

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2005326770 B2**

- (54) Title
Peptide antibiotics and methods for making same
- (51) International Patent Classification(s)
C07K 7/62 (2006.01)
- (21) Application No: **2005326770** (22) Date of Filing: **2005.07.01**
- (87) WIPO No: **WO06/083317**
- (30) Priority Data
- (31) Number (32) Date (33) Country
10/881,160 **2004.07.01** **US**
- (43) Publication Date: **2006.08.10**
(44) Accepted Journal Date: **2011.11.03**
- (71) Applicant(s)
Biosource Pharm, Inc.
- (72) Inventor(s)
Curran, William V.;Francis, Noreen;Leese, Richard A.;Borders, Donald B.;Jarolmen, Howard
- (74) Agent / Attorney
Cullens Patent and Trade Mark Attorneys, Level 32 239 George Street, Brisbane, QLD, 4000
- (56) Related Art
Storm D.R. et al., Annual Review of Biochemistry, 1977, vol. 46, pages 723-763
Nakajima K., Chemical & Pharmaceutical Bulletin, 1967, vol. 15, pages 1219-1224
de Visser P.C. et al., Journal of Peptide Research, 2003, vol. 61, pages 298-306

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 August 2006 (10.08.2006)

PCT

(10) International Publication Number
WO 2006/083317 A3

(51) International Patent Classification:
C07K 7/62 (2006.01)

(21) International Application Number:
PCT/US2005/023343

(22) International Filing Date: 1 July 2005 (01.07.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/881,160 1 July 2004 (01.07.2004) US

(71) Applicant (*for all designated States except US*):
BIOSOURCE PHARM, INC. [US/US]; 135 Route
59 East, Spring Valley, New York 10977 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **LEESE, Richard, A.** [US/US]; 7 Marget Ann Lane, Suffern, New York 10901 (US). **FRANCIS, Noreen** [US/US]; 85 Wilder Road, Suffern, New York 10901 (US). **CURRAN, William, V.** [US/US]; 27 Harding Street, Pearl River, New York 10965 (US). **BORDERS, Donald, B.** [US/US]; 13 Heatherhill Lane, Suffern, New York 10901 (US). **JAROLMEN, Howard** [US/US]; 900 Ridgewood Blvd. E., Township of Washington, New Jersey 07676 (US).

(74) Agent: **LILLING, Bruce**; LILLING & LILLING, PC,
P.O. Box 560, Goldens Bridge, New York 10526 (US).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM,

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:
18 January 2007

(48) Date of publication of this corrected version:
1 March 2007

(15) Information about Correction:
see PCT Gazette No. 09/2007 of 1 March 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PEPTIDE ANTIBIOTICS AND METHODS FOR MAKING SAME

(57) Abstract: Disclosed herein are novel peptides and protected peptides. These compounds can be derived from naturally occurring peptides, such as those selected from colistin, circulin A, polymyxin A, polymyxin B, polymyxin D, octapeptin B, octapeptin C, and [Ile⁷]polymyxin B₁. Also disclosed are pharmaceutical compositions containing the new peptides, as well as methods for preparing the novel peptides and protected peptides.



WO 2006/083317 A3

PEPTIDE ANTIBIOTICS AND METHODS FOR MAKING SAME**RELATED APPLICATION**

[001] This Application claims the benefit of U.S. Application Serial No. 10/881,160, filed July 1, 2004, which is being converted to a Provisional Application, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[002] Disclosed herein are novel peptides and novel protected peptides derived from polymyxin and octapeptin peptides, including, e.g., colistin, circulin A, polymyxin A, polymyxin B, polymyxin D, octapeptin B, octapeptin C, and [Ile⁷]polymyxin B₁. The novel peptides and novel protected peptides have antibacterial properties. Also disclosed are pharmaceutical compositions containing the novel peptides and novel protected peptides, as well as methods for preparing the novel peptides and novel protected peptides.

BACKGROUND

[003] Gram-negative bacteria that are resistant to aminoglycoside, β -lactam, and fluoroquinolone antibiotics are increasingly common. These bacteria are often susceptible to the polymyxins and related peptides having antibacterial properties (Refs. 1, 10, 23). As a result, there is interest in the use of polymyxins for multidrug-resistant gram-negative bacterial infections in humans (Ref. 23).

[004] Peptides such as polymyxin B and the related colistin (polymyxin E) have been administered to humans as antibacterial agents. However, their use has been previously restricted because of their toxicity. These peptides comprise a seven amino acid cyclic peptide attached to an exocyclic three amino acid chain, wherein the N-terminal amine of the exocyclic chain is linked to a "side chain" or "tail". The tail is most commonly an acyl group.

[005] Some renal toxicity has been observed with the recommended dosing of polymyxin B in patients. Neurotoxicity has also been observed in patients with compromised renal functions, with an overall incidence of 7.3%

reported in one large study with colistin (Ref. 1). The acyl exocyclic chain and the adjacent N-terminal 2,4-diaminobutanoic acid (Dab) residue can be enzymatically removed from polymyxin, thereby yielding the corresponding nonapeptide. The *in vivo* toxicity of the nonapeptide of polymyxin B is significantly less than that of polymyxin B itself (Ref. 16). The toxicity of the nonapeptide in cell culture is reduced by about 100-fold relative to polymyxin B; however, the antibacterial activity of the nonapeptide is also reduced by about 2 - 64 fold relative to polymyxin B (Ref. 11).

[006] Attempts have been made to chemically modify polymyxin and colistin in order to obtain peptides with improved antibacterial properties. For example, the total synthesis of polymyxin B and four analogs was previously accomplished by a combination of solid phase peptide syntheses to obtain linear structures, followed by removal from the resin and condensation in solution at high dilution to obtain the cyclic peptide structure (Ref. 7). The derivatives, however, were less active than polymyxin B. A more recent total synthesis of polymyxin B and a few closely related compounds was accomplished only by solid phase peptide synthesis (Refs. 15, 26). Although both of these solid phase total synthetic approaches can provide new derivatives of polymyxin, these methods appear limited since the quantities of antibiotic produced are small and require large amounts of amino acid precursors. Any scale up of these methods for clinical studies may prove to be difficult and costly.

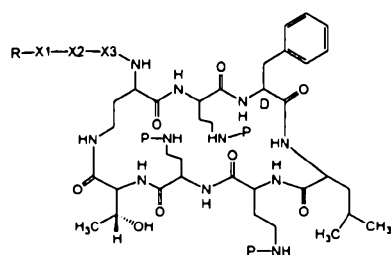
[007] Accordingly, there remains an ongoing need for new peptide compounds having antibacterial properties, and new methods for preparing such compounds.

SUMMARY

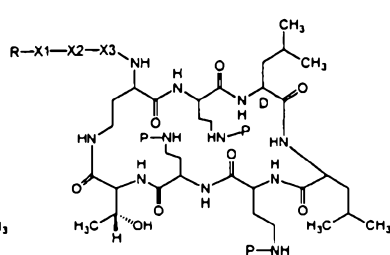
[008] Disclosed herein are novel peptides, such as peptide antibiotics and/or other peptides having antibacterial properties, and methods for preparing the peptides. The compounds disclosed herein can provide structural diversity in the exocyclic region (the exocyclic amino acids and tail) of the cyclic peptide.

[008a] Definitions of the specific embodiments of the invention as claimed herein follow.

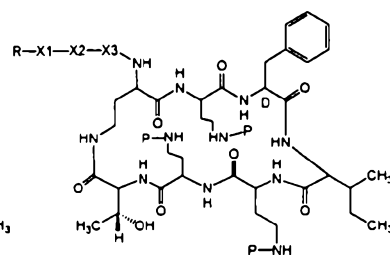
[008b] According to a first embodiment of the invention, there is provided an antibiotic compound or protected antibiotic compound selected from the following structures wherein R, X1, X2, X3, and P are as specified below:



PBpeptide derivatives



Cpeptide derivatives



ILpeptide derivatives

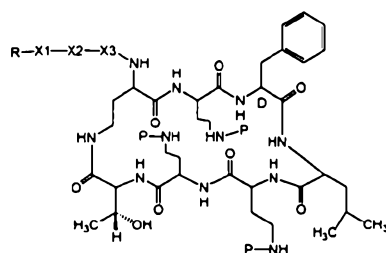
Compound	R	Peptide	X1*	X2	X3*	P
1	n-C ₉ H ₁₉ CO-	PBpeptide	Dab	Thr	Dab	H
1P	n-C ₉ H ₁₉ CO-	PBpcptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
3	n-C ₈ H ₁₇ NHCO-	PBpeptide	Dab	Thr	Dab	H
3P	n-C ₈ H ₁₇ NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
4	phenyl-NHCS-	PBpcptide	Dab	Thr	Dab	H
4P	phenyl-NHCS-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
5	phenyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
5P	phenyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
6	phenyl-CO-	PBpeptide	Dab	Thr	Dab	H
6P	phenyl-CO-	PBpcptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
7	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab	Thr	Dab	H
7P	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab	Thr	Dab	H
8P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
16	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab	Thr	Dab	H
16P	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
19	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab	Thr	Dab	H
19P	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpeptide	Dab	Thr	Dab	H
20P	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpcptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
21	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab	Thr	Dab	H
21P	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
22	benzyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
22P	benzyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
23	cyclohexyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
23P	cyclohexyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
48	cyclohexyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
48P	cyclohexyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-

Compound	R	Peptide	X1*	X2	X3*	P
51	$n\text{-C}_8\text{H}_{17}\text{NHCO-}$	Cpeptide	Dab	Thr	Dab	H
51P	$n\text{-C}_8\text{H}_{17}\text{NHCO-}$	Cpeptide	Dab-P	Thr	Dab-P	$\text{HSO}_3\text{-Fmoc-}$
53	$4\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_2\text{-}$	Cpeptide	Dab	Thr	Dab	H
53-P	$4\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_2\text{-}$	Cpeptide	Dab-P	Thr	Dab-P	$\text{HSO}_3\text{-Fmoc-}$
54	$n\text{-C}_9\text{H}_{19}\text{CO-Lys}$	PBpeptide	Dab	Thr	Dab	H
54P	$n\text{-C}_9\text{H}_{19}\text{CO-Lys}$	PBpeptide	Dab-P	Thr	Dab-P	$\text{HSO}_3\text{-Fmoc-}$

*Dab-P is 4-N-($\text{HSO}_3\text{-Fmoc}$)-diaminobutyl, Lys-P is 6-N-($\text{HSO}_3\text{-Fmoc}$)-lysyl

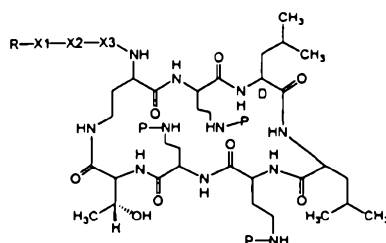
All amino acids are the L-isomers unless indicated otherwise.

[008c] According to a second embodiment of the invention, there is provided an antibiotic compound having one of the following structures:



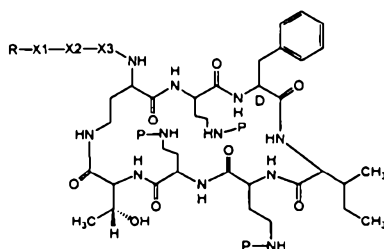
PBpeptide derivatives

(PB);



Cpeptide derivatives

(C); or



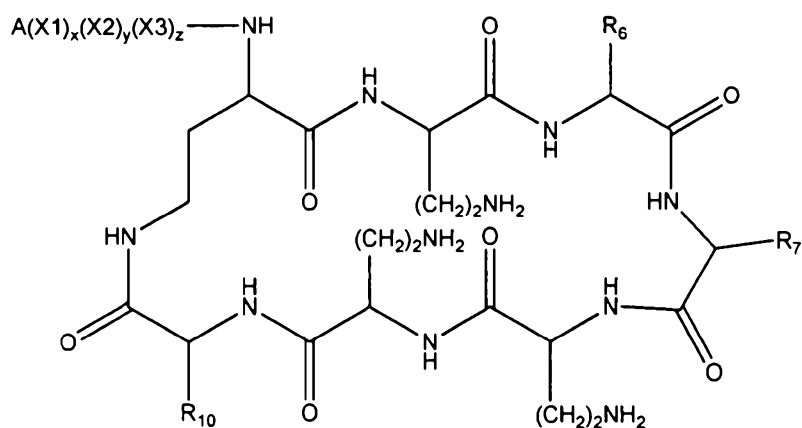
ILpeptide derivatives

(IL)

for use against gram-negative and gram-positive bacteria, wherein the cyclic polypeptide, R, X1, X2, and X3 are as specified below:

Compound	R	X1*	X2	X3*	Cyclic peptide
1	n-C ₉ H ₁₉ CO-	Dab	Thr	Dab	PB
3	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	PB
4	phenyl-NHCS-	Dab	Thr	Dab	PB
5	phenyl-NHCO-	Dab	Thr	Dab	PB
6	phenyl-CO-	Dab	Thr	Dab	PB
7	2-naphthyl-OCH ₂ -CO-	Dab	Thr	Dab	PB
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	PB
16	n-C ₉ H ₁₉ CO-Phe-	Dab	Thr	Dab	PB
19	C ₆ H ₅ CH ₂ OCH ₂ -CO-	Dab	Thr	Dab	PB
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	Dab	Thr	Dab	PB
21	4-Cl-C ₆ H ₄ -NHCO-	Dab	Thr	Dab	PB
22	benzyl-NHCO-	Dab	Thr	Dab	PB
23	cyclohexyl-NHCO-	Dab	Thr	Dab	PB
48	cyclohexyl-NHCO-	Dab	Thr	Dab	IL
51	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	C
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	C
54	n-C ₉ H ₁₉ CO-Lys-	Dab	Thr	Dab	PB

[008d] According to a third embodiment of the invention, there is provided an antibiotic compound of the formula:



or a pharmaceutically acceptable salt thereof, wherein:

A is selected from R'-(C=O)-(p-aminophenylacetyl)-, R'-(C=O)-Lys-, R'-(C=O)-Phe-, R'-NH-(C=O)-, R'-NH(C=S)-, R'-SO₂-, R'-(C=NH)-, R'-O-(C=O)-, R'-O-CH₂-(C=O)-, R'-, R'-alkyl, R'-P(O)OH, R'-O-(C=S)-, R'-NH-(C=NH)-, and R'-(C=S)-;

R' is selected from aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl, wherein each aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl are each optionally substituted with one or more groups selected from halogen, (C₁-C₂₀) alkyl, aryl, heteroaryl and aryloxy;

or

A is $R'-(C=O)-$, and R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and substituted alkyl, wherein at least one hydrogen on said substituted alkyl is replaced by a substituent group selected from halogen, aryl, heteroaryl and aryloxy;

X_1 is selected from Dab, Phe, Ala, Lys and Gly;

X_2 is Thr;

X_3 is Dab;

x and y are integers each independently selected from 0 and 1;

z is 1; and

R_6 , R_7 and R_{10} are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl,

provided that when R_6 and R_7 are each isobutyl and R_{10} is 1-hydroxy-1-ethyl, then x and y are each 1 and X_1 is Dab.

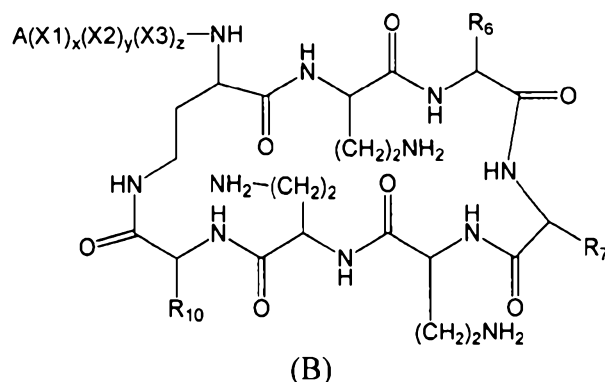
[008e] According to a fourth embodiment of the invention, there is provided a pharmaceutical composition formulated for parenteral administration, the pharmaceutical composition comprising one or more compounds of any one of the first to third embodiments in a pharmaceutically-acceptable solution, dispersion, suspension, emulsion, or sterile powder for reconstitution.

[008f] According to a fifth embodiment of the invention, there is provided a method of treating a Gram-negative infection in a subject, the method comprising administering to said subject one or more compounds of any one of the first to third embodiments or a pharmaceutical composition of the fourth embodiment.

[008g] According to a sixth embodiment of the invention, there is provided a method for preparing a compound, the method comprising:

- (a) treating a polymyxin peptide having a sidechain comprising an amino group with an amino protecting group reagent to form a protected polymyxin peptide, wherein the polymyxin peptide comprises a cyclic heptapeptide attached to an exocyclic peptide chain comprising an acyl group, and the protecting group comprises at least one acidic substituent;
- (b) reacting the protected peptide with a deacylase enzyme in an aqueous solution to form a protected, deacylated peptide comprising a free amino terminus on the side chain; and

(c) reacting the free amino terminus on the side chain of the protected, deacylated peptide with an addition reagent and removing the protecting group to form a peptide antibiotic having a structure of formula (B):



wherein:

A is selected from R'-(C=O)-(p-aminophenylacetyl)-, R'-(C=O)-Lys-, R'-(C=O)-Phe-, R'-NH-(C=O)-, R'-NH(C=S)-, R'-SO₂-, R'-(C=NH)-, R'-O-(C=O)-, R'-O-CH₂-(C=O)-, R'-, R'-alkyl, R'-P(O)OH, R'-O-(C=S)-, R'-NH-(C=NH)-, and R'-(C=S)-; and

R' is selected from aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl, wherein each aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl are each optionally substituted with one or more groups selected from halogen, (C₁-C₂₀) alkyl, aryl, heteroaryl and aryloxy;

or

A is R'-(C=O)-, and R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and substituted alkyl, wherein at least one hydrogen on said substituted alkyl is replaced by a substituent group selected from halogen, aryl, heteroaryl and aryloxy;

X1 is selected from Dab, Phe, Ala, Lys, and Gly;

X2 is Thr:

X3 is Dab;

x and y are integers each independently selected from 0 and 1;

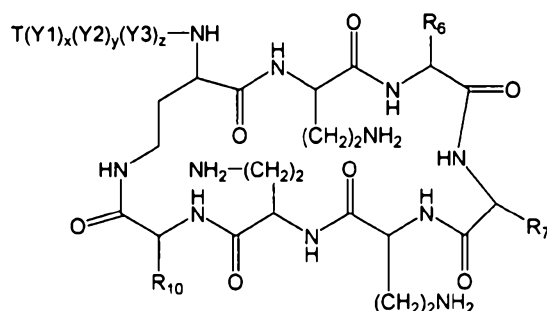
z is 1;

R₆, R₇, and R₁₀ are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, hydroxymethyl and 1-hydroxy-1-ethyl,

provided that when R₆ and R₇ are each isobutyl and R₁₀ is 1-hydroxy-1-ethyl, then x and y are each 1 and X1 is Dab.

[008h] According to a seventh embodiment of the invention, there is provided a method for preparing an antibiotic compound, the method comprising the steps of:

(a) treating a peptide of formula (A)

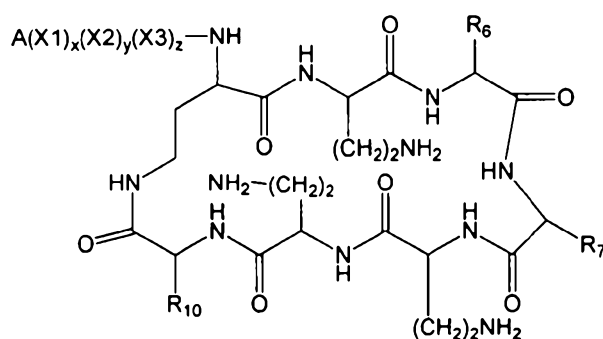


(A)

with 2-sulfo-9-fluorenylmethoxycarbonyl;

(b) treating the protected peptide with a deacylating enzyme in an aqueous system to form a protected, deacylated peptide; and

(c) reacting the protected, deacylated peptide of (b) with an addition reagent and then removing the protecting group to form the peptide antibiotic having a structure of formula (B):



(B)

wherein:

T is $R-(C=O)-$, where R is alkyl;

A is $R'-NH-(C=O)-$ where R' is aryl;

X1, X3, Y1 and Y3 are each 2,4-diaminobutanoic acid residue;

X2 and Y2 are each threonine residue;

x, y, and z are each 1;

R_6 is benzyl;

R_7 is selected from isopropyl and sec-butyl; and

R_{10} is 1-hydroxy-1-ethyl.

Definitions

[008i] The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

[008j] Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

[Text continues on page 3.]

[009] "Acyl," as used herein, refers to a carbonyl radical attached to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl group. Exemplary acyls include, but are not limited to: (1) "unsubstituted alkanoyl," which is defined as carbonyl radical attached to an unsubstituted alkyl group; (2) "unsubstituted alkenoyl," which is defined as carbonyl radical attached to an unsubstituted alkenyl group; (3) "substituted alkanoyl," which is defined as a carbonyl radical attached to a substituted alkyl group, in which one or more hydrogen atoms is replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido; and (4) "substituted alkenoyl," which is defined as carbonyl radical attached to a substituted alkenyl group, in which one or more hydrogen atoms is replaced by a substituent group as described above. Nonlimiting examples of acyls include radicals such as acetyl, n-octanoyl, n-nonanoyl, benzoyl, and isonicotinoyl.

[010] "Acylamino," as used herein, refers to an amino group bonded to an acyl group.

[011] "Acyloxy," as used herein, refers to an oxygen radical substituted with an acyl group. In some embodiments, acyloxy is substituted with an acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, or ureido group.

[012] An "addition reagent," as used herein, is a compound that can react with an amino group such as the N-terminus of a peptide, thereby chemically modifying the amino group by addition of all, or a component, of the addition reagent to the amino group. For example, an addition reagent may be an acylamino reagent such as $R'-(C=O)-LG$ or a sulfonating reagent such as $R'-SO_2-LG$, where LG is a leaving group, that can react with an amino group to

form an acylamino group or a sulfonamino group, respectively. An addition reagent may also be, for example, an isocyanate, isothiocyanate, activated ester, acid chloride, sulfonyl chloride, activated sulfonamide, activated heterocycle, activated heteroaryl, chloroformate, cyanoformate, thioacyl ester, phosphoryl chloride, phosphoramidate, imidate, or lactone. An addition reagent may also be an aldehyde or ketone that reacts with an amine under reductive conditions to form an alkylated amine. An addition reagent may also be an activated amino acid, or an amino acid and a peptide coupling reagent, such as, e.g., PyBOP[®] (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HBTU/HOBt (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate / N-hydroxybenzotriazole), or DCC (dicyclohexylcarbodiimide).

[013] "Alkenyl," as used herein, refers to linear or branched radicals having 2-20 carbon atoms, such as 2-12, 2-10, or 2-6 carbon atoms, and containing at least one carbon-carbon double bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. The double bond portion(s) of the unsaturated hydrocarbon chain may be either in the cis or trans configuration. Nonlimiting examples of alkenyls include "unsubstituted alkenyl," which is defined as an alkenyl group that bears no substituent groups. Other nonlimiting examples of alkenyl groups include ethenyl, 2-phenyl-1-ethenyl, prop-1-en-2-yl, prop-2-en-1-yl (allyl), prop-1-en-1-yl, cycloprop-1-en-1-yl; cycloprop-2-en-1-yl, but-1-en-1-yl, but-1-en-2-yl, 2-methyl-prop-1-en-1-yl, but-2-en-1-yl, but-2-en-2-yl, buta-1,3-dien-1-yl, buta-1,3-dien-2-yl, cyclobut-1-en-1-yl, cyclobut-1-en-3-yl, cyclobuta-1,3-dien-1-yl.

[014] "Alkoxy," as used herein, refers to an oxygen radical substituted with an alkyl, alkenyl, alkynyl, cycloalkyl, or heterocyclyl group. Nonlimiting examples include methoxy, tert-butoxy, benzyloxy, and cyclohexyloxy.

[015] "Alkyl," as used herein, refers to a linear or branched saturated radicals having at least one carbon atom, such as 1-20 carbon atoms, 1-12, 1-10, or 1-6 carbon atoms, or at least 6 carbon atoms, at least 7 carbon atoms, at least 8 carbon atoms, at least 9 carbon atoms, or at least 10 carbon atoms, unless otherwise specified. A "lower alkyl" is defined as an alkyl group containing 1-4 carbon atoms. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Nonlimiting examples of alkyl groups include methyl, butyl, tert-butyl, isopropyl, trifluoromethyl, nonyl, undecyl, octyl, dodecyl, methoxymethyl, 2-(2'-aminophenacyl), 3-indolylmethyl, benzyl, and carboxymethyl. Other exemplary alkyls include, but are not limited to: (1) "unsubstituted alkyl," which is defined as an alkyl group that bears no substituent groups; and (2) "substituted alkyl," which denotes an alkyl radical in which one or more hydrogen atoms is replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Exemplary alkyl groups include, but are not limited to, methyl, ethyls such as ethanyl (ethyl), propyls such as propan-1-yl (n-propyl), propan-2-yl (isopropyl), butyls such as butan-1-yl (n-butyl), butan-2-yl (s-butyl), 2-methyl-propan-1-yl (iso-butyl), 2-methyl-propan-2-yl (tert-butyl), , trifluoromethyl, nonyl, undecyl, octyl, dodecyl, methoxymethyl, 2-(2'-aminophenacyl), 3-indolylmethyl, benzyl, and carboxymethyl.

[016] "Alkynyl," as used herein, refers to linear and branched radicals having from 2-10 carbon atoms, and containing at least one carbon-carbon triple bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy,

iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Examples of alkynyl groups include, but are not limited to, ethynyl, prop-1-yn-1-yl, prop-2-yn-1-yl, but-1-yn-1-yl, but-1-yn-3-yl, and but-3-yn-1-yl.

[017] "Amino," as used herein, refers to an NR_1R_2 radical, in which R_1 and R_2 may be selected from hydrido, acyl, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, formyl, heteroaryl, heterocyclyl, hydroxy, imino, nitro, oxo, sulfinyl, sulfonyl, and thio. A monosubstituted amino refers to an NR_1R_2 radical wherein R_1 is hydrido and R_2 is selected from acyl, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, formyl, heteroaryl, heterocyclyl, hydroxy, imino, nitro, oxo, sulfinyl, sulfonyl, and thio. A disubstituted amino refers to an NR_1R_2 radical wherein R_1 and R_2 are each independently selected from acyl, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, formyl, heteroaryl, heterocyclyl, hydroxy, imino, nitro, oxo, sulfinyl, sulfonyl, and thio.

[018] An "amino acid," as used herein, refers to a compound containing a carboxylic acid group and an amino group and having the formula $\text{H}_2\text{N}[\text{C}(\text{R})(\text{R}')]\text{n}-\text{C}(\text{O})\text{OH}$, where n is an integer greater than equal to one, and R and R' are independently selected from hydrogen and amino acid side chains. For example, when n is equal to one, the amino acid of the formula $\text{H}_2\text{N}[\text{C}(\text{R})(\text{R}')]\text{C}(\text{O})\text{OH}$ is an alpha amino acid, and when n is equal to two, the amino acid of the formula $\text{H}_2\text{N}-\text{C}(\text{R}_1)(\text{R}_1')-\text{C}(\text{R}_2)(\text{R}_2')-\text{C}(\text{O})\text{OH}$ is a beta amino acid, where R_1 , R_1' , R_2 , and R_2' are each independently chosen from amino acid side chains. An "amino acid residue," as used herein, refers to an amino acid that is part of a peptide or protein, and having the formula $-\text{N}(\text{H})\text{C}(\text{R})(\text{R}')\text{C}(\text{O})-$. An "amino acid side chain" as used herein, refers to any side chain from a naturally-occurring or synthetic amino acid. For example, methyl may be referred to as an alanine side chain, and 2-amino-1-ethyl may be referred to as the side chain of 2,4-diaminobutanoic acid.

[019] Exemplary amino acids may be chosen from the twenty encoded amino acids and derivatives thereof, as well as from, e.g., other α -amino acids, β -amino acids, γ -amino acids, δ -amino acids, and ω -amino acids. An amino acid may have R or S chirality at any chiral atom. An amino acid may be chosen from, e.g., alanine, β -alanine, α -aminoadipic acid, 2-aminobutanoic acid,

4-aminobutanoic acid, 1-aminocyclopentanecarboxylic acid, 6-aminohexanoic acid, 2-aminoheptanedioic acid, 7-aminoheptanoic acid, 2-aminoisobutyric acid, aminomethylpyrrole carboxylic acid, 8-amino-3,6-dioxaoctanoic acid, aminopiperidinecarboxylic acid, 3-amino-propionic acid, aminoserine, aminotetrahydropyran-4-carboxylic acid, arginine, asparagine, aspartic acid, azetidine carboxylic acid, benzothiazolylalanine, butylglycine, carnitine, 4-chlorophenylalanine, citrulline, cyclohexylalanine, cyclohexylstatine, cysteine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid, dihydroxyphenylalanine, dimethylthiazolidine carboxylic acid, glutamic acid, glutamine, glycine, histidine, homoserine, hydroxyproline, isoleucine, isonipecotic acid, leucine, lysine, methanoproline, methionine, norleucine, norvaline, ornithine, p-aminobenzoic acid, penicillamine, phenylalanine, phenylglycine, piperidinylalanine, piperidinylglycine, proline, pyrrolidinylalanine, sarcosine, selenocysteine, serine, statine, tetrahydropyranglycine, thienylalanine, threonine, tryptophan, tyrosine, valine, allo-isoleucine, allo-threonine, 2,6-diamino-4-hexanoic acid, 2,6-diaminopimelic acid, 2,3-diaminopropionic acid, dicarboxidine, homoarginine, homocitrulline, homocysteine, homocystine, homophenylalanine, homoproline, and 4-hydrazinobenzoic acid.

[020] N-protected α -amino acids for peptide synthesis having L- or D-chirality at C α are commercially available, e.g., from Novabiochem[®] (San Diego, CA) and Bachem (Bubendorf, Switzerland). The synthesis of chiral α -amino acids and other amino acids is also well known to those of ordinary skill in the art, and is described, e.g., in Arnstein *Synthesis of amino acids and proteins*, University Park Press, 1975; *Enantioselective Synthesis of Beta-Amino Acids*, Juaristi et al., Eds., Wiley-VCH: New York, 2005; and Williams *Synthesis of optically active α -amino acids*, Pergamon Press, 1989.

[021] "Amino protecting group," as used herein, refers to any substituent that may be used to prevent an amino group on a molecule from undergoing a chemical reaction while chemical change occurs elsewhere in the molecule. An amino protecting group can be removed under the appropriate chemical conditions. Numerous amino protecting groups are known to those skilled in the art, and examples of amino protecting groups, methods for their addition, and methods for their removal can be found in "Protective Groups in Organic

Synthesis" by Theodora W. Greene, John Wiley and Sons, New York, 1991, the disclosure of which is incorporated herein by reference. Nonlimiting examples of amino protecting groups include phthalimido, trichloroacetyl, STA-base, benzyloxycarbonyl, t-butoxycarbonyl, t-amylloxycarbonyl, isobornylloxycarbonyl, adamantylloxycarbonyl, chlorobenzyloxycarbonyl, and nitrobenzyloxycarbonyl. Other exemplary amino protecting groups include "carbamate amino protecting groups," which are defined as a carbonyl containing protecting group that when bound to an amino group forms a carbamate. Nonlimiting exemplary amino carbamate protecting groups include 9-fluorenylmethoxycarbonyl (Fmoc), allyloxycarbonyl (Alloc), carbobenzyloxy (CBZ), and t-butoxycarbonyl (Boc) protecting groups. Other exemplary protecting groups include 9-fluorenylmethoxycarbonyl (Fmoc) substituted with acidic substituents, such as 2-sulfo-9-fluorenylmethoxycarbonyl, 2-carboxymethyl-9-fluorenylmethoxycarbonyl, and 4-carboxy-9-fluorenylmethoxycarbonyl.

[022] "Amino protecting group reagents," as used herein, refer to addition reagents that can react with an amino group such as the N-terminus of a peptide, thereby chemically modifying said amino group by addition of an amino protecting group.

[023] "Aryl," as used herein, refers to a mono-, bi-, or other multi-carbocyclic, aromatic ring system. The aryl group can optionally be fused to one or more rings selected from aryls, cycloalkyls, and heterocyclyls. Aryls can have from 5-14 ring members, such as from six to ten ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, azido, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, cycloalkyl, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Nonlimiting examples of aryl groups include phenyl, naphthyl, biphenyl, and anthracenyl.

[024] "Aryloxy," as used herein, refers to an oxygen radical substituted with an aryl or heteroaryl group. An exemplary aryloxy includes, but is not limited to, phenoxy.

[025] "Carbamoyl," as used herein, refers to a nitrogen radical of the formula

$-N(R^{x2})-C(O)-OR^{x3}$, wherein R^{x2} is selected from hydrido, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl, and R^{x3} is selected from alkyl, aryl, cycloalkyl, heteroaryl, and heterocyclyl.

[026] "Carboalkoxy," as used herein, refers to a carbonyl radical bonded to an alkoxy or aryloxy group.

[027] "Carboxy," as used herein, refers to a COOH radical.

[028] "Carboxyamino," as used herein, refers to a CONH₂ radical.

[029] "Carboxyamido," as used herein, refers to a carbonyl radical bonded to a monosubstituted amino or disubstituted amino group.

[030] "Cycloalkyl," as used herein, refers to a saturated or partially unsaturated carbocyclic ring in a single or fused carbocyclic ring system having from three to twelve ring members, such as a ring system having from three to seven ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, cycloalkyl, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Nonlimiting exemplary cycloalkyl groups include cyclopropyl, cyclobutyl, cyclohexyl, and cycloheptyl.

[031] "Fmoc" is a 9-fluorenylmethoxycarbonyl group.

[032] "Halo," as used herein, refers to a bromo, chloro, fluoro or iodo radical.

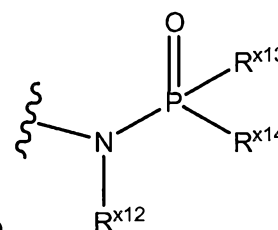
[033] "Heteroaryl," as used herein, refers to an aromatic radical having from one to four hetero atoms or hetero groups selected from O, N, NH, S, or SO in a single or fused heterocyclic ring system, having from five to fifteen ring members, such as a heteroaryl ring system having from six to ten ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, cycloalkyl, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl,

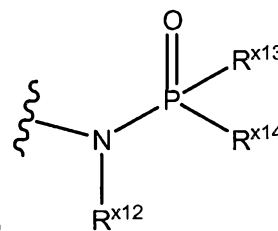
sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Nonlimiting examples of heteroaryl groups include indolyl, pyridinyl, thiazolyl, thiadiazoyl, isoquinolinyl, pyrazolyl, oxazolyl, oxadiazolyl, triazolyl, and pyrrolyl groups.

[034] "Heterocyclyl" or "heterocyclic," as used herein, refers to a saturated or partially unsaturated ring containing one to four hetero atoms or hetero groups selected from O, N, NH, N(alkyl, such as lower alkyl), S, SO or SO₂, in a single or fused heterocyclic ring system having from three to twelve ring members, such as a heterocyclyl ring system having from three to seven ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkyl, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, cycloalkyl, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido. Nonlimiting examples of heterocyclyl groups include morpholinyl, piperidinyl, pyrrolidinyl and succinimidyl.

[035] "Hydroxy," as used herein, refers to -OH.

[036] "Iminoamino," as used herein, refers to -N(H)C(=NR^{x26})R^{x27}, wherein R^{x26} and R^{x27} are selected from hydrido, alkyl, aryl, cycloalkyl, heteroaryl, and heterocyclyl.

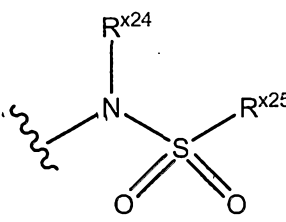


[037] "Phosphonamino," as used herein, refers to , wherein R^{x13} and R^{x14} are independently selected from alkoxy, alkyl, amino, aryl, aryloxy, cycloalkyl, disubstituted amino, halo, heteroaryl, heterocyclyl, hydroxy, monosubstituted amino, and thio.

[038] "Sulfinyl," as used herein, refers to -S(=O)OH.

[039] "Sulfo," as used herein, refers to -SO₃H.

[040] "Sulfonamino," as used herein, refers to an amino radical of the

formula , wherein each of R^{x24} is selected from hydrido, alkyl, cycloalkyl, aryl, heteroaryl, and heterocyclyl, and R^{x25} is selected from alkyl, aryl, cycloalkyl, heteroaryl, and heterocyclyl.

[041] "Sulfonyl," as used herein, refers to a hexavalent sulfur radical substituted with two oxo substituents and a third substituent selected from alkyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl.

[042] "Thio," as used herein, refers to a radical containing a substituent group independently selected from hydrido, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, attached to a divalent sulfur atom, such as methylthio and phenylthio.

[043] "Thioacyl," as used herein, refers to a thiocarbonyl radical attached to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl or heteroaryl group.

[044] "Thioacylamino," as used herein, refers to an amino radical bonded to a thioacyl group.

[045] "Thioacylester," as used herein, refers to a thiocarbonyl radical attached to an alkoxy group.

[046] "Thioureido," as used herein, refers to a nitrogen radical of the formula

$-N(R^{x5})-C(S)-N(R^{x6})(R^{x7})$, wherein each of R^{x5} and R^{x6} is independently selected from hydrido, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl; and R^{x7} is selected from an alkyl, aryl, cycloalkyl, heteroaryl, and heterocyclyl.

[047] "Ureido," as used herein, refers to a nitrogen radical of the formula $-N(R^{x21})-C(O)-NR^{x22}R^{x23}$, wherein each of R^{x21} and R^{x22} is independently selected from hydrido, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl; and R^{x23} is selected from an alkyl, aryl, cycloalkyl, heteroaryl, and heterocyclyl.

[048] The compounds of the present invention may be used in the form of salts or pharmaceutically-acceptable salts derived from inorganic or organic acids. The present invention includes all such salts and all crystalline forms of such salts. By "pharmaceutically-acceptable salt" is meant those salts that are, within the scope of sound medical judgment, suitable for use in contact with the

tissues of humans and lower animals without undue toxicity, irritation, and allergic response, and are commensurate with a reasonable benefit/risk ratio.

Pharmaceutically-acceptable salts are well known in the art. For example, S. M. Berge, et al. describes pharmaceutically-acceptable salts in *J. Pharm. Sci.*, 1977, 66:1-19. All of these salts may be prepared by conventional means from the corresponding compound of the invention by treating, for example, the compound with the appropriate acid or base.

[049] The salts or pharmaceutically-acceptable salts may be prepared in situ during the final isolation and purification of the compounds of the invention, or separately by reaction of a free base function with a suitable acid. For example, basic addition salts can be prepared in situ during the final isolation and purification of compounds of this invention by combining a carboxylic acid-containing group with a suitable base such as the hydroxide, carbonate, or bicarbonate of a pharmaceutically-acceptable metal cation, or with ammonia or an organic primary, secondary, or tertiary amine.

[050] Nonlimiting examples of organic acids may be selected from aliphatic, cycloaliphatic, aromatic, aryl, heterocyclic, carboxylic and sulfonic classes of organic acids, examples of which include, without limitation, formic, acetic, propionic, succinic, glycolic, gluconic, maleic, embonic (pamoic), methanesulfonic, ethanesulfonic, 2-hydroxyethanesulfonic, pantothenic, benzenesulfonic, toluenesulfonic, sulfanilic, mesylic, cyclohexylaminosulfonic, stearic, algenic, β -hydroxybutyric, malonic, galactic, and galacturonic acid. Representative organic acid addition salts include acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate (isethionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, phosphate, glutamate, bicarbonate, p-toluenesulfonate and undecanoate addition salts. Examples of acids that may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, hydrobromic acid, sulfuric acid and phosphoric acid and such organic acids as oxalic acid, maleic acid, succinic acid, and citric acid.

[051] Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides (e.g., methyl, ethyl, propyl, and butyl chlorides, bromides and iodides); dialkyl sulfates (e.g., dimethyl, diethyl, dibutyl and diamyl sulfates); long-chain halides (e.g., decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides); or arylalkyl halides (e.g., benzyl and phenethyl chloride bromides and iodides) and others. Water- or oil-soluble or -dispersible products are thereby obtained.

[052] For example, suitable pharmaceutically-acceptable base addition salts of compounds of the invention include, but are not limited to, metallic salts made from aluminum, calcium, lithium, magnesium, potassium, sodium and zinc or organic salts made from N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, N-methylglucamine, lysine and procaine. Pharmaceutically-acceptable basic addition salts include cations based on alkali metals or alkaline earth metals such as lithium, sodium, potassium, calcium, magnesium, and aluminum salts, and nontoxic quaternary ammonia and amine cations including ammonium, tetramethylammonium, tetraethylammonium, methylammonium, dimethylammonium, trimethylammonium, triethylammonium, diethylammonium, and ethylammonium. Other representative organic amines useful for the formation of base addition salts include ethylenediamine, ethanolamine, diethanolamine, piperidine, and piperazine.

[053] The compounds of the invention can possess one or more asymmetric carbon atoms and are thus capable of exhibiting optical activity. The compounds of the invention can exist in enantiomeric and/or diastereomeric forms, as well as in the form of racemic or non-racemic mixtures thereof. The compounds disclosed herein can be utilized in the present invention as a single isomer or as a mixture of stereochemical isomeric forms.

[054] Diastereomers can be separated by conventional means such as chromatography, distillation, crystallization or sublimation. Enantiomers can be obtained by resolution of the racemic mixtures according to conventional processes, for example by formation of diastereomeric salts by treatment with an optically active acid or base. Nonlimiting examples of appropriate acids include tartaric, diacetyltartaric, dibenzoyltartaric, ditoluoyltartaric and camphorsulfonic acid. The mixture of diastereomers can be separated by crystallization followed by liberation of the optically active bases from the optically active salts. An

alternative process for separation of enantiomers includes the use of a chiral chromatography column optimally chosen to maximize the separation of the enantiomers. Another method involves synthesis of covalent diastereomeric molecules by treating compounds of the invention with an activated form of an enantiomerically enriched acid or with an enantiomerically enriched isocyanate. The synthesized diastereomers can be separated by conventional means such as chromatography, distillation, crystallization or sublimation, and then hydrolyzed to obtain the enantiomerically enriched compound. Optically active compounds can likewise be obtained by utilizing optically active starting materials. These isomers may be in the form of a free acid, a free base, an ester or a salt.

[055] Geometric isomers can also exist in the compounds of the present invention. The present invention encompasses the various geometric isomers and mixtures thereof resulting from the arrangement of substituents around a carbon-carbon double bond or arrangement of substituents around a carbocyclic ring. Substituents around a carbon-carbon double bond are designated as being in the "Z" or "E" configuration wherein the terms "Z" and "E" are used in accordance with IUPAC standards. Substituents around a carbon-carbon double bond alternatively can be referred to as "cis" or "trans," where "cis" represents substituents on the same side of the double bond and "trans" represents substituents on opposite sides of the double bond.

[056] The arrangement of substituents around a carbocyclic ring is also designated as "cis" or "trans." The term "cis" represents substituents on the same side of the plane of the ring and the term "trans" represents substituents on opposite sides of the plane of the ring. Mixtures of compounds wherein the substituents are disposed on both the same and opposite sides of plane of the ring are designated "cis/trans".

[057] An isolated, pure, or purified compound refers to a composition containing at least 10%, such as at least 20%, at least 50%, at least 80%, or at least 90% of the compound. In one embodiment, a pharmaceutically acceptable salt thereof or a pharmaceutical composition comprising any of the compounds disclosed herein exhibits a detectable (e.g., statistically significant) antimicrobial activity when tested in conventional biological assays such as those described herein.

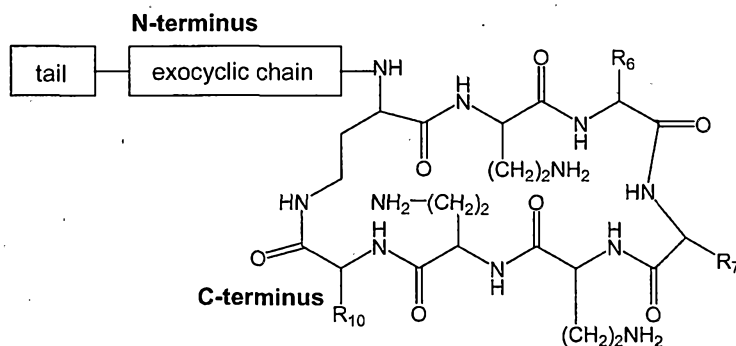
[058] The term “pharmaceutically-acceptable prodrugs” as used herein, represents those prodrugs of the compounds of the present invention that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention.

[059] The term “pro-drug,” as used herein, represents compounds that are rapidly transformed in vivo to the parent compound of the formulas described herein, for example, by hydrolysis in blood. A discussion is provided in T. Higuchi and V. Stella, *Pro-drugs as Novel Delivery Systems*, Vol. 14 of the ACS Symposium Series, and in Edward B. Roche, ed., *Bioreversible Carriers in Drug Design*, American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated herein by reference.

DETAILED DESCRIPTION

[060] Disclosed herein are novel peptides and methods for preparing peptides. The peptides can have applications as antibacterial agents.

[061] One embodiment discloses new peptides comprising a cyclic heptapeptide attached to an exocyclic peptide chain and tail. In one embodiment, the new peptides are derived from naturally occurring peptides having the following structure:



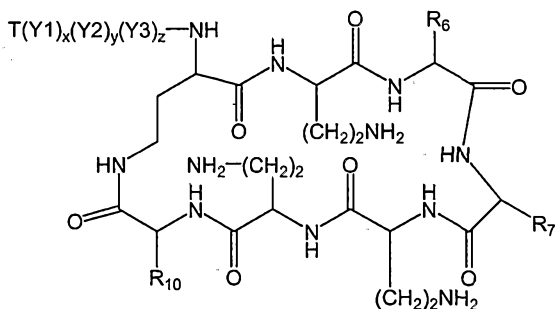
[062] These naturally occurring peptides have an exocyclic peptide chain attached to an N-terminal “side chain” or “tail.” In one embodiment, such peptides are related to naturally occurring peptides including polymyxins or octapeptins. These peptides have the common structure shown above with variation at amino acids 6, 7, and 10 (polymyxin numbering), where R_6 depicts the side chain of the

amino acid at position 6, R_7 depicts the side chain of the amino acid at position 7, and R_{10} depicts the side chain of the amino acid at position 10. In one embodiment, R_6 , R_7 , and R_{10} may each be independently chosen from iso-propyl (to give Val), benzyl (to give Phe), iso-butyl (to give Leu), sec-butyl (to give Ile), 1-hydroxy-1-ethyl (to give Thr), and hydroxymethyl (to give Ser). In another embodiment, R_6 and R_7 are each independently chosen from iso-propyl, benzyl, iso-butyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl and R_{10} is chosen from iso-propyl, iso-butyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl. The exocyclic chain and tail provide variation as well. In one embodiment, the exocyclic chain comprises 1 to 3 amino acid residues:

[063] In one embodiment, the exocyclic chain comprises a chain of amino acid residues. In another embodiment, the exocyclic chain is represented by $-(Y1)_x(Y2)_y(Y3)_z-$, where $Y1$, $Y2$, and $Y3$ are each independently selected from amino acid residues including non-encoded amino acid residues, and x , y , and z are integers independently selected from 0 and 1.

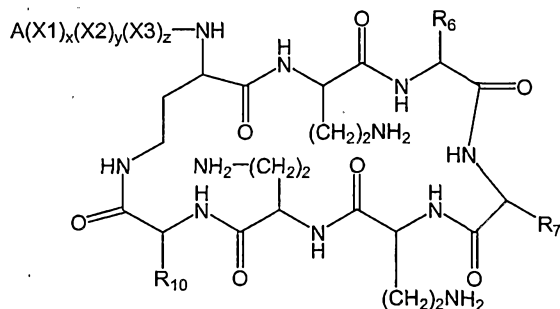
[064] In one embodiment, the tail is depicted as "T", where the "tail-exocyclic chain" portion has the formula $T(Y1)_x(Y2)_y(Y3)_z-$. In one embodiment, T is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl, and hydrogen wherein R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[065] Accordingly, one embodiment provides new peptides derived from naturally occurring peptides, where the naturally occurring peptides have the structure of formula (A):



(A)

[066] Also disclosed are methods for preparing new peptides containing either free amino groups or protected amino groups. In one embodiment, the new peptides are related to, for example, naturally occurring peptides described herein. In one embodiment, the new peptides have the structure of formula (B):



(B)

[067] In one embodiment, the exocyclic chain is represented by $-(X1)_x(X2)_y(X3)_z-$, where $X1$, $X2$, and $X3$ are each independently selected from amino acid residues, including any residue described herein, and x , y , and z are integers independently selected from 0 and 1, and "A" represents the tail where A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl, and hydrogen wherein R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[068] One embodiment disclosed herein is a method for preparing a compound, comprising:

- (a) treating a peptide having at least one side chain comprising an amino group with an amino protecting group reagent to form a protected peptide, wherein the peptide comprises a cyclic heptapeptide attached to an exocyclic peptide chain comprising an acyl group, and the protecting group contains at least one acidic substituent; and
- (b) treating the protected peptide with a deacylating agent to form a protected, deacylated peptide.

[069] In one embodiment, the peptide to be treated with the protecting group reagent in (a) is selected from naturally occurring peptides having the structure of formula (A), such as polymyxins and octapeptins. Representative polymyxins and octapeptins include polymyxin A (PA), polymyxin B (PB), [Ile⁷]-

polymyxin B₁ (IL), polymyxin D, polymyxin E, polymyxin F, polymyxin M (mattacin), polymyxin S, polymyxin T, circulin A, polymyxin D (PD), octapeptin A, octapeptin B (OB), octapeptin C (OC), and octapeptin D. Polymyxin E is conventionally called colistin. Letters (e.g., polymyxin A, polymyxin B, polymyxin C, etc.) generally refer to polymyxins having variations in amino acid sequence. Numerical subscripts (e.g., octapeptin A₁, octapeptin A₂, octapeptin A₃, etc. or polymyxin B₁, polymyxin B₂, polymyxin B₃, etc.) generally refer to variations in the tail. Polymyxins and octapeptins can be isolated from natural sources with a variety of lipid tails, as shown in Table 1 for polymyxin B. One exception is colistin (polymyxin E): colistin A (polymyxin E₁) and colistin B (polymyxin E₂) differ in lipid tail.

Table 1

<u>Peptide</u>	<u>Tail</u>
Polymyxin B ₁	6-methyloctanoyl
Polymyxin B ₂	6-methylheptanoyl
Polymyxin B ₃	octanoyl
Polymyxin B ₄	heptanoyl
Polymyxin B ₅	nonanoyl
Polymyxin B ₆	3-hydroxy-6-methyloctanoyl
Polymyxin E ₁ (colistin A)	6-methyloctanoyl
Polymyxin E ₂ (colistin B)	6-methylheptanoyl

[070] Table 2 shows the peptide sequences of several peptides disclosed herein.

Table 2

<u>Peptide</u>	<u>Exocyclic chain</u>	<u>Cyclic Peptide</u>
	1 - 2 - 3	4 - 5 - 6 - 7 - 8 - 9 - 10
Polymyxin A	Dab-Thr-Dab-	[Dab-Dab-DLeu-Leu-Dab-Dab-Thr-]
Polymyxin B (PB)	Dab-Thr-Dab-	[Dab-Dab-DPhe-Leu-Dab-Dab-Thr-]
Polymyxin D	Dab-Thr-DSer-	[Dab-Dab-DLeu-Thr-Dab-Dab-Thr-]
Polymyxin M	Dab-Thr-Dab	[Dab-Dab-Leu-Thr-Dab-Dab-Thr-]
Polymyxin S	Dab-Thr-Ser	[Dab-Dab-DPhe-Thr-Dab-Dab-Thr-]
Polymyxin T	Dab-Thr-Dab	[Dab-Dab-DPhe-Leu-Dab-Dab-Leu-]
Colistin (C)	Dab-Thr-Dab-	[Dab-Dab-DLeu-Leu-Dab-Dab-Thr-]
Circulin A (CA)	Dab-Thr-Dab-	[Dab-Dab-DLeu-Ile-Dab-Dab-Thr-]
Polymyxin A (PA)	Dab-Thr-Dab-	[Dab-Dab-DLeu-Leu-Dab-Dab-Thr-]
Polymyxin D (PD)	Dab-Thr-DSer-	[Dab-Dab-DLeu-Thr-Dab-Dab-Thr-]
Octapeptin A	Dab-	[Dab-Dab-DLeu-Leu-Dab-Dab-Leu-]
Octapeptin B (OB)	Dab-	[Dab-Dab-DLeu-Leu-Dab-Dab-Leu-]
Octapeptin C (OC)	Dab-	[Dab-Dab-DLeu-Phe-Dab-Dab-Leu-]
Octapeptin D	DSer-	[Dab-Dab-DLeu-Leu-Dab-Dab-Leu-]
[Ile ⁷]-polymyxin B (IL)	Dab-Thr-Dab-	[Dab-Dab-DPhe-Ile-Dab-Dab-Thr-]

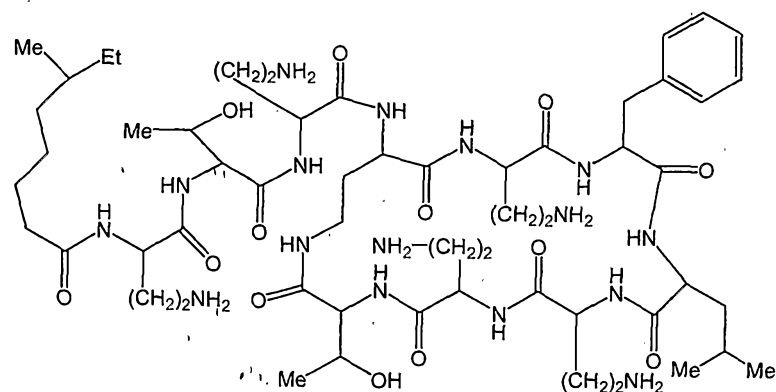
*All amino acids are L-isomers unless designated as the D-isomer. Dab is 2,4-diaminobutanoic acid.

[071] In one embodiment, the peptide to be treated is selected from polymyxin A, polymyxin B (e.g., polymyxin B₁, polymyxin B₂, and polymyxin B₃), [Ile⁷]-polymyxin B₁, polymyxin C, polymyxin D, polymyxin E (also called colistin), polymyxin F, polymyxin M (also called mattacin), polymyxin P, polymyxin S, polymyxin T (e.g., polymyxin T₁), colistin (e.g. colistin A and colistin B), circulin A, octapeptin A (e.g., octapeptin A₁, octapeptin A₂, and octapeptin A₃) octapeptin B (e.g., octapeptin B₁, octapeptin B₂, and octapeptin B₃), octapeptin C (e.g., octapeptin C₁), and octapeptin D. Polymyxin F is also known, where the amino acid composition is 5:1:1:1:2 Dab:Thr:Ser:Ile:Leu.

[072] In one embodiment, the peptide to be treated is selected from polymyxin B, polymyxin A, polymyxin D, [Ile⁷]-polymyxin B₁, colistin, circulin A, octapeptin B, and octapeptin C.

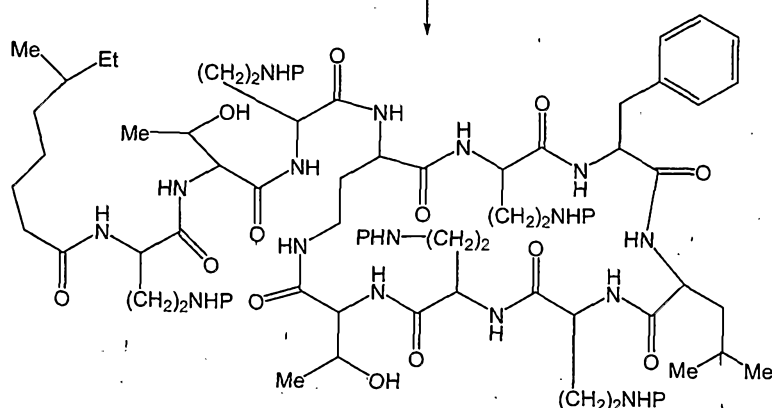
[073] In one embodiment, the resulting protected deacylated peptide can be further modified to form new protected peptide compounds. New peptide compounds can then be formed following deprotection.

[074] Scheme I below shows one embodiment of a method of protecting and deacylating a peptide. For illustrative purposes, the method is depicted with polymyxin B₁. However, this method is applicable to all peptides having the common heptapeptide cyclic core structure of formula (A), such as the polymyxins and octapeptins.



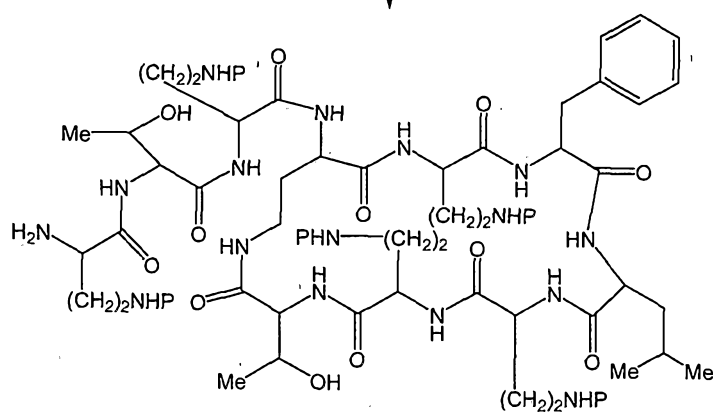
Polymyxin B

amino protecting group
reagent



Protected PBpeptide-3

deacylating agent



Protected Deacylated PBpeptide-3

Scheme I

[075] In Scheme I, "P" depicts the protecting group containing at least one acidic substituent. The peptide of Scheme I (polymyxin B, or PB) has three amino acids in the exocyclic chain, and is thus termed "PBpeptide-3."

[076] Although Scheme I depicts protecting all of the amino groups of polymyxin B, the present invention also encompasses protecting one or more amino groups. One of ordinary skill in the art can readily appreciate that the method depicted in Scheme I may equally apply to any peptide disclosed herein.

[077] In one embodiment, the protecting group contains at least one "acidic substituent," which, as used herein, refers to a substituent containing a donatable hydrogen. Exemplary acidic substituents include the acid or salt form of sulfo, sulfate, sulfonate, carboxy, carboxylate, phosphonate, and phosphate. In one embodiment, the protecting group comprises an aryl or heteroaryl substituted with the acidic substituent.

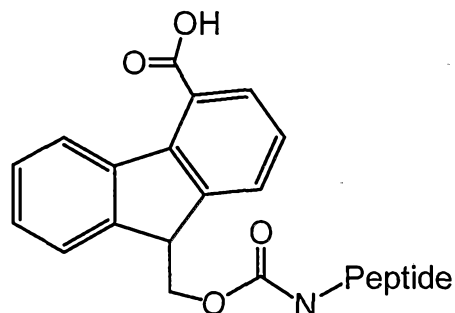
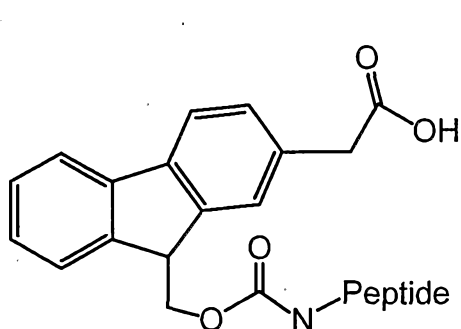
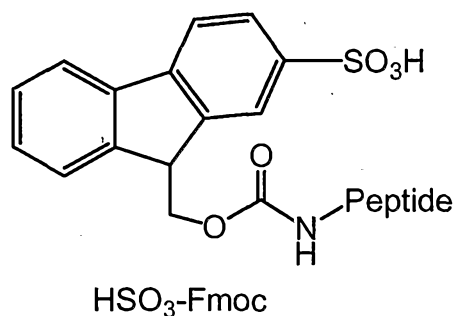
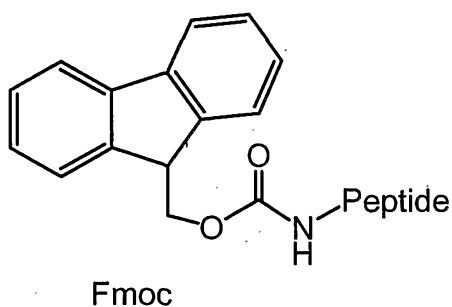
[078] In one embodiment, the protecting group is selected from the acid or salt form of sulfo, sulfate, sulfonate, carboxy, carboxylate, phosphonate, and phosphate derivatives of amino protecting groups, such as carbamate amino protecting groups. Exemplary carbamate amino protecting groups include, but are not limited to, the protecting groups disclosed in "Protective Groups in Organic Synthesis" by Theodora W. Greene, John Wiley and Sons, New York, 1991 at pp. 315-348, the disclosure of which is incorporated herein by reference.

Nonlimiting examples of carbamate amino protecting groups include

9-(2,7-dibromo)fluorenylmethyl carbamate,
2,7-di-*t*-butyl-[9-(10,10-dioxo-10,10,10,10-tetrahydrothioxanthyl)] methyl carbamate (DBD-Tmoc), 4-methoxyphenacyl carbamate (phenoc), 2-phenylethyl carbamate (hZ), 1-methyl-1-(4-biphenyl)ethyl carbamate (Bpoc), 1-(3,5-di-*t*-butylphenyl)-1-methylethyl carbamate (*t*-bumeoc), 2-(2'-and 4'-pyridyl)ethyl carbamate (pyoc), 8-quinolyl carbamate, benzyl carbamate (Cbz- or Z), *p*-nitrobenzyl carbamate, *p*-bromobenzyl carbamate, *p*-chlorobenzyl carbamate, 2,4-dichlorobenzyl carbamate, 4-methylsulfinylbenzyl carbamate (MsZ), 9-anthrylmethyl carbamate, diphenylmethyl carbamate, 2-(*p*-toluenesulfonyl)ethyl carbamate, 4-methylthiophenyl carbamate (Mtpc), 2-triphenylphosphonioisopropyl carbamate (Ppoc), *m*-chloro-*p*-acyloxybenzyl carbamate, *p*-(dihydroxyboryl)benzyl carbamate (Dobz), 5-benzisoxazolylmethyl carbamate (Bic), 2-(trifluoromethyl)-6-chromonylmethyl carbamate (Tcroc),

m-nitrophenyl carbamate, 3,5-dimethoxybenzyl carbamate, *o*-nitrobenzyl carbamate, 3,4-dimethoxy-6-nitrobenzyl carbamate, phenyl(*o*-nitrophenyl)methyl carbamate, phenothiazinyl-(10)-carbonyl derivative, *N'*-*p*-toluenesulfonylaminocarbonyl derivative, *t*-amyl carbamate, *p*-cyanobenzyl carbamate, *p*-decyloxybenzyl carbamate, *o*-(*N,N*-dimethylcarboxamido)benzyl carbamate, di(2-pyridyl)methyl carbamate, 2-furanylmethyl carbamate, *p*-(*p'*-methoxyphenylazo)benzyl carbamate, 1-methyl-1-(3,5-dimethoxyphenyl)ethyl carbamate, 1-methyl-1-(*p*-phenylazophenyl)ethyl carbamate, 1-methyl-1-phenylethyl carbamate, 1-methyl-1-(4-pyridyl)ethyl carbamate, phenyl carbamate, *p*-(phenylazo)benzyl carbamate, 2,4,6-tri-*t*-butylphenyl carbamate, 4-(trimethylammonium)benzyl carbamate, and 2,4,6-trimethylbenzyl carbamate.

[079] Another exemplary protecting group includes 9-fluorenylmethoxycarbonyl (Fmoc) substituted with acidic substituents or salts thereof. Depicted below are examples of protecting groups substituted with acidic substituents for polymyxin peptides and other related peptides, where 2-(sulfo)-9-fluorenylmethoxycarbonyl is abbreviated as HSO₃-Fmoc, and its sodium salt is abbreviated as NaSO₃-Fmoc:



[080] Carboxylic acid derivatives of Fmoc suitable as amino protecting group reagents for the protection of the peptides of the invention can be prepared according to Refs. 8 and 9, the disclosures of which are disclosed herein by reference. Sulfated derivatives of Fmoc can be prepared according to Refs. 28, 29, 32, and 33, the disclosures of which are incorporated herein by reference.

[081] In one embodiment, the protected peptide is water soluble. The water solubility may allow the protected peptide (for example, the protected peptide PBpeptide-3 prior to deacylation in Scheme I), to react with a biologically-based deacylase, e.g., an enzyme, in an aqueous system. In one embodiment, the protected peptide is polyanionic, water soluble as a salt, and can react in aqueous or partially aqueous solution with a deacylase enzyme.

[082] In another embodiment, the protected peptide is water soluble and can undergo an enzyme-mediated transformation other than, or in addition to, deacylation.

[083] In one embodiment, "water soluble" refers to the peptide being substantially completely water soluble. In another embodiment, "water soluble" refers to the peptide being sufficiently water soluble such that any reaction that consumes the peptide in an aqueous system enables the previously insoluble portion of peptide to dissolve in water, and thus, may cause the reaction to be driven to completion. In one embodiment, the protecting group has a sufficient number and/or type of acidic substituents to confer water solubility to the protected peptide.

[084] In one embodiment, the protected peptide is water soluble and can be administered as a prodrug (vide infra).

[085] New peptide compounds can be prepared from polymyxin A, polymyxin B (e.g., polymyxin B₁, polymyxin B₂, and polymyxin B₃), [Ile⁷]-polymyxin B₁, polymyxin C, polymyxin D, polymyxin E (also called colistin), polymyxin F, polymyxin M (also called mattacin), polymyxin P, polymyxin S, polymyxin T (e.g., polymyxin T₁), circulin A, octapeptin A (e.g., octapeptin A₁, octapeptin A₂, and octapeptin A₃), octapeptin B (e.g., octapeptin B₁, octapeptin B₂, and octapeptin B₃), octapeptin C (e.g., octapeptin C₁), and octapeptin D.

[086] In one embodiment, the peptide is naturally occurring. Peptides having the common heptapeptide cyclic core of structure **(A)** can be isolated from *Bacillus* spp. (e.g., *Bacillus circulans*, *Bacillus polymyxa*, *Bacillus colistinus*),

Aerohacter aerogenes, *Paenibacillus kobensis* M, and other bacterial species (Refs. 30-31). For example, polymyxins can be isolated from the fermentation of *Bacillus polymyxa* according to procedures described in Ref. 14.

[087] In another embodiment, the peptide is chemically synthesized. Chemical synthesis of peptides is well known to those of ordinary skill in the art, and is described in, e.g., *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Chan et al., Eds., Oxford University Press: New York, 2000; Bodanszky *Principles of Peptide Synthesis*, Springer Verlag: New York, 1993; Lloyd-Williams et al. *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press: Boca Raton, FL, 1997; and the Novabiochem® (San Diego, CA) Catalog.

[088] In one embodiment, the deacylating agent is an enzymatic deacylating agent. One example of an enzyme useful for deacylation of the protected peptide is produced by certain microorganisms of the genus family Actinoplanaceae. Some of the known species and varieties of this family include *Actinoplanes philippinensis*, *Actinoplanes armeniacus*, *Actinoplanes utahensis*, *Actinoplanes missouriensis*, *Spirillospora albida*, *Streptosporangium roseum*, *Streptosporangium vulgare*, *Streptosporangium roseum var hollandensi*, *Streptosporangium album*, *Streptosporangium viridialbum*, *Amorphosporangium auranticolor*, *Ampullariella regularis*, *Ampullariella campanulata*, *Ampullariella lobata*, *Ampullariella digitata*, *Pilimelia terevasa*, *Pimelia anulata*, *Planomonospora parontospora*, *Planomonospora venezuelensis*, *Planobispora longispora*, *Planobispora rosea*, *Dactylosporangium aurantiacum*, and *Dactylosporangium thailandense*. All natural and artificial variants and mutants which are obtained from the *Actinoplanaceae* family and which produce the enzyme may be used in this invention.

[089] Suitable methods for enzymatic deacylation can be found in U.S. Patent Nos. 4,524,135, 4,537,717, 4,482,487, RE 32,310, RE 32,311, 5,039,789, and 5,028,590, International Publication Nos. WO03/014147, WO 01/44272, WO 01/44274, and WO 01/44271, the disclosures of which are incorporated herein by reference.

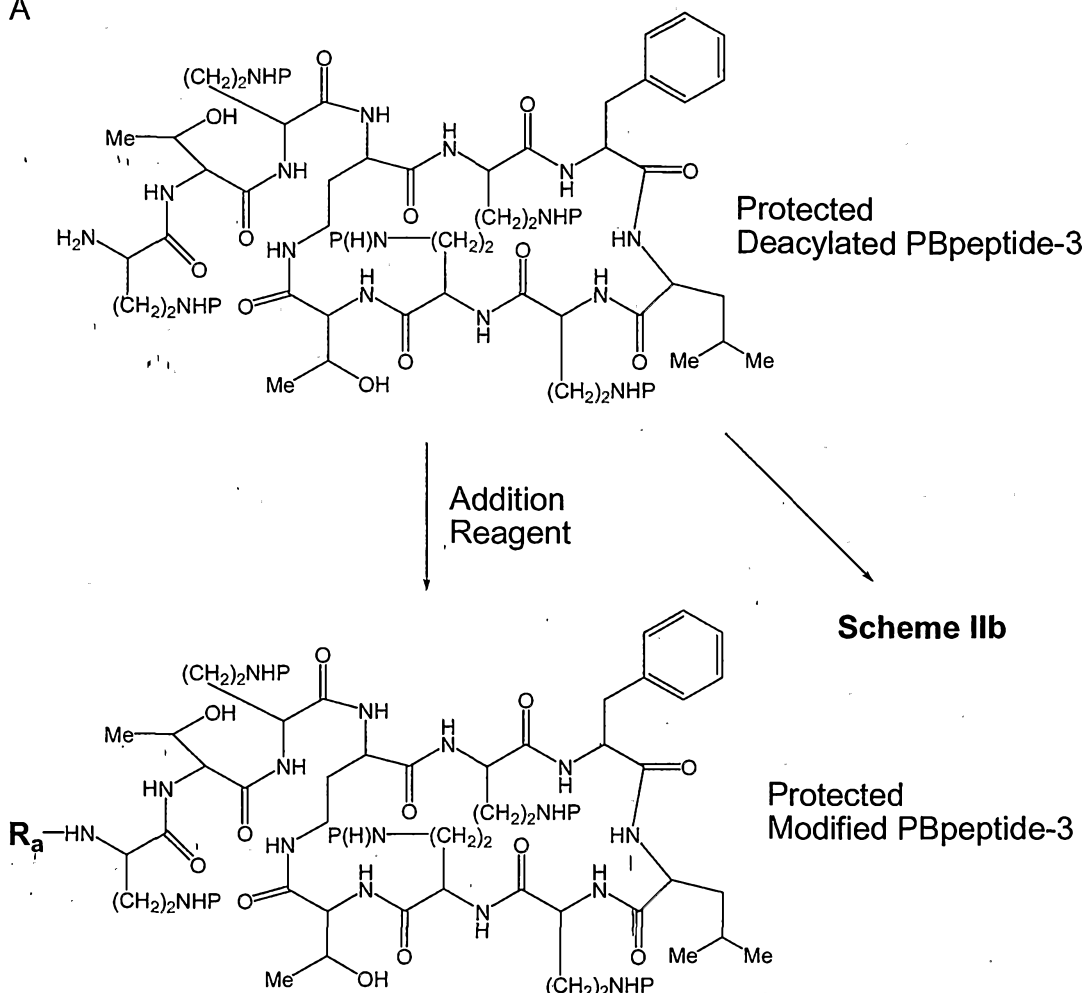
[090] The deacylase enzyme can be obtained as a water-soluble freeze-dried solid. In one embodiment, the deacylase is obtained by fermenting *Actinoplanes utahensis*, separating the cells from the fermentation medium,

washing the cells with water, extracting the cells with basic buffer at pH 8-11 for about 20 minutes, adjusting the extract to pH 7-8 and freeze-drying. The powdered form of the enzyme resulting from this process can be relatively stable and can be readily re-dissolved in water for use. Further purification can be obtained by gel filtration or other types of chromatography. This enzyme can deacylate, for example, N-[(2-sulfo)-9-fluorenylmethoxycarbonyl]₅ polymyxin B to obtain the protected polymyxin B decapeptide (protected deacylated PBpeptide-3). In other embodiments, the enzyme from *Actinoplanes utahensis* can be used as the whole broth from the fermentation or as the washed cells.

[091] The enzyme from *Actinoplanes utahensis* can also be used as a water-solubilized preparation. The water-solubilized enzyme preparation can be obtained by a relatively strong basic extraction of the washed cells, followed by adjustment of the pH of the clear extract to pH 7-8. This water-solubilized enzyme preparation can be freeze-dried to a solid form.

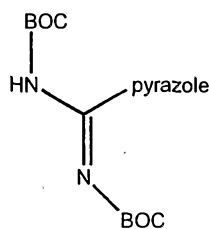
[092] Deacylation of the protected peptide provides a free amino terminus that can be modified by reaction with an addition reagent (Scheme IIa).

A



[093] Scheme IIa illustrates the direct modification of the deacylated PBpeptide-3. Treatment of the deacylated PBpeptide-3 with an addition agent results in the formation of protected modified PBpeptide-3 compound. The reaction of an amine with an addition reagent, as defined herein, is well known to those skilled in the art. For example, treatment of deacylated protected PBpeptide-3 with an isocyanate gives compounds in which $R_a\text{-NH-}$ is ureido. Similarly, treatment of deacylated protected PBpeptide-3 with an activated ester, lactone or acid chloride yields compounds in which $R_a\text{-NH-}$ is acylamino. Treatment of deacylated protected PBpeptide-3 with a sulfonyl chloride or activated sulfonamide results compounds in which $R_a\text{-NH-}$ is a sulfonamino. Treatment of deacylated protected PBpeptide-3 with an activated heterocycle results in a compound in which $R_a\text{-NH-}$ is a heterocyclyl amino. Treatment of

deacylated protected PBpeptide-3 with an activated heteroaryl results in a compound in which $R_a\text{-NH-}$ is a heteroaryl amino. Treatment of deacylated protected PBpeptide-3 with a carbonate, chloroformate, or cyanoformate gives compounds in which $R_a\text{-NH-}$ is a carbamate. Treatment of deacylated protected PBpeptide-3 with a thioacyl ester gives compounds in which $R_a\text{-NH-}$ is thioacylamino. Treatment of deacylated protected PBpeptide-3 with a phosphoryl chloride or phosphoramidate, gives compounds in which $R_a\text{-NH-}$ is phosphonamino. Treatment of deacylated protected PBpeptide-3 with an imidate or a ketenamine ($R''_2\text{C=C=NH}$) gives compounds in which $R_a\text{-NH-}$ is iminoamino. Treatment of deacylated protected PBpeptide-3 with an isocyanate gives compounds in which $R_a\text{-NH-}$ is ureido. Treatment of deacylated protected PBpeptide-3 with a thioisocyanate gives compounds in which $R_a\text{-NH-}$ is thioureido. Treatment of deacylated protected PBpeptide-3 with an aldehyde or ketone under reductive conditions gives compounds in which $R_a\text{-NH-}$ is a monosubstituted amino group or a disubstituted amino group. Nonlimiting examples of reducing agents include H_2/Ni (cat.), Zn/HCl , NaBH_3CN , $\text{NaBH}(\text{OAc})_3$, NaBH_4 , $\text{BH}_3\cdot\text{pyridine}$, and formic acid. Treatment of deacylated protected PBpeptide-3 with a guanidinylation agent such as



provides compounds in which $R_a\text{-NH-}$ is guanidino.

[094] It will be understood by those skilled in the art that if an addition agent contains substituents that are incompatible with the reaction conditions under which the protected modified PBpeptide is formed, said substituents can be protected prior to use in the reaction. Suitable protecting groups and methods of making them can be found in Greene (*vide supra*).

[095] In another embodiment, one or more N-terminal amino acids may be removed from the exocyclic chain of the deacylated protected peptide. For example, a sequence of N-terminal amino acid hydrolyses, e.g., Edman degradations, modified Edman degradations or enzymatic reactions (e.g., catalyzed by an aminopeptidase), can be used to obtain the protected forms of

other peptides, designated as the "peptide-0," "peptide-1," or "peptide-2," which contain zero, one or two amino acids, respectively, in the exocyclic chain.

[096] The Edman degradation is a well-established reaction known to those skilled in the art (see, for example, P. Edman, 1950, *Acta Chem. Scan.* 4: 283-93 and P. Edman, 1956, *Acta Chem. Scan.* 10: 761-768). Exemplary methods for performing N-terminal amino acid hydrolyses are described in Voet et al., *Biochemistry*, 2nd Edition, John Wiley & Sons, New York, 1995, pp. 107-109, Creighton et al., *Proteins*, 2nd Edition, W.H. Freeman, New York, 1993, pp. 31-35, and Loudon et al., *Organic Chemistry*, 2nd Edition, Benjamin/Cummings, Menlo Park, CA, 1988, pp. 1154-1161, the disclosures of which are incorporated herein by reference.

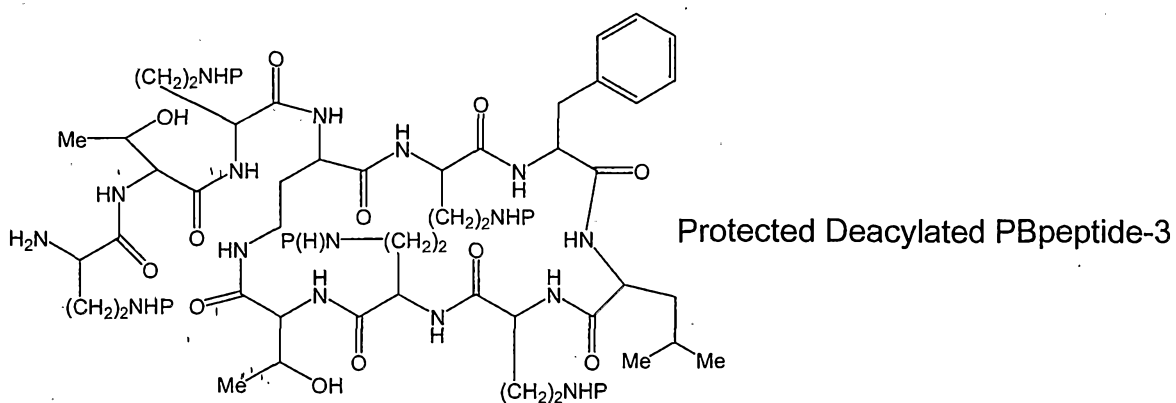
[097] The Edman degradation can be carried out under a variety of conditions. In the first step of the Edman degradation, an isothiocyanate reacts with the terminal amine under neutral to mildly basic (pH < 9.5) conditions in solvents such as, but not limited to, tetrahydrofuran, N,N'-dimethylformamide, dichloromethane, dioxane or ethanol to form a thiourea derivative of the peptide. A variety of isothiocyanates can be used (see K. K. Han et al. *Biochemie* 1977,59 : 557-576).

[098] Upon treatment with acid or base, the thiourea peptide undergoes a cyclization reaction, giving a thiohydantoin and a peptide shorter by one amino acid residue. Subsequent cyclization and cleavage can be accomplished under a variety of conditions. Typically, anhydrous trifluoroacetic acid, heptafluorobutyric acid (see, for example, W. F. Brandt et al., 1976, *Z. Physiol. Chem.* 357: 1505-1508) or concentrated hydrochloric acid (see, for example, G. E. Tarr, 1977, *Methods Enzymol.* 47: 335-337) are used. Mild basic conditions such as triethylamine or N,N-dimethylallylamine / acetic acid (pH ~9) can also be used (see G.C. Barrett et al., 1985, *Tetrahedron Lett.* 26(36): 4375-4378). For a review of this reaction see K.K. Han, 1985, *Int. J. Biochem.* 17(4): 429-445). In one embodiment, cyclization and cleavage are accomplished under basic conditions.

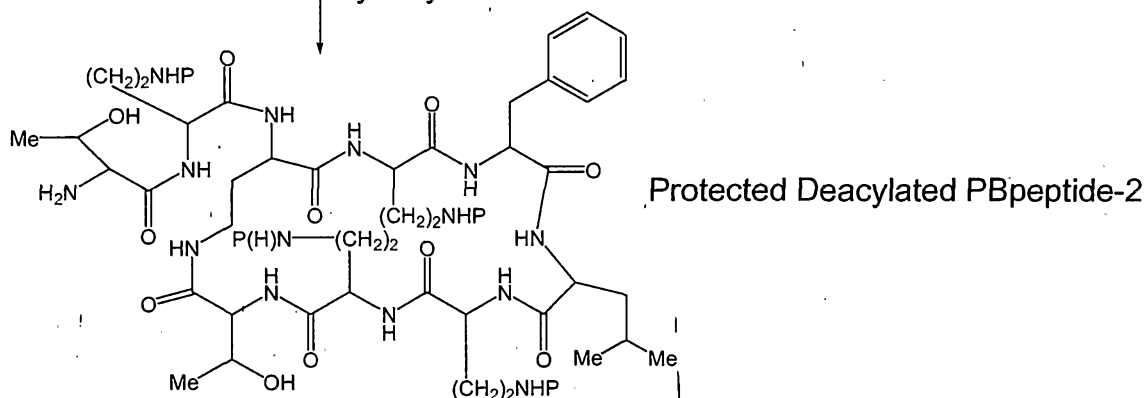
[099] In another embodiment, Scheme 11a illustrates modification of the protected deacylated PBpeptide-3 directly, for example, where the addition reagent is an acylamino reagent selected from R'-(C=O)-LG and R'-SO₂-LG, where LG is a leaving group. In some embodiments, an addition reagent may be an activated amino acid, or an amino acid and a peptide coupling reagent, such

as, e.g., PyBOP[®] (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HBTU/HOBt (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate / N-hydroxybenzotriazole), or DCC (dicyclohexylcarbodiimide). In one embodiment, R' and R'' are selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl. Nonlimiting examples of R'-(C=O)-LG include acyl halides, such as acyl chlorides, acyl cyanides, esters (e.g., succinimidyl esters), acyl azides, lactones, and anhydrides. Nonlimiting examples of R'-SO₂-LG include sulfonyl chlorides. When R_a is an imine, R'-(C=NH)-, a modified PBpeptide-3 containing an iminoamino group may be formed by reacting deacylated PBpeptide-3 with a ketenamine R''₂C=C=NH, where R'' is selected from R' and hydrogen. When R_a is an amide R'-NH-(C=O)- or a thioamide R'-NH-(C=S)-, a modified PB peptide 3 containing a urea or thiourea may be formed by reacting deacylated PB peptide 3 with an isocyanate or a isothiocyanate, respectively. When R_a is a substituted alkyl, R'-alkyl-, a modified PBpeptide 3 containing the substituted alkyl may be formed by reacting deacylated PBpeptide 3 with R'C(O)R'' and a reducing agent. Nonlimiting examples of reducing agents include H₂/Ni (cat.), Zn/HCl, NaBH₃CN, NaBH(OAc)₃, NaBH₄, BH₃·pyridine, and formic acid.

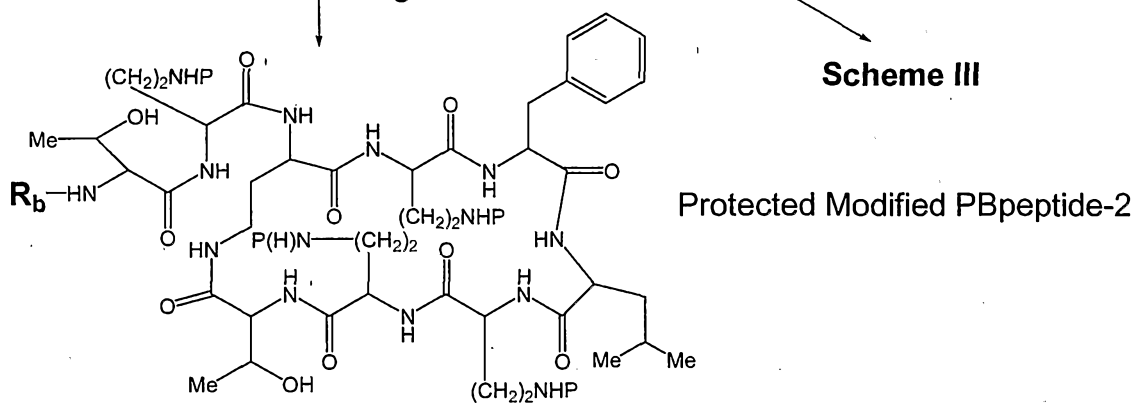
[0100] Schemes IIb, III, and IV, schematically depict how "peptide-0," "peptide-1," or "peptide-2" versions of polymyxin B can be prepared (*vide infra*). Scheme IIb shows removal of an amino acid to convert protected deacylated PBpeptide-3 to protected deacylated PBpeptide-2. The protected deacylated PBpeptide-2 can then be chemically modified with the procedures described for R_a above to install the R_b substituent.



N-terminal
amino acid
hydrolysis



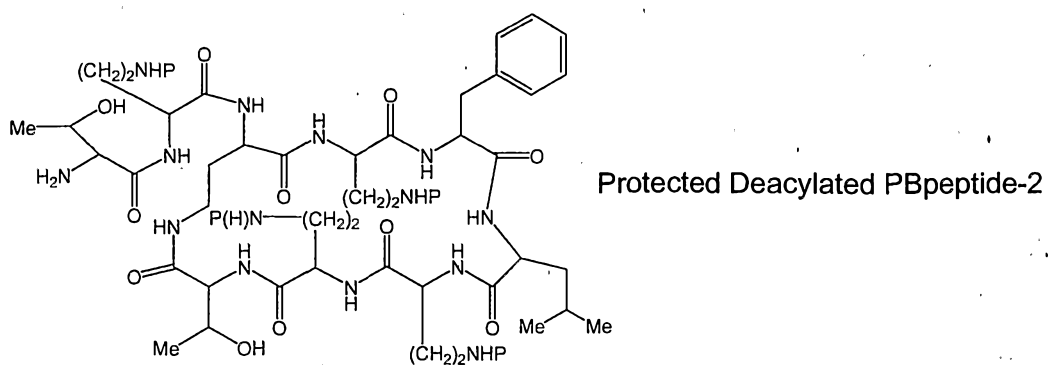
Addition
Reagent



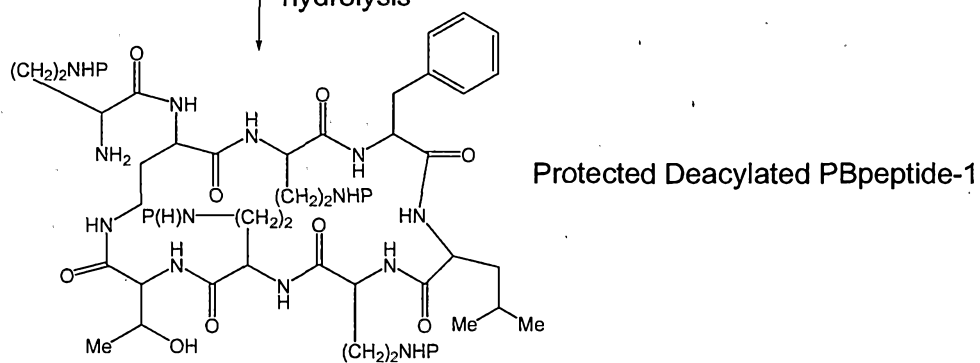
Scheme III

Scheme IIb

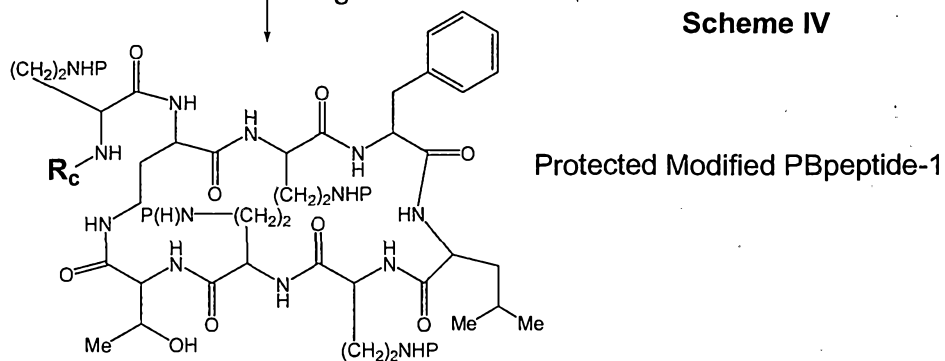
[0101] Scheme III similarly shows conversion of protected deacylated PBpeptide-2 to protected deacylated PBpeptide-1, which can then be chemically modified with addition reagents, as described above, to install the R_c substituent.



N-terminal
amino acid
hydrolysis



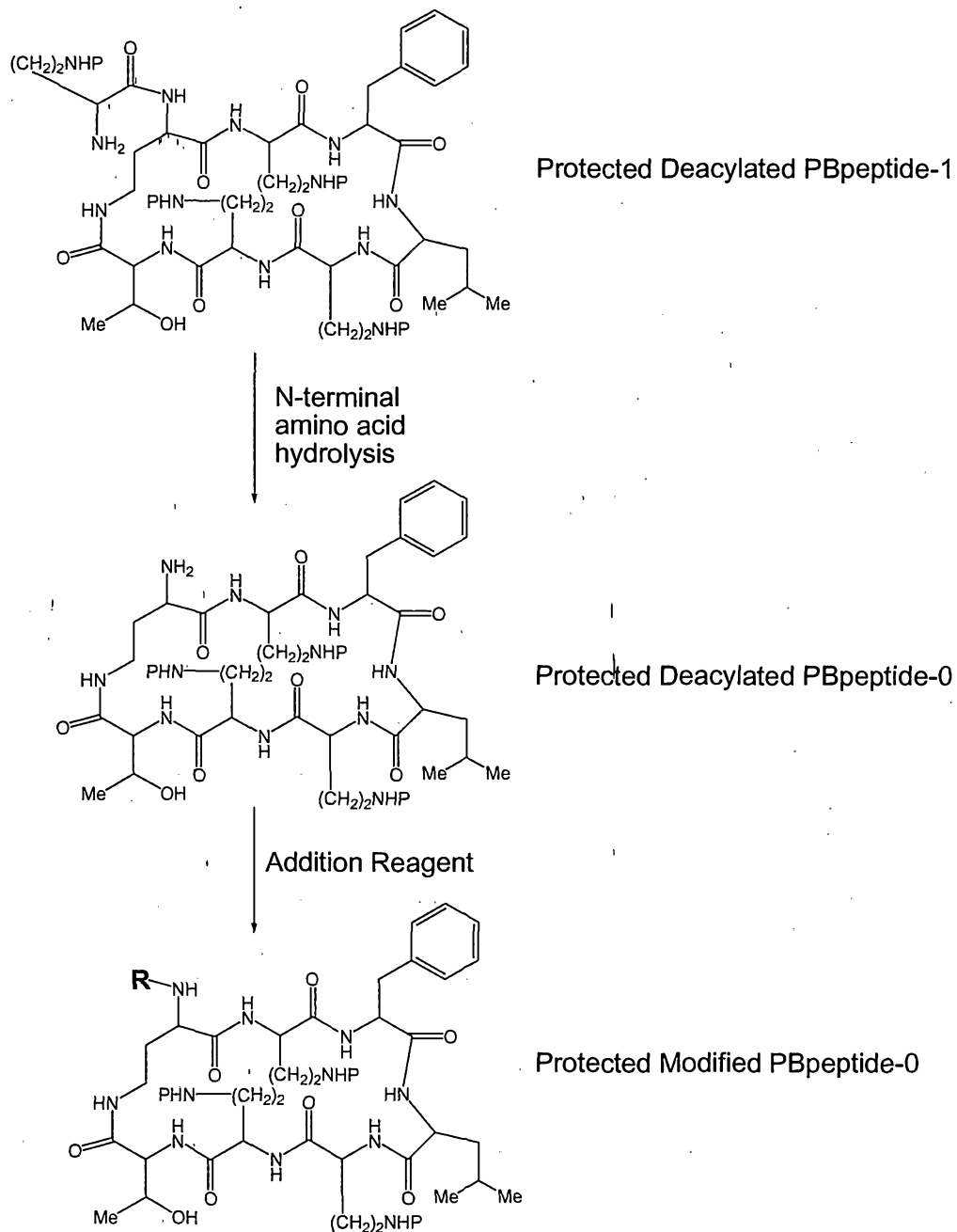
Addition
Reagent



Scheme IV

Scheme III

[0102] Alternatively, deacylated protected PBpeptide-1 can be subjected to another N-terminal amino acid hydrolysis reaction (Scheme IV), to form deacylated protected PBpeptide-0, which does not contain an exocyclic peptide chain.



Scheme IV

[0103] Although the Schemes above illustrate reactions involving polymyxin B derivatives, one of ordinary skill in the art can appreciate that these methods are equally applicable to any of the peptides disclosed herein.

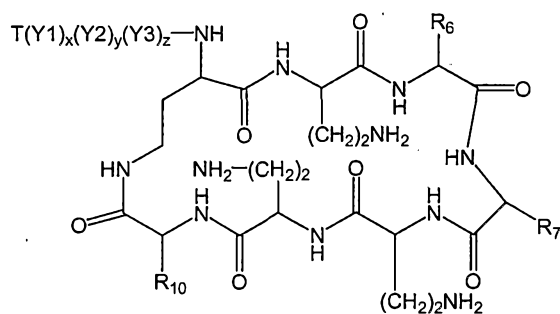
[0104] One of ordinary skill in the art can appreciate that the hydrolysis/chemical modification steps need not strictly occur in separate reactions. For example, the hydrolysis and chemical modification steps can occur nearly simultaneously or can be performed in a one-pot reaction mixture.

[0105] Each of the protected peptides, such as the protected deacylated PBpeptide-2, can be used to make new peptides having antibacterial activity. For example, reagents that form the tail, e.g., alkyl or aromatic acids, can be converted to activated species and then coupled to the protected peptides, followed by deprotection to give new peptides, such as compounds 1, 2, 6, 7, 11, and 12 in Table 3. The protected peptides can also be treated directly with alkyl or aromatic isocyanates or isothiocyanates to give the corresponding ureas and thioureas, and the products deprotected to give another new series of novel peptides, as represented by compound 35. Ureas can also be prepared by treatment with N-carboxyloxysuccinimidyl derivatives of the appropriate amines. The latter derivatives are readily prepared from the reaction of amines and disuccinimidyl carbonate.

[0106] One embodiment provides a method for preparing a compound, comprising:

- (a) treating a peptide having at least one side chain comprising an amino group with an amino protecting group reagent to form a protected peptide, wherein the peptide comprises a cyclic heptapeptide attached to an exocyclic peptide chain comprising an acyl group, and the protecting group comprises at least one acidic substituent; and
- (b) treating the protected peptide with a deacylating agent to form a protected, deacylated peptide.

[0107] In one embodiment, the peptide in step (a) has a structure of formula (A):



(A)

wherein:

Y1, Y2, and Y3 are each independently selected from amino acid residues; T is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, R' -, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; and R_6 , R_7 , and R_{10} are each independently chosen from isopropyl, benzyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl. In another embodiment, R_6 and R_7 are each independently chosen from isopropyl, benzyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl and R_{10} is chosen from isopropyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl. In another embodiment, Y1, Y2, and Y3 are each independently selected from 2,4-diaminobutanoic acid residue, threonine residue, and serine residue. In a further embodiment, T is chosen from 6-methyloctanoyl, 6-methylheptanoyl, octanoyl, heptanoyl, nonanoyl, and 3-hydroxy-6-methyloctanoyl.

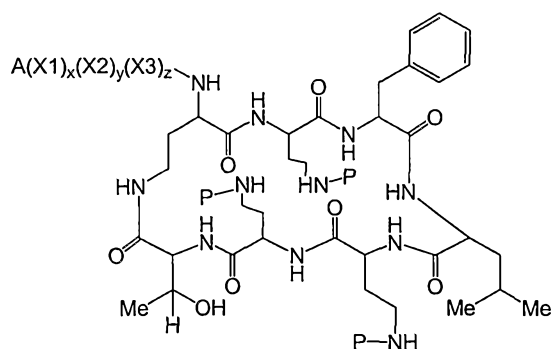
[0108] In another embodiment, the peptide in step (a) is selected from polymyxin A, polymyxin B, [Ile⁷]-polymyxin B, polymyxin C, polymyxin D, colistin, polymyxin F, polymyxin M, polymyxin P, polymyxin S, polymyxin T, circulin A, octapeptin A, octapeptin B, octapeptin C, and octapeptin D. In another embodiment, the peptide in step (a) is selected from polymyxin B, polymyxin A, polymyxin D, [Ile⁷]-polymyxin B, colistin, circulin A, octapeptin B, and octapeptin C. In a further embodiment, the peptide in step (a) is selected from polymyxin A, polymyxin D, [Ile⁷]-polymyxin B, colistin, circulin A, octapeptin B, and octapeptin C. In another embodiment, the peptide in step (a) is polymyxin B.

[0109] In one embodiment, the at least one acidic substituent is selected from carboxy, carboxylate, sulfo, sulfate, phosphonate, and salts thereof. Another

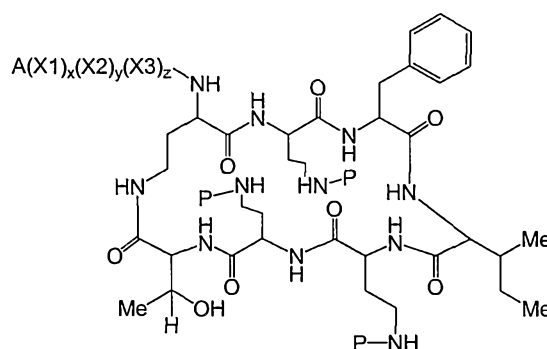
embodiment provides a protecting group comprising an aryl or heteroaryl substituted with the at least one acidic substituent. In a further embodiment, the protecting group is a sulfonic acid of 9-fluorenylmethoxycarbonyl, such as 2-sulfo-9-fluorenylmethoxycarbonyl.

[0110] In one embodiment, the deacylating agent is an enzyme. The source of the enzyme is *Actinoplanes utahensis* in another embodiment.

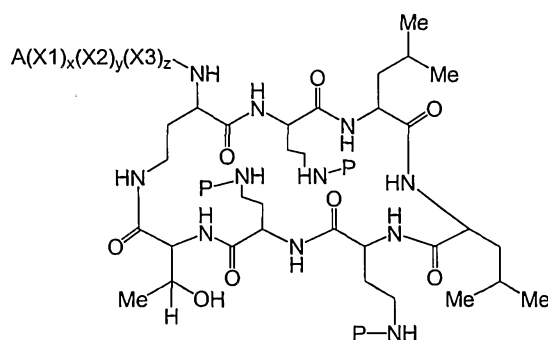
[0111] In one embodiment, the method for preparing a compound further comprises step (c): forming from the protected deacylated peptide compounds having the following formulae:



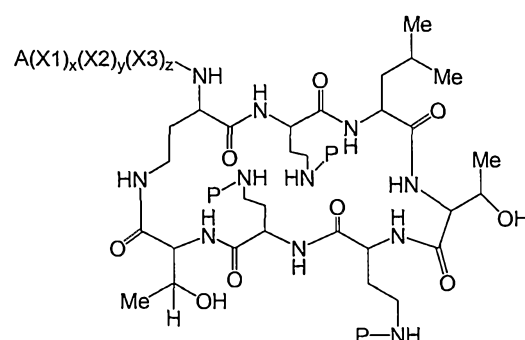
(I)



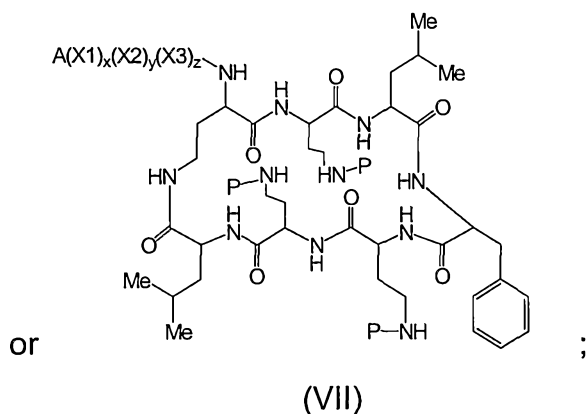
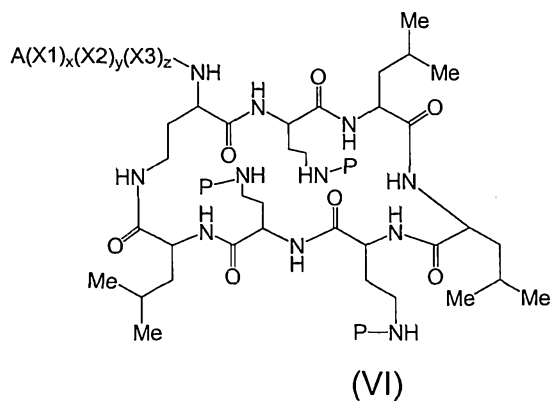
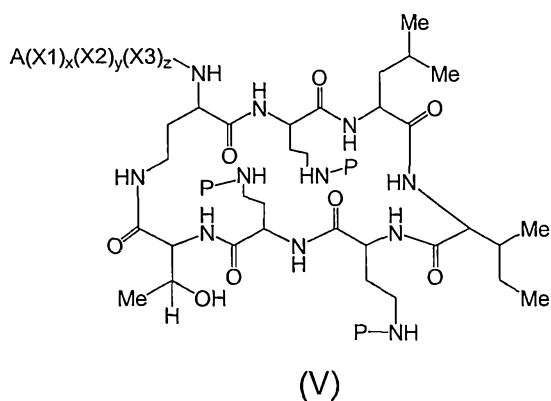
(II)



(III)



(IV)



wherein A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, R' -, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X_1 , X_2 , and X_3 are each independently selected from amino acid residues; x , y , and z are integers independently selected from 0 and 1; and P is the protecting group containing at least one acidic substituent.

[0112] In another embodiment, the forming comprises treating the protected peptide with a reagent having the formula $A-LG$, wherein LG is a leaving group. In another embodiment, the forming comprises treating the protected peptide with a reagent selected from isocyanate, thioisocyanate, lactone, an activated heterocycle, an activated heteroaryl, imidate, ketenamine, an aldehyde and a reducing agent, and a ketone and a reducing agent. In a further embodiment, the

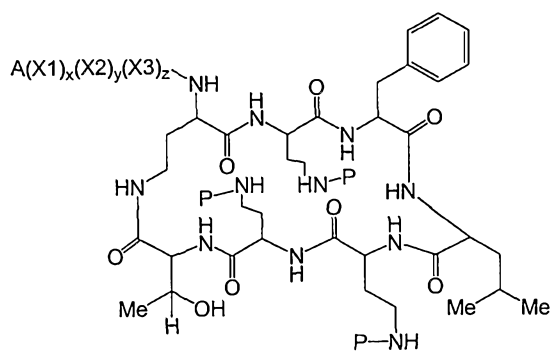
forming comprises treating the protected peptide with an acylating reagent selected from acyl halides, acyl cyanides, esters, lactones, and anhydrides. In another embodiment, the forming comprises treating the protected peptide with a sulfonating reagent selected from sulfonyl chloride and activated sulfonamides.

[0113] In one embodiment, the forming comprises subjecting the protected deacylated peptide to an N-terminal amino acid hydrolysis reaction such that x is 0 and each of y and z is independently 1. In another embodiment, the forming comprises subjecting the protected deacylated peptide to a second N-terminal amino acid hydrolysis reaction such that each of x and y is independently 0 and z is 1. Another embodiment includes subjecting the protected deacylated peptide to a third N-terminal amino acid hydrolysis reaction such that each of x , y and z is 0.

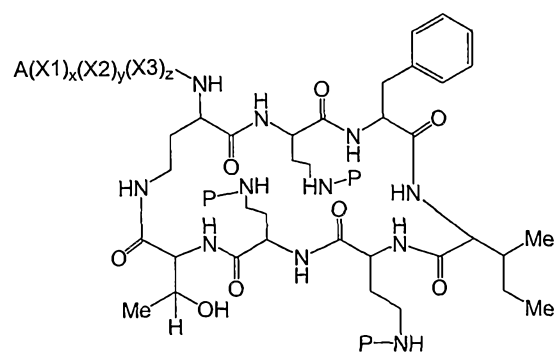
[0114] One embodiment discloses a method for preparing a compound, comprising:

- (a) treating a peptide having at least one side chain comprising an amino group with an amino protecting group reagent to form a protected peptide, wherein the peptide comprises a cyclic heptapeptide attached to an exocyclic peptide chain comprising an acyl group, and the protected peptide is water soluble; and
- (b) treating the protected peptide with a deacylating agent to form a protected, deacylated peptide.

