting compound.

21. A method for sensitizing clinically important Gram-negative bacteria to a host defence mechanism complement present in the serum, wherein said bacteria are subjected to the action of a derivative according to any one of claims 1 to 7 during a clinical infection.

22. The method according to claim 21, wherein said bacteria are selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

23. Use of a derivative according to any one of claims 1 to 7 in the manufacture of medicament for treating infections caused by Gram-negative bacteria.

24. The use according to claim 23, wherein said bacteria are selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

25. Use of a derivative according to any one of claims 1 to 7 for the manufacture of a medicament for sensitizing Gram-negative bacteria against antibacterial agents.

26. The use according to claim 25, wherein said bacteria are selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

27. The use according to claim 25, wherein said antibacterial agent is selected from the group consisting of clarithromycin, azithromycin, erythromycin and other macrolides, ketolides, clindamycin and other linco-samines, streptogramins, rifampin, rifabutin, rifalazile and other rifamycins, fusidic acid, mupirocin, oxazolidinones, vancomycin, dalbavancin, telavancin, oritavancin and other glycopeptide antibiotics, fluoroquinolones, bacitracin, tetracycline derivatives, betalactam antibiotics, novobiocin, pleuromutilins, folate synthesis inhibitors, deformylase inhibitors, and bacterial efflux pump inhibitors.

28. The use according to claim 27, wherein said antibacterial agent is selected from the group consisting of: clarithromycin, azithromycin, erythromycin, clindamycin, the streptogramin combination quinupristin-dalfopristin, rifampin, fusidic acid, mupirocin, the oxazolidinone linezolid, vancomycin, the fluoroquinolone moxifloxacin, and the folate synthesis inhibitor trimetoprim.

29. Use of a derivative according to any one of claims 1 to 7 for the manufacture of a medicament for sensitizing Gram-negative bacteria to a host defence mechanism complement present in the serum.

30. The use according to claim 29, wherein said bacteria are selected from the group consisting of: *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

31. A process for preparing a polymyxin derivative of formula (I) as defined in claim 1 comprising modifying a natural or synthetic polymyxin or octapeptin compound or a derivative thereof having 4 to 6 positively charged residues by replacing 1 to 4 of said residues by neutral residues, or by a covalent bond, or converting 1 to 4 of said residues into neutral residues in order to obtain a polymyxin derivative of formula (I) according to claim 1, having 2 or 3 positively charged residues.

32. The process according to claim 31 comprising carrying out the process as a total synthetic process.

33. The process according to claim 31 comprising carrying out the process as a semisynthetic process.

34. The process according to claim 33 comprising the steps of:
a) subjecting a natural or synthetic polymyxin or octapeptin compound or a derivative thereof to cleavage in order to remove the side chain

of said polymyxin compound, and recovering the cyclic part of said compound, and

b) coupling to the cyclic part obtained in step a) a synthetically prepared side chain in order to obtain a polymyxin derivative of formula (I) according to claim 1.

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35. The process according to claim 34 comprising carrying out the cleavage in step a) enzymatically.

36. The process according to claim 34 comprising carrying out the cleavage in step a) chemically.

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37. The process according to claim 34 comprising carrying out the cleavage in step a) using a combination of both chemical and enzymatic treatments.