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(54) Titre : DEFENSINES THETA ANTIMICROBIENNES ET METHODES D'UTILISATION CORRESPONDANTES
 (54) Title: ANTIMICROBIAL THETA DEFENSINS AND METHODS OF USING SAME

(57) **Abrégé/Abstract:**

The present invention relates to an isolated cyclic peptide, theta defensin, having antimicrobial activity, and to theta defensin analogs. A theta defensin can have the amino acid sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8, wherein Xaa1 to Xaa8 are defined; wherein Xaa1 can be linked through a peptide bond to Xaa8; and wherein crosslinks can be formed between Xaa3 and Xaa3, between Xaa5 and Xaa5, and between Xaa7 and Xaa7. For example, the invention provides a theta defensin having the amino acid sequence Gly-Phe-Cys-Arg-Cys-Leu- Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1), wherein the Gly at position 1 (Gly-1) is linked through a peptide bond to Arg-18, and wherein disulfide bonds are present between Cys-3 and Cys-16, between Cys-5 and Cys-14, and between Cys-7 and Cys-12. The invention also provides nucleic acids encoding theta defensins and antibodies that specifically bind a theta defensin. In addition, the invention relates to methods of using theta defensin to reduce or inhibit microbial growth or survival.





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(57) Abstract		
<p>The present invention relates to an isolated cyclic peptide, theta defensin, having antimicrobial activity, and to theta defensin analogs. A theta defensin can have the amino acid sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8, wherein Xaa1 to Xaa8 are defined; wherein Xaa1 can be linked through a peptide bond to Xaa8; and wherein crosslinks can be formed between Xaa3 and Xaa3, between Xaa5 and Xaa5, and between Xaa7 and Xaa7. For example, the invention provides a theta defensin having the amino acid sequence Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1), wherein the Gly at position 1 (Gly-1) is linked through a peptide bond to Arg-18, and wherein disulfide bonds are present between Cys-3 and Cys-16, between Cys-5 and Cys-14, and between Cys-7 and Cys-12. The invention also provides nucleic acids encoding theta defensins and antibodies that specifically bind a theta defensin. In addition, the invention relates to methods of using theta defensin to reduce or inhibit microbial growth or survival.</p>		

ANTIMICROBIAL THETA DEFENSINS AND METHODS OF USING SAME**BACKGROUND OF THE INVENTION****FIELD OF THE INVENTION**

The present invention relates generally to antimicrobial agents and, more specifically, to cyclic
10 theta defensin peptides and methods of using a
theta defensin to reduce or inhibit microbial growth or
survival.

BACKGROUND INFORMATION

Infections by microorganisms, including
15 bacteria, viruses and fungi, are a major cause of human
morbidity and mortality. Although anyone can be a victim
of such infection, the sick and elderly are particularly
susceptible. For example, hospitalized patients
frequently acquire secondary infections due to a
20 combination of their weakened condition and the
prevalence of microorganisms in a hospital setting. Such
opportunistic infections result in increased suffering of
the patient, increased length of hospitalization and,
consequently, increased costs to the patient and the
25 health care system. Similarly, the elderly, particularly
those living in nursing homes or retirement communities,
are susceptible to infections because of their close
living arrangement and the impaired responsiveness of
their immune systems.

Numerous drugs are available for treating infections by certain microorganisms. In particular, various bacterial infections have been amenable to treatment by antibiotics. However, the prolonged use of antibiotics since their discovery has resulted in the selection of bacteria that are relatively resistant to these drugs. Furthermore, few if any drugs are effective against microorganisms such as viruses. As a result, continuing efforts are being made to identify new and effective agents for treating infections by a variety of microorganisms.

The identification of naturally occurring compounds that act as antimicrobial agents has provided novel and effective drugs. Many organisms protect themselves by producing natural products that are toxic to other organisms. Frogs, for example, produce a class of peptides, magainins, which provide a defense mechanism for the frog against potential predators. Magainins have been purified and shown to have antimicrobial activity, thus providing a natural product useful for reducing or inhibiting microbial infections.

Natural products useful as antimicrobial agents also have been purified from mammalian organisms, including humans. For example, the defensins are a class of peptides that have been purified from mammalian neutrophils and demonstrated to have antimicrobial activity. Similarly, indolicidin is a peptide that has been isolated from bovine neutrophils and has antimicrobial activity, including activity against viruses, bacteria, fungi and protozoan parasites. Thus, naturally occurring compounds provide a source of drugs that are potentially useful for treating microbial infections.

Upon identifying naturally occurring peptides useful as antimicrobial agents, efforts began to chemically modify the peptides to obtain analogs having improved properties. Such efforts have resulted, for example, in the identification of indolicidin analogs which, when administered to an individual, have increased selectivity against the infecting microorganisms as compared to the individual's own cells. Thus, the availability of naturally occurring antimicrobial agents has provided new drugs for treating microbial infections and has provided a starting material to identify analogs of the naturally occurring molecule that have desirable characteristics.

Although such natural products and their analogs have provided new agents for treating microbial infections, it is well known that microorganisms can become resistant to drugs. Thus, a need exists to identify agents that effectively reduce or inhibit the growth or survival of microorganisms. The present invention satisfies this need and provides additional advantages.