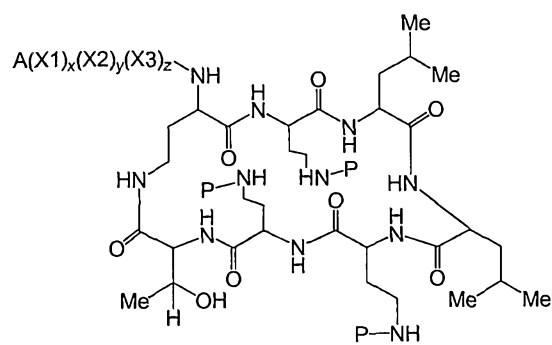
[0115] One embodiment provides new protected peptides having a structure chosen from formulae (I) – (VII):



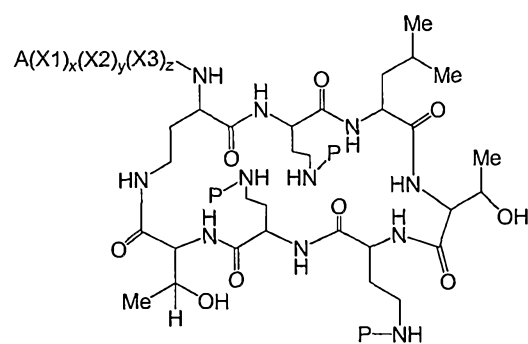
(I)



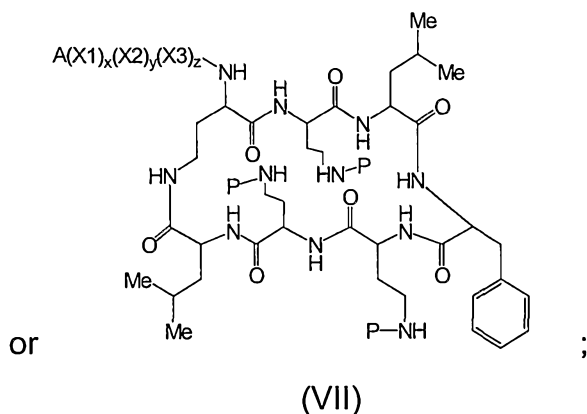
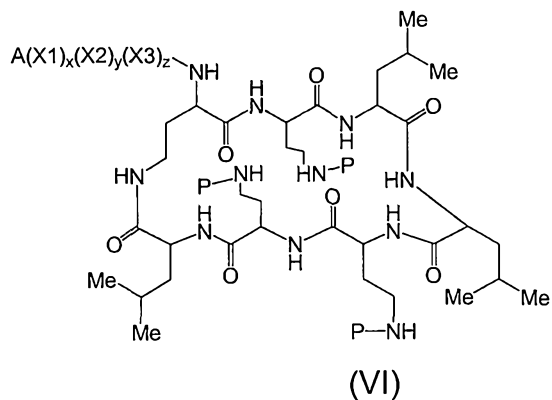
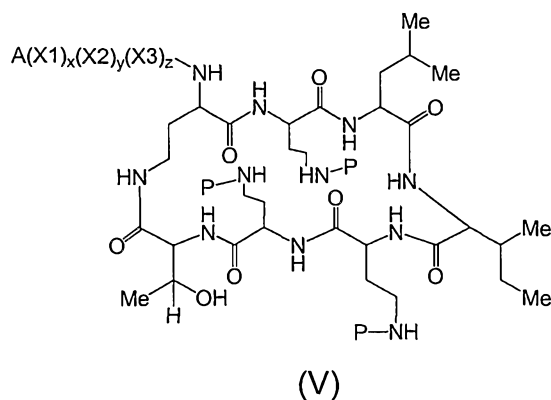
(II)



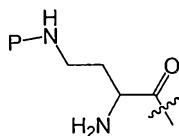
(III)



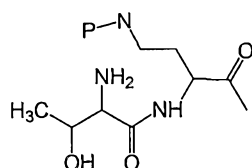
(IV)



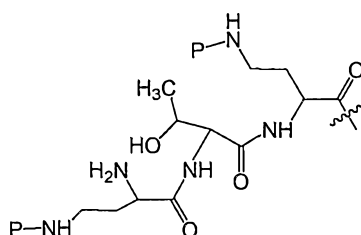
wherein A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, $R'-$, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X_1 , X_2 , and X_3 are each independently selected from amino acid residues; x , y , and z are integers independently selected from 0 and 1; and P is a protecting group containing at least one acidic substituent. In another embodiment, P is a 9-fluorenylmethoxycarbonyl group substituted with at least one acidic substituent, such as a 2-sulfo-9-fluorenylmethoxycarbonyl group. Another embodiment discloses the compound where x and y are each independently 0, z is 1, and X_3 is:



[0116] A further embodiment includes the compound where x is 0, each of y and z is independently 1, and X2-X3 is:

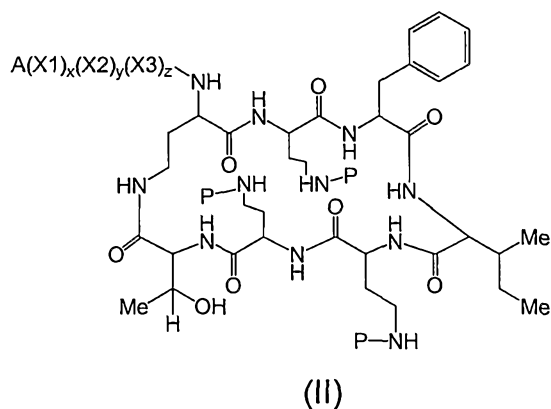
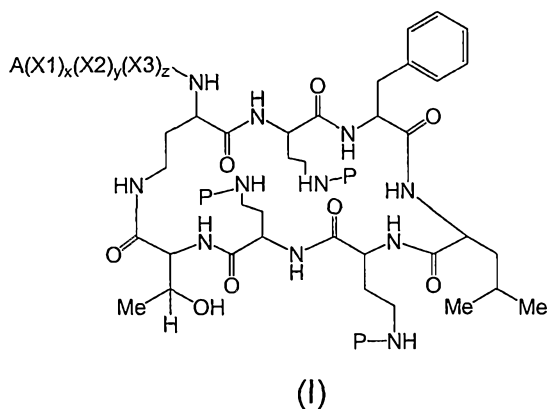


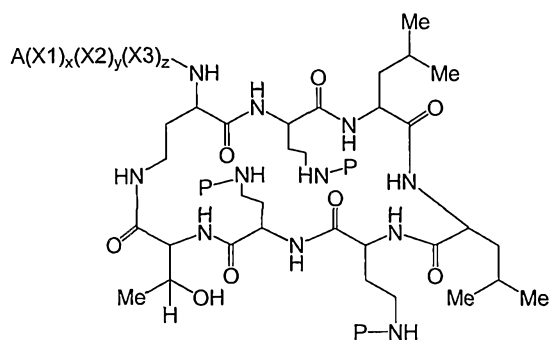
[0117] Another embodiment provides the compound where x, y, and z are each independently 1, and X1-X2-X3 is:



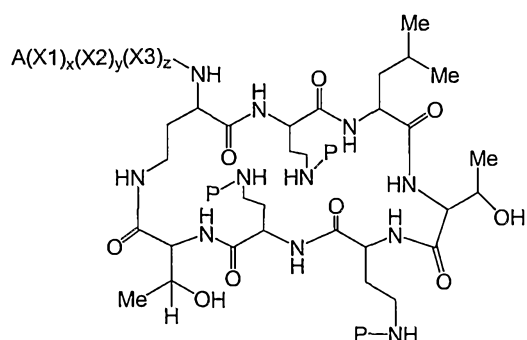
[0118] In a further embodiment, each of x, y, and z is independently 0 and A is hydrogen. In another embodiment, the compound is a prodrug.

[0119] One embodiment discloses a compound having a structure chosen from formulae (I) – (VII):

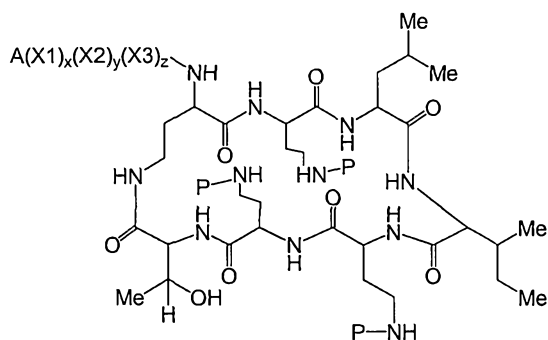




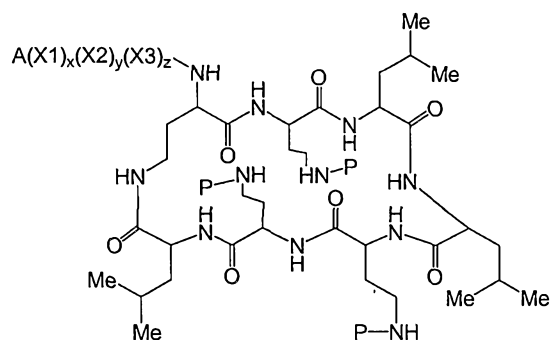
(III)



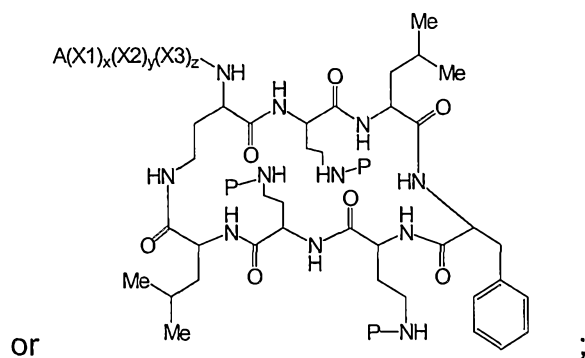
(IV)



(V)



(VI)



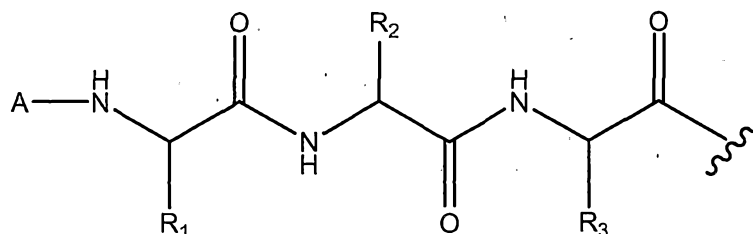
(VII)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently

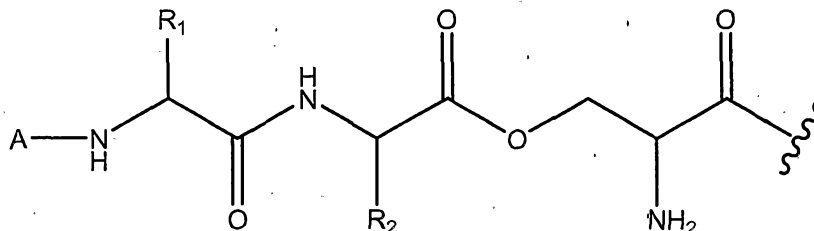
selected from amino acid residues; x, y, and z are integers independently selected from 0 and 1; P is a protecting group; and the compound having a structure chosen from formulae (I) – (VII) is water-soluble.

[0120] Nonlimiting examples of amino acid residues for X1, X2, and X3 include those derived from the twenty encoded amino acids and derivatives thereof, other α -amino acids, β -amino acids, γ -amino acids, δ -amino acids, and ω -amino acids. X1, X2, and X3 may have either R or S chirality at any chiral atom. In one embodiment, X1, X2, and X3 are chosen from alanine, β -alanine, α -aminoadipic acid, α -aminobutanoic acid, γ -aminobutanoic acid, ϵ -aminocaproic acid, 1-aminocyclopentanecarboxylic acid, ϵ -aminohexanoic acid, 2-aminoheptanedioic acid, 7-aminoheptanoic acid, α -aminoisobutyric acid, aminomethylpyrrole carboxylic acid, 8-amino-3,6-dioxa-octanoic acid, aminopiperidinecarboxylic acid, 3-amino-propionic acid, aminoserine, aminotetrahydropyran-4-carboxylic acid, arginine, asparagine, aspartic acid, azetidine carboxylic acid, benzothiazolylalanine, butylglycine, carnitine, 4-chlorophenylalanine, citrulline, cyclohexylalanine, cyclohexylstatine, cysteine, diaminobutanoic acid, diaminopropionic acid, dihydroxyphenylalanine, dimethylthiazolidine carboxylic acid, glutamic acid, glutamine, glycine, histidine, homoserine, hydroxyproline, isoleucine, isonipecotic acid, leucine, lysine, methanoproline, methionine, norleucine, norvaline, ornithine, p-aminobenzoic acid, penicillamine, phenylalanine, phenylglycine, piperidinylalanine, piperidinyglycine, proline, pyrrolidinylalanine, sarcosine, selenocysteine, serine, statine, tetrahydropyranglycine, thienylalanine, threonine, tryptophan, tyrosine, valine, allo-isoleucine, allo-threonine, 2,6-diamino-4-hexanoic acid, 2,6-diaminopimelic acid, 2,3-diaminopropionic acid, dicarboxidine, homoarginine, homocitrulline, homocysteine, homocystine, homophenylalanine, homoproline, and 4-hydrazinobenzoic acid.

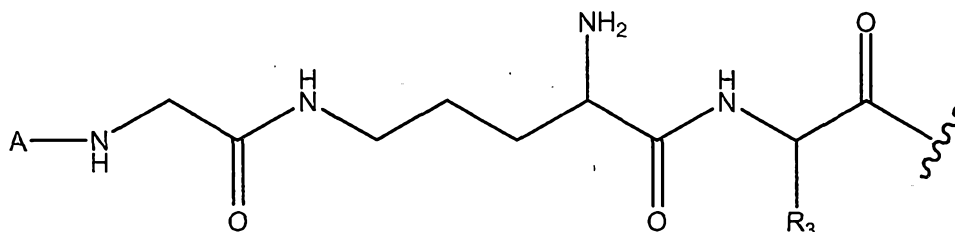
[0121] X1 and X2; X2 and X3; and X3 and the N-terminal residue of the cyclic heptapeptide may be joined by amide bonds. For example, in the embodiment wherein X1, X2, and X3 are α -amino acids, X1, X2, and X3 (having side chains R1, R2, and R3, respectively) may be connected as depicted below.



[0122] In some embodiments, X1 and X2 and/or X2 and X3 may be joined by ester bonds, as depicted below in the embodiment wherein X3 is serine.



[0123] In embodiments wherein X1 or X2 has a side chain amino group, X1 and X2 and/or X2 and X3 may be connected through a side chain amide, as depicted below in the embodiment wherein X2 is ornithine.



[0124] In one embodiment, the tail of the new peptide is depicted as "AB", where the "tail-exocyclic chain" portion has the formula $AB(X1)_x(X2)_y(X3)_z$. In one embodiment, AB is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, R' -alkyl, and hydrogen wherein R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[0125] In one embodiment, new compounds can be formed by removing the protecting groups from the protected peptide, i.e., by deprotection, by the methods described in "Protective Groups in Organic Synthesis" by Theodora W. Greene, John Wiley and Sons, New York, 1991, the disclosure of which is incorporated herein by reference.

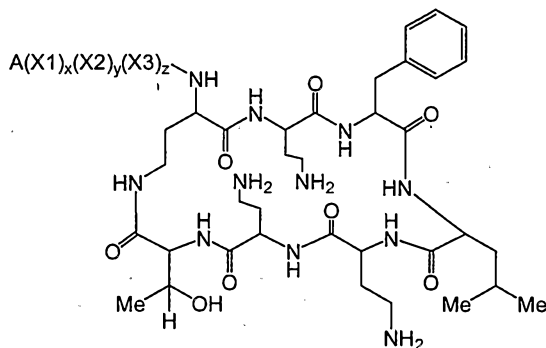
[0126] Removal of amino protecting groups can be accomplished according to procedures described in Greene (*vide supra*). As those skilled in the art will recognize, the choice of amino protecting group employed in the first step

of the process will dictate the reagents and procedures used in removing said amino protecting group.

[0127] When the reagent for the chemical modification contains one or more protecting group(s), those protecting group(s) must also be removed. The choice of the protecting group(s) utilized on the chemical modifying agent substituent(s) will dictate the reagents and procedures used in removing said protecting group(s). When the protecting group(s) utilized on the modifying agent substituent(s) and the protecting group utilized for the protected peptide are compatible, the protecting groups may be removed in a single step. However, when the protecting group(s) are incompatible multiple steps may be required to remove all of the protecting groups.

[0128] In one embodiment, the deprotected peptides are purified by gel filtration, chromatography, or reverse-phase HPLC.

[0129] One embodiment includes a compound of formula (1):



(1)

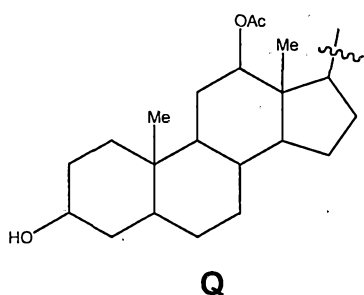
wherein A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, R' -, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; $X1$, $X2$, and $X3$ are each independently selected from amino acid residues; and x , y , and z are integers independently selected from 0 and 1,

with the proviso that:

- 1) A does not comprise at least one amino acid residue;
- 2) if $X3$ is 2,4-diaminobutanoic acid, each of x and y is independently selected from 0 and 1, and A is $R'-(C=O)-$, then R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and

substituted alkyl, wherein at least one hydrogen is replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido,

provided that the substituted alkyl is not selected from alkyl-CHOH-CH₂-, phenyl-CH₂-, adamantyl-CH₂-, substituted aryloxy-CH₂-, and CH₃-CHQ-CH₂-CH₂- wherein Q is the structure:

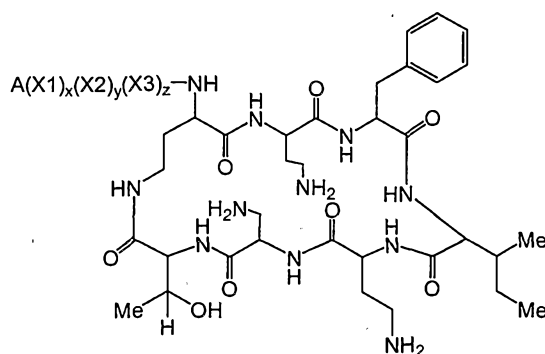


3) if x, y and z are each 1, X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is aryl, then the aryl is not a 6-membered ring having three hydroxy substituents; and

4) if each of x, y, and z is independently 0 and A is R'-(C=O)-, then R' is selected from C₈₋₂₀-alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[0130] In another embodiment, if X3 is 2,4-diaminobutanoic acid, x and y are independently selected from 0 and 1, and A is R'-(C=O)-, then R' is selected from C₉₋₂₀ unsubstituted alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl and substituted alkyl, wherein at least one hydrogen is replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkynyl, amino, substituted aryl, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, iminoamino, monosubstituted amino, nitro, oxo, phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido.

[0131] One embodiment provides a compound of formula (2):



(2)

wherein A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, $R'-$, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; $X1$, $X2$, and $X3$ are each independently selected from amino acid residues; and x , y , and z are integers independently selected from 0 and 1,

with the proviso that:

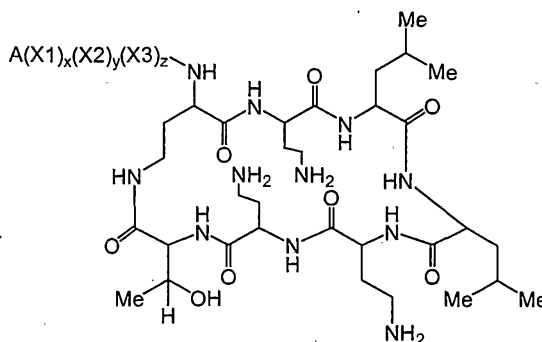
- 1) A does not comprise at least one amino acid residue; and
- 2) if each of x , y and z is independently 1, $X1$ is 2,4-diaminobutanoic acid, $X2$ is threonine, $X3$ is 2,4-diaminobutanoic acid, and A is $R'-(C=O)-$, then R' is not branched C_8 -alkyl.

[0132] One embodiment discloses a compound selected from formulae (I) – (VII), (1) and (2), wherein $X1$ is 2,4-diaminobutanoic acid, $X2$ is threonine, $X3$ is 2,4-diaminobutanoic acid, A is $R'-O-(C=O)-$ and R' is phenyl. Another embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein $X1$ is 2,4-diaminobutanoic acid, $X2$ is threonine, $X3$ is 2,4-diaminobutanoic acid, A is $R'-(C=O)-$ and R' is n - C_9 -alkyl. Another embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein $X1$ is 2,4-diaminobutanoic acid, $X2$ is threonine, $X3$ is 2,4-diaminobutanoic acid, A is $R'-(C=O)-$ and R' is an alkyl substituted with a group selected from $N-(C_{1-10}$ -alkyl)-4-aminophenyl and a benzyloxy group. A further embodiment includes a compound selected from formulae (I) – (VII), (1) and (2), wherein $X1$ is 2,4-diaminobutanoic acid, $X2$ is threonine, $X3$ is 2,4-diaminobutanoic acid, A is $R'-NH-(C=O)-$ and R' is selected from n - C_8 -alkyl, C_2 -alkyl substituted with a 3-indolyl group, cyclohexyl, unsubstituted phenyl, benzyl, 4'-biphenyl, and phenyl substituted with a group selected from 4- C_{10} -alkyl, 4'-phenyloxy, and 4-chloro.

[0133] Another embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-NH-(C=S)- and R' is phenyl. Another embodiment discloses a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is selected from phenyl, 4-pyridinyl, and alkyl substituted with a 2-naphthoxy group. A further embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-SO₂- and R' is 4-methylphenyl. Another embodiment includes a compound selected from formulae (I) – (VII), (1) and (2), wherein x is 0, each of y and z is independently 1, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-NH-(C=O)- and R' is n-C₈-alkyl.

[0134] Another embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein x is 0, y and z are each 1, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-NH-(C=S)- and R' is phenyl. A further embodiment includes a compound selected from formulae (I) – (VII), (1) and (2), wherein each of x and y is independently 0, z is 1, X3 is 2,4-diaminobutanoic acid, A is R'-NH-(C=O)- and R' is n-C₉-alkyl. Another embodiment discloses a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is glycine, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-SO₂- and R' is n-C₁₀-alkyl. Another embodiment provides a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is lysine, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is n-C₉-alkyl. A further embodiment includes a compound selected from formulae (I) – (VII), (1) and (2), wherein X1 is phenylalanine, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is n-C₉-alkyl.

[0135] One embodiment provides a compound of formula (3):



(3)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

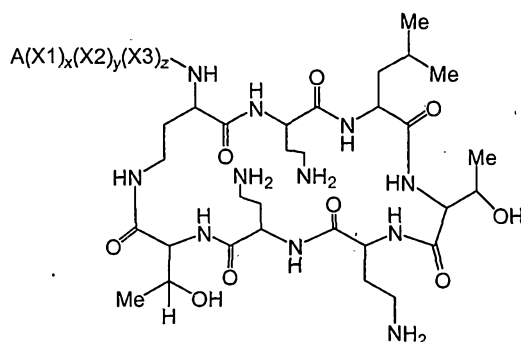
with the proviso that:

- 1) A does not comprise at least one amino acid residue;
- 2) if x is selected from 0 or 1, each of y and z is independently 1, X1 is an amino acid residue, X2 is threonine, X3 is 2,4-diaminobutanoic acid and A is R'-(C=O)-, then R' is selected from unbranched unsubstituted C₁₋₄-alkyl, cycloalkyl, alkenyl, aryl, heteroaryl and heterocyclyl, provided that: a) the alkenyl is not furanyl-CH=CH-, b) the aryl is not selected from naphthyl and 4-nitrophenyl, and c) the heteroaryl is not 2-thiophenyl; and
- 3) if x is selected from 0 or 1, each of y and z is independently 1, X1 is an amino acid residue, X2 is threonine, X3 is 2,4-diaminobutanoic acid and A is R'-SO₂-, then R' is selected from C₁₋₇-alkyl, C₉₋₂₀-alkyl, cycloalkyl, alkenyl, heteroaryl, and heterocyclyl.

[0136] Another embodiment includes a compound of formula (3), wherein if x is selected from 0 or 1, y and z are each 1, X1 is an amino acid residue, X2 is threonine, X3 is 2,4-diaminobutanoic acid and A is R'-(C=O)-, then R' is selected from unbranched unsubstituted C₁₋₄ alkyl, cycloalkyl, and heterocyclyl. Another embodiment provides a compound of formula (3), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-NH-(C=O)- and R' is n-C₈-alkyl. A further embodiment discloses a compound

of formula (3), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-SO₂- and R' is 4-methylphenyl.

[0137] One embodiment provides a compound of formula (4):

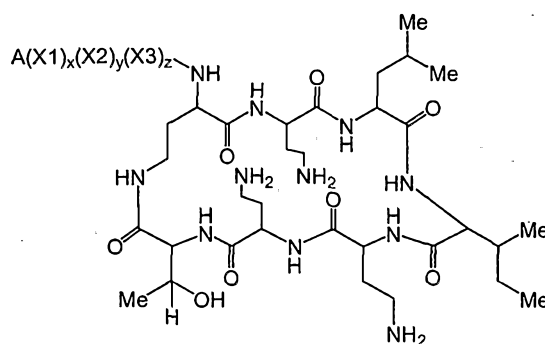


(4)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

with the proviso that if x, y and z are each 1, X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is not branched C₈₋₉-alkyl. In another embodiment, A does not comprise at least one amino acid residue.

[0138] One embodiment includes a compound of formula (5):



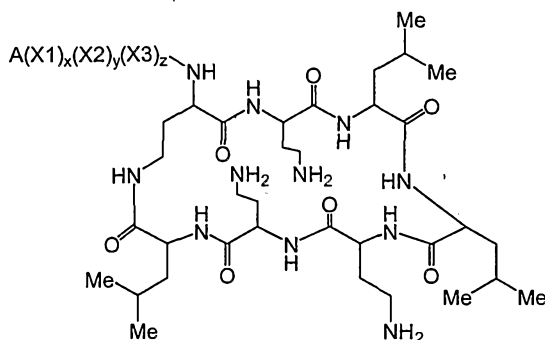
(5)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected

from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

with the proviso that if x, y and z are each 1, X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is not branched C₇₋₈-alkyl. In another embodiment, A does not comprise at least one amino acid residue.

[0139] One embodiment provides a compound of formula (6):

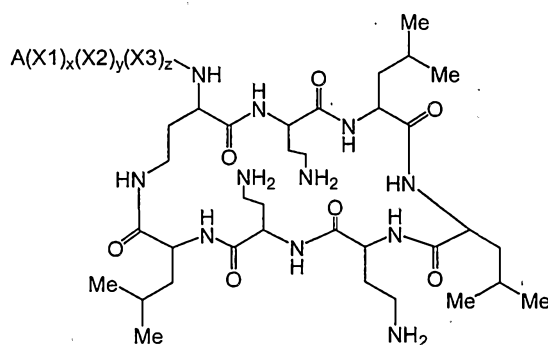


(6)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

with the proviso that if each of x and y is independently 0, z is 1, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is selected from substituted alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl, provided that the substituted alkyl a) is not substituted with an oxazolidine, or b) is not of the formula alkyl-CHOH-CH₂-. In another embodiment, A does not comprise at least one amino acid residue.

[0140] One embodiment discloses a compound of formula (6):

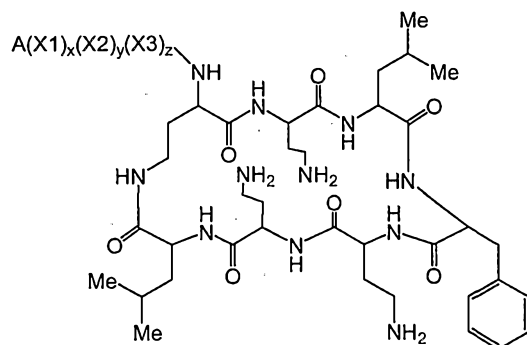


(6)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

with the proviso that if each of x and y is independently 0, z is 1, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is selected from substituted alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl, provided that the substituted alkyl a) is not substituted with an oxazolidine, or b) is not of the formula alkyl-CHOH-CH₂-. In another embodiment, A does not comprise at least one amino acid residue. In a further embodiment, if each of x and y is independently 0, z is 1, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is selected from substituted C₁₋₇-alkyl, substituted C₁₂₋₂₀-alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[0141] One embodiment provides a compound of formula (7):



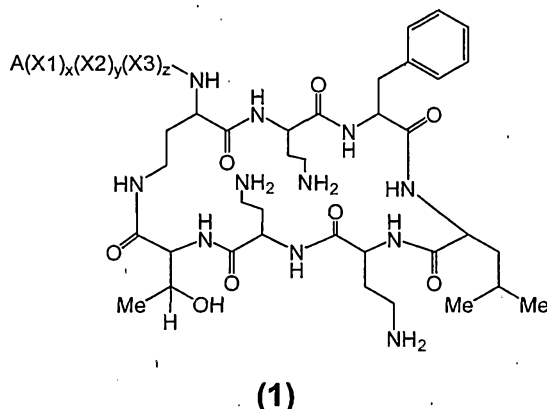
(7)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and each of x, y, and z is an integer independently selected from 0 and 1,

with the proviso that if each of x and y is independently 0, z is 1, X3 is 2,4-diaminobutanoic acid, and A is R'-(C=O)-, then R' is not branched C₉₋₁₁-alkyl with a hydroxyl substituent. In another embodiment, A does not comprise at least one amino acid residue.

[0142] One embodiment includes a compound of formula (1):



(1)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

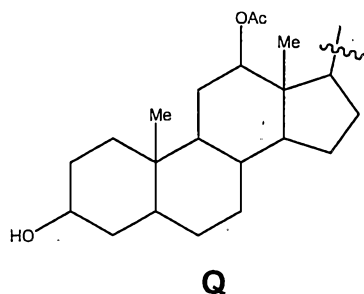
with the proviso that:

1) if X3 is 2,4-diaminobutanoic acid, each of x and y is independently selected from 0 and 1, and A is R'-(C=O)-, then R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and

substituted alkyl, wherein at least one hydrogen is replaced by a substituent group selected from acyl, acylamino, acyloxy, alkenyl, alkoxy, alkynyl, amino, aryl, aryloxy, carbamoyl, carboalkoxy, carboxy, carboxyamido, carboxyamino, cyano, disubstituted amino, formyl, guanidino, halo, heteroaryl, heterocyclyl, hydroxy, iminoamino, monosubstituted amino, nitro, oxo,

phosphonamino, sulfinyl, sulfonamino, sulfonyl, thio, thioacylamino, thioureido, and ureido,

provided that the substituted alkyl is not selected from alkyl-CHOH-CH₂-, phenyl-CH₂-, adamantyl-CH₂-, substituted aryloxy-CH₂-, and CH₃-CHQ-CH₂-CH₂- wherein Q is the structure:

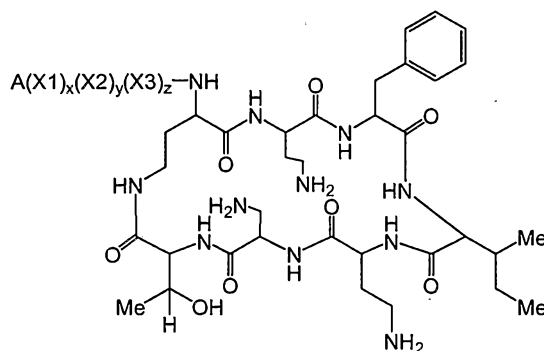


2) if x, y and z are each 1, X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is aryl, then the aryl is not a 6-membered ring having three hydroxy substituents; and

3) if each of x, y, and z is independently 0, then A is selected from C₈₋₂₀-alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl.

[0143] Another embodiment provides a compound selected from formulae (I) – (VII) and (1), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, and A is an N^α-(n-C₉-alkanoyl)lysine.

[0144] One embodiment includes a compound of formula (2):

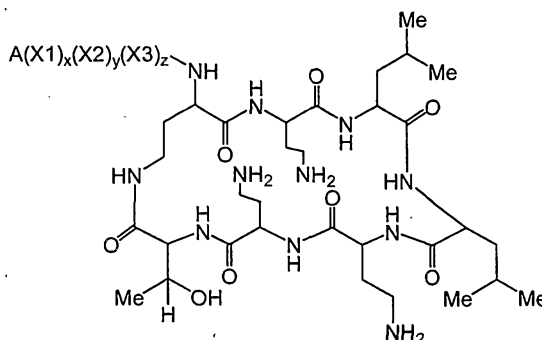


(2)

wherein A is selected from R'-(C=O)-, R'-SO₂-, R'-(C=NH)-, R'-NH-(C=S)-, R'-NH-(C=O)-, R'-NH-(C=NH)-, R'-O-(C=O)-, R'-O-(C=S)-, R'-P(O)OH-, R'-(C=S)-, R'-alkyl-, R'-, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X1, X2, and X3 are each independently selected from amino acid residues; and x, y, and z are integers independently selected from 0 and 1,

with the proviso that if each of x , y and z is independently 1, X_1 is 2,4-diaminobutanoic acid, X_2 is threonine, X_3 is 2,4-diaminobutanoic acid, and A is $R'-(C=O)-$, then R' is not branched C_8 -alkyl.

[0145] One embodiment discloses a compound of formula (3):



(3)

wherein:

A is selected from $R'-(C=O)-$, $R'-SO_2-$, $R'-(C=NH)-$, $R'-NH-(C=S)-$, $R'-NH-(C=O)-$, $R'-NH-(C=NH)-$, $R'-O-(C=O)-$, $R'-O-(C=S)-$, $R'-P(O)OH-$, $R'-(C=S)-$, R' -alkyl-, $R'-$, and hydrogen; R' is selected from alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, and heterocyclyl; X_1 , X_2 , and X_3 are each independently selected from amino acid residues; and x , y , and z are integers independently selected from 0 and 1,

with the proviso that:

1) if x is selected from 0 or 1, each of y and z is independently 1, X_1 is an amino acid residue, X_2 is threonine, X_3 is 2,4-diaminobutanoic acid and A is $R'-$, then R' is selected from N^α -alkanoylphenylalanine, N^α -alkenoylphenylalanine, N^α -arylcarbonylphenylalanine, N^α -heteroarylcarbonylphenylalanine;

2) if x is selected from 0 or 1, each of y and z is independently 1, X_1 is an amino acid residue, X_2 is threonine, X_3 is 2,4-diaminobutanoic acid and A is $R'-(C=O)-$, then R' is selected from unbranched unsubstituted C_{1-4} -alkyl, cycloalkyl, alkenyl, aryl, heteroaryl and heterocyclyl, provided that: a) the alkenyl is not furanyl- $CH=CH-$, b) the aryl is not selected from naphthyl and 4-nitrophenyl, and c) the heteroaryl is not 2-thiophenyl; and

[0146] 3) if x is selected from 0 or 1, each of y and z is independently 1, X_1 is an amino acid residue, X_2 is threonine, X_3 is 2,4-diaminobutanoic acid and A is $R'-SO_2-$, then R' is selected from C_{1-7} -alkyl, C_{9-20} -alkyl, cycloalkyl, alkenyl, heteroaryl, and heterocyclyl.

[0147] Another embodiment includes a compound selected from formulae (I) – (VII) and (1)–(3), wherein X1 is 2,4-diaminobutanoic acid, X2 is threonine, X3 is 2,4-diaminobutanoic acid, A is R'-(C=O)- and R' is an N^α-(n-C₉-alkanoyl)phenylalanine.

[0148] One embodiment includes a method for treating an infection in a subject by administering a therapeutically-effective amount of a pharmaceutical composition comprising a compound selected from formulae (I) – (VII) and (1)–(7) and a pharmaceutically acceptable carrier.

Antibacterial Activity

[0149] The antibacterial activities of certain new compounds are indicated in Table 4 as their minimum inhibitory concentrations (MICs) against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. MICs can be determined by the conditions disclosed in Table 4, as well as in Jarolmen, H. et al., "Activity of Minocycline Against R-Factor Carrying Enterobacteriaceae," *Infectious Immunity*, Vol. 1, No. 4, pp. 321-326, 1970, the disclosure of which is incorporated herein by reference.

Pro-drugs

[0150] In one embodiment, pro-drugs of disclosed peptides are provided. Peptides having the heptapeptide structure of formula (B) and protected with HSO₃-Fmoc and/or other amino protecting groups comprising at least one acidic group are antibacterial pro-drugs. The HSO₃-Fmoc group is cleaved after introduction into an animal, such as, e.g., a mammal, including a human, liberating the biologically active peptide. Administration of a biologically active peptide as the protected pro-drug may result in a slow release mechanism for the antibacterial compound. A discussion of pro-drugs is provided in Refs. 32-33.

Pharmaceutical Compositions

[0151] Also disclosed are pharmaceutical compositions or formulations comprising compounds disclosed herein, or salts thereof.

[0152] The pharmaceutical compositions can be formulated for oral, intravenous, intramuscular, subcutaneous or parenteral administration for the therapeutic or prophylactic treatment of diseases, such as bacterial infections.

[0153] The pharmaceutical preparations disclosed herein may be prepared in accordance with standard procedures and are administered at dosages that are selected to reduce, prevent or eliminate infection (see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA and Goodman and Gilman's "The Pharmaceutical Basis of Therapeutics," Pergamon Press, New York, NY, the contents of which are incorporated herein by reference, for a general description of the methods for administering various antimicrobial agents for human therapy).

[0154] The pharmaceutical compositions can comprise one or more of the compounds disclosed herein in association with one or more nontoxic, pharmaceutically-acceptable carriers and/or diluents and/or adjuvants and/or excipients. As used herein, the phrase "pharmaceutically-acceptable carrier" refers to any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Nonlimiting examples of carriers and excipients include corn starch or gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride and alginic acid. The compositions may contain croscarmellose sodium, microcrystalline cellulose, corn starch, sodium starch glycolate and alginic acid.

[0155] Tablet binders that can be included are acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone (Povidone), hydroxypropyl methylcellulose, sucrose, starch and ethylcellulose.

[0156] Lubricants that can be used include magnesium stearate or other metallic stearates, stearic acid, silicone fluid, talc, waxes, oils and colloidal silica.

[0157] Flavoring agents such as peppermint, oil of wintergreen, cherry flavoring or the like can also be used. It may also be desirable to add a coloring agent to make the dosage form more aesthetic in appearance or to help identify the product.

[0158] For oral or parenteral administration, compounds of the present invention can be mixed with conventional pharmaceutical carriers and excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, wafers and the like. The compositions comprising a compound of this invention may contain

from about 0.1% to about 99% by weight of the active compound, such as from about 10% to about 30%.

[0159] For oral use, solid formulations such as tablets and capsules are useful. Sustained release or enterically coated preparations may also be devised. For pediatric and geriatric applications, one embodiment provides suspensions, syrups and chewable tablets. For oral administration, the pharmaceutical compositions are in the form of, for example, a tablet, capsule, suspension or liquid.

[0160] The pharmaceutical compositions may be made in the form of a dosage unit containing a therapeutically-effective amount of the active ingredient. Examples of such dosage units are tablets and capsules. For therapeutic purposes, the tablets and capsules which can contain, in addition to the active ingredient, conventional carriers such as binding agents, for example, acacia gum, gelatin, polyvinylpyrrolidone, sorbitol, or tragacanth; fillers, for example, calcium phosphate, glycine, lactose, maize-starch, sorbitol, or sucrose; lubricants, for example, magnesium stearate, polyethylene glycol, silica, or talc; disintegrants, for example, potato starch, flavoring or coloring agents, or acceptable wetting agents. Oral liquid preparations generally are in the form of aqueous or oily solutions, suspensions, emulsions, syrups or elixirs, preparations of the invention may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous agents, preservatives, coloring agents and flavoring agents. Nonlimiting examples of additives for liquid preparations include acacia, almond oil, ethyl alcohol, fractionated coconut oil, gelatin, glucose syrup, glycerin, hydrogenated edible fats, lecithin, methyl cellulose, methyl or propyl para-hydroxybenzoate, propylene glycol, sorbitol, or sorbic acid.

[0161] For intravenous (IV) use, the pharmaceutical composition can be dissolved or suspended in any of the commonly used intravenous fluids and administered by infusion. Intravenous fluids include, without limitation, physiological saline or Ringer's solution. Intravenous administration may be accomplished by using, without limitation, syringe, minipump or intravenous line.

[0162] Pharmaceutical compositions of this invention for parenteral injection comprise pharmaceutically-acceptable aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, benzyl alcohol, polyols (such as glycerol, propylene glycol, and polyethylene glycol), and suitable mixtures thereof, vegetable oils (such as corn oil or olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. The compositions can include various buffers.

[0163] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents. They may also contain taggants or other anti-counterfeiting agents, which are well known in the art. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, and phenol sorbic acid. It may also be desirable to include isotonic agents such as sugars and sodium chloride. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

[0164] Injectable depot forms can be made by forming microencapsulating matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations can also be prepared by entrapping the drug in liposomes or microemulsions, which are compatible with body tissues.

[0165] The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions, which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

[0166] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. Such forms may include forms that dissolve or disintegrate quickly in the oral environment. In such solid dosage forms, the active compound can be mixed with at least one inert, pharmaceutically-acceptable excipient or carrier. Suitable excipients include, for example, (a) fillers or extenders such as starches, lactose, sucrose, glucose,

mannitol, and silicic acid; (b) binders such as cellulose and cellulose derivatives (such as hydroxypropylmethylcellulose, hydroxypropylcellulose, and carboxymethylcellulose), alginates, gelatin, polyvinylpyrrolidone, sucrose, and açacia; (c) humectants such as glycerol; (d) disintegrating agents such as sodium starch glycolate, croscarmellose, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (e) solution retarding agents such as paraffin; (f) absorption accelerators such as quaternary ammonium compounds; (g) wetting agents, such as cetyl alcohol and glycerol monostearate, fatty acid esters of sorbitan, poloxamers, and polyethylene glycols; (h) absorbents such as kaolin and bentonite clay; (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (j) glidants such as talc, and silicone dioxide. Other suitable excipients include, for example, sodium citrate or dicalcium phosphate. The dosage forms may also comprise buffering agents.

[0167] Solid dosage forms, including those of tablets, dragees, capsules, pills, and granules, can be prepared with coatings and shells such as functional and aesthetic enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and colorants. They may also be in a form capable of controlled or sustained release. Examples of embedding compositions that can be used for such purposes include polymeric substances and waxes.

[0168] The pharmaceutical compositions can be delivered using controlled (e.g., capsules) or sustained release (e.g., bioerodable matrices) delivery systems. Exemplary delayed release delivery systems for drug delivery that are suitable for administering the pharmaceutical compositions are described in U.S. Patent Nos. 4,452,775 (issued to Kent), 5,239,660 (issued to Leonard), and 3,854,480 (issued to Zaffaroni).

[0169] In some cases, in order to prolong the effect of the drug, it may be desirable to slow the absorption of the drug following subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. Amorphous material may be used alone or together with stabilizers as necessary. The rate of absorption of the drug then depends upon its rate of dissolution, which in turn, may depend upon crystal size and crystalline form

[0170] Alternatively, delayed absorption of a parenterally administered drug form can be accomplished by dissolving or suspending the drug in an oil vehicle.

[0171] For intramuscular preparations, a sterile formulation of a compound of the present invention, or a suitable soluble salt form of the compound, for example the hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as Water-for-Injection (WFI), physiological saline or 5% glucose. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g., an ester of a long chain fatty acid such as ethyl oleate.

[0172] A dose of an intravenous, intramuscular, or parental formulation of a compound of the present invention may be administered as a bolus or by slow infusion. A bolus is a dose that is administered in less than 30 minutes. In one embodiment, a bolus is administered in less than 15 or less than 10 minutes. In another embodiment, a bolus is administered in less than 5 minutes. In yet another embodiment, a bolus is administered in one minute or less. An infusion is a dose that is administered at a rate of 30 minutes or greater. In one embodiment, the infusion is one hour or greater. In another embodiment, the infusion is substantially constant.

[0173] For topical use the pharmaceutical compositions can also be prepared in suitable forms to be applied to the skin, or mucus membranes of the nose and throat, and can take the form of creams, ointments, liquid sprays or inhalants, lozenges, or throat paints. Such topical formulations further can include chemical compounds such as dimethylsulfoxide (DMSO) to facilitate surface penetration of the active ingredient.

[0174] For application to the eyes or ears, the pharmaceutical composition can be presented in liquid or semiliquid form formulated in hydrophobic or hydrophilic bases as ointments, creams, lotions, paints or powders.

[0175] For rectal administration, the pharmaceutical compositions can be administered in the form of suppositories admixed with conventional carriers such as cocoa butter, polyethylene glycol or a suppository wax or other glyceride that are solid at room temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active compound.

[0176] Alternatively, the pharmaceutical compositions can be in powder form for reconstitution in the appropriate pharmaceutically acceptable carrier at the time of delivery. In another embodiment, the unit dosage form of the compound can be a solution of the compound, or a salt thereof, in a suitable diluent in sterile, hermetically sealed ampoules or sterile syringes. The concentration of the compound in the unit dosage may vary, e.g. from about 1 percent to about 50 percent, depending on the compound used and its solubility and the dose desired by the physician. If the compositions contain dosage units, each dosage unit can contain from 1-500 mg of the active material. For adult human treatment, the dosage employed can range from 5 mg to 10 g, per day, depending on the route and frequency of administration.

[0177] The pharmaceutical compositions disclosed herein can be placed in a pharmaceutically acceptable carrier and are delivered to a recipient subject (e.g., a human) in accordance with known methods of drug delivery. In general, the methods of delivering the pharmaceutical compositions *in vivo* utilize art-recognized protocols for delivering the agent with the only substantial procedural modification being the substitution of the compounds of the present invention for the drugs in the art-recognized protocols. Likewise, methods for using the claimed compositions for treating cells in culture, for example, to eliminate or reduce the level of bacterial contamination of a cell culture, utilize art-recognized protocols for treating cell cultures with antibacterial agent(s) with the only substantial procedural modification being the substitution of the compounds of the present invention for the drugs in the art-recognized protocols.

Methods of Use

[0178] In one embodiment, the invention provides a method for treating an infection in a subject by administering a therapeutically-effective amount of a compound or composition of the invention. In one embodiment, the method comprises administering to a subject in need thereof a pharmaceutical composition comprising at least one of the compounds described herein. In one embodiment, the pharmaceutical composition can comprise any one of the compounds described herein as the sole active compound or in combination with another compound, composition, or biological material.

[0179] The terms "treatment," "therapeutic method," and their cognates refer to both therapeutic treatment and prophylactic/preventative measures. Those in need of treatment may include individuals already having a particular medical disease as well as those at risk for the disease (*i.e.*, those who are likely to ultimately acquire the disorder). A therapeutic method results in the prevention or amelioration of symptoms or an otherwise desired biological outcome and may be evaluated by improved clinical signs, delayed onset of disease, reduced/elevated levels of lymphocytes and/or antibodies, etc.

[0180] In one embodiment, the pharmaceutical is therapeutically effective in humans while not being not cross resistant with aminoglycosides, β -lactams, and fluoroquinolones.

[0181] Exemplary procedures for delivering an antibacterial agent are described in U. S. Patent No. 5,041,567, and PCT patent application number EP94/02552 (publication no. WO 95/05384), the disclosures of which are incorporated herein by reference in their entirety. As used herein, the phrases "therapeutically effective dose" and "therapeutically-effective amount" refer to an amount of a compound of the present invention that prevents the onset, alleviates the symptoms, stops the progression of a bacterial infection, or results in another desired biological outcome such as, *e.g.*, improved clinical signs or reduced/elevated levels of lymphocytes and/or antibodies. The term "treating" is defined as administering, to a subject, a therapeutically-effective amount of a compound of the invention both to prevent the occurrence of an infection and to control or eliminate an infection. The term "subject," as used herein, refers to a mammal, a plant, a lower animal, or a cell culture. In one embodiment, a subject is a human or other animal patient in need of antibacterial treatment.

[0182] The present invention also provides methods of administering a compound disclosed herein or a pharmaceutical composition thereof to a subject in need thereof in an amount that is efficacious in reducing or eliminating the bacterial infection. The pharmaceutical compositions of this invention can be administered to humans and other animals orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, or drops), buccally, as an oral or nasal spray, or by an implanted reservoir, external pump or catheter. The compositions may also be administered through the lungs by inhalation. The term "parenteral administration" as used

herein, refers to modes of administration, which include intravenous, intramuscular, intraperitoneal, intracisternal, subcutaneous and intraarticular injection and infusion. The compound or composition may be prepared for ophthalmic or aerosolized uses.

[0183] In one embodiment, the pharmaceutical composition is an antibacterial agent, antibiotic, or antifungal agent.

[0184] In one embodiment, the method of the instant invention may be used to treat a subject having a bacterial infection in which the infection is caused or exacerbated by any type of bacteria, such as gram-negative or gram-positive bacteria. In one embodiment, the bacterial infection may be caused or exacerbated by gram-negative bacteria. These gram-negative bacteria include, but are not limited to, *Acinetobacter* spp. (including *Acinetobacter baumannii*), *Citrobacter* spp., *Enterobacter* spp., *Escherichia* spp. (including *Escherichia coli*), *Haemophilus influenzae*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Klebsiella* spp. (including *Klebsiella pneumoniae*), *Salmonella* spp., *Shigella* spp., *Yersinia pseudotuberculosis*, and all species of *Enterobacter*, *Pasteurella*, *Brucella*, *Bordetella*, *Proteus*, *Serratia*, *Providencia*, and *Edwardsiella*.

[0185] In one embodiment, the bacterial infection may be caused or exacerbated by gram-positive bacteria. These gram-positive bacteria include, but are not limited to, methicillin-susceptible and methicillin-resistant staphylococci (including *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, and coagulase-negative staphylococci), glycopeptide intermediary-susceptible *S. aureus* (GISA), vancomycin-resistant *Staphylococcus aureus* (VRSA), penicillin-susceptible and penicillin-resistant streptococci (including *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae*, *S. avium*, *S. bovis*, *S. lactis*, *S. sanguis* and *Streptococci* Group C, *Streptococci* Group G and viridans streptococci), enterococci (including vancomycin-susceptible and vancomycin-resistant strains such as *Enterococcus faecalis* and *E. faecium*), *Clostridium difficile*, *C. clostridiiforme*, *C. innocuum*, *C. perfringens*, *C. ramosum*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Corynebacterium jeikeium*, *Bifidobacterium* spp., *Eubacterium aerofaciens*, *E. lentum*, *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *Lactococcus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Peptostreptococcus anaerobius*, *P. asaccarolyticus*, *P.*

magnus, *P. micros*, *P. prevotii*, *P. productus*, *Propionibacterium acnes*, *Actinomyces* spp., and *Moraxella* spp. (including *M. catarrhalis*).

[0186] In one embodiment, the antibacterial activity of the compounds disclosed herein against classically "resistant" strains is comparable to that against classically "susceptible" strains in *in vitro* experiments. In one embodiment, a compound according to this invention or a pharmaceutical composition thereof is administered according to the methods of this invention to a patient in need of rapidly acting antibiotic therapy.

[0187] The method of the instant invention may be used for any bacterial infection of any organ or tissue in the body. In one embodiment, the bacterial infection is caused by gram-negative bacteria. These organs or tissue include, without limitation, skeletal muscle, skin, bloodstream, kidneys, heart, lung and bone. The method of the invention may be used to treat, without limitation, skin and soft tissue infections, bacteremia and urinary tract infections. The method of the invention may be used to treat community acquired respiratory infections, including, without limitation, otitis media, sinusitis, chronic bronchitis and pneumonia, including pneumonia caused by drug-resistant *S. pneumoniae* or *H. influenzae*. The method of the invention also may be used to treat mixed infections that comprise different types of gram-negative bacteria, or which comprise both gram-positive and gram-negative bacteria. These types of infections include intra-abdominal infections and obstetrical/gynecological infections. The method of the invention also may be used to treat an infection including, without limitation, endocarditis, nephritis, septic arthritis, intra-abdominal sepsis, bone and joint infections and osteomyelitis. In one embodiment, any of the above-described diseases may be treated using compounds according to this invention or pharmaceutical compositions thereof.

[0188] The method of the present invention may also be practiced while concurrently administering one or more other antimicrobial agents, such as antibacterial agents (antibiotics) or antifungal agents. In one aspect, the method may be practiced by administering more than one compound according to this invention. In another embodiment, the method may be practiced by administering a compound according to this invention with another peptide compound described herein, or peptide compounds described, for example in International Patent Applications WO 01/44272; WO 01/44274; and WO 01/44271.

[0189] Antibacterial agents and classes thereof that may be co-administered with a compound of the present invention include, without limitation, penicillins and related drugs, carbapenems, cephalosporins and related drugs, aminoglycosides, bacitracin, gramicidin, mupirocin, chloramphenicol, thiamphenicol, fusidate sodium, lincomycin, clindamycin, macrolides, novobiocin, polymyxins, rifamycins, spectinomycin, tetracyclines, vancomycin, teicoplanin, streptogramins, anti-folate agents including sulfonamides, trimethoprim and its combinations and pyrimethamine, synthetic antibacterials including nitrofurans, methenamine mandelate and methenamine hippurate, nitroimidazoles, quinolones, fluoroquinolones, isoniazid, ethambutol, pyrazinamide, para-aminosalicylic acid (PAS), cycloserine, capreomycin, ethionamide, prothionamide, thiacetazone, viomycin, everninomicin, glycopeptide, glycylcycline, ketolides, oxazolidinone; imipenen, amikacin, netilmicin, fosfomycin, gentamicin, ceftriaxone, ZIRACIN[®] (56-deacetyl-57-demethyl-45-O-de(2-methyl-1-oxopropyl)-12-O-(2,3,6-trideoxy-3-C-methyl-4-O-methyl-3-nitro-alpha-L-arabino-hexopyranosyl)flambamycin), LY 333328, CL 331002, HMR 3647 ZYVOX[®] (linezolid), SYNERCID[®] (dalbapristin-quinupristin), Aztreonam, Metronidazole, Epiroprim, OCA-983, GV-143253, Sanfetrinem sodium, CS-834, Biapenem, A-99058.1, A-165600, A-179796, KA 159, Dynemicin A, DX8739, DU 6681; Cefluprenam, ER 35786, Cefoselis, Sanfetrinem celexetil, HGP-31, Cefpirome, HMR-3647, RU-59863, Mersacidin, KP 736, Rifalazil; Kosan, AM 1732, MEN 10700, Lenapenem, BO 2502A, NE-1530, K130, OPC 20000, OPC 2045, Venepriam, PD 138312, PD 140248, CP 111905, Sulopenem, ritipenam acoxyl, RO-65-5788, Cyclothialidine, Sch-40832, SEP-132613, micacocidin A, SB-275833, SR-15402, SUN A0026, TOC 39, carumonam, Cefozopran, Cefetamet pivoxil, and T 3811.

[0190] Antifungal agents that may be co-administered with a compound according to the invention include, without limitation, Caspofungen, Voriconazole, Sertaconazole, IB-367, FK-463, LY-303366, Sch-56592, Sitafloracin, DB-289, polyenes, such as Amphotericin, Nystatin, Primaricin; azoles, such as Fluconazole, Itraconazole, and Ketoconazole; allylamines, such as Naftifine and Terbinafine; and anti-metabolites such as Flucytosine. Other antifungal agents include without limitation, those disclosed in Foster et al., *Drug Discovery Today* 5:25-32 (2000), herein incorporated by reference. Foster et al. discloses antifungal

compounds including Corynecandin, Mer-WF3010, Fusacandins, Artrichitin/LL 15G256, Sordarins, Cispentacin, Azoxybacillin, Aureobasidin and Khafrefungin.

[0191] The compounds of the present invention can be administered as an aerosol for the treatment of pneumonia or other lung-based infections. In one embodiment, the aerosol delivery vehicle is an anhydrous or dry powder inhaler. The compounds or pharmaceutical compositions thereof may also be directly injected or administered into an abscess, ventricle or joint. Parenteral administration includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, cisternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion. In another embodiment, the compounds of the present invention are administered intravenously, subcutaneously or orally. In yet another embodiment, the compound or composition is administered to a cell culture, such as by administering in a nutrient medium.

Dosing

[0192] Actual dosage levels of active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain a therapeutically effective amount of the active compound(s) to achieve the desired therapeutic response for a particular patient, compositions, and mode of administration. The effective amount can be determined as described herein. The selected dosage level will depend upon the activity of the particular compound, the route of administration, the severity of the condition being treated, and the condition and prior medical history of the patient being treated. However, it is within the skill of the art to start doses of the compound at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. In one embodiment, the data obtained from the assays can be used in formulating a range of dosage for use in humans.

[0193] The method can also comprise administering to the subject an effective dose of a compound of the present invention. An effective dose can range from about 0.1 to about 100 mg/kg of a compound of the invention or a pharmaceutically acceptable salt thereof. In one embodiment, the dose ranges from about 0.1 to about 50 mg/kg of a compound of the invention or a pharmaceutically acceptable salt thereof. In another embodiment, the dose

ranges from about 1 to 25 mg/kg of a compound of the invention or a pharmaceutically acceptable salt thereof. An effective dose for cell culture can range from 0.1 to 1000 µg/mL, such as from 0.1 to 200 µg/mL.

[0194] Generally dosage levels of about 0.1 µg/kg to about 50 mg/kg, such as a level ranging from about 5 to about 20 mg of active compound per kilogram of body weight per day, can be administered topically, orally or intravenously to a mammalian patient. Other dosage levels range from about 1 µg/kg to about 20 mg/kg, from about 1 µg/kg to about 10 mg/kg, from about 1 µg/kg to about 1 mg/kg, from 10 µg/kg to 1 mg/kg, from 10 µg/kg to 100 µg/kg, from 100 µg to 1 mg/kg, and from about 500 µg/kg to about 5 mg/kg per day. If desired, the effective daily dose may be divided into multiple doses for purposes of administration, e.g., two to four separate doses per day. In one embodiment, the pharmaceutical composition can be administered once per day.

[0195] Compositions containing the compounds of the invention can be administered as a single daily dose or in multiple doses per day. The treatment regime may require administration over extended periods of time, e.g., for several days or for from two to four weeks. The amount per administered dose or the total amount administered will depend on such factors as the nature and severity of the infection, the age and general health of the patient, the tolerance of the patient to the compound and the microorganism or microorganisms involved in the infection.

[0196] A compound of the present invention may also be administered in the diet or feed of a patient or animal. If administered as part of a total dietary intake, the amount of compound employed can be less than 1% by weight of the diet, such as no more than 0.5% by weight. The diet for animals can be normal foodstuffs to which the compound can be added or it can be added to a premix.

[0197] Other embodiments disclosed herein include:

[0198] 1. A method for preparing an intermediate for use in the synthesis of a new peptide antibiotic, including the steps of:

[0199] (a) Protecting the amino groups of the polymyxin B, colistin, [Ile⁷]-polymyxin B₁, circulin, and an octapeptin with (2-sulfo)-9-fluorenyl methoxycarbonyl or another acidic derivative of 9-fluorenylmethoxycarbonyl;

[0200] (b) Treating the product from the reaction of step (a) with a deacylase to provide a protected peptide intermediate; and

[0201] (c) Using a modified Edman degradation method or peptidase enzymatic reaction to obtain another protected intermediate by reducing, by one to three amino acids, in the side chain of the protected peptide intermediate; and

[0202] (d) Purifying the protected peptide intermediate by chromatography.

[0203] Another embodiment discloses:

[0204] 2. A method for producing an antibiotic active against gram-negative and gram-positive bacteria, including strains resistant to clinically used antibiotics, comprising the steps of:

[0205] (a) Protecting the amino groups of the polymyxins or other related antibiotics chosen from the group consisting of colistin, [Ile7]-polymyxin B₁, circulin, and an octapeptin, with (2-sulfo)-9-fluorenylmethoxycarbonyl or another acidic derivative of 9-fluorenylmethoxycarbonyl;

[0206] (b) Treating the product from the reaction of step (a) with a deacylase to provide a protected peptide intermediate;

[0207] (c) Using a modified Edman degradation method or peptidase enzymatic reaction to obtain another protected intermediate peptide by reducing in size by one to three amino acids, the exocyclic peptide side chain of the protected peptide.

[0208] (d) Chemically modifying the intermediate to produce a protected antibacterial derivative; and

[0209] (e) Removing the acidic protecting groups to produce the antibiotic.

[0210] Another embodiment discloses:

[0211] 3. An intermediate, which is a chemically protected form of a peptide derived from the polymyxins, [Ile7]-polymyxin B₁, octapeptins, colistin, circulins, or related antibiotics and selected from a group consisting of the following, or their corresponding salts:

- 1) H-(X1) (X2) (X3)-peptide-[(2-sulfo)-9-Fmoc]_n
- 2) H-(X2) (X3)-peptide-[(2-sulfo)-9-Fmoc]_n
- 3) H-(X3)-peptide-[(2-sulfo)-9-Fmoc]_n
- 4) H-peptide-[(2-sulfo)-9-Fmoc]₃

wherein for Case 1) H-(X1)(X2)(X3)-peptide-[(2-sulfo)Fmoc]_n: H is hydrogen, X1 is L-Dab or another amino acid, X2 is L-Thr or another amino acid, X3 is L-Dab or D-Dab or another amino acid and n=3-6;

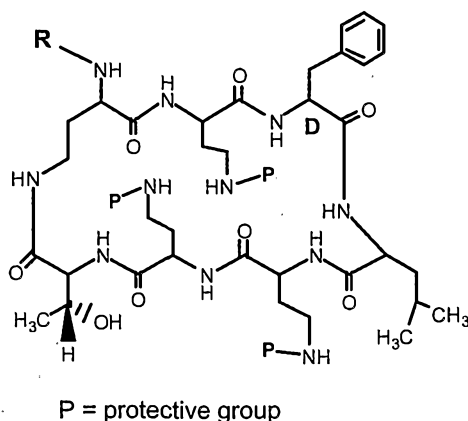
for Case 2) H-(X2)(X3)-peptide-[(sulfo)-9-Fmoc]_n: H is hydrogen, X2 is L-Thr or another amino acid, X3 is L-Dab or D-Dab or another amino acid, and n=3-5;

for Case 3) H-(X3)-[peptide-[(2-sulfo)-9-Fmoc]_n: H is hydrogen, X3 is L-Dab or D-Dab or another amino acid and n=3-4; and

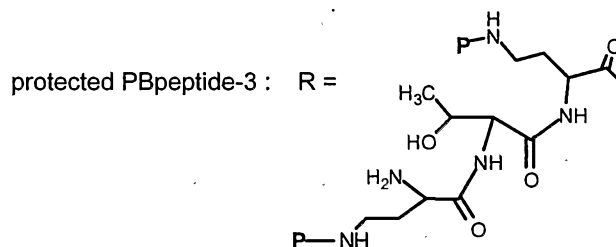
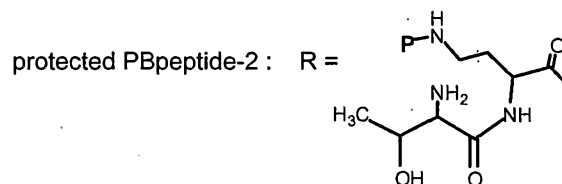
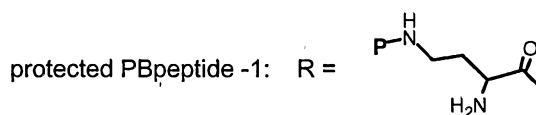
for Case 4) H-peptide-[(sulfo)-9-Fmoc]_n: H is hydrogen.

[0212] Another embodiment discloses:

[0213] 4. Acidic protected peptide intermediates, derived from the corresponding protected polymyxin B, which can be used to synthesize new peptide antibiotics or their prodrugs where the protecting group is preferably (2-sulfo)-9-Fmoc and the protected peptide intermediates have the structure:

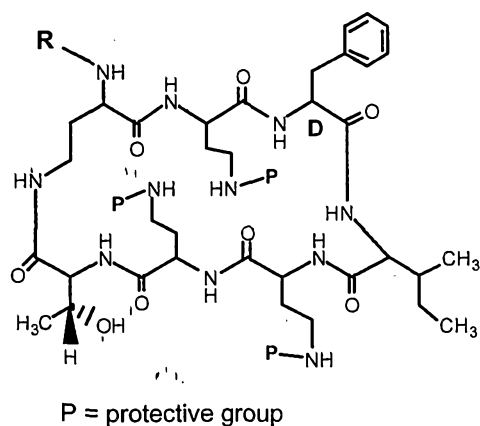


protected PBpeptide : R = H

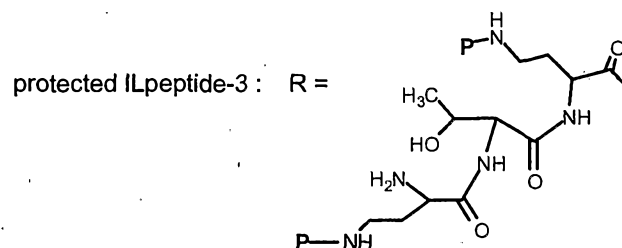
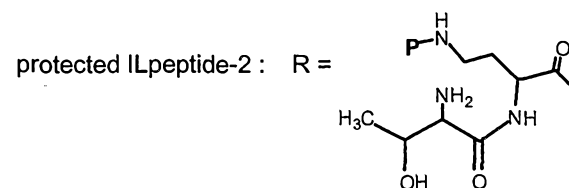
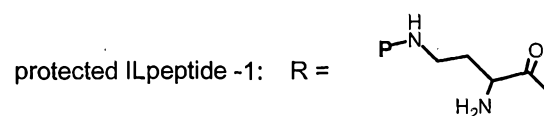


[0214] Another embodiment discloses:

[0215] 5. Acidic protected peptide intermediates, derived from the corresponding protected [Ile7]-polymyxin B₁, which can be used to synthesize new peptide antibiotics or their prodrugs where the protecting group is preferably (2-sulfo)-9-Fmoc and the protected peptide intermediates have the structure:

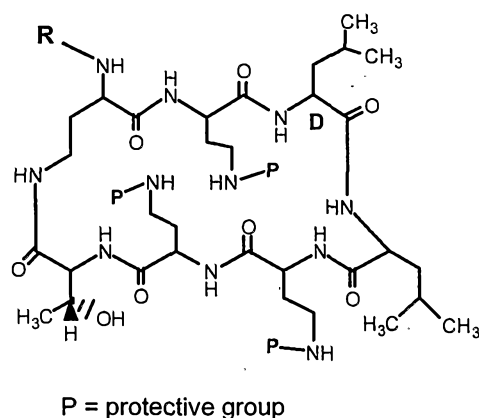


protected ILpeptide : R = H

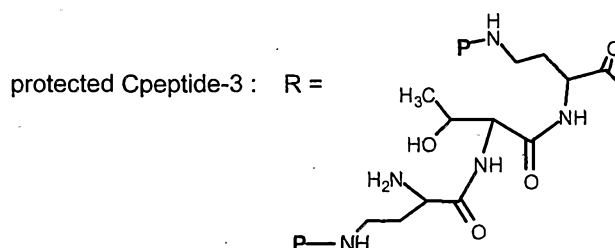
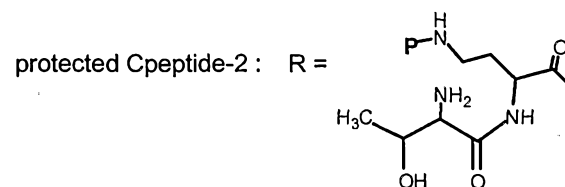
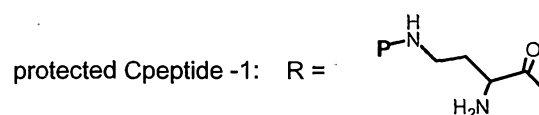


[0216] Another embodiment discloses:

[0217] 6. Acidic protected peptide intermediates, derived from the corresponding protected colistin, which can be used to synthesize new peptide antibiotics or their prodrugs where the protecting group is preferably (2-sulfo)-9-Fmoc and the protected peptide intermediates have the structure:

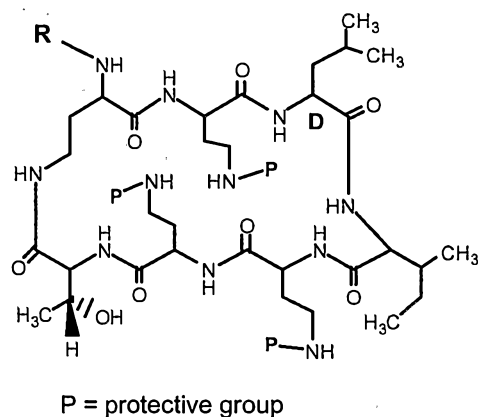


protected Cpeptide : R = H

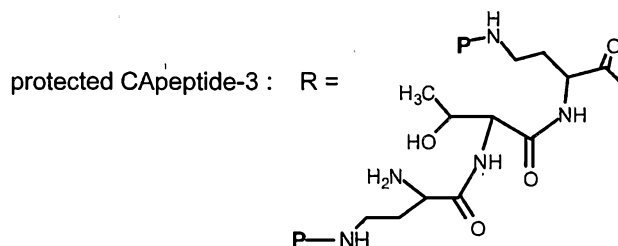
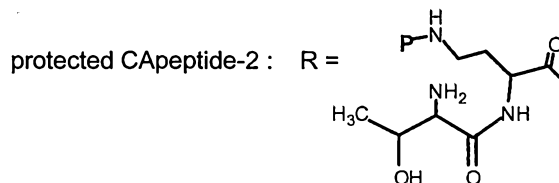
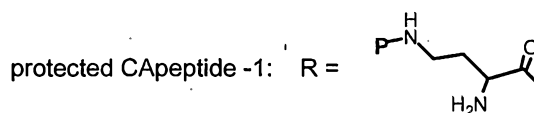


[0218] Another embodiment discloses:

[0219] 7. Acidic protected peptide intermediates, derived from the corresponding protected circulin A, which can be used to synthesize new peptide antibiotics or their prodrugs where the protecting group is preferably (2-sulfo)-9-Fmoc and the protected intermediates have the structure:

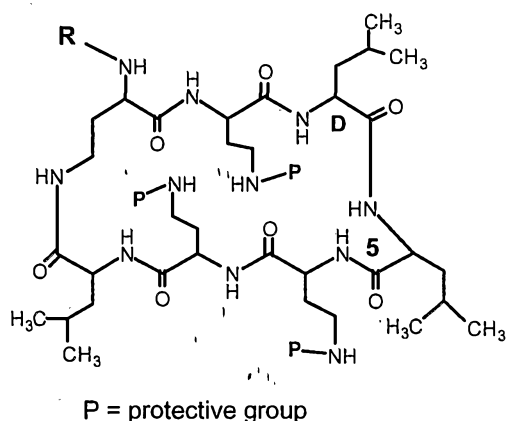


protected CApeptide : R = H

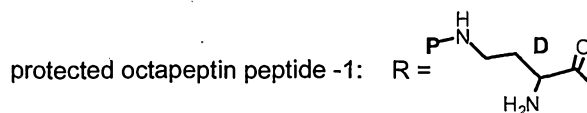


[0220] Another embodiment discloses:

[0221] 8. Acidic protected peptide intermediates, derived from the corresponding protected octapeptin, which can be used to synthesize new peptide antibiotics or their prodrugs where the protecting group is preferably (2-sulfo)-9-Fmoc and the protected peptide intermediates have the structure:



protected octapeptin peptide : R = H



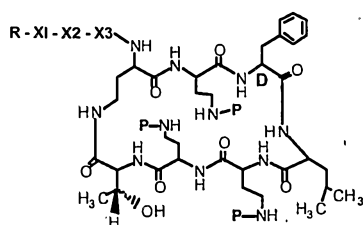
[0222] Another embodiment discloses:

[0223] 9. A protected peptide intermediate, derived from the corresponding octapeptin, wherein another component of the octapeptin antibiotic includes L-phenylalanine instead of L-leucine at the 5-position and wherein the component forms a similar, but alternative, protected peptide intermediate.

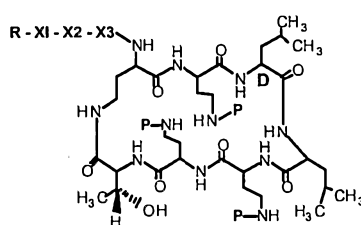
[0224] Another embodiment discloses:

[0225] 10. An antibacterial compound or protected compound prepared from a chemically protected form of PBpeptides, Cpeptides or ILpeptides having the following structure where P equals the protective group (2-sulfo)-9-Fmoc or hydrogen:

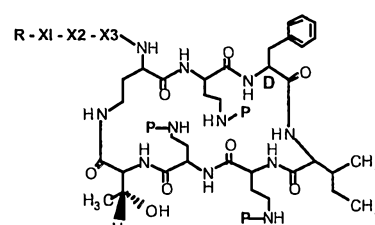
Table 3



PBpeptide derivatives



Cpeptide derivatives



ILpeptide derivatives

Compound	R	Peptide	X1*	X2	X3*	P
(P) ₅ Polymyxin B	C ₈ H ₁₇ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
I	n-C ₉ H ₁₉ CO-	PBpeptide	Dab	Thr	Dab	H
1P	n-C ₉ H ₁₉ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
2	n-C ₉ H ₁₉ CO-PAPA-**	PBpeptide	Dab	Thr	Dab	H
2P	n-C ₉ H ₁₉ CO-PAPA-**	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
3	n-C ₈ H ₁₇ NHCO-	PBpeptide	Dab	Thr	Dab	H

<u>Compound</u>	<u>R</u>	<u>Peptide</u>	<u>X1*</u>	<u>X2</u>	<u>X3*</u>	<u>P</u>
3P	n- C ₈ H ₁₇ NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
4	phenyl-NHCS-	PBpeptide	Dab	Thr	Dab	H
4P	phenyl-NHCS-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
5	phenyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
5P	phenyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
6	phenyl-CO-	PBpeptide	Dab	Thr	Dab	H
6P	phenyl-CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
7	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab	Thr	Dab	H
7P	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab	Thr	Dab	H
8P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
9	n- C ₈ H ₁₇ NHCO-	PBpeptide	----	Thr	Dab	H
9P	n- C ₈ H ₁₇ NHCO-	PBpeptide	----	Thr	Dab-P	HSO ₃ -Fmoc-
10	n- C ₁₀ H ₂₁ SO ₂ -	PBpeptide	Gly	Thr	Dab	H
10P	n- C ₁₀ H ₂₁ SO ₂ -	PBpeptide	Gly	Thr	Dab-P	HSO ₃ -Fmoc
11	n- C ₉ H ₁₉ CO-	PBpeptide	Lys	Thr	Dab	H
11P	n- C ₉ H ₁₉ CO-	PBpeptide	Lys-P	Thr	Dab-P	HSO ₃ -Fmoc
12	n- C ₉ H ₁₉ CO-	PBpeptide	Phe	Thr	Dab	H
12P	n- C ₉ H ₁₉ CO-	PBpeptide	Phe	Thr	Dab-P	HSO ₃ -Fmoc-
13	iso-nicotinoyl-	PBpeptide	Dab	Thr	Dab	H
13P	iso-nicotinoyl-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
14	3-indolyethylNHCO-	PBpeptide	Dab	Thr	Dab	H
14P	3-indolyethylNHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
15	N-Acetyl-PAPA-	PBpeptide	Dab	Thr	Dab	H
15P	N-Acetyl-PAPA-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
16	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab	Thr	Dab	H
16P	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
17	p-(n-C ₁₀ H ₂₁)-Phenyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
17P	p-(n-C ₁₀ H ₂₁)-Phenyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
18	4-biphenylNHCO-	PBpeptide	Dab	Thr	Dab	H
18P	4-biphenylNHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
19	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab	Thr	Dab	H
19P	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpeptide	Dab	Thr	Dab	H
20P	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
21	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab	Thr	Dab	H
21P	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
22	benzyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
22P	benzyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
23	cyclohexyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
23P	cyclohexyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
24	phenyl-NHCS-	PBpeptide	----	Thr	Dab	H
24P	phenyl-NHCS-	PBpeptide	----	Thr	Dab-P	HSO ₃ -Fmoc-

<u>Compound</u>	<u>R</u>	<u>Peptide</u>	<u>X1*</u>	<u>X2</u>	<u>X3*</u>	<u>P</u>
25	n-C ₉ H ₁₉ CO-	PBpeptide	----	----	Dab	H
25P	n-C ₉ H ₁₉ CO-	PBpeptide	----	----	Dab-P	HSO ₃ -Fmoc-
26	n-C ₉ H ₁₉ CO-	ILpeptide	Dab	Thr	Dab	H
26P	n-C ₉ H ₁₉ CO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
27	n-C ₉ H ₁₉ CO-PAPA-**	ILpeptide	Dab	Thr	Dab	H
27P	n-C ₉ H ₁₉ CO-PAPA-**	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
28	n-C ₈ H ₁₇ NHCO-	ILpeptide	Dab	Thr	Dab	H
28P	n-C ₈ H ₁₇ NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
29	phenyl-NHCS-	ILpeptide	Dab	Thr	Dab	H
29P	phenyl-NHCS-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
30	phenyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
30P	phenyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
31	phenyl-CO-	ILpeptide	Dab	Thr	Dab	H
31P	phenyl-CO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
32	2-naphthyl-OCH ₂ -CO-	ILpeptide	Dab	Thr	Dab	H
32P	2-naphthyl-OCH ₂ -CO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
33	4-CH ₃ -C ₆ H ₄ -SO ₂ -	ILpeptide	Dab	Thr	Dab	H
33P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
34	n-C ₈ H ₁₇ NHCO-	ILpeptide	----	Thr	Dab	H
34P	n-C ₈ H ₁₇ NHCO-	ILpeptide	----	Thr	Dab-P	HSO ₃ -Fmoc-
35	n-C ₁₀ H ₂₁ SO ₂ -	ILpeptide	Gly	Thr	Dab	H
35P	n-C ₁₀ H ₂₁ SO ₂ -	ILpeptide	Gly	Thr	Dab-P	HSO ₃ -Fmoc-
36	n-C ₉ H ₁₉ CO-	ILpeptide	Lys	Thr	Dab	H
36P	n-C ₉ H ₁₉ CO-	ILpeptide	Lys-P	Thr	Dab-P	HSO ₃ -Fmoc-
37	n-C ₉ H ₁₉ CO-	ILpeptide	Phe	Thr	Dab	H
37P	n-C ₉ H ₁₉ CO-	ILpeptide	Phe	Thr	Dab-P	HSO ₃ -Fmoc-
38	iso-nicotinoyl-	ILpeptide	Dab	Thr	Dab	H
38P	iso-nicotinoyl-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
39	3-indolylethylNHCO-	ILpeptide	Dab	Thr	Dab	H
39P	3-indolylethylNHCO-	ILpeptide	Dab	Thr	Dab	HSO ₃ -Fmoc-
40	N-Acetyl-PAPA-	ILpeptide	Dab	Thr	Dab	H
40P	N-Acetyl-PAPA-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
41	n-C ₉ H ₁₉ CO-Phe-	ILpeptide	Dab	Thr	Dab	H
41P	n-C ₉ H ₁₉ CO-Phe-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
42	p-(n-C ₁₀ H ₂₁)-Phenyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
42P	p-(n-C ₁₀ H ₂₁)-Phenyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
43	4-biphenylNHCO-	ILpeptide	Dab	Thr	Dab	H
43P	4-biphenylNHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
44	C ₆ H ₅ CH ₂ OCH ₂ CO-	ILpeptide	Dab	Thr	Dab	H
44P	C ₆ H ₅ CH ₂ OCH ₂ CO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
45	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	ILpeptide	Dab	Thr	Dab	H
45P	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
46	4-Cl-C ₆ H ₄ -NHCO-	ILpeptide	Dab	Thr	Dab	H

<u>Compound</u>	<u>R</u>	<u>Peptide</u>	<u>X1*</u>	<u>X2</u>	<u>X3*</u>	<u>P</u>
46P	4-Cl-C ₆ H ₄ -NHCO-	ILpeptide	Dab	Thr	Dab	HSO ₃ -Fmoc-
47	benzyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
47P	benzyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
48	cyclohexyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
48P	cyclohexyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
49	phenyl-NHCS-	ILpeptide	----	Thr	Dab	H
49P	phenyl-NHCS-	ILpeptide	----	Thr	Dab-P	HSO ₃ -Fmoc-
50	n-C ₉ H ₁₉ CO-	ILpeptide	----	----	Dab	H
50P	n-C ₉ H ₁₉ CO-	ILpeptide	----	----	Dab-P	HSO ₃ -Fmoc-
51	n-C ₈ H ₁₇ NHCO-	Cpeptide	Dab	Thr	Dab	H
51P	n-C ₈ H ₁₇ NHCO-	Cpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
52	n-C ₉ H ₁₉ CO-Phe-	Cpeptide	Dab	Thr	Dab	H
52P	n-C ₉ H ₁₉ CO-Phe-	Cpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Cpeptide	Dab	Thr	Dab	H
53-P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Cpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
54	n-CH ₉ H ₁₉ CO-Lys	PBpeptide	Dab	Thr	Dab	H
54P	n-CH ₉ H ₁₉ CO-Lys	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-

* Dab-P is 4-N-(HSO₃-Fmoc)-diaminobutyl, Lys-P is 6-N-(HSO₃-Fmoc)-lysyl

** PAPA is p-aminophenylacetyl

All amino acids are the L-isomers unless indicated otherwise.

[0226] Another embodiment discloses:

[0227] 11. An antibiotic prepared from an intermediate, which is a chemically protected form of a peptide derived from the polymyxins, octapeptins, colistin, [Ile⁷]polymyxin B₁, said antibiotic selected from a group consisting of the following, or their corresponding salts:

Case 1 A-(X1) (X2) (X3) – Peptide

Case 2 A-(X2) (X3) – Peptide

Case 3 A-(X3)- Peptide

Case 4 A-Peptide

wherein for Case 1) A-(X1) (X2) (X3) – Peptide: A = R'-(C=O) - , R'-SO₂ - , R'-(C=NH) - , R'-NH-(C=S) - , R'-NH-(C=O) - , where R' is alkyl, cycloalkyl, alkenyl, aryl, heteroaryl, or heterocyclyl and X1 is L-Dab or another amino acid, X2 is L-Thr or another amino acid, and X3 is L-Dab or another amino acid;

for Case 2) A-(X2)(X3) - Peptide: "A" is the same as described for Case 1, X2 is L-Thr or another amino acid and X3 is L-Dab or another amino acid;

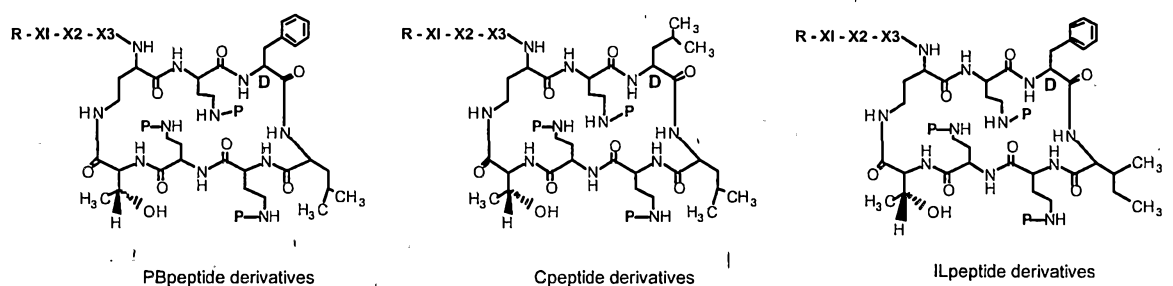
for Case 3) A-(X3)-peptide: "A" is the same as described in Case 1 and X3 is L-Dab or another amino acid;

for Case 4) A-peptide: "A" is the same as described for Case 1.

[0228] Another embodiment discloses:

[0229] 12. Peptide antibiotics having the following structure and minimum inhibitory concentrations for use against gram-negative and gram-positive bacteria:

Table 4



<u>Compound</u>	<u>R</u>	<u>X1*</u>	<u>X2</u>	<u>X3*</u>	<u>cyclic peptide</u>	<u>E. coli MIC*</u>	<u>Staph MIC*</u>	<u>Pseudo MIC*</u>
Polymyxin B	C ₈ H ₁₇ CO-	Dab	Thr	Dab	PB	0.6	>10	0.6
Polymyxin B ₁	C ₈ H ₁₇ CO-	Dab	Thr	Dab	PB	0.6	10	0.6
[Ile ⁷]-polymyxin B ₁	C ₈ H ₁₇ CO-	Dab	Thr	Dab	IL	0.6	20	1.25
Colistin	C ₈ H ₁₇ CO-	Dab	Thr	Dab	C	0.6	>20	0.6
1	n-C ₉ H ₁₉ CO-	Dab	Thr	Dab	PB	0.6		
2	n-C ₁₀ H ₂₁ CO-PAPA-*	Dab	Thr	Dab	PB	1.25	2.5	1.25
3	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	PB	1.25	10	0.6
4	phenyl-NHCS-	Dab	Thr	Dab	PB	0.6	>10	0.6
5	phenyl-NHCO-	Dab	Thr	Dab	PB	0.6	>10	1.25
6	phenyl-CO-	Dab	Thr	Dab	PB	1.25	>10	0.6
7	2-naphthyl-OCH ₂ -CO-	Dab	Thr	Dab	PB	1.25	>10	0.6
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	PB	1.25	>20	0.6
9	n-C ₈ H ₁₇ NHCO-	----	Thr	Dab	PB	2.5	>10	1.25
10	n-C ₁₀ H ₂₁ SO ₂ -	Gly	Thr	Dab	PB	2.5	5	2.5
11	n-C ₉ H ₁₉ CO-	Lys	Thr	Dab	PB	2.5	10	1.25
12	n-C ₉ H ₁₉ CO-	Phe	Thr	Dab	PB	2.5	2.5	2.5
13	iso-nicotinoyl-	Dab	Thr	Dab	PB	5.0	>20	1.25
14	3-indolyethylNHCO-	Dab	Thr	Dab	PB	2.5	>20	1.25
15	N-Acetyl-PAPA-	Dab	Thr	Dab	PB	2.5	>20	2.5
16	n-C ₉ H ₁₉ CO-Phe-	Dab	Thr	Dab	PB	1.25	2.5	0.6

<u>Compound</u>	<u>R</u>	<u>X1*</u>	<u>X2</u>	<u>X3*</u>	<u>cyclic</u> <u>peptide</u>	<u>E. coli</u> <u>MIC*</u>	<u>Staph</u> <u>MIC*</u>	<u>Pseudo</u> <u>MIC*</u>
17	4-n-C ₁₀ H ₂₁ -Phenyl-NHCO-	Dab	Thr	Dab	PB	5.0	2.5	2.5
18	4-biphenylNHCO-	Dab	Thr	Dab	PB	2.5	2.5	1.2
19	C ₆ H ₅ CH ₂ OCH ₂ -CO-	Dab	Thr	Dab	PB	0.6	>20	1.25
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	Dab	Thr	Dab	PB	0.6	5.0	1.25
21	4-Cl-C ₆ H ₄ -NHCO-	Dab	Thr	Dab	PB	0.6	20	0.6
22	benzyl-NHCO-	Dab	Thr	Dab	PB	1.25	>20	0.6
23	cyclohexyl-NHCO-	Dab	Thr	Dab	PB	0.6	>20	0.6
24	phenyl-NHCS-	--	Thr	Dab	PB	>5.0	>20	5.0
25	n-C ₉ H ₁₉ CO-	--	--	Dab	PB	>5.0	10	1.25
48	cyclohexyl-NHCO-	Dab	Thr	Dab	IL	0.6		0.6
51	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	C	1.25	20	0.6
52	n-C ₉ H ₁₉ CO-Phe-	Dab	Thr	Dab	C	1.25	2.5	2.5
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	C	1.25	>20	0.6
54	n-C ₉ H ₁₉ CO-Lys-	Dab	Thr	Dab	PB	0.6	2.5	0.6

*MIC values were determined by serial twofold broth dilution method using *Escherichia coli*, ATCC #26, *Staphylococcus aureus* Smith, and *Pseudomonas aeruginosa*, ATCC 27853, as assay organisms which were grown in Mueller Hinton broth.

** PAPA = p-aminophenylacetyl

[0230] Another embodiment discloses:

[0231] A process for preparing a water-soluble, stable, solid form of a deacylase enzyme including the steps of:

[0232] (a) Fermenting a strain of *Actinoplanes utahensis* to obtain cells of the organism.

[0233] (b) Washing the cells with water to remove impurities.

[0234] (c) Extracting the washed cells with an aqueous base, pH 8 – 11.

[0235] (d) Adjusting the extract to pH 7-8 and freeze drying the solution to obtain the solid form of the enzyme.

[0236] Another embodiment discloses:

[0237] 14. The process further comprises step (e):

[0238] (e) thereafter, further purifying said enzyme by use of chromatography.

[0239] A series of new antibiotics is represented by the sulfonyl derivatives (Table 3, compounds 8 and 10). The side chains from these compounds are not attached to the PBpeptides by an acyl group but instead by a

sulfonyl group. Other linkers such as ureas or thioreas have been made and resulted in compounds with good bacterial activity. Potent activity was also shown with aromatic acyl exocyclic chains and aromatic groups linked through urea and thiourea linkages (compounds 4 and 5) as shown in Table 3.

[0240] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

EXAMPLES

[0241] The polymyxin B₁ and the [Ile⁷]-polymyxin B₁ (Table 3) were isolated from the polymyxin B complex. The new peptides derived from ILpeptide-3 could be prepared by purifying [Ile⁷]-polymyxin B₁ from the polymyxin B complex and protecting this peptide, deacylating this protected product, adding back a new side chain and deprotecting. An alternative procedure involved protecting the polymyxin B complex, deacylating to obtain the protected PBpeptide-3 and ILpeptide-3 mixture, adding the new side chain, deprotecting, and then resolving the mixture to obtain new derivatives of both peptides. A summary of HPLC retention times observed for the new peptides derived from the PBpeptide and ILpeptide is given in Table 5.

Table 5

<u>Compound Numbers</u>	<u>Isocratic system %Acetonitrile</u>	<u>Respective Retention Times (minutes)</u>
Polymyxin B ₁ , IL variant	58	4.79, 4.16
1,26	60	5.45, 4.85
2,27	48	4.52, 4.20
3,28	55	4.04, 3.64
4,29	65	4.39, 3.75
5,30	64	3.34, 2.94
6,31	65	3.61, 3.16

<u>Compound Numbers</u>	<u>Isocratic system</u> <u>%Acetonitrile</u>	<u>Respective Retention</u> <u>Times (minutes)</u>
7,32	60	4.10, 3.64
8,33	64	3.91, 3.40
9,34	53	4.80, 4.29
10,35	60	4.23, 3.92
11,36	54	3.89, 3.55
12,37	60	6.23, 5.93
13,38	70	3.88, 3.37
14,39	64	5.52, 4.65
15,40	68	4.15, 3.56
16,41	48	5.38, 4.97
17,42	43	5.74, 5.35
18,43	58	4.79, 4.21
19,44	64	4.34, 3.74
20,45	57	4.14, 3.70
21,46	62	4.64, 3.98
22,47	65	4.08, 3.52
23,48	65	5.03, 4.25
24,49	60	3.68, 3.23
25,50	53	3.80, 3.47

* HPLC analyses were performed using a reverse phase C8 column. Isocratic eluents were prepared from various ratios of Eluent B and Eluent C to give the % acetonitrile listed.

Eluent B: 0.05 M (NH₄)₂SO₄ + 0.005 M H₂SO₄ pH~2.44

Eluent C: 85% acetonitrile and 15% water

[0242] Analysis by HPLC of peptides protected with at least one (2-Sulfo)-9-fluorenylmethoxycarbonyl group can be performed using a reverse phase C8 column using a gradient of 35% to 75% MeCN/0.2M (NH₄)₃PO₄ at pH

7.2. Analysis by HPLC of deprotected peptides can be performed using the conditions given in Table

[0243] Unless otherwise noted, N-hydroxysuccinimide reagents were prepared according to the methods disclosed in Gershonov, E., Goldwasser, I., Fridkin, M., Schechter, Y, **2000**, "A Novel Approach for a Water-Soluble Long-Acting Insulin ProDrug: Design, Preparation, and Analysis of [(2-Sulfo)-9-Fluorenylmethoxycarbonyl]₃-Insulin," Journal of Medicinal Chemistry 43: (13), 2530-2537, and Schechter, Y.Tsudbery, H., Fridkin, M., **2002**, N-[(2-Sulfo)-9-Fluorenylmethoxycarbonyl]₃-Gentamicin Is a Long-Acting Prodrug Derivative," Journal of Medicinal Chemistry 45: (19), 4264-4270.

Example 1. N-[(2-Sulfo)-9-fluorenylmethoxycarbonyl]₅- polymyxin B

[0244] Polymyxin B sulfate (1.0 g., 0.841 mmol) was dissolved in a solution of 25 mL, saturated sodium bicarbonate, 25 mL of water, and 25 mL of tetrahydrofuran. Polymyxin B sulfate is commercially available, e.g., from Sigma (Milwaukee, WI). A solution of (2-sulfo)-9-fluorenylmethoxy-N-hydroxysuccinimide (2.0 g., 4.8 mmol) in 25 mL of tetrahydrofuran was added in several portions over 45 min. The reaction mixture was stirred at room temperature over night and diluted with 50 mL of water, then acidified with 25 mL of 6 N hydrochloric acid to give an oily precipitate. The mixture was chilled and the aqueous layer was decanted and the oily residue was dissolved in 100 mL of ethanol. The ethanol was evaporated under vacuum (35 °C) and the resulting solid was triturated with ethyl acetate, filtered and dried to afford 1.74 g. of product. HPLC with a gradient on a reverse phase C8 column showed a single peak, N-[2-(sulfo)-9-fluorenylmethoxycarbonyl]₅-polymyxin B, C₁₃₁H₁₅₀N₁₆O₃₈S₅, when the column eluent was monitored at 215 nm. ESIMS: calc. m/z for C₁₃₁H₁₅₂N₁₆O₃₈S₅, (M+2H)⁺² = 1358.4. Found 1358.5

[0245] The above procedure can be used with polymyxin free base and 2(sulfo)-9-fluorenylmethoxycarbonyl chloride with similar results.

Example 2. Preparation of the Deacylase

[0246] The deacylase is produced by culturing *Actinoplanes utahensis* NRRL 12052 under submerged aerobic fermentation conditions. The fermentation

protocol employed is known (Boeck, L.D. et al., Journal of Antibiotics 41:(8), 1085-1092). A stock culture of the NRRL 12052 variant, preserved in 20% glycerol at -70°C, was introduced into a 25x150 mm test tube with a glass rod and Morton closure containing 10 mL of a medium composed of sucrose 2.0%, pre-cooked oatmeal 2.0%, distiller's grains and solubles 0.5%, yeast extract 0.25%, K₂HPO₄ 0.1%, KCl 0.05%, MgSO₄.7H₂O 0.05% and FeSO₄.7H₂O 0.0002% in deionized water. After incubation at 30 °C for 72 hrs on a rotary shaker orbiting at 250 rpm the resulting mycelial suspension was transferred into 50 mL of PM3 medium in a 250-mL Erlenmeyer flask. This medium contained sucrose 2.0%, peanut meal 1.0%, K₂HPO₄ 0.12%, KH₂PO₄ 0.05% and MgSO₄.7H₂O 0.025% in tap water. The flask was incubated at a temperature of 30 °C for a period of 60 to 90 hrs. The harvest time was determined by an assay which involved HPLC analyses of the deacylation of (2-Sulfo-9-fluorenylmethoxycarbonyl)]₅ polymyxin B by the whole broth at different times during the fermentation.

[0247] Because single-colony isolates from a lyophile of the culture were heterogeneous for both morphology and enzyme production capability, selections were made to recover a stable, high-producing variant. Initially, multiple fermentations were carried out using inocula prepared from strain 12052. Vegetative growth from the flask yielding the best deacylating activity was plated on a differential agar (CM). CM agar contained corn steep liquor 0.5%, Bacto peptone 0.5%, soluble starch 1.0%, NaCl 0.05%, CaCl₂.2H₂O 0.05% and Bacto agar 2.0%. Colonies were then selected for further evaluation. Isolate No. 18 was selected as a small colony type and shown to be the best deacylase producer of all colonies selected. Comparison was based on conversion of protected polymyxin B to deacylated protected polymyxin B as determined by HPLC. This isolate was routinely used for the production of the deacylase enzyme.

Example 3. Deacylation of the Protected Polymyxin B by the Enzyme in Cells

[0248] Precipitation Method: Cells from 450 mL of deacylase enzyme from the original fermentation according to Example 2 were washed 3X with water, then brought back to original volume with 0.02 M ammonium phosphate buffer and adjusted to pH 8.0. N-[(2-Sulfo)-9-fluorenylmethoxycarbonyl)]₅-polymyxin B,

897 mg, was added and the mixture was placed on a shaker at 174 rpm and maintained at 30 °C. After five hours the mixture was separated by centrifugation. The clear decant was adjusted to pH 2.3 with 1 N HCl solution to induce precipitation and allowed to stand at room temperature. The precipitate was separated from the mixture, slurried in 80 mL water, adjusted to pH 6.5 to obtain a clear solution, and freeze-dried to obtain 400 mg, the salt of the protected peptide, N-[(2-Sulfo)-9-fluorenylmethoxycarbonyl]₅-polymyxin peptide [(NaSO₃-Fmoc)₅-PBP-3] as a tan powder. The cells remaining after centrifugation were extracted with methanol/water to obtain additional material.

[0249] **Resin Method:** Cells from 1 liter of deacylase enzyme from the original fermentation according to Example 2 were washed 3X with water, brought back to original volume with 0.02 M ammonium phosphate buffer, and combined with 2.0 g of N-(2-sulfo)-9-fluorenylmethoxycarbonyl]₅-polymyxin B. The mixture was placed on a shaker at 175 rpm and maintained at 30 °C for 17 hours. The mixture was then separated by centrifugation and the decant was combined with 20 mL of Amberchrom® CG-161m resin. The resin was washed with 150 mL water, 100 mL 10% CH₃CN:H₂O (3X), and 100 mL 20% CH₃CN:H₂O (2x). The peptide was then eluted 2X with 30% CH₃CN:H₂O, evaporated to remove CH₃CN, and freeze-dried to obtain 283 mg powder, the protected peptide(HSO₃-Fmoc)₅-PBP-3. A second amount of peptide was then eluted 3X with 100 mL 50% CH₃CN:H₂O. The eluates were combined, evaporated and freeze-dried to obtain 460 mg of the protected peptide as a powder.

[0250] A third amount of peptide was extracted from the cells remaining after centrifugation using MeOH: H₂O, 6 x 100 mL each. The extracts were combined, brought to four liters with water, adjusted to pH 2.1 with sulfuric acid and combined with Amberchrom® CG-161m resin. The resin was rinsed with water and then the peptide was eluted with 100 mL 35% CH₃CN:H₂O, which was evaporated and freeze-dried to obtain 94 mg of purified peptide. The remaining peptide was eluted with 50% CH₃CN:H₂O, evaporated to remove CH₃CN, and freeze-dried to obtain 539 mg of the protected peptide. The protected peptide [(2-Sulfo)-9-fluorenylmethoxycarbonyl]₅ polymyxin B peptide[(HSO₃-Fmoc)₅-PBP-3]] was isolated from this procedure as tan powder, C₁₂₂H₁₃₄N₁₆O₃₇S₅. Materials prepared by both the resin method and the precipitation method were about 75% pure when analyzed by HPLC with the

column eluent monitored at 215 nm. ESIMS: calc. m/z for $C_{122}H_{136}N_{16}O_{37}S_5$: $(M+2H)^{+2} = 1288.4$; Found: 1288.

Example 4. Deacylation of the Protected Polymyxin B by the Solubilized Enzyme

[0251] Water washed cells from 250 mL of *Actinoplanes utahensis* fermentation as performed according to Example 2 were combined with 125 mL of 0.02 M ammonium phosphate buffer, adjusted to pH 10.1 and stirred thirty minutes. The solution containing the enzyme was separated by centrifuge and adjusted to pH 8.0. This solution was used directly for deacylations or freeze-dried to obtain a powder form for storage. The decant containing the solubilized enzyme at pH 8.0, was combined with 100 mg $(HSO_3-Fmoc)_5$ -polymyxin B, dissolved in 10 mL of $CH_3CN:H_2O$ (1:1), and placed on a shaker at 175 rpm, 84 °C. After two hours the completed reaction was removed from the shaker and adjusted to pH 2.0. The precipitate was mixed with 40 mL methanol and the soluble product was separated from a dark precipitate. The solution, containing 80 mg of the $(HSO_3-Fmoc)_5$ -PBP-3, was evaporated to 2 mL and added to 10 mL of EtOAc to precipitate the $(HSO_3-Fmoc)_5$ -PBP-3 as a tan powder.

Example 5. Purification of $(NaSO_3-Fmoc)_5$ -PBP-3 (protected decapeptide)

[0252] $(NaSO_3-Fmoc)_5$ -PBP-3, 143 mg, was prepared according to the procedure of Example 3. The protected decapeptide was dissolved in 40 mL of 20% CH_3CN 0.05M in sodium phosphate at pH 6.7. Insolubles were removed by centrifugation. The decant was applied to a styrene-divinylbenzene resin cartridge (Supelco EnviChrom-P®, 25 x 35 mm) which had been slurry packed and rinsed with 20 mL 20% CH_3CN – 0.05M pH 6.7 buffer. Flow rate was about 2 mL/min at RT. The cartridge was eluted with incrementally increasing concentrations of CH_3CN of about 0.05M in sodium phosphate at pH 6.7. Collected fractions were evaluated by analytical HPLC. The desired product was eluted with 33% and 40% CH_3CN eluents. Product-containing fractions were pooled and CH_3CN removed under vacuum. The product pool was desalted by adsorption onto a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P®) which was then rinsed with four 1.0 mL portions of distilled water. Product was eluted

from the cartridge with 16 mL of 67% CH₃CN, the solvent evaporated under vacuum, the pH of the resulting aqueous solution adjusted to about 5.8 with dilute NaOH, then the product was freeze dried. Yield: 72 mg of a light tan solid, (NaSO₃-Fmoc)₅-PBP-3, C₁₂₂H₁₃₄N₁₆O₃₇S₅. ESIMS: calc. *m/z* for C₁₂₂H₁₃₆N₁₆O₃₇S₅ : (M+2H)⁺² = 1288.4. Found: 1288.2.

Example 6. Preparation of N-Phenylthiocarbamyl-(NH₄SO₃-Fmoc)₅-PBP-3 (Compound 4P)

[0253] Purified (NaSO₃-Fmoc)₅-PBP-3, 3.9 mg, was prepared according to the procedure of Example 5. The protected peptide was dissolved in 0.20 mL of 75% MeOH 0.25 M in pH 8.8 potassium borate, and 0.003 mL phenylisothiocyanate was added and stirred at RT. Phenylisothiocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). After about 90 min, the reaction mixture was diluted with 4 mL of 0.4 M ammonium phosphate at pH 7.2. The product solution was applied to a 0.5g styrene-divinylbenzene resin cartridge (EnviChrom-P®) which was rinsed with 6 mL of 20% CH₃CN 0.10 M in sodium phosphate at pH 6.7. Product was eluted using 6 mL of 40% CH₃CN 0.05 M in sodium phosphate at pH 6.7. Solvent was evaporated under vacuum and the product was desalted using the procedure of Example 5. Yield: 3.5 mg of a white solid, N-phenylthiocarbamyl-(NaSO₃-Fmoc)₅-PBP-3, C₁₂₉H₁₃₇N₁₇O₃₇S₆. ESIMS: calc. *m/z* for C₁₂₉H₁₃₉N₁₇O₃₇S₆ : (M+2H)⁺² = 1355.4. Found: 1355.6.

Example 7. Preparation of N-Phenylthiocarbamyl-PBP-3 (PTC-PBP-3, Compound 4)

[0254] N-Phenylthiocarbamyl-(NH₄SO₃-Fmoc)₅-PBP-3, 8.4 mg, was prepared according to the procedure of Example 6. The protected peptide was dissolved in 0.20 mL dimethylformamide (DMF), 0.010 mL piperidine was added and stirred at RT for 60 min. The reaction mixture was diluted with 4 mL 0.10 M ammonium acetate – 0.050 M acetic acid (pH 5.05), 0.006 mL acetic acid, and 4 mL MeOH. The clear solution was applied to a CM-Sepharose® column (10 x 20 mm, ca. 2 mL volume) which had been conditioned with 50% MeOH 0.05 M in ammonium acetate buffer at pH 5.0. The sample-loaded column was rinsed with 4 mL 50% MeOH – 0.05 M ammonium acetate pH 5.0 then with 4 mL 0.05M

ammonium acetate pH 5.0 buffer. Product was eluted with 8 mL of 0.27 M sodium sulfate at pH 2.3. The product was further purified by application onto a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P®) which was eluted with incrementally increasing concentrations of CH₃CN 0.05M in pH 2.3 sodium sulfate; product was eluted with 20% CH₃CN. Solvent was removed under vacuum from the product-containing fraction pool which was then desalted using the procedure of Example 5, pH adjusted to 6.3 then freeze dried. Yield: 2.7 mg of a white solid, PTC-PBP-3, C₅₄H₈₇N₁₇O₁₂S. FABMS: calc. for C₅₄H₈₈N₁₇O₁₂S : (M+H)⁺ = 1198.7. Found: 1198.5(M+H)⁺, 1220.4(M+Na)⁺.

Example 8. (NH₄SO₃-Fmoc)₄-PBP-2 (protected nonapeptide)

[0255] N-Phenylthiocarbamyl-(NH₄SO₃-Fmoc)₅-PBP-3, 2.9 mg, was prepared according to the procedure of Example 6. The protected peptide was dissolved in 0.30 mL of anhydrous trifluoroacetic acid (TFA) and heated in a 50° C water bath for 15 min. The TFA was evaporated with a stream of dry nitrogen and the residue was dissolved in 12 mL 0.20M ammonium phosphate at pH 7.2 and 6 mL CH₃CN containing 49 mg triglycine (as an acylating agent scavenger). To remove potentially reactive intermediates, the product solution was first applied to a 0.5 g styrene-divinylbenzene resin cartridge (EnviChrom-P®) which was eluted with 10 mL of 40% CH₃CN 0.04M in ammonium phosphate at pH 7.2. Product-containing fractions were pooled (~18 mL), diluted with 12 mL distilled water, then applied to a fresh 0.5 g EnviChrom-P® resin cartridge which was eluted with incrementally increasing concentrations of CH₃CN about 0.05M in ammonium phosphate at pH 7.2. Product-containing fractions were pooled (~12 mL), 8 mL of 0.54M sodium sulfate pH 2.3 buffer added, and the product was desalted using the procedure of Example 5. The solution pH was adjusted to 5.9 and the sample freeze dried. Yield: 1.8 mg of a white solid, (NaSO₃-Fmoc)₄-PBP-2, C₁₀₃H₁₁₆N₁₄O₃₁S₄. ESIMS: calc. m/z for C₁₀₃H₁₁₈N₁₄O₃₁S₄ (M+2H)²⁺ = 1087.3. Found: 1087.0.

Example 9. n-Decanoyl-PBP-3 (Compounds 1 and 1P)

[0256] (NaSO₃-Fmoc)₅-PBP-3, 21.8 mg, was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.20 mL DMF,

0.020 mL distilled water, and 0.030 mL saturated NaHCO_3 (pH~8.9); 4.5 mg n-decanoyl-N-hydroxysuccinimide (activated ester) was added and stirred at RT for 55 min (about 82% conversion by HPLC). An additional 1.8 mg of activated ester was added; after 20 min at RT conversion to Compound 1P was at least 95%. To the reaction mix was added 0.010 mL piperidine. After 35 min at RT the reaction mixture was diluted with 4 mL 0.25 M ammonium sulfate at pH 2.3 yielding a very milky mixture at pH 3.0 which was extracted with 4 mL ethyl acetate; the product-containing aqueous phase was diluted with 4 mL distilled water. Product was initially isolated by size exclusion chromatography on a Sephadex[®] G-25 column (2.5 x 40 cm) eluted with 0.10 M ammonium sulfate at pH 2.3. Product-containing fractions were pooled and further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) by elution with incrementally increasing concentrations of CH_3CN about 0.05M in sodium phosphate at pH 6.7; product was eluted with 25% CH_3CN . Product was desalted and freeze dried using the procedure of Example 5. Yield: 2.5 mg of a white solid, Compound 1, $\text{C}_{57}\text{H}_{100}\text{N}_{16}\text{O}_{13}$. FABMS: calc. for $\text{C}_{57}\text{H}_{100}\text{N}_{16}\text{O}_{13}$, $(\text{M}+\text{H})^+ = 1217.8$. Found: 1217($\text{M}+\text{H})^+$, 1239($\text{M}+\text{Na})^+$.

Example 10. N-(n-Decanoyl)-p-aminophenylacetyl-PBP-3 (Compounds 2 and 2P)

[0257] $(\text{NH}_4\text{SO}_3\text{-Fmoc})_5\text{-PBP-3}$, 18.5 mg, was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.20 mL DMF and 0.030 mL saturated NaHCO_3 (pH~8.9); 5.8 mg N-(n-decanoyl)-p-aminophenylacetyl-N-hydroxysuccinimide ($\text{C}_{10}\text{-PAPA-OSu}$) was added and stirred at RT for 50 min (about 69% conversion by HPLC). An additional 2.5 mg of $\text{C}_{10}\text{-PAPA-OSu}$ was added; after 30 min at RT conversion to Compound 2P was about 87%. To the reaction mix was added 0.010 mL piperidine. After 25 min at RT the reaction mixture was diluted with 5 mL 0.20 M ammonium sulfate at pH 2.3 yielding a very milky mixture at pH 2.8 which was extracted with 5 mL ethyl acetate. Product was initially isolated by size exclusion chromatography on a Sephadex[®] G-25 column (2.5 x 40 cm) eluted with 0.10 M ammonium sulfate at pH 2.3. Product-containing fractions were pooled and further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) by

elution with incrementally increasing concentrations of CH₃CN about 0.10M in ammonium sulfate at pH 2.3; product was eluted with 30% CH₃CN. Further purification was achieved using size exclusion chromatography on a Sephadex[®] LH-20 column (2.5 x 40 cm) eluted with 0.026 M ammonium acetate / 0.053 M acetic acid in MeOH. Product-containing fractions were evaporated under vacuum to near dryness, the residue dissolved in 10 mL distilled water, then desalted and freeze dried using the procedure of Example 5. Yield: 2.5 mg of a white solid, Compound 2, C₆₅H₁₀₇N₁₇O₁₄. FABMS: calc. for C₆₅H₁₀₈N₁₇O₁₄, (M+H)⁺ = 1350.8. Found: 1351(M+H)⁺, 1373(M+Na)⁺.

Example 11. n-Octanoylcarbamy-PBP-3 (Compounds 3 and 3P)

[0258] (NaSO₃-Fmoc)₅-PBP-3, 10.2 mg, was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.20 mL DMF and 0.020 mL distilled water; 0.003 mL octyl isocyanate was added and stirred at RT for 60 min (about 31% conversion by HPLC). Octyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). To the reaction mixture was added 0.020 mL saturated NaHCO₃ pH 8.9; after 20 min at RT conversion to Compound 3P was about 91%. After another 35 min at RT 0.010 mL piperidine was added. After 30 min at RT the reaction mixture was diluted with 4 mL 0.026 M ammonium acetate / 0.053 M acetic acid in MeOH and the product was isolated by size exclusion chromatography on a Sephadex[®] LH-20 column as described in Example 10. Product-containing fractions were evaporated under vacuum to near dryness, the residue dissolved in 8 mL distilled water, then desalted and freeze dried using the procedure of Example 5. Yield: 2.3 mg of a white solid, Compound 3, C₅₆H₉₉N₁₇O₁₃. FABMS: calc. for C₅₆H₁₀₀N₁₇O₁₃, (M+H)⁺ = 1218.8. Found: 1219(M+H)⁺, 1241(M+Na)⁺.

Example 12. Phenylcarbamy-PBP-3 (Compounds 5 and 5P)

[0259] (NaSO₃-Fmoc)₅-PBP-3, 19.8 mg, (ca. 85% pure) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.20 mL DMF and 0.020 mL saturated NaHCO₃ pH 8.7; 0.005 mL phenyl isocyanate was added and stirred at RT to obtain Compound 5P. Phenyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). After 45

min 0.020 mL of piperidine was added to remove protecting groups. After 45 min at RT the reaction mixture was diluted with 4 mL 0.10 M ammonium acetate – 0.05 M acetic acid, 0.012 mL acetic acid and 4 mL MeOH yielding a clear solution at apparent pH 6.2. The product was isolated on a CM-Sepharose[®] column as described in Example 7. The product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN 0.05 M in sodium sulfate at pH 2.3; product was eluted with 20% CH₃CN. Product-containing fractions were desalted and freeze dried using the procedure of Example 5. Yield: 4.4 mg of a white solid, Compound 5, C₅₄H₈₇N₁₇O₁₃. FABMS: calc. for C₅₄H₈₈N₁₇O₁₃, (M+H)⁺ = 1182.7. Found: 1183(M+H)⁺, 1205(M+Na)⁺, 1221(M+K)⁺.

Example 13. Benzoyl-PBP-3 (Compounds 6 and 6P)

[0260] (NaSO₃-Fmoc)₅-PBP-3, 20.4 mg; (ca. 85% pure) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.20 mL DMF and 0.020 mL saturated NaHCO₃ pH 8.0; 8.4 mg of benzoyl-N-hydroxysuccinimide was added and stirred at 29 °C to obtain Compound 6P. After 60 min 0.020 mL of piperidine was added. After 20 min at RT the reaction mixture was diluted with 4 mL 0.10 M ammonium acetate – 0.05 M acetic acid, 0.013 mL acetic acid and 4 mL MeOH yielding a clear solution. The product was isolated on a CM-Sepharose[®] column as described in Example 7. The product was further purified by preparative HPLC using a Delta-Pak[®] C18 column (25 x 210 mm, Waters Corp.) eluted with an isopropanol gradient (18% - 23% over 100 min, linear, at 5 mL/min) buffered with 0.05M sodium sulfate at pH 2.5. Product-containing fractions were pooled, solvent removed under vacuum, and product was desalted and freeze dried using the procedure of Example 5. Yield: 1.6 mg of a white solid, Compound 6, C₅₄H₈₆N₁₆O₁₃. FABMS: calc. for C₅₄H₈₇N₁₆O₁₃, (M+H)⁺ = 1166.7. Found: 1167(M+H)⁺, 1189(M+Na)⁺.

Example 14. of 2-Naphthoxyacetyl-PBP-3 (Compounds 7 and 7P)

[0261] (NaSO₃-Fmoc)₅-PBP-3, 19.0 mg, was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.40 mL of 75% MeOH 0.25 M in pH 8.8 potassium borate; 6.3 mg of

2-naphthoxyacetyl-N-hydroxysuccinimide was added and stirred at RT to produce Compound 7P. After 35 min 0.020 mL piperidine was added. After 30 min at RT the reaction mixture was diluted and product isolated on a CM-Sepharose[®] column as in Example 7. Product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 25% CH₃CN. Product-containing fractions were pooled, diluted with an equal volume of distilled water, and product was desalted and freeze dried using the procedure of Example 5. Yield: 4.7 mg of a white solid, Compound 7, C₅₉H₉₀N₁₆O₁₄.

[0262] FABMS: calc. for C₅₉H₉₁N₁₆O₁₄, (M+H)⁺ = 1247.7. Found: 1247(M+H)⁺, 1269(M+Na)⁺.

Example 15. p-Toluenesulfonyl-PBP-3 (Compounds 8 and 8P)

[0263] (NaSO₃-Fmoc)₅-PBP-3, 21.7 mg, was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.50 mL of 75% MeOH 0.25 M in pH 8.8 potassium borate; 5.1 mg of p-toluenesulfonyl chloride was added and stirred at RT to produce Compound 8P. p-Toluenesulfonyl chloride is commercially available, e.g., from Aldrich (Milwaukee, WI). After 60 min 0.020 mL piperidine was added. After 30 min at RT the reaction mixture was diluted and product isolated on a CM-Sepharose[®] column as described in Example 7. Product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 20% CH₃CN. Product-containing fractions were pooled, diluted with an equal volume of distilled water, and product was desalted and freeze dried using the procedure of Example 5. Yield: 4.9 mg of a white solid, Compound 8, C₅₄H₈₈N₁₆O₁₄S. FABMS: calc. for C₅₄H₈₉N₁₆O₁₄S (M+H)⁺ = 1217.6, Found: 1217(M+H)⁺, 1239(M+Na)⁺.

Example 16. n-Octanoylcarbamyl-PBP-2 (Compounds 9 and 9P)

[0264] (NaSO₃-Fmoc)₄-PBP-2, 10.1 mg, was prepared according to the procedure of Example 8. The protected peptide was dissolved in 0.20 mL DMF and 0.020 mL saturated NaHCO₃ pH 8.7; 0.003 mL octyl isocyanate was added

and stirred at RT to produce Compound 9P. Octyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). After 45 min at RT 0.020 mL piperidine was added. After 40 min at RT the reaction mixture was diluted with 10 mL 20% CH₃CN containing 0.014 mL H₂SO₄. Product was isolated on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 25% and 30% CH₃CN. Product-containing fractions were pooled, solvent removed under vacuum, 2 mL 1.0 M ammonium acetate pH 5.0 buffer was added, pH adjusted to 5.0 by addition of ca. 0.6 mL 1.5 M NH₄OH, and then diluted with an equal volume of MeOH. Product was isolated via CM-Sepharose[®] chromatography as described in Example 7. The product was further purified on a 0.5 g styrene-divinylbenzene cartridge as above but using eluents at pH 9.9 (0.10 M NH₄OH – 0.01 M (NH₄)₂SO₄); product eluted with 30% CH₃CN. Product-containing fractions were pooled, solvent removed under vacuum, and product was desalted and freeze dried using the procedure of Example 5. Yield: 2.7 mg of a white solid, C₅₂H₉₂N₁₆O₁₁. FABMS: calc. for C₅₂H₉₃N₁₆O₁₁, (M+H)⁺ = 1118.7, Found: 1119(M+H)⁺, 1141(M+Na)⁺, 1157(M+K)⁺.

Example 17. N-(n-Decylsulfonyl)glycyl-PBP-2 (Compounds 10 and 10P)

[0265] Purified (NaSO₃-Fmoc)₄-PBP-2, 9.7 mg, was prepared according to the procedure of Example 8. The protected peptide was dissolved in 0.20 mL DMF and 0.020 mL saturated NaHCO₃ pH 8.7; 5.2 mg of n-decylsulfonamidoglycyl-N-hydroxy succinimide was added and stirred at RT to produce Compound 10P. After 45 min 0.020 mL piperidine was added. After another 40 min at RT the reaction mixture was diluted and product isolated on a CM-Sepharose[®] column as described in Example 7. Product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 33% CH₃CN. Product-containing fractions were pooled, solvent removed under vacuum, and product was desalted and freeze dried using the procedure of Example 5. Yield: 1.5 mg of a white solid, Compound 10, C₅₅H₉₇N₁₅O₁₄S. FABMS: calc. for C₅₅H₉₈N₁₅O₁₄S, (M+H)⁺ = 1224.7. Found: 1224.5(M+H)⁺, 1246.5(M+Na)⁺.

Example 18. 2-N-(n-Decanoyl)lysyl-PBP-2 (Compounds 11 and 11P)

[0266] Purified (NaSO₃-Fmoc)₄-PBP-2, 10.3 mg, was prepared according to the procedure of Example 8. The protected peptide was dissolved in 0.20 mL of DMF and 0.020 mL of saturated NaHCO₃ pH 8.3; 8.7 mg of 6-N-Fmoc-2-N-(n-decanoyl)-lysyl-N-hydroxysuccinimide ester was added and stirred at RT to produce Compound 11P. After 50 min 0.020 mL piperidine was added to remove protecting groups. After 45 min at RT the reaction mixture was diluted and product isolated on a CM-Sepharose[®] column as described in Example 7. Product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 25% CH₃CN. Product-containing fractions were pooled, diluted with an equal volume of distilled water, and product was desalted and freeze dried using the procedure of Example 5. Yield: 2.0 mg of a white solid, Compound 11, C₅₉H₁₀₄N₁₆O₁₃. FABMS: calc. for C₅₉H₁₀₅N₁₆O₁₃, (M+H)⁺ = 1245.8, Found: 1245(M+H)⁺, 1267(M+Na)⁺.

Example 19. N-(n-Decanoyl)phenylalanyl-PBP-2 (Compounds 12 and 12P)

[0267] About 10 mg of purified (NaSO₃-Fmoc)₄-PBP-2 was prepared according to the procedure of Example 8. The protected peptide was dissolved in 0.40 mL of 75% MeOH 0.25M in pH 8.8 potassium borate; 6 mg of N-(n-decanoyl)-phenylalanyl-N-hydroxysuccinimide ester was added and stirred at RT to produce Compound 12P. After 50 min 0.020 mL piperidine was added. After 45 min at RT the reaction mixture was diluted and product isolated on a CM-Sepharose[®] column as in Example 7. Product was further purified on a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P[®]) using incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 33% CH₃CN. Product-containing fractions were pooled, diluted with an equal volume of distilled water, and product was desalted and freeze dried using the procedure of Example 5. Yield: 3.0 mg of a white solid, Compound 12, C₆₂H₁₀₁N₁₅O₁₃. FABMS: calc. for C₆₂H₁₀₂N₁₅O₁₃ (M+H)⁺ = 1264.8. Found: 1265(M+H)⁺, 1287(M+Na)⁺.

Example 20. N-Phenylthiocarbamyl-(NaSO₃-Fmoc)₄PBP-2 (Compound 24P)

[0268] Purified (NaSO₃-Fmoc)₄-PBP-2, 90.2 mg, was prepared according to the procedure of Example 8. The protected peptide was dissolved in 2.0 mL reaction solvent as in Example 23; 0.050 mL of phenylisothiocyanate was added. Phenylisothiocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). After 87 min stirring at RT the reaction mixture was diluted with 50 mL of 20% CH₃CN 0.1M in pH 6.76 sodium phosphate buffer. This solution was applied to a 2.5 g EnviChrom-P[®] column which was eluted with incrementally increasing concentrations of CH₃CN about 0.05M in sodium phosphate at pH 6.76; product was eluted with 30-35% CH₃CN. The product was desalted on a 3.1 g EnviChrom-P[®] column and freeze dried using the procedure of Example 23. Yield: 85 mg of a white solid, Compound 24P.

Example 21. (NaSO₃-Fmoc)₄PBP-1 (Protected octapeptide)

[0269] N-phenylthiocarbamyl-(NaSO₃-Fmoc)₄-PBP-2 (70.0 mg) was prepared according to the procedure of Example 20. The protected peptide was dissolved in 0.70 mL of anhydrous trifluoroacetic acid (TFA) and heated in a 50 °C water bath for 15 min. TFA was removed using a stream of dry nitrogen. The residue was dissolved in 40 mL of 25% CH₃CN 0.05 M in sodium sulfate at pH 2.3. The product was purified by application onto a 2.06 g EnviChrom-P[®] column which was eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 40% CH₃CN. Product-containing fractions were pooled and CH₃CN was removed under vacuum at 35 °C. The product was desalted by adsorption onto a 3.1 g EnviChrom-P[®] column which was then rinsed with 8, 8 and 4 mL portions of distilled water. Product was stripped from the column with 40 mL of 60% CH₃CN, solvent evaporated under vacuum, and the aqueous solution was freeze dried. Yield: 60 mg of a white solid, protected octapeptide, C₉₉H₁₀₉N₁₃O₂₉S₄. ESIMS: calc. m/z for (M+2H)⁺² = 1036.8. Found: 1036.4(M+2H)⁺².

Example 22. (NaSO₃-Fmoc)₃PBP (Protected heptapeptide)

[0270] (NaSO₃-Fmoc)₄-PBP-2 (0.35 mg) was prepared according to the procedure of Example 8. The protected peptide was dissolved in 0.40 mL of buffer (0.02M sodium citrate, 0.006M beta-mercaptoethylamine, adjusted to pH 5.5 with HCl).

[0271] Cathepsin C (Sigma product code C8511, dipeptidyl aminopeptidase, EC 3.4.14.1) was dissolved in the same buffer at a concentration of 10 units/mL. The cathepsin C solution (0.10 mL) was then added to 0.40 mL of the (NaSO₃-Fmoc)₄-PBP-2 solution and the mixture was incubated at 37 °C. The reaction was monitored by HPLC at 3.0 hrs (about 43% conversion to protected heptapeptide) and at 24 hrs (about 90% conversion). The relative retention time and ultraviolet absorption spectrum of the product peak was consistent with the production of protected heptapeptide; the time-dependent appearance of a short retention time peak with characteristic Fmoc absorption spectrum is consistent with liberation of the N-terminal dipeptide H₂N-Thr-Dab(Fmoc)-CO₂H from the protected nonapeptide.

Example 23. Isonicotinoyl-PBP-3 (Compounds 13 and 13P)

[0272] (NaSO₃-Fmoc)₅-PBP-3 (20.5 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.40 mL of 75% MeOH 0.25M in pH 8.8 potassium borate; 8.7 mg of isonicotinoyl-N-hydroxysuccinimide ester was added. The solution was stirred 63 min at RT to form Compound 13P and then 0.030 mL piperidine was added to remove the protecting groups. After stirring 45 min at RT the reaction mixture was diluted with 10 mL 60% MeOH 0.04 M in pH 5.0 ammonium acetate and 0.019 mL acetic acid. The clear solution was applied to a CM-Sepharose column (10 x 20 mm, ca. 2 mL volume) which had been conditioned by rinsing with 20 mL of 60% MeOH 0.04M in pH 5.0 ammonium acetate buffer. The sample-loaded column was rinsed with 10 mL of 60% MeOH 0.04M in pH 5.0 ammonium acetate, and then with 4 mL of aqueous 0.05 M pH 5.0 ammonium acetate buffer. Product was eluted with 8 mL of 0.27 M sodium sulfate buffer at pH 2.3. Product was further purified by application onto a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P) which was eluted with incrementally increasing concentrations of

CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 15% CH₃CN. Product-containing fractions were pooled and CH₃CN was removed under vacuum at 35 °C. The product solution was desalted by adsorption onto a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P) which was then rinsed with four 1.0 mL portions of distilled water. Product was stripped from the cartridge with 12 mL of 67% CH₃CN, solvent evaporated under vacuum, and the aqueous solution was freeze dried. Yield: 9.4 mg of a white solid, Compound 13, C₅₃H₈₅N₁₇O₁₃. FABMS: calc. for C₅₃H₈₅N₁₇O₁₃Na (M+Na)⁺ = 1190.7. Found: 1190 (M+Na)⁺.

Example 24. 3-Indolyethylcarbamyl-PBP-3 (Compounds 14 and 14P)

[0273] (NaSO₃-Fmoc)₅-PBP-3 (22.5 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.30 mL of collidine/acetic acid/water (40:1:4); 18.6 mg of 3-indolyethylcarbamyl-N-hydroxysuccinimide, prepared according to the procedure of Example 43, was added in two increments. The solution was stirred 130 min at RT to form Compound 14P, the reaction mixture was flushed with argon, 0.050 mL piperidine was added to remove the protecting groups, and the mixture again flushed with argon. After stirring 25 min at RT the reaction mixture was diluted with 10 mL 60% MeOH 0.04 M in pH 5.0 ammonium acetate and 0.042 mL acetic acid. Product was isolated from the clear reaction dilution on a CM-Sepharose column as in Example 23. Product was further purified by application onto a 0.5 g styrene-divinylbenzene cartridge (EnviChrom-P) which was eluted with incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 20% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 7.3 mg of a white solid, Compound 14, C₅₈H₉₂N₁₈O₁₃. FABMS: calc. for C₅₈H₉₃N₁₈O₁₃ (M+H)⁺ = 1249.7. Found: 1250 (M+H)⁺.

Example 25. N-Acetyl-p-aminophenylacetyl-PBP-3 (Compounds 15 and 15P)

[0274] (NaSO₃-Fmoc)₅-PBP-3 (20.7 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 23; 5.8 mg of N-acetyl-p-aminophenylacetyl-N-hydroxysuccinimide was added. The

solution was stirred 48 min at RT to obtain Compound 15P and then 0.020 mL piperidine was added to remove protecting groups. After stirring 30 min at RT the reaction mixture was diluted with 10 mL 50% MeOH 0.05 M in pH 5.0 ammonium acetate and 0.013 mL acetic acid. Product was isolated from the clear reaction dilution on a CM-Sepharose column in similar fashion as in Example 23. Product was further purified on a 0.5 g EnviChrom-P cartridge which was eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 15% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 3.2 mg of a white solid, Compound 15, C₅₇H₉₁N₁₇O₁₄. FABMS: calc. for C₅₇H₉₂N₁₇O₁₄ (M+H)⁺ = 1238.7 Found: 1238(M+H)⁺.

Example 26. N-(n-Decanoyl)phenylalanyl-PBP-3 (Compounds 16 and 16P)

[0275] (NaSO₃-Fmoc)₅-PBP-3 (24.0 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; 12.9 mg of N-(n-decanoyl)phenylalanyl-N-hydroxysuccinimide was added. The solution was stirred 48 min at RT to form compound 16P, and then 0.050 mL piperidine was added to remove protecting groups. After stirring 30 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 30% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 7.5 mg of a white solid, Compound 16, C₆₆H₁₀₉N₁₇O₁₄. FABMS: calc. for C₆₆H₁₁₀N₁₇O₁₄, (M+H)⁺ = 1364.8. Found: 1365 (M+H)⁺.

Example 27. 4-n-Decylphenylcarbamyl-PBP-3 (Compounds 17 and 17P)

[0276] (NaSO₃-Fmoc)₅-PBP-3 (12.1 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.40 mL of collidine/acetic acid/water (20:1:2); 9.7 mg of 4-n-decylphenylcarbamyl-N-hydroxysuccinimide, prepared according to the procedure of Example 43, was added in two increments along with 0.030 mL of diisopropylethylamine and stirred about 2 hrs at RT to form Compound 17P.

Piperidine, 0.055 mL was added to remove blocking groups. After stirring 40 min at RT the reaction mixture was diluted with 10 mL 60% MeOH 0.04 M in pH 5.0 ammonium acetate and 0.060 mL acetic acid. Product was isolated from the reaction dilution filtrate (PVDF membrane) on a CM-Sepharose column as in Example 23. The product was desalted and freeze dried as in Example 23. Yield: 5.0 mg of a white solid, Compound 17, $C_{64}H_{105}N_{17}O_{13}$. FABMS: calc. for $C_{64}H_{106}N_{17}O_{13} (M+H)^+$ = 1320.8. Found: 1322 (M+H)⁺.

Example 28. 4-Biphenylcarbamyl-PBP-3 (Compounds 18 and 18P)

[0277] $(NaSO_3-Fmoc)_5$ -PBP-3 (11.9 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.30 mL of collidine/acetic acid/water (20:1:2); 7.0 mg of 4-biphenyl isocyanate (commercially available, e.g., from Aldrich (Milwaukee, WI)) was added in two increments. After stirring about 2 hrs at RT to form Compound 18P, 0.050 mL piperidine was added to remove protecting groups. After stirring 32 min at RT the reaction mixture was diluted with 10 mL 60% MeOH 0.04M in pH 5.0 ammonium acetate and 0.035 mL acetic acid. Product was isolated from the reaction dilution filtrate (PVDF membrane) on a CM-Sepharose column as in Example 23. The product was desalted and freeze dried as in Example 23. Yield: 5.1 mg of a white solid, Compound 18, $C_{60}H_{91}N_{17}O_{13}$. FABMS: calc. for $C_{60}H_{92}N_{17}O_{13} (M+H)^+$ = 1258.7. Found: 1258 (M+H)⁺.

Example 29. Benzyloxyacetyl-PBP-3 (Compounds 19 and 19P)

[0278] $(NaSO_3-Fmoc)_5$ -PBP-3 (23.5 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; 12.5 mg of benzyloxyacetyl-N-hydroxysuccinimide ester was added and the solution was stirred 56 min at RT to form Compound 19P. Piperidine, 0.050 mL, was then added to remove protecting groups. After stirring 30 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 17.5% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 7.7 mg of a

white solid, Compound 19, $C_{56}H_{90}N_{16}O_{14}$. FABMS: calc. for $C_{56}H_{91}N_{16}O_{14}$, $(M+H)^+ = 1211.7$. Found: 1211 $(M+H)^+$.

Example 30. 4-Phenyloxyphenylcarbamyl-PBP-3 (Compounds 20 and 20P)

[0279] $(NaSO_3-Fmoc)_5$ -PBP-3 (16.6 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; 7.7 mg of 4-phenyloxyphenyl isocyanate was added and the solution was stirred 85 min at RT to form Compound 20P. 4-phenyloxyphenyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). Piperidine, 0.040 mL, was added to remove the protecting groups. After stirring 15 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column in similar fashion as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH_3CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 25% CH_3CN . The product was desalted and freeze dried as in Example 23. Yield: 5.1 mg of a white solid, Compound 20, $C_{60}H_{91}N_{17}O_{14}$. FABMS: calc. for $C_{60}H_{92}N_{17}O_{14}$, $(M+H)^+ = 1274.7$. Found: 1274 $(M+H)^+$.

Example 31. 4-Chlorophenylcarbamyl-PBP-3 (Compounds 21 and 21P)

[0280] $(NaSO_3-Fmoc)_5$ -PBP-3 (12.0 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; about 8.2 mg of 4-chlorophenyl isocyanate was added in two increments. 4-Chlorophenylisocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). The solution was stirred 80 min at RT to form Compound 21P, and then 0.040 mL piperidine was added to remove protecting groups. The reaction mixture was stirred 27 min at RT and diluted as in Example 23. The product was isolated from the diluted reaction filtrate (PVDF membrane) on a CM-Sepharose column as in Example 23. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH_3CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 25% CH_3CN . The product was desalted and freeze dried as in Example 23. Yield: 3.6 mg of a white solid, Compound 21, $C_{54}H_{86}N_{17}O_{13}Cl$. FABMS: calc. for $C_{54}H_{87}N_{17}O_{13}Cl$, $(M+H)^+ = 1216.6$. Found: 1216 $(M+H)^+$.

Example 32. Benzylcarbamyl-PBP-3 (Compounds 22 and 22P)

[0281] (NaSO₃-Fmoc)₅-PBP-3 (12.2 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; about 0.004 mL of benzyl isocyanate (commercially available, e.g., from Aldrich (Milwaukee, WI)) was added. The solution was stirred 45 min at RT to form Compound 22P, and then 0.040 mL piperidine was added to remove the protecting groups. After stirring 35 min at RT the reaction mixture was diluted as in Example 23 and product was isolated from the reaction dilution on a CM-Sepharose column as in Example 23. The product was desalted and freeze dried as in Example 23. Yield: 5.5 mg of a white solid, Compound 22, C₅₅H₈₉N₁₇O₁₃. FABMS: calc. for C₅₅H₉₀N₁₇O₁₃, (M+H)⁺ = 1196.7. Found: 1196 (M+H)⁺.

Example 33. Cyclohexylcarbamyl-PBP-3 (Compounds 23 and 23P)

[0282] (NaSO₃-Fmoc)₅-PBP-3 (19.3 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; 0.008 mL of cyclohexyl isocyanate was added. Cyclohexyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). The solution was stirred 30 min at RT to form Compound 23P, and then 0.050 mL piperidine was added to remove protecting groups. The reaction mixture was stirred 35 min at RT and diluted. Product was isolated on a CM-Sepharose column as in Example 24 and then further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 18% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 5.1 mg of a white solid, Compound 23, C₅₄H₉₃N₁₇O₁₃. FABMS: calc. for C₅₄H₉₄N₁₇O₁₃, (M+H)⁺ = 1188.7. Found: 1189 (M+H)⁺.

Example 34. Phenylthiocarbamyl-PBP-2 (Compound 24)

[0283] Purified phenylthiocarbamyl-(NaSO₃-Fmoc)₄-PBP-2 (13.7 mg, compound 24P from Example 20) was dissolved in 0.20 mL of DMF and 0.010 mL of piperidine. After stirring 15 min at RT the reaction mixture was diluted with 8 mL 50% MeOH 0.05M in pH 5.0 ammonium acetate and 0.006 mL of acetic acid.

Product was isolated from the reaction dilution on a CM-Sepharose column in similar fashion as in Example 23. The product was desalted and freeze dried as in Example 23. Yield: 6.9 mg of a white solid, Compound 24, $C_{50}H_{80}N_{15}O_{11}S$ FABMS: calc. for $C_{50}H_{81}N_{15}O_{11}S$ (M+H)⁺ = 1098.6. Found: 1099(M+H)⁺.

Example 35. n-Decanoyl-PBP-1 (Compounds 25 and 25P)

[0284] (NaSO₃-Fmoc)₄-PBP-1 (16.2 mg) was prepared according to example to Example 20. The protected peptide was dissolved as in Example 24; 12.6 mg of n-decanoyl-N-hydroxysuccinimide ester was added in two increments. The solution was stirred about 2 hrs at RT to form Compound 25P and then 0.050 mL of piperidine was added to remove the protecting groups. After stirring 37 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column as in Example 24. The product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 24% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 2.6 mg of a white solid, Compound 25, $C_{49}H_{85}N_{13}O_{10}$. FABMS: calc. for $C_{49}H_{86}N_{13}O_{10}$, (M+H)⁺ = 1016.7. Found: 1017 (M+H)⁺.

Example 36. n-Octylcarbamyl-colistin decapeptide (Compounds 51 and 51P)

[0285] (NaSO₃-Fmoc)₅-colistin decapeptide (20.2 mg), prepared according to the procedure of Example 45, was dissolved as in Example 24; 0.006 mL of n-octyl isocyanate was added. Octyl isocyanate is commercially available, e.g., from Aldrich (Milwaukee, WI). The solution was stirred 48 min at RT to form Compound 51P, and then 0.050 mL piperidine was added to remove the protecting groups. After stirring 25 min at RT the reaction mixture was diluted and filtered through a PVDF-membrane. The product was isolated from the filtrate on a CM-Sepharose column as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 22% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 3.0 mg of a white solid, Compound 51, $C_{53}H_{101}N_{17}O_{13}$. FABMS: calc. for $C_{53}H_{102}N_{17}O_{13}$, (M+H)⁺ = 1184.8. Found: 1185 (M+H)⁺.

Example 37. N-(n-Decanoyl)phenylalanyl-colistin decapeptide (Compounds 52 and 52P)

[0286] (NaSO₃-Fmoc)₅-colistin decapeptide (20.7 mg), prepared according to the procedure of Example 45, was dissolved as in Example 24; 9.5 mg of N-(n-decanoyl)phenylalanyl-N-hydroxysuccinimide ester was added. The solution was stirred 46 min at RT to form Compound 52P, and then 0.050 mL piperidine was added to remove protecting groups. The reaction mixture was stirred 25 min at RT and diluted. Product was isolated on a CM-Sepharose column as in Example 24 and further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 30.5% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 2.5 mg of a white solid, Compound 52, C₆₃H₁₁₁N₁₇O₁₄. FABMS: calc. for C₆₃H₁₁₂N₁₇O₁₄, (M+H)⁺ = 1330.9. Found: 1331 (M+H)⁺.

Example 38. P-Toluenesulfonyl-Colistin decapeptide (Compounds 53 and 53P)

[0287] (NaSO₃-Fmoc)₅-colistin decapeptide (20.8 mg), prepared according to the procedure of Example 45, was dissolved as in Example 24; 6.8 mg of p-toluenesulfonyl chloride (commercially available, e.g., from Aldrich (Milwaukee, WI)) was added. The reaction was stirred 51 min at RT to form Compound 53P and then 0.050 mL piperidine was added to remove protecting groups. After stirring 47 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05 M in sodium sulfate at pH 2.3; product was eluted with 20% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 1.1 mg of a white solid, Compound 53, C₅₁H₉₀N₁₆O₁₄S. FABMS: calc. for C₅₁H₉₁N₁₆O₁₄S, (M+H)⁺ = 1183.7. Found: 1184 (M+H)⁺.

Example 39. Isolation of [Ile⁷]-Polymyxin B₁ and [Leu⁷]-Polymyxin B₁

[0288] Commercial polymyxin B sulfate (46 mg) was dissolved in 10 mL of 0.05 M sodium sulfate pH 2.3 buffer. About 9 mL of the sample solution was

injected onto a Waters Delta-Pak® C-18 Radial-Pak column (2.5 x 21 cm) which was eluted at room temperature at 5.0 mL/min with a 22% to 32% CH₃CN linear gradient over 120 min; eluents were buffered with about 0.04 M sodium sulfate at pH 2.3. UV absorption of the eluate was monitored at 225 nm. The [Ile⁷] variant eluted at about 65 min and the major component, polymyxin B₁ ([Leu⁷]-polymyxin B₁), eluted at about 77 min. As determined by analytical HPLC, fractions containing the desired products were separately pooled, CH₃CN was evaporated under vacuum at 35 °C, and the aqueous solutions were desalted and freeze dried as in Example 23. Yields: 3.8 mg of a white solid, [Ile⁷]-polymyxin B₁, C₅₆H₉₈N₁₆O₁₃. FABMS: calc. for C₅₆H₉₉N₁₆O₁₃, (M+H)⁺ = 1203.8. Found: 1204(M+H)⁺; 18 mg of a white solid, [Leu⁷]-polymyxin B₁, C₅₆H₉₈N₁₆O₁₃. FABMS: calc. for C₅₆H₉₉N₁₆O₁₃, (M+H)⁺ = 1203.8. Found: 1204(M+H)⁺. About 2 mg each of the products were separately dissolved in 1.0 mL of 6M HCl and heated at about 120 °C for 22 hrs. The solvent was removed by evaporation under vacuum at 50 °C and the residues were dissolved in 1.0 mL of distilled water. The samples were derivatized with Fmoc chloride in pH 10.1 sodium borate buffered aqueous CH₃CN for 5 min at 50 °C. Amino acid Fmoc derivatives were separated at pH 6.7 on an analytical reversed phase column (C18, 15 cm) eluted with a CH₃CN gradient at 40 °C. Peak areas were integrated at 264 nm and amino acids were identified by correspondence of retention times to reference Fmoc amino acids.

		<u>Mole Ratios (Phe = 1.00)</u>			
		<u>[Ile⁷]-PB₁</u>		<u>[Leu⁷]-PB₁</u>	
<u>Amino Acid</u>	<u>Retn t(min)</u>	<u>Expected</u>	<u>Found</u>	<u>Expected</u>	<u>Found</u>
Unknown	3.63	0.00	0.07	0.00	0.09
Ser	3.82	0.00	0.07	0.00	0.00
Thr	4.39	2.00	1.76	2.00	1.82
Ile	8.25	1.00	0.88	0.00	0.00
Leu	8.57	0.00	0.13	1.00	1.00
Phe	9.38	1.00	1.00	1.00	1.00
Dab	16.35	6.00	5.82	6.00	5.75

Example 40. Cyclohexylcarbamyl-[Ile⁷]-Polymyxin Decapeptide
(Compounds 48 and 48P)

[0289] (NaSO₃-Fmoc)₅-PBP-3 (55.8 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved in 0.60 mL of reaction solvent as in Example 24; 0.012 mL of cyclohexyl isocyanate was added. Cyclohexyl isocyanate is commercially available, e.g., from Aldrich. The solution was stirred 50 min at RT to form Compound 48P and then 0.10 mL piperidine was added to remove the protecting groups. After stirring 30 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column in similar fashion as in Example 24. The product-containing fractions were pooled (8 mL) and injected onto a Waters Delta-Pak[®] C-18 Radial-Pak column (2.5 x 21 cm) which was eluted at room temperature at 5.0 mL/min with a 15% to 25% CH₃CN linear gradient over 120 min; eluents were buffered with about 0.04 M sodium sulfate at pH 2.3. UV absorption of the eluate was monitored at 225 nm. The cyclohexylcarbamyl-[Ile⁷] variant (Compound 48) eluted at about 83 min and the major component, cyclohexylcarbamyl-[Leu⁷]-polymyxin decapeptide (Compound 23), eluted at about 93 min. Product fractions were pooled, desalted, and freeze dried as in Example 39. Yields: 2.6 mg of a white solid, Compound 48, C₅₄H₉₃N₁₇O₁₃. FABMS: calc. for C₅₄H₉₄N₁₇O₁₃, (M+H)⁺ = 1188.7. Found: 1189 (M+H)⁺; and 20.1 mg of a white solid, Compound 23. Amino acid analyses were performed on both samples in similar fashion as in Example 39.

Mole Ratios (Phe = 1.00)					
Amino Acid	Retn t(min)	[Ile ⁷]-variant		[Leu ⁷]-variant	
		Expected	Found	Expected	Found
Unknown	3.12	0.00	0.09	0.00	0.14
Thr	3.72	2.00	1.86	2.00	1.82
Ile	7.06	1.00	0.96	0.00	0.00
Leu	7.34	0.00	0.04	1.00	1.00
Phe	8.09	1.00	1.00	1.00	1.00
Dab	14.96	6.00	5.49	6.00	5.44

Example 41. Preparation of 2-N-(n-Decanoyl)lysyl-PBP-3 (Compounds 54 and 54P)

[0290] (NaSO₃-Fmoc)₅-PBP-3 (21.7 mg) was prepared according to the procedure of Example 3. The protected peptide was dissolved as in Example 24; 11.8 mg of 6-N-Fmoc-2-N-(n-decanoyl) lysyl-N-hydroxysuccinimide was added. The solution was stirred 45 min at RT to form Compound 54P and then 0.050 mL of piperidine was added to remove protecting groups. After stirring 30 min at RT the reaction mixture was diluted and product was isolated on a CM-Sepharose column as in Example 24. Product was further purified on a 0.5 g EnviChrom-P cartridge eluted with incrementally increasing concentrations of CH₃CN about 0.05M in sodium sulfate at pH 2.3; product was eluted with 22.5% CH₃CN. The product was desalted and freeze dried as in Example 23. Yield: 7.2 mg of a white solid, Compound 54, C₆₃H₁₁₂N₁₈O₁₄. FABMS: calc. for C₆₃H₁₁₃N₁₈O₁₄ (M+H)⁺ = 1345.9. Found: 1346(M+H)⁺.

Example 42. 4-n-Decylphenylcarbamyl-N-hydroxysuccinimide

[0291] A mixture of p-decylaniline (0.4504 g, 1.93 mmols), disuccinimidyl carbonate (0.5840 g, 2.28 mmols) and triethylamine (0.26 mL, 1.93 mmols) in 10 mL of acetonitrile was stirred at room temperature overnight. A small amount of insoluble solid was filtered off and the filtrate was evaporated to dryness. The residue was taken up in ethyl acetate and extracted with 0.5 N hydrochloric acid, water, saturated sodium bicarbonate solution and brine, dried over magnesium sulfate and evaporated to the product, C₂₁H₃₀N₂O₄, a beige solid, yield 0.3784 g., FABMS: calc. for C₂₁H₃₁N₂O₄, (M+H)⁺ = 375.2. Found 375 (M+H)⁺. This intermediate was used in Example 27 to prepare Compounds 17 and 17P.

Example 43. 3-Indolyethylcarbamyl-N-hydroxysuccinimide

[0292] A solution of tryptamine (0.1730 g, 1.08 mmol) and disuccinimidyl carbonate (0.5213 g, 2.03 mmol) in 20 mL of acetonitrile was stirred at room temperature overnight. The reaction mixture was evaporated to dryness and slurried in ethyl acetate and filtered from insoluble solid. Evaporation of the ethyl acetate gave a glass-like solid which was purified by gradient-elution chromatography on silica gel using ethyl acetate: hexane. The product was eluted

with ethyl acetate:hexane (8:2) to afford 0.1402 g of the desired compound, $C_{15}H_{15}N_3O_4$, FABMS: calc. for $C_{15}H_{15}N_3O_4$, $M^+ = 301.1$, Found 301(M)⁺. This intermediate was used to prepare Compounds 14 and 14P in Example 24.

**Example 44. N-[(2-Sulfo)-9-fluorenylmethoxycarbonyl]₅-colistin[
(HSO₃-Fmoc)₅-colistin]**

[0293] Colistin sulfate (polymyxin E) (0.2481g, 0.1956 mmol) was dissolved in a solution of 7.0 mL, saturated sodium bicarbonate, 7.0 mL of water and 7.0 mL of tetrahydrofuran. Colistin sulfate is commercially available from Sigma (Milwaukee, WI). A solution of (2-sulfo)-9-fluorenylmethoxy-N-hydroxysuccinimide (0.5017 g, 1.203 mmol) in 5 mL of tetrahydrofuran was added in several portions over 45 min. The reaction was stirred at room temperature over night, then diluted with 20 mL of water, and acidified with 10 mL of 6N hydrochloric acid to give an oily precipitate. The mixture was chilled and the aqueous layer decanted and the oily residue was dissolved in 100 mL of ethanol. The ethanol was evaporated under vacuum (35 °C) and the resulting solid was triturated with ethyl acetate, filtered and dried to afford 0.431 g of product. HPLC with a gradient on a reverse phase column showed a single major peak,

N-[2-(sulfo)-9-fluorenylmethoxycarbonyl]₅-colistin, $[C_{128}H_{152}N_{16}O_{38}S_5]$, ESIMS: calc. m/z for $C_{128}H_{154}N_{16}O_{38}S_5$ ($M+2H$)²⁺ = 1341.4. Found: 1341.6

[0294] The above procedure can be used with 2(sulfo-9-)fluorenylmethoxycarbonyl chloride with similar results.

Example 45. (NaSO₃-Fmoc)₅-colistin decapeptide

[0295] Solubilized deacylase enzyme, 940 mg, representing 400 mL original enzyme fermentation volume, prepared according to Example 2, was combined with 320 mL of 0.02 molar ammonium phosphate buffer, pH 7.2, 80 mL ethanol, 2.96 grams EDTA disodium salt and 200 mg (HSO₃-Fmoc)₅-colistin, prepared according to the procedure of Example 44. The mixture was adjusted to pH 8 and placed on a shaker at 140 rpm, 30 °C. Progress of the deacylation was monitored by HPLC. After three hours the completed reaction was removed from the shaker and allowed to stand at room temperature for one hour before being

adjusted to pH 2.0 with 1N HCl. The precipitate was mixed with 50 mL water, dissolved by adjusting to pH 6.46 and allowed to stand at 4 °C overnight resulting in a small amount of precipitate of the protected decapeptide. The precipitate was combined with methanol, which was then evaporated to dryness, re-dissolved in water and freeze-dried to obtain 12.5 mg of light tan powder, C₁₂₂H₁₃₄N₁₆O₃₇S₅. ESIMS: calc. m/z for C₁₂₂H₁₃₅N₁₆O₃₇S₅Na: (M+H+Na)⁺² = 1282.4. Found: 1282.2. The remaining decant was freeze-dried to obtain an additional 192 mg of tan powder, the protected colistin decapeptide.

Example 46. Other Peptides

[0296] Other novel peptides and novel protected peptides can be made by the methods described above. Any peptide having the common heptapeptide cyclic core structure of the polymyxins and the octapeptins, including, e.g., polymyxin A, polymyxin B (e.g., polymyxin B1, polymyxin B2, and polymyxin B3), [Ile⁷]-polymyxin B₁, polymyxin C, polymyxin D, polymyxin E (also called colistin), polymyxin F, polymyxin M (also called mattacin), polymyxin P, polymyxin S, polymyxin T (e.g., polymyxin T1), colistin, circulin A, octapeptin A (e.g., octapeptin A1, octapeptin A2, and octapeptin A3) octapeptin B (e.g., octapeptin B1, octapeptin B2, and octapeptin B3), octapeptin C (e.g., octapeptin C1), and octapeptin D, can be amino protected as described in Example 1, deacylated as described in Examples 3 and 4, purified as in Example 5, modified as described in Examples 7 and 9, and subjected to Edman degradation as described in Example 8.

References

- [0297] 1. Evans, M.E., Feola, D.J., Rapp, R.P. **1999**. Polymyxin B Sulfate and Colistin: Old Antibiotics for Emerging Multiresistant Gram-Negative Bacteria. *Ann. Pharmacother.* 33:960-967.
- [0298] 2. McCallister, S.M., Alpar H.O., Brown, M.R.W. **1999**. *Antimicrob. Chemother.* 43:203-210.
- [0299] 3. Chihara, S., Ito, A., Yahata, M., Tobita, T., Koyama, Y. **1974**. Chemical Synthesis and Characterization of n-Fattyacyl Mono-Aminoacyl Derivatives of Colistin Nonapeptide. *Agr. Biol. Chem.* 38: (10), 1767-1777.

- [0300] 4. Chihara, S., Ito, A., Yahata, M., Tobita, T., Koyama, Y. **1973**. Chemical Synthesis and Characterization of α -N-Octanoyl and Other α -N-Acyl Nonapeptide Derivatives. *Agr. Biol. Chem.* 37: (12), 2709-2717.
- [0301] 5. Chichara, S., Ito, A., Yahata, M., Tobita, T., Koyama, Y. **1974**. Chemical Synthesis, Isolation and Characterization of n-N-Fattyacyl Colistin Nonapeptide with Special Reference to the Correlation Between Antimicrobial Activity and Carbon Number of Fattyacyl Moiety. *Agr. Biol. Chem.* 38: (3), 521-529.
- [0302] 6. Weinstein, J., Afonso, A., Moss, E.JR., Miller, G.H. **1988**. Selective Chemical Modifications of Polymyxin B. *Biorg. Med. Chem. Lett.* 8:3991-3996.
- [0303] 7. Tsubery, H., Ofek, I., Cohen, S., Fridkin, M. **2001**. N-Terminal Modifications of Polymyxin B Nonapeptide and Their Effect on Antibacterial Activity. *Peptides*. 22: 1675-1681.
- [0304] 8. Mutter, M., and Bellof, D. **1984**. A New Base-labile Anchoring Group for Polymer-supported peptide synthesis, *Helv. Chim. Acta*, 67, 2009.
- [0305] 9. Liu, Y.-Z., Ding, S.H., Chu, J.-Y. and Felix, A.M. **1990**. A Novel Fmoc-based Anchorage for the Synthesis of protected Peptide on Solid Phase, *Int. J. Pept. Protein Res.*, 35, 95.
- [0306] 10. Markou, N., Apostolakos, H., Koumoudiou, C., Anthanasiou, M., Koutsoukou, A., Alamanos, I., and Gregorakos, L. **2003**. Intravenous colistin in the treatment of sepsis from multiresistant Gram- negative bacilli in critically ill patients, *Critical Care* 7, R78-R83.
- [0307] 11. Duwe, A.K., Rupar, C.A., Horsman, G.B., and Vas, S.I., **1986**. In Vitro Cytotoxicity and Antibiotic Activity of Polymyxin B Nonapeptide, *Antimicrob. Agents Chemother*, 30:340-341.
- [0308] 12. Kurihara T., Takeda, H., and Ito, H. **1972**. Compounds related to colistin. V. Synthesis and pharmacological activity of colistin analogs, *Yakugaku Zasshi* 92:129-34.
- [0309] 13. Parker, W.L., Rathnum, M.L., **1975**. EM49, A New Peptide Antibiotic IV. The Structure of EM49. *J. Antibiot.* 28: (5), 379-389.

[0310] 14. Hausmann, W., et al., **1954**. Polymyxin B1. Fractionation, molecular-weight determination, amino acid and fatty acid composition, *J. Am. Chem. Soc.* 76, 4892-4896.

[0311] 15. DeVisser Kriek, N.M.A.J., van Hooft, P.A.V., Van Schepdael A., Fillipov, D.V., van der Marel, G.A., Overcleef, H.S., van Boom, J.H., and Noort, D.M. **2003**. Synthesis of polymyxin B and analogues, *J. Peptide Res.* 61, 298-306.

[0312] 16. Kimura, Y., Matsunaga, and Vaara, M., **1992**. Polymyxin B Octapeptide and Polymyxin B Heptapeptide are Potent Outer Membrane Permeability-Increasing Agents. *J. Antibiot.*, 45, 742-749.

[0313] 17. Boeck, L.D., Fukuda, D.S., Abbott, B.J., Debono, M. **1988**. Deacylation of A21978C, an acidic Lipopeptide Antibiotic Complex, by *Actinoplanes utahensis*. *J. Antibiot.* 41: (8), 1085-1092.

[0314] 18. Kreuzman, A.J., Hodges, R.L., Swartling, J.R., Ghag, S.K., Baker, P.J., McGilvray, D., Yeh, W.K. **2000**. Membrane-Associated Echinocandin B Deacylase of *Actinoplanes utahensis*: Purification, Characterization, Heterologous Cloning

[0315] 19. Boeck, L.D., Fukuda, D.S., Abbott, B.J., Debono, M. **1989**. Deacylation of Echinocandin B by *Actinoplanes utahensis*. *J. Antibiot.* 42: (3), 382-388.

[0316] 20. Borders, D. B. Curran, W. V., Fantini, A. A., Francis,

[0317] N.D., Jarolmen, H., Leese, R. A., **2003**. Derivatives of Laspartomycin and Preparation and Use Thereof, U.S. Patent No. 6,511,962.

[0318] 21. Elverdam, I., Larsen, P., Lund, E. **1981**. Isolation and Characterization of Three New Polymyxins in Polymyxins B and E by High-Performance Liquid Chromatography. *J. Chromatogr.* 218: 653-661.

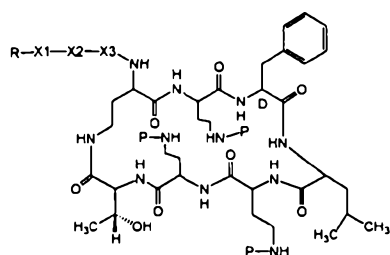
[0319] 22. Sakura, N., Itoh, T., Uchida, Y., Ohki, K., Okimura, K., Chiba, K., Sato, Y., Sawanishi, H. **2004**. The Contribution of the N-Terminal Structure of Polymyxin B Peptides to Antimicrobial and Lipopolysaccharide Binding Activity, *Bull. Chem. Soc. Jpn.* 77: 1915-1924.

[0320] 23. Falagas, M. E., Kasiakou, S. K. **2005**. Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Rev. Anti-Infect. Agents.* 40: 1333-1341.

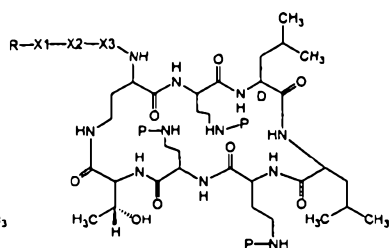
- [0321] 24. Salem, E. M., El-Gammal, A. A. **1980**. Synthesis of Pelargonoyl-Cyclic Decapeptide Analog of the Antibiotic Polymyxin B₁. *Pharmazie* 35: 540-541.
- [0322] 25. Srinivasa, B. R., Ramachandran, L. K. **1980**. Essential Amino Groups of Polymyxin B. *Indian J. Biochem. Biophys.* 17: 112-118.
- [0323] 26. Kline, T., Holub, D., Therrien, J., Leung, T., Ryckman, D. **2001**. Synthesis and characterization of the colistin peptide polymyxin E₁ and related antimicrobial peptides. *J. Pept. Res.*, 57: 175-187.
- [0324] 27. Bouchaudon, J., Jolles, G. **1973**. Cyclopeptides Derived From Polymyxins and Their Preparation. U.S. Patent No. 3,753,970.
- [0325] 28. Shechter, Y., Preciado-Patt, L., Schreiber, G., Fridkin, M. **2001**. Prolonging the half-life of human interferon- α_2 in circulation: Design, preparation, and analysis of (2-sulfo-9-fluorenylmethoxycarbonyl)7-interferon- α_2 . *Proc. Natl. Acad. Sci., U.S.A.* 98: 1212-1217.
- [0326] 29. Merrifield, R. B., Bach, A. E. **1978**. 9-(2-Sulfo)fluorenylmethoxycarbonyl chloride, a new reagent for the purification of synthetic peptides. *J. Org. Chem.*, **43**: 4808-4816.
- [0327] 30. Kleinkauf, H., van Dohren, H. **1990**. Nonribosomal synthesis of peptide antibiotics. *Eur. J. Biochem.* **192**: 1-15.
- [0328] 31. Martin, N. I., Hu, H., Moakes, M. M., Churey, J. J., Whittall, R., Worobo, R. W., Vederas, J. C. **2003**. Isolation, structural characterization, and properties of mattacin (polymyxin M), a cyclic peptide antibiotic produced by *Paenibacillus kobensis* M. *J. Biol. Chem.* **278**: 13124-13132.
- [0329] 32. Gershonov, E., Goldwaser, I., Fridkin, M., Schechter, Y. , **2000**, "A Novel Approach for a Water-Soluble Long-Acting Insulin ProDrug: Design, Preparation, and Analysis of [(2-Sulfo)-9-Fluorenylmethoxycarbonyl]₃-Insulin," *J. Med. Chem.* 43: (13), 2530-2537, and
- [0330] 33. Schechter, Y., Tsudbery, H., Fridkin, M., **2002**, N-[(2-Sulfo)-9-Fluorenylmethoxycarbonyl]₃-Gentamicin Is a Long-Acting Prodrug Derivative," *J. Med. Chem.* 45: (19), 4264-4270.

The claims defining the invention are as follows:

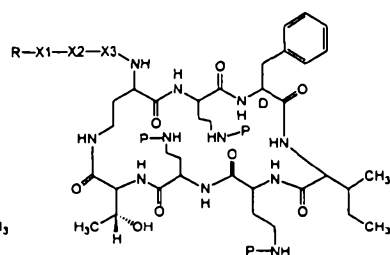
1. An antibiotic compound or protected antibiotic compound selected from the following structures wherein R, X1, X2, X3, and P are as specified below:



PBpeptide derivatives



Cpeptide derivatives



ILpeptide derivatives

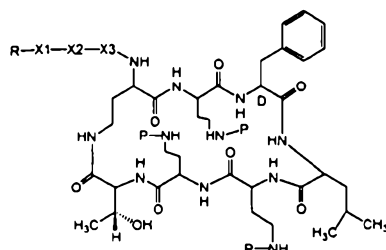
Compound	R	Peptide	X1*	X2	X3*	P
I	n-C ₉ H ₁₉ CO-	PBpeptide	Dab	Thr	Dab	H
1P	n-C ₉ H ₁₉ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
3	n- C ₈ H ₁₇ NHCO-	PBpeptide	Dab	Thr	Dab	H
3P	n- C ₈ H ₁₇ NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
4	phenyl-NHCS-	PBpeptide	Dab	Thr	Dab	H
4P	phenyl-NHCS-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
5	phenyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
5P	phenyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
6	phenyl-CO-	PBpeptide	Dab	Thr	Dab	H
6P	phenyl-CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
7	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab	Thr	Dab	H
7P	2-naphthyl-OCH ₂ -CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab	Thr	Dab	H
8P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
16	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab	Thr	Dab	H
16P	n-C ₉ H ₁₉ CO-Phe-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
19	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab	Thr	Dab	H
19P	C ₆ H ₅ CH ₂ OCH ₂ CO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpeptide	Dab	Thr	Dab	H
20P	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
21	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab	Thr	Dab	H
21P	4-Cl-C ₆ H ₄ -NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
22	benzyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
22P	benzyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
23	cyclohexyl-NHCO-	PBpeptide	Dab	Thr	Dab	H
23P	cyclohexyl-NHCO-	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
48	cyclohexyl-NHCO-	ILpeptide	Dab	Thr	Dab	H
48P	cyclohexyl-NHCO-	ILpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc
51	n-C ₈ H ₁₇ NHCO-	Cpeptide	Dab	Thr	Dab	H
51P	n-C ₈ H ₁₇ NHCO-	Cpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc

Compound	R	Peptide	X1*	X2	X3*	P
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Cpeptide	Dab	Thr	Dab	H
53-P	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Cpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-
54	n-C ₉ H ₁₉ CO-Lys	PBpeptide	Dab	Thr	Dab	H
54P	n-C ₉ H ₁₉ CO-Lys	PBpeptide	Dab-P	Thr	Dab-P	HSO ₃ -Fmoc-

*Dab-P is 4-N-(HSO₃-Fmoc)-diaminobutyl, Lys-P is 6-N-(HSO₃-Fmoc)-lysyl

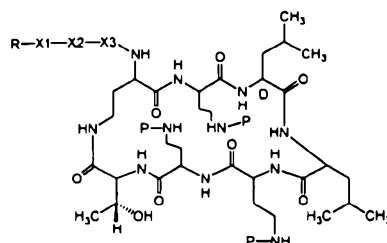
All amino acids are the L-isomers unless indicated otherwise.

2. An antibiotic compound having one of the following structures:



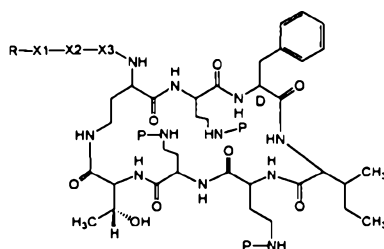
PBpeptide derivatives

(PB);



Cpeptide derivatives

(C); or



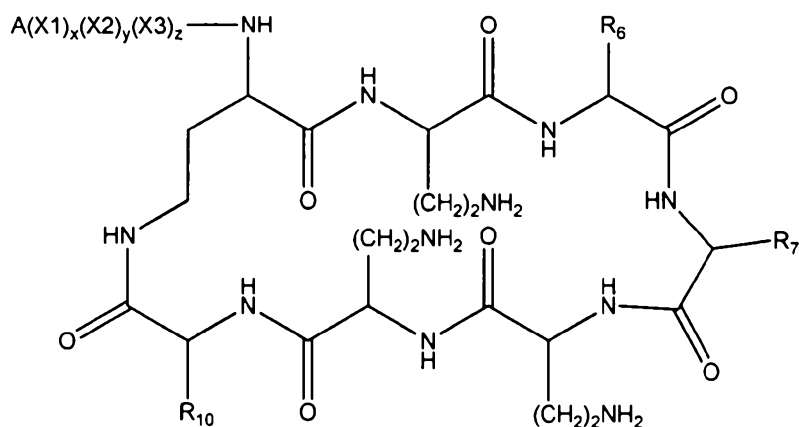
ILpeptide derivatives

(IL)

for use against gram-negative and gram-positive bacteria, wherein the cyclic polypeptide, R, X1, X2, and X3 are as specified below:

Compound	R	X1*	X2	X3*	Cyclic peptide
1	n-C ₉ H ₁₉ CO-	Dab	Thr	Dab	PB
3	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	PB
4	phenyl-NHCS-	Dab	Thr	Dab	PB
5	phenyl-NHCO-	Dab	Thr	Dab	PB
6	phenyl-CO-	Dab	Thr	Dab	PB
7	2-naphthyl-OCH ₂ -CO-	Dab	Thr	Dab	PB
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	PB
16	n-C ₉ H ₁₉ CO-Phe-	Dab	Thr	Dab	PB
19	C ₆ H ₅ CH ₂ OCH ₂ -CO-	Dab	Thr	Dab	PB
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	Dab	Thr	Dab	PB
21	4-Cl-C ₆ H ₄ -NHCO-	Dab	Thr	Dab	PB
22	benzyl-NHCO-	Dab	Thr	Dab	PB
23	cyclohexyl-NHCO-	Dab	Thr	Dab	PB
48	cyclohexyl-NHCO-	Dab	Thr	Dab	IL
51	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	C
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	C
54	n-C ₉ H ₁₉ CO-Lys-	Dab	Thr	Dab	PB

3. An antibiotic compound of the formula:



or a pharmaceutically acceptable salt thereof, wherein:

A is selected from R'-(C=O)-(p-aminophenylacetyl)-, R'-(C=O)-Lys-, R'-(C=O)-Phe-, R'-NH-(C=O)-, R'-NH(C=S)-, R'-SO₂-, R'-(C=NH)-, R'-O-(C=O)-, R'-O-CH₂-(C=O)-, R'-, R'-alkyl, R'-P(O)OH, R'-O-(C=S)-, R'-NH-(C=NH)-, and R'-(C=S)-;

R' is selected from aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl, wherein each aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl are each optionally substituted with one or more groups selected from halogen, (C₁-C₂₀) alkyl, aryl, heteroaryl and aryloxy;

or

A is R'-(C=O)-, and R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and substituted alkyl, wherein at least one hydrogen on said substituted alkyl is replaced by a substituent group selected from halogen, aryl, heteroaryl and aryloxy;

X1 is selected from Dab, Phe, Ala, Lys and Gly;

X2 is Thr;

X3 is Dab;

x and y are integers each independently selected from 0 and 1;

z is 1; and

R₆, R₇ and R₁₀ are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl,

provided that when R₆ and R₇ are each isobutyl and R₁₀ is 1-hydroxy-1-ethyl, then x and y are each 1 and X1 is Dab.

4. The antibiotic compound of claim 3, or a pharmaceutically acceptable salt thereof, wherein A is selected from R'-(C=O)-, R'-(C=O)-(p-aminophenylacetyl)-, R'-(C=O)-Lys-, R'-(C=O)-Phe-, R'-NH-(C=O)-, R'-NH(C=S)-, R'-SO₂-, or R'-O-CH₂-(C=O)-.

5. The antibiotic compound of claim 3 or claim 4, or a pharmaceutically acceptable salt thereof, wherein A is selected from

R'-(C=O)-, wherein R' is a) an unsubstituted alkyl having at least 9 carbon atoms, b) aryl or c) heteroaryl;

R'-(C=O)-(p-aminophenylacetyl)-, wherein R' is alkyl;

R'-(C=O)-Lys-, wherein R' is alkyl;

R'-(C=O)-Phe-, wherein R' is alkyl;

R'-NH-(C=O)-, wherein R' is a) alkyl optionally substituted with aryl or heteroaryl; b) aryl optionally substituted with halogen, aryl, alkyl or aryloxy; or c) cycloalkyl;

R'-NH(C=S)-, wherein R' is aryl;

R'-SO₂-, wherein R' is a) alkyl or b) aryl optionally substituted with alkyl; and

R'-O-CH₂-(C=O)-, wherein R' is a) aryl or b) alkyl optionally substituted with aryl.

6. The antibiotic compound of claim 3 or claim 4, or a pharmaceutically acceptable salt thereof, wherein:

A is selected from $R'-(C=O)-(p\text{-aminophenylacetyl})-$, $R'-(C=O)\text{-Lys-}$, $R'-(C=O)\text{-Phe-}$, $R'\text{-NH-(C=O)-}$, $R'\text{-NH(C=S)-}$, $R'\text{-SO}_2\text{-}$ and $R'\text{-O-CH}_2\text{-(C=O)-}$, and R' is selected from phenyl, methylphenyl, benzyl, naphthyl, pyridinyl, indolylethyl, $(C_8\text{-}C_{10})$ alkyl, $(n\text{-}C_{10}H_{21})\text{-phenyl}$, biphenyl, phenoxyphenyl, chlorophenyl, and cyclohexyl; or

A is $R'-(C=O)-$, wherein R' is selected from $C_9\text{-alkyl}$, phenyl and pyridinyl.

7. The antibiotic compound of any one of claims 3 to 6, or a pharmaceutically acceptable salt thereof, wherein:

R_6 and R_7 are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl; and

R_{10} is selected from isopropyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl.

8. The antibiotic compound of any one of claims 3 to 7, or a pharmaceutically acceptable salt thereof, wherein:

R_6 is benzyl or isobutyl;

R_7 is isobutyl or sec-butyl; and

R_{10} is 1-hydroxy-1-ethyl.

9. The antibiotic compound of any one of claims 3 to 8, or a pharmaceutically acceptable salt thereof, wherein R_6 is benzyl.

10. The antibiotic compound of claim 9, or a pharmaceutically acceptable salt thereof, wherein:

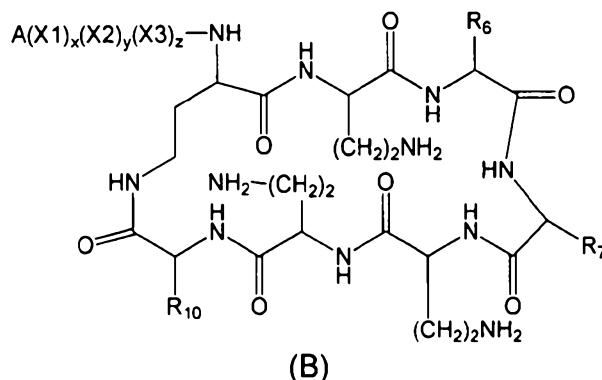
A is $R'\text{-NH-(C=O)-}$; and

R' is phenyl with one hydrogen atom replaced by one chloro substituent.

11. The antibiotic compound of any one of claims 3 to 10, or a pharmaceutically acceptable salt thereof, wherein R_7 is isobutyl.

12. The antibiotic compound of any one of claims 3 to 10, or a pharmaceutically acceptable salt thereof, wherein R_7 is sec-butyl.
13. The antibiotic compound of any one of claims 3 to 8, 11 and 12, or a pharmaceutically acceptable salt thereof, wherein R_6 is isobutyl.
14. The antibiotic compound of any one of claims 3 to 13, or a pharmaceutically acceptable salt thereof, wherein:
X1 is Dab;
X2 is Thr;
X3 is Dab; and
x, y and z are each 1.
15. A pharmaceutical composition formulated for parenteral administration, the pharmaceutical composition comprising one or more compounds of any one of claims 1 to 14 and in a pharmaceutically-acceptable solution, dispersion, suspension, emulsion, or sterile powder for reconstitution.
16. A method of treating a Gram-negative infection in a subject, the method comprising administering to said subject one or more compounds of any one of claims 1 to 14, or a composition of claim 15.
17. A method for preparing a compound, the method comprising:
 - (a) treating a polymyxin peptide having a sidechain comprising an amino group with an amino protecting group reagent to form a protected polymyxin peptide,
wherein the polymyxin peptide comprises a cyclic heptapeptide attached to an exocyclic peptide chain comprising an acyl group, and the protecting group comprises at least one acidic substituent;
 - (b) reacting the protected peptide with a deacylase enzyme in an aqueous solution to form a protected, deacylated peptide comprising a free amino terminus on the side chain; and

(c) reacting the free amino terminus on the side chain of the protected, deacylated peptide with an addition reagent and removing the protecting group to form a peptide antibiotic having a structure of formula (B):



wherein:

A is selected from R'-(C=O)-(p-aminophenylacetyl)-, R'-(C=O)-Lys-, R'-(C=O)-Phe-, R'-NH-(C=O)-, R'-NH(C=S)-, R'-SO₂-, R'-(C=NH)-, R'-O-(C=O)-, R'-O-CH₂-(C=O)-, R'-, R'-alkyl, R'-P(O)OH, R'-O-(C=S)-, R'-NH-(C=NH)-, and R'-(C=S)-; and

R' is selected from aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl, wherein each aryl, heteroaryl, alkyl, alkenyl, heterocyclyl and cycloalkyl are each optionally substituted with one or more groups selected from halogen, (C₁-C₂₀) alkyl, aryl, heteroaryl and aryloxy;

or

A is R'-(C=O)-, and R' is selected from unsubstituted alkyl having at least 9 carbon atoms, cycloalkyl, alkenyl, aryl, heteroaryl, heterocyclyl, and substituted alkyl, wherein at least one hydrogen on said substituted alkyl is replaced by a substituent group selected from halogen, aryl, heteroaryl and aryloxy;

X1 is selected from Dab, Phe, Ala, Lys, and Gly;

X2 is Thr;

X3 is Dab;

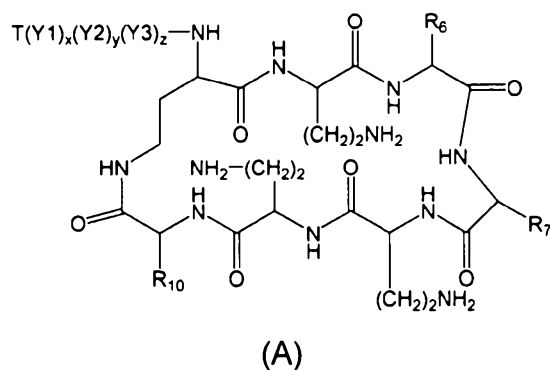
x and y are integers each independently selected from 0 and 1;

z is 1; and

R₆, R₇, and R₁₀ are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, hydroxymethyl and 1-hydroxy-1-ethyl.

provided that when R₆ and R₇ are each isobutyl and R₁₀ is 1-hydroxy-1-ethyl, then x and y are each 1 and X1 is Dab.

18. The method of claim 17, wherein the polymyxin peptide in (a) has a structure of formula (A):



wherein:

Y1 is selected from Dab, Phe, Ala, Lys, and Gly;

Y2 is Thr;

Y3 is Dab;

x, y and z are each 1;

T is R-(C=O)-;

R is alkyl; and

R₆, R₇, and R₁₀ are each independently selected from isopropyl, benzyl, isobutyl, sec-butyl, hydroxymethyl and 1-hydroxy-1-ethyl, provided that when R₆ and R₇ are each isobutyl and R₁₀ is 1-hydroxy-1-ethyl, then x is 1.

19. The method of claim 17 or claim 18, wherein

R₆ is selected from benzyl or isobutyl;

R₇ is selected from isopropyl, isobutyl, sec-butyl, 1-hydroxy-1-ethyl, and hydroxymethyl; and

R₁₀ is selected from 1-hydroxy-1-ethyl and hydroxymethyl.

20. The method of claim 18 or claim 19, wherein Y1 and Y3 are each 2,4-diaminobutanoic acid residue and Y2 is threonine residue.

21. The method of any one of claims 18 to 20, wherein T is R-(C=O)- and R is selected from 6-methyloctanoyl, 6-methylheptanoyl, octanoyl, heptanoyl, nonanoyl, and 3-hydroxy-6-methyloctanoyl.

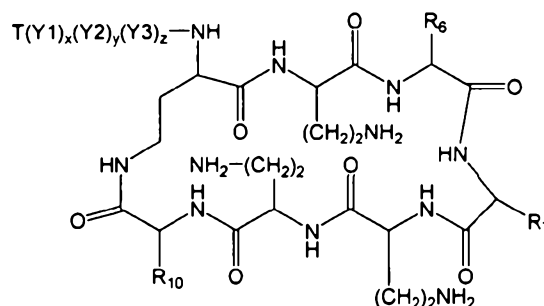
22. The method of any one of claims 17 to 21, wherein the at least one acidic substituent is selected from carboxy, carboxylate, sulfo, sulfate, phosphate, phosphonate, and salts thereof.

23. The method of any one of claims 17 to 22, wherein the protecting group is a sulfonic acid of 9-fluorenylmethoxycarbonyl.

24. The method of any one of claims 17 to 23, wherein a source of the enzyme is *Actinoplanes utahensis*.

25. A method for preparing an antibiotic compound, the method comprising the steps of:

(a) treating a peptide of formula (A)

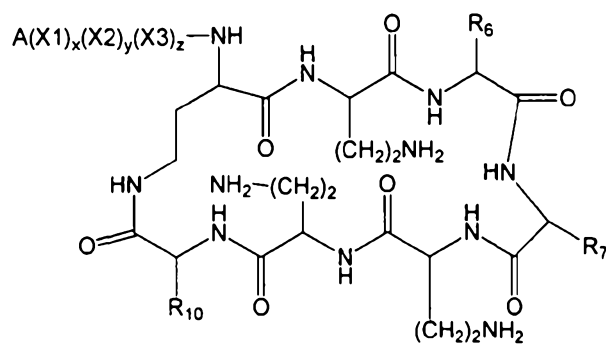


(A)

with 2-sulfo-9-fluorenylmethoxycarbonyl;

(b) treating the protected peptide with a deacylating enzyme in an aqueous system to form a protected, deacylated peptide; and

(c) reacting the protected, deacylated peptide of (b) with an addition reagent and then removing the protecting group to form the peptide antibiotic having a structure of formula (B):



(B)

wherein:

T is R-(C=O)-, where R is alkyl;

A is R'-NH-(C=O)- where R' is aryl;

X1, X3, Y1 and Y3 are each 2,4-diaminobutanoic acid residue;

X2 and Y2 are each threonine residue;

x, y, and z are each 1;

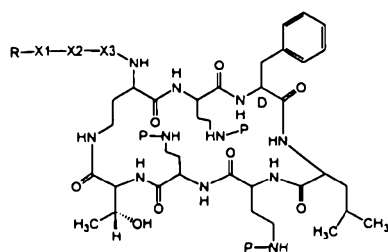
R₆ is benzyl;

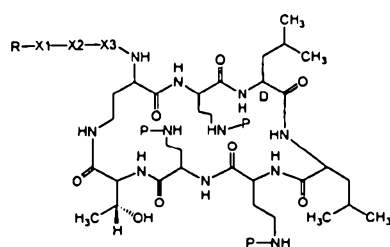
R₇ is selected from isopropyl and sec-butyl; and

R₁₀ is 1-hydroxy-1-ethyl.

26. The method of any one of claims 17 to 25, where the prepared compound is an antibiotic active against gram-negative bacteria.

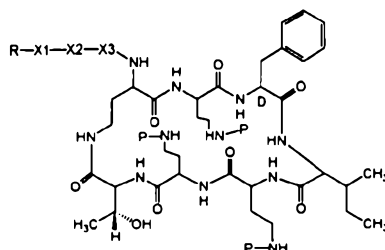
27. The method of any one of claims 17 to 24, where the prepared compound has one of the following structures:





Cpeptide derivatives

(C); or



ILpeptide derivatives

(IL)

for use against gram-negative and gram-positive bacteria, wherein the cyclic polypeptide, R, X1, X2, and X3 are as specified below:

Compound	R	X1*	X2	X3*	Cyclic peptide
1	n-C ₉ H ₁₉ CO-	Dab	Thr	Dab	PB
3	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	PB
4	phenyl-NHCS-	Dab	Thr	Dab	PB
5	phenyl-NHCO-	Dab	Thr	Dab	PB
6	phenyl-CO-	Dab	Thr	Dab	PB
7	2-naphthyl-OCH ₂ -CO-	Dab	Thr	Dab	PB
8	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	PB
16	n-C ₉ H ₁₉ CO-Phe-	Dab	Thr	Dab	PB
19	C ₆ H ₅ CH ₂ OCH ₂ -CO-	Dab	Thr	Dab	PB
20	4-(C ₆ H ₅ O)C ₆ H ₄ NHCO-	Dab	Thr	Dab	PB
21	4-Cl-C ₆ H ₄ -NHCO-	Dab	Thr	Dab	PB
22	benzyl-NHCO-	Dab	Thr	Dab	PB
23	cyclohexyl-NHCO-	Dab	Thr	Dab	PB
48	cyclohexyl-NHCO-	Dab	Thr	Dab	IL
51	n-C ₈ H ₁₇ NHCO-	Dab	Thr	Dab	C
53	4-CH ₃ -C ₆ H ₄ -SO ₂ -	Dab	Thr	Dab	C
54	n-C ₉ H ₁₉ CO-Lys-	Dab	Thr	Dab	PB.

Date: 14 September 2011