SUMMARY OF THE INVENTION

The present invention relates to an isolated cyclic theta defensin peptide, which exhibits broad spectrum antimicrobial activity, and to theta defensin analogs. In general, a theta defensin or theta defensin analog has the amino acid sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8, wherein Xaa1 independently is Gly, Ile, Leu, Val or Ala; Xaa2 is Phe, Trp or Tyr; Xaa3 is Cys or Trp; Xaa4 independently is Arg or Lys; Xaa5 is Cys or Trp; Xaa6 is Cys or Trp; Xaa7 is Thr or Ser; and Xaa8 is

Arg or Lys. Xaa1 can be linked through a peptide bond to Xaa8. Furthermore, crosslinks can be formed between Xaa3 and Xaa3, between Xaa5 and Xaa5, and between Xaa7 and Xaa7. For example, the invention provides theta defensin
5 having the amino acid sequence
Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1), wherein the Gly at position 1 (Gly-1) is linked through a peptide bond to Arg-18, and wherein disulfide bonds are present between
10 Cys-3 and Cys-16, Cys-5 and Cys-14, and Cys-7 and Cys-12.

The invention also relates to methods of using a theta defensin or an analog thereof to reduce or inhibit microbial growth or survival in an environment capable of sustaining microbial growth or survival by
15 contacting the environment with theta defensin. As such, the invention provides methods of reducing or inhibiting microbial growth or survival on a solid surface, for example, surgical instruments, hospital surfaces, and the like.

20 The invention further relates to methods for reducing or inhibiting microbial growth or survival in an individual, particularly a mammal such as a human. Thus, the invention provides methods of treating an individual suffering from a pathology characterized, at least in
25 part, by microbial infection, by administering theta defensin or an analog thereof to the individual, thereby reducing the severity of the pathologic condition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows purification of RTD-1. Panel A shows reverse phase HPLC (RP-HPLC) of peripheral blood leukocyte extracts. An α -defensin-enriched extract of 5 6×10^6 leukocytes (91 % PMNs) was fractionated by RP-HPLC on a 0.46 x 25 cm C-18 column equilibrated in 0.1% aqueous TFA and developed with a linear acetonitrile gradient (dotted line). RTD-1 eluted in the peak marked with an arrow. Panel B shows analytical RP-HPLC of 10 purified RTD-1. The purity of RTD-1 was assessed by RP-HPLC of RTD-1 obtained from the peak marked by an arrow in panel A on an analytical C-18 column developed with acetonitrile at 0.5% per min.

Figure 2 shows the peptide backbone structure 15 of RTD-1. Panel A shows the amino acid sequence of the peptide chain, determined by Edman sequencing. The corresponding MALDI-TOF MS analysis of purified proteolytic fragments is also shown. Residues in parentheses were assigned based on MALDI-TOF MS data. 20 Calculated MALDI-TOF MS values are in parentheses. The peptides shown in Panel A (top to bottom) correspond to SEQ ID NOS:2-9, respectively. Panel B shows a schematic of RTD-1 (SEQ ID NO:1) cyclized peptide backbone.

Figure 3 shows disulfide analysis of RTD-1. A 25 tridisulfide-containing 17-residue oligopeptide generated by trypsin digestion was purified by RP-HPLC and further digested with thermolysin. MS analysis (calculated values in parentheses) of the digest or of HPLC-purified fragments disclosed thermolytic cleavage at Cys-14/Ile-15 30 and at Cys-5/Leu-6 (arrows), producing four major thermolytic fragments (Th-1 to Th-4). The masses of all

fragments were consistent with the disulfide assignments shown.

Figure 4 shows the structure of RTD-1. Panel A shows a schematic of the covalent structure of RTD-1 compared with that of circulin A (SEQ ID NO:10), an antiviral peptide isolated from the plant *Chassalia parvifolia*. Panel B shows a theoretical model of RTD-1 obtained by molecular dynamics and energy minimization in water. The model shows a high degree of structural similarity to porcine protegrin 1 (PG-1 SEQ ID NO:11) for those residues defined in the PG-1 solution structure. Panel C shows the alignment of the PG-1 and RTD-1 sequences and disulfide motifs.

Figure 5 shows the coordinates used to generate the molecular model shown in Figure 4.

Figure 6 shows synthesis and characterization of RTD-1. Panel A shows the scheme for solid phase peptide synthesis and cyclization entailed chain assembly, cleavage/deprotection, purification of the reduced linear chain, oxidation and cyclization. Panel B shows co-elution of synthetic and natural RTD-1 on RP-HPLC. Panel C shows circular dichroic spectra of synthetic and natural RTD-1 determined in water, 10 mM sodium phosphate buffer, and methanol at a peptide concentration of 111 µg/ml (53.3 µM).

Figure 7 shows the zone of inhibition (mm inhibition) of growth of *Staphylococcus aureus* 502A (closed circles), *Escherichia coli* ML35 (open circles), *Listeria monocytogenes* EGD (open triangles), and *Cryptococcus neoformans* 271A (closed triangles) at various concentrations of theta defensin.

Figure 8 shows a comparison of staphylocidal activity of natural and synthetic RTD-1. *S. aureus* 502a was incubated with increasing concentrations of natural or synthetic theta defensin peptide. Killing was
5 quantified by colony counts.

Figure 9 shows microbicidal activity of RTD-1. Panel A shows incubation of *S. aureus* 502a with increasing concentrations of natural or synthetic peptide. Killing was quantified by colony counts.
10 Panels B-D show incubation of the indicated organisms with RTD-1 peptide: *Listeria monocytogenes* and *Staphylococcus aureus* (Panel B); *Salmonella typhimurium* and *Escherichia coli* (Panel C); and *Cryptococcus neoformans* and *Candida albicans* (Panel D). The limit of
15 detection (1 colony per plate) was equal to 1×10^3 colony forming units in the incubation mixture. Panel E shows killing of *S. aureus* 502a with natural or synthetic RTD-1 supplemented with increasing concentrations of NaCl.

Figure 10 shows microbicidal activity of
20 acyclic RTD-1. *S. aureus* 502a was incubated with increasing concentrations of acyclic RTD-1 with (solid circles) or without (open circles) 130 mM NaCl. Killing activity was quantified by colony counts after 18 hrs.

Figure 11 shows RTD1a and RTD1b cDNAs. Figure
25 11A shows full length cDNA sequence of RTD1a (SEQ ID NO:13) with the deduced amino acid sequence (SEQ ID NO:14). Figure 11B shows full length cDNA sequence of RTD1b (SEQ ID NO:15) with the deduced amino acid sequence (SEQ ID NO:16). Underlined amino acids are found in
30 RTD-1, and superscript numbers correspond to the residue numbering of RTD-1 shown in Figure 2. The underlined sequences in Figure 11A correspond to nucleotides 287 to

313 (SEQ ID NO:17) and amino acids 65 to 73 (SEQ ID NO:18) of RTD1a. The underlined sequences in Figure 11B correspond to nucleotides 282 to 308 (SEQ ID NO:19) and amino acids 65 to 73 (SEQ ID NO:20) of RTD1b. ATG of the initiation methionines are in bold, as are the polyadenylation sites at the 3' ends of the sequences.

Figure 12 shows the amino acid sequences of RTD1a, RTD1b, and human neutrophil defensin HNP-4. Panel A shows covalent structures of mature RTD-1 (SEQ ID NO:1) and HNP-4 (SEQ ID NO:12). Panel B shows amino acid sequences of precursors of RTD1a (SEQ ID NO:21), RTD1b (SEQ ID NO:22) and HNP-4 (SEQ ID NO:23). Identical amino acids are indicated with a period. In-frame stops in the coding sequence are indicated as "^". Hyphens are inserted to maximize sequence alignments. Shading is used to demarcate signal, pro-segment, mature peptide and untranslated regions.

Figure 13 shows genomic sequences of RTD1.1 (RTD1a) (Figure 14A; SEQ ID NO:24) and RTD1.2 (RTD1b) (Figure 13B; SEQ ID NO:25). Exon sequences are in uppercase, intron sequences in lower case.

Figure 14 shows the DNA probes used for specific hybridization of RTD1a (Panel A; SEQ ID NO:26); complementary sequence, SEQ ID NO:30 and RTD1b (Panel B; SEQ ID NO:27; complementary sequence, SEQ ID NO:31).

Figure 15 shows human theta defensin cDNA. The nucleotide sequence (SEQ ID NO:28) and deduced amino acid sequence (SEQ ID NO:29) are shown.

Figure 16 shows the sequence and disulfide bonding pattern of RTD-1 (SEQ ID NO:1), RTD-2 (SEQ ID NO:32) and RTD-3 (SEQ ID NO:33).

DETAILED DESCRIPTION OF THE INVENTION

5 The invention provides theta defensin peptides, or a functional fragment thereof, having antimicrobial activity. The theta defensin peptides of the invention include theta defensin and theta defensin analogs, having the amino acid sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-
10 Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa5, wherein Xaa1 independently is an aliphatic amino acid; Xaa2 is an aromatic amino acid; Xaa3 is Cys or Trp; Xaa4 independently is Arg or Lys; Xaa5 is Cys or Trp; Xaa6 is Cys or Trp; Xaa7 is Thr or Ser; and Xaa8 is
15 Arg or Lys. For example, Xaa1 can be an aliphatic amino acid such as Gly, Ile, Leu, Val or Ala and Xaa2 can be an aromatic amino acid such as Phe, Trp or Tyr. In general, a theta defensin is a cyclic peptide, wherein Xaa1 is linked through a peptide bond to Xaa8, and contains three
20 intrachain crosslinks, which are formed between Xaa3 and Xaa3, between Xaa5 and Xaa5, and between Xaa7 and Xaa7. However, as disclosed herein, the invention also encompasses linear theta defensin precursors as well as peptide portions of a theta defensin.

25 As used herein, the term "independently," when used in reference to the selection of an amino acid at a position in the generic structure of a theta defensin, means that the selection of one amino acid at a position, for example, Xaa1 at position 1 of the theta defensin
30 sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa5, has no influence on the selection, for example, of Xaa1 at

position 6 or 10 or the like. For example, Xaa1 in can be Gly at position 1 and can be Leu at position 6.

A composition of the invention is exemplified by an isolated cyclic theta defensin, which lacks free amino and carboxyl termini and, therefore, is resistant to exopeptidases and is thus relatively stable to proteolytic degradation. The theta defensins of the invention exhibit broad spectrum antimicrobial activity. The exemplified theta defensin is an 18 amino acid cyclic peptide having the amino acid sequence Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1), wherein the Gly at position 1 (Gly-1) is linked through a peptide bond to Arg-18, and wherein three intrachain crosslinks are present due to disulfide bonds between Cys-3 and Cys-16, between Cys-5 and Cys-14, and between Cys-7 and Cys-12.

As used herein, the term "isolated," when used in reference to theta defensin, means that the peptide is relatively free of proteins, lipids, nucleic acids or other molecules it normally is associated with in a cell. In general, an isolated theta defensin peptide constitutes at least about 75% by weight of a sample containing the theta defensin, and usually constitutes about 90% of a sample, particularly about 95% of the sample or 99% or more. An isolated theta defensin can be obtained by isolation from a cell expressing the theta defensin (see Example I), can be chemically synthesized (see Example II), or can be expressed from a recombinant nucleic acid molecule (see Example V). Following chemical synthesis or recombinant expression, the theta defensin precursor peptide generally is linear and, therefore, can be further subjected to appropriate

conditions for cyclizing the peptide and forming the intrachain crosslinks (see Example II).

The theta defensin peptide shown as SEQ ID NO:1 constitutes the first member of a new class of defensins and is the basis for constructing theta defensin analogs as disclosed herein. Previously described defensins are cationic, arginine-rich peptides having 29 to 42 amino acids and containing three disulfide bonds (see Lehrer et al., Cell 64:229-230 (1991); Lehrer and Ganz, Current Opin. Immunol. 11:23-27 (1999)). The β defensins, for example, contain 38 to 42 amino acids and have a net charge of +4 to +10 (see U.S. Patent No. 5,459,235, issued October 17, 1995).

The disulfide bonds in β defensins are formed in a characteristic pattern between the first and fifth Cys residues, the second and fourth Cys residues, and the third and sixth Cys residues. In addition, some β defensins contain a pyroglutamate residue at the amino terminus (U.S. Patent No. 5,459,235, *supra*, 1995).

Defensins and defensin-like peptides are endogenously expressed in various organisms. In mammals, defensins generally are expressed in neutrophils, macrophages and intestinal cells (see Lehrer et al., *supra*, 1991; Lehrer and Ganz, *supra*, 1999). Defensins can exhibit potent antimicrobial activity against a broad spectrum of microorganisms, including gram negative and gram positive bacteria, fungi, protozoans such as *Acanthamoeba* and *Giardia*, enveloped viruses such as herpes simplex viruses and human immunodeficiency viruses, and helminths. Defensins also have other properties, including chemotactic activity for human monocytes and the ability to interfere with

adrenocorticotropin binding to its receptor (see Lehrer et al., *supra*, 1991).

A new class of defensins, termed theta defensins, is disclosed herein. Theta defensins have been classified as members of the defensin family of peptides based on their cationicity, arginine-rich composition and the presence of three intrapeptide disulfide bonds, as well as their broad spectrum antimicrobial activity. However, theta defensins are distinguishable from previously described defensins in that theta defensins are cyclic peptides, which lack a free amino or carboxyl terminus, and are shorter than previously described defensins.

The theta defensins are exemplified by the peptide shown as SEQ ID NO:1, which contains 18 amino acids, wherein the amino terminus of the first amino acid (Gly) is linked to the carboxyl terminus of the last amino acid (Arg) through a peptide bond, and wherein disulfide bonds are formed between Cys-3 and Cys-16, Cys-5 and Cys-14, and Cys-7 and Cys-12. For convenience of discussion, reference to an amino acid position in a theta defensin, or an analog thereof, is made with respect to the amino acid position in the linear form of theta defensin shown as SEQ ID NO:1 or of the theta defensin sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa5. As such, the amino acids are referred to as positions 1 through 18, starting with the Gly residue in (position 1; SEQ ID NO:1) and ending with Arg (position 18).

A theta defensin having the amino acid sequence of SEQ ID NO:1 can be obtained by purification of the native peptide from a natural source (see Example I). A

theta defensin having the amino acid sequence of
SEQ ID NO:1, or of the theta defensin sequence Xaa1-Xaa2-
Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-
Xaa5-Xaa1-Xaa3-Xaa7-Xaa5, also can be chemically
5 synthesized using routine methods of solid phase
synthesis (see Example II) or can be expressed from a
recombinant nucleic acid molecule encoding the
theta defensin (see Example V).

The invention additionally provides a theta
10 defensin comprising the amino acid sequence Arg-Cys-Ile-
Cys-Thr-Arg-Gly-Phe-Cys (SEQ ID NO:18) or Arg-Cys-Leu-
Cys-Arg-Arg-Gly-Val-Cys (SEQ ID NO:20). Further provided
is a theta defensin having the amino acid sequence Gly-
Phe-Cys-Arg-Cys-Ile-Cys-Thr-Arg-Gly-Phe-Cys-Arg-Cys-Ile-
15 Cys-Thr-Arg (SEQ ID NO:32). The invention also provides
a theta defensin having the amino acid sequence Gly-Val-
Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Leu-Cys-
Arg-Arg (SEQ ID NO:33).

As disclosed herein, the RTD1a and RTD1b
20 peptides can form homodimers (see Example VI). The
homodimers can be linked by a peptide bond and contain
intrachain disulfide crosslinks (see Example VI and
Figure 16).

In general, a precursor theta defensin is
25 obtained following chemical synthesis of the peptide,
since the newly synthesized peptide is not cyclized and
does not contain the appropriate intrachain crosslinking.
Similarly, expression of a recombinant nucleic acid
molecule encoding a theta defensin generally results in
30 the production of a precursor theta defensin peptide,
unless the peptide is expressed in a cell that can effect
formation of the appropriate bonds. Accordingly, the

term "precursor," when used in reference to a theta defensin peptide, means a form of the peptide that lacks a peptide bond between the amino terminal and carboxyl terminal amino acids or lacks at least one of the three disulfide bonds characteristic of a theta defensin. Such precursor peptides can be converted into a mature cyclic theta defensin containing, for example, one, two or three disulfide bonds by exposing the precursor peptide to the appropriate conditions for effecting formation of the intrapeptide crosslinks, for example, the conditions disclosed in Example II. However, as disclosed herein, precursor theta defensins also are contemplated within the present invention.

A theta defensin or theta defensin analog can be prepared by solid phase methods (Example II). Theta defensin analogs, which are encompassed within SEQ ID NO:5, are synthesized based on SEQ ID NO:1, but substituting one or more amino acids of SEQ ID NO:1 as desired, particularly by incorporating conservative amino acid substitutions. Such conservative amino acid substitutions are well known and include, for example, the substitution of an amino acid having a small hydrophobic side chain with another such amino acid (for example, Ala for Gly) or the substitution of one basic residue with another basic residue (for example, Lys for Arg). Similar conservative amino acid substitutions in other antimicrobial peptides such as indolicidin resulted in the production of indolicidin analogs that maintained their broad spectrum antimicrobial activity (see U.S. Patent No. 5,547,939, issued August 20, 1996).

Thus, a theta defensin analog having, for example, a substitution of Leu-6 with a Val, Ile or Ala residue, or a substitution of Arg-8 or Arg-9 or Arg-13 or Arg-18 with a

Lys residue similarly can be expected to maintain broad spectrum antimicrobial activity.

A theta defensin analog also can have substitutions of the cysteine residues involved in a disulfide bond, with amino acids that can form an intrachain crosslink, for example, with tryptophan residues, which can form a di-tryptophan crosslink. Similarly to naturally occurring indolicidin, which is a linear antimicrobial peptide, indolicidin analogs having an intrachain di-tryptophan crosslink also have antimicrobial activity. Furthermore, substitution of the Trp residues involved in the di-tryptophan crosslink in an indolicidin analog with Cys residues results in an indolicidin analog that has an intrachain disulfide crosslink and exhibits broad spectrum antimicrobial activity. By analogy to such indolicidin analogs, a theta defensin analog can contain, in place of one or more of the characteristic disulfide bonds, one or more corresponding di-tryptophan, lactam or lanthionine crosslinks. For example, a crosslink in a theta defensin analog can be formed, for example, between two Trp residues, which form a di-tryptophan crosslink. In addition, a crosslink can be a monosulfide bond formed by a lanthionine residue. A crosslink also can be formed between other amino acid side chains, for example, a lactam crosslink formed by a transamidation reaction between the side chains of an acidic amino acid and a basic amino acid, such as between the γ -carboxyl group of Glu (or β -carboxyl group of Asp) and the ϵ -amino group of Lys; or can be a lactone produced, for example, by a crosslink between the hydroxy group of Ser and the γ -carboxyl group of Glu (or β -carboxyl group of Asp); or a covalent bond formed, for example, between two amino acids, one or both of which have a modified side chain.

The invention additionally provides a theta defensin peptide, or a functional fragment thereof, having the amino acid sequence Xaa1-Xaa2-Xaa9-Xaa4-Xaa10-Xaa1-Xaa11-Xaa4-Xaa4-Xaa1-Xaa1-Xaa12-Xaa4-Xaa13-Xaa1-Xaa14-Xaa7-Xaa8, wherein Xaa1 independently is an aliphatic amino acid such as Gly, Ile, Leu, Val or Ala; Xaa2 is an aromatic amino acid such as Phe, Trp or Tyr; Xaa4 independently is Arg or Lys; Xaa7 is Thr or Ser; Xaa8 is Arg or Lys; Xaa9 is Glu, Asp, Lys or Ser; Xaa10 is Glu, Asp, Lys or Ser; Xaa11 is Glu, Asp, Lys or Ser; Xaa12 is Glu, Asp, Lys or Ser; Xaa13 is Glu, Asp, Lys or Ser; Xaa14 is Glu, Asp, Lys or Ser. In such a theta defensin peptide, an intrachain crosslink can be formed between two amino acids, Xaa9 and Xaa14; Xaa10 and Xaa13; or Xaa11 and Xaa12, which correspond to the same position as disulfide crosslinks in natural theta defensin. The intrachain crosslink can be, for example, a lactam or lactone.

In theta defensin peptides having less than three crosslinks, as found in native theta defensin, the amino acids at the positions corresponding to the native crosslinks, amino acids Xaa3, Xaa5 and Xaa6 in SEQ ID NO:1, can be modified. For example if positions Xaa3 are disulfide crosslinked, the amino acids at position Xaa5 and Xaa6 can be non cysteine residues, for example, a hydrophobic amino acid such as Tyr, Val, Ile, Leu, Met, Phe or Trp; a small amino acid such as Gly, Ser, Ala, or Thr; or a large polar amino acid such as Asn or Gln.

If desired, a theta defensin analog of the invention can have one or more amino acid deletions or additions as compared to SEQ ID NO:1, again, by analogy to indolicidin analogs, which can have a carboxyl terminal amino acid deletion or as many as five amino

terminal amino acid deletions, yet still maintain broad spectrum antimicrobial activity. Thus, it can be expected that theta defensin analogs having one or a few deletions or additions at selected positions in the theta defensin sequence also will maintain broad spectrum antimicrobial activity and, as such, are considered functional fragments of a theta defensin. As used herein, a "functional fragment" when used in reference to a theta defensin is a portion of a theta defensin that still retains some or all of the antimicrobial activity of a theta defensin. The antimicrobial activity of a theta defensin analog, or a functional fragment thereof, containing one or more amino acid substitutions, deletions or additions as compared to SEQ ID NO:1 can be confirmed using assays as disclosed herein (Example III) or otherwise known in the art.

As used herein, the term "amino acid" is used in its broadest sense to mean the naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs. Thus, reference herein to an amino acid includes, for example, naturally occurring proteogenic (L)-amino acids, as well as (D)-amino acids, chemically modified amino acids such as amino acid analogs, naturally occurring non-proteogenic amino acids such as norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid. As used herein, the term "proteogenic" indicates that the amino acid can be incorporated into a protein in a cell through a metabolic pathway.

Theta defensin having the amino acid sequence of SEQ ID NO:1 was chemically synthesized as a linear precursor peptide using solid phase Fmoc chemistry (see

Example II). The linear peptide was subjected to reducing conditions, then oxidized to allow formation of the disulfide bonds, and treated with ethylenediaminecarbodiimide to cyclize the peptide. The synthesized cyclic theta defensin was characterized by reverse phase-high performance liquid chromatography (RP-HPLC), MALDI-TOF mass spectrometry and circular dichroism (CD) and comigrated with native theta defensin by acid-urea PAGE (Example II). The synthetic cyclic theta defensin also demonstrated broad spectrum antimicrobial activity (see Example III).

The invention additionally provides a method of preparing theta defensin. The method of synthesis includes the steps of synthesizing a linear peptide of an amino acid sequence corresponding to the amino acid sequence of theta defensin, forming one or more crosslink bonds within the linear peptide, and cyclizing the peptide by linking the carboxyl and amino termini to form a cyclic peptide. The crosslink formed can be a disulfide, lanthionine, lactam or lactone. The cysteine residues used in the linear peptide can be in a pre-formed activated ester form. If a disulfide crosslink is formed between two cysteines, the crosslink can be formed by oxidation. The formation of a peptide bond between the amino and carboxyl termini can be advantageously mediated by placing the carboxyl terminus and amino terminus of the linear peptide each approximately the same number of amino acids from the nearest cysteine.

The cyclization step can be performed with ethylenediaminecarbodiimide and N-hydroxybenzotriazole, for example, 60 equivalents and 20 equivalents,

respectively, in a solvent. The synthesis can be performed in dimethylsulfoxide as the solvent.

Cyclized versions of the theta defensin peptides of the invention are resistant to exo-peptidases such as aminopeptidases and carboxypeptidases because there is no amino or carboxyl terminus to serve as a substrate for the exo-peptidases. The invention further provides a method of enhancing protease resistance of a peptide by synthesizing a peptide, wherein the amino-terminal amino acid and carboxyl-terminal amino acid of the peptide are positioned by intrachain crosslinks and whereby a peptide bond is formed between the amino-terminal and carboxyl-terminal amino acids.

An advantage of using chemical synthesis to prepare a theta defensin is that (D)-amino acids can be substituted for (L)-amino acids, if desired. The incorporation of one or more (D)-amino acids into a theta defensin analog can confer, for example, additional stability of the peptide *in vitro* or, particularly, *in vivo*, since endogenous endoproteases generally are ineffective against peptides containing (D)-amino acids. Naturally occurring antimicrobial peptides that have been chemically synthesized to contain (D)-amino acids maintain their antimicrobial activity (Wade et al., Proc. Natl. Acad. Sci. USA 87:4761-4765 (1990)).

If desired, the reactive side group of one or more amino acids in a theta defensin can be modified or amino acid derivatives can be incorporated into the peptide (see, for example, Protein Engineering: A practical approach (IRL Press 1992); Bodanszky, Principles of Peptide Synthesis (Springer-Verlag 1984)).

Selective modification of a reactive group, other than those involved in formation of the three intrachain crosslinks characteristic of a defensin, can impart desirable characteristics upon a theta defensin analog, although modifications that allow the formation of intrachain crosslinks at the appropriate positions also can be effected. The choice of including such a modification is determined, in part, by the characteristics required of the peptide. Such modifications can result, for example, in theta defensin analogs having greater antimicrobial selectivity or potency than naturally occurring theta defensin (SEQ ID NO:1).

The theta defensins of the invention are polypeptides having antimicrobial activity. As used herein, the term "polypeptide" when used in reference to a theta defensin is intended to refer to a peptide or polypeptide of two or more amino acids. The term is similarly intended to refer to derivatives, analogues and functional mimetics thereof. For example, derivatives can include chemical modifications of the polypeptide such as alkylation, acylation, carbamylation, iodination, or any modification which derivatizes the polypeptide. Analogues can include modified amino acids, for example, hydroxyproline or carboxyglutamate, and can include amino acids that are not linked by peptide bonds. Mimetics encompass chemicals containing chemical moieties that mimic the function of the polypeptide. For example, if a polypeptide contains two charged chemical moieties having functional activity, a mimetic places two charged chemical moieties in a spatial orientation and constrained structure so that the charged chemical function is maintained in three-dimensional space. Thus,

a mimetic, which orients functional groups that provide the antimicrobial function of a theta defensin, are included within the meaning of a theta defensin derivative. All of these modifications are included within the term "polypeptide" so long as the polypeptide retains its antimicrobial function.

A theta defensin can incorporate polypeptide derivatives. Peptide derivatives are well known in the art (see, for example, U.S. patent 5,804,558, issued September 8, 1998). For example, certain commonly encountered amino acids, which are not encoded by the genetic code, include, for example, beta-alanine (beta-Ala), or other omega-amino acids, such as 3-aminopropionic, 2,3-diaminopropionic (2,3-diaP), 4-aminobutyric and so forth, alpha-aminisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine (2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β -2-thienylalanine (Thi); methionine sulfoxide (MSO); and homoarginine (Har).

In peptides of the invention, one or more amide linkages (--CO--NH--) can be replaced with another linkage which is an isostere such as --CH₂NH--, --CH₂S--, --CH₂CH₂-, --CH=CH-- (cis and trans), --COCH₂--, --CH(OH)CH₂-- and --CH₂SO--. This replacement can be made by methods known in the art (see, for example, Spatola, Vega Data Vol. 1, Issue 3, (1983); Spatola, in Chemistry and Biochemistry of Amino Acids Peptides and Proteins, Weinstein, ed., Marcel Dekker, New York, p. 267 (1983); Morley, J. S., Trends Pharm. Sci. pp. 463-468 (1980); Hudson et al., Int. J. Pept. Prot. Res. 14:177-185

(1979); Spatola et al., Life Sci. 38:1243-1249 (1986);
Hann, J. Chem. Soc. Perkin Trans. I 307-314 (1982);
Almquist et al., J. Med. Chem. 23:1392-1398 (1980);
Jennings-White et al., Tetrahedron Lett. 23:2533 (1982);
5 Szelke et al., EP 45665 (1982); Holladay et al.,
Tetrahedron Lett. 24:4401-4404 (1983); and Hruby, Life
Sci. 31:189-199 (1982)).

In addition to polypeptide derivatives of a
theta defensin, the invention additionally provides a
10 chemical mimetic of a theta defensin peptide. As
described above, mimetics contain chemical functional
groups that mimic the function of a theta defensin. Such
a mimetic chemical can orient functional groups on a
theta defensin peptide sufficient for antimicrobial
15 activity. A mimetic places the functional chemical
moieties in a spatial orientation and constrained
structure so that the chemical function is maintained in
three-dimensional space. Thus, a mimetic orients
chemical functional groups that provide the theta
20 defensin function of antimicrobial activity in an
orientation that mimics the structure of a theta
defensin.

As disclosed herein, a molecular model of a
theta defensin has been determined (Example III). Using
25 the molecular model of theta defensin, one skilled in the
art can identify a chemical such as a peptidomimetic. As
used herein, the term "peptidomimetic" is used broadly to
mean a peptide-like molecule that has a similar structure
and activity as a theta defensin. With respect to the
30 theta defensin peptides of the invention,
peptidomimetics, which include chemically modified
peptides, peptide-like molecules containing non-naturally
occurring amino acids, peptoids and the like, have the

antimicrobial activity upon which the peptidomimetic is derived (see, for example, "Burger's Medicinal Chemistry and Drug Discovery" 5th ed., vols. 1 to 3 (ed. M.E. Wolff; Wiley Interscience 1995)). Peptidomimetics
5 provide various advantages over a peptide, including that a peptidomimetic can be more stable during passage through the digestive tract and, therefore, useful for oral administration.

Methods for identifying a peptidomimetic are
10 well known in the art and include, for example, the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures
15 (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). This structural depository is continually updated as new crystal structures are determined and can be screened for compounds having suitable shapes, for example, the same shape as a theta defensin peptide.
20 Another database, the Available Chemicals Directory (Molecular Design Limited, Information Systems; San Leandro CA), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of a theta defensin
25 peptide.

As used herein, the term "antimicrobial selectivity" refers to the relative amount of antimicrobial activity of theta defensin, or a theta defensin analog, against a microorganism as
30 compared to its activity against the environment to which it is administered, particularly its activity against normal cells in a treated individual. For example, a theta defensin analog that is characterized by having

antimicrobial activity that is equivalent to native theta defensin, but having decreased hemolytic activity as compared to native theta defensin, is considered to have greater antimicrobial selectivity than native
5 theta defensin.

As disclosed herein, theta defensin (SEQ ID NO:1) and analogs thereof have broad spectrum antimicrobial activity (see Example III). As used herein, the term "broad spectrum," when used in reference
10 to the antimicrobial activity of theta defensin or an analog thereof, refers to the ability of the peptide to reduce or inhibit the survival or proliferative ability of various viruses, prokaryotic and eukaryotic microorganisms. For example, theta defensin
15 (SEQ ID NO:1) and analogs thereof can exhibit antimicrobial activity against protozoans such as *Giardia lamblia*, *Chlamydia sp.* and *Acanthamoeba sp.*; viruses, particularly enveloped viruses such as herpes simplex virus and HIV-1; fungi such as *Cryptococcus* and *Candida*;
20 various genera of gram negative and gram positive bacteria, including *Escherichia*, *Salmonella* and *Staphylococcus* and *Listeria*; and parasitic helminths such as liver flukes. Antimicrobial activity can occur through "microbicidal inhibition," which refers to the
25 ability of a theta defensin to reduce or inhibit the survival of a microorganism by killing or irreversibly damaging it, or through "microbistatic inhibition," which refers to the ability of the theta defensin to reduce or inhibit the growth or proliferative ability of a target
30 microorganism without necessarily killing it. The invention theta defensins are also active in the presence of physiological salt and serum (Example III).

A precursor theta defensin or theta defensin analog can be expressed from a recombinant nucleic acid molecule encoding the peptide. Thus, the invention also provides isolated nucleic acid molecules encoding a
5 theta defensin or theta defensin analog having the sequence Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa5. For example, the invention provides an isolated nucleic acid molecule encoding the linear form of SEQ ID NO:1, which
10 is a precursor of the cyclic theta defensin peptide.

As used herein, the term "isolated," when used in reference to a nucleic acid molecule, means the nucleic acid molecule is relatively free of proteins, lipids, nucleic acids or other molecules it normally is
15 associated with in a cell. In general, an isolated nucleic acid molecule encoding a theta defensin constitutes at least about 75% by weight of a sample containing the nucleic acid molecule, and usually constitutes about 90% of a sample, particularly about 95%
20 of the sample or more. It is recognized, however, that an isolated nucleic acid molecule encoding a theta defensin can be contained in a vector. For purposes of the present definition of "isolated," vector DNA is not considered to be part of a sample when determining the
25 degree of isolation of the nucleic acid molecule encoding the theta defensin, since the encoding nucleic acid molecule generally can be readily purified from the vector. An isolated nucleic acid molecule encoding a theta defensin can be chemically synthesized or can be
30 cloned from a cell that contains a theta defensin gene or encodes a theta defensin mRNA, which can be converted to a cDNA.

An isolated nucleic acid molecule of the invention, which encodes a precursor theta defensin, can be prepared by chemical synthesis, based on the disclosed theta defensin amino acid sequence and knowledge in the art of codons encoding each amino acid. Thus, a nucleic acid molecule encoding SEQ ID NO:1, for example, can be synthesized by the steps of 1) selecting one of the four codons for Gly, 2) linking to the Gly encoding triplet one of the two codons for Phe, 3) linking to the Gly-Phe encoding hexamer one of the two codons for Cys, and so forth until a complete coding sequence has been synthesized. Since a nucleic acid sequence encoding SEQ ID NO:1 only is about 54 nucleotides in length (60 nucleotides if an initiator methionine and a STOP codon are included), synthesis of the sequence readily can be prepared using routine methods and, if desired, can be purchased from a commercial source. Similarly, nucleic acid molecules encoding theta defensin analogs can be synthesized based on the amino acid sequence of the analog.

Theta defensin cDNA was cloned from rhesus macaque bone marrow mRNA using 3' RACE with degenerate primers (see Example V). RTD1 is encoded by two similar cDNAs, termed RTD1a (SEQ ID NO:13) and RTD1b (SEQ ID NO:15), each of which contains 9 of the 18 amino acid residues in the mature RTD-1 peptide (see Example V and Figure 11). The cDNAs encode separate peptides, which become cyclized by formation of peptide bonds that join the two peptides. The use of two genes to encode separate prepropeptides that are processed to form a cyclized peptide has not been previously described.

The invention additionally provides a nucleic acid molecule encoding the genomic DNA for rhesus macaque theta defensin RTD1a, also called RTD1.1 (SEQ ID NO:24), and RTD1b, also called RTD1.2 (SEQ ID NO:25) (see Example 5 V and Figure 13). The invention further provides a nucleic acid molecule encoding a human theta defensin (SEQ ID NO:28), which corresponds to a human theta defensin cDNA (Figure 15). The human theta defensin peptide region corresponds to amino acid residues 65 to 10 73 in the precursor (amino acids RCICTRGFC; SEQ ID NO:18). In addition, the invention provides highly specific probes for RTD1a (SEQ ID NO:26) and RTD1b (SEQ ID NO:27).

Additional nucleic acid molecules encoding 15 theta defensin can also be cloned from other mammalian cells. For example, degenerate oligonucleotide probes can be prepared based on the amino acid sequence of theta defensin (SEQ ID NO:1) and used to screen a cDNA or genomic DNA library to obtain cloned nucleic acid 20 molecules encoding the theta defensin, as described in Example V. The peptide of SEQ ID NO:1 originally was isolated from leukocytes of *Rhesus* macaques. Thus, a DNA library prepared from leukocytes from other organisms can be screened to identify and clone a nucleic acid molecule 25 encoding the theta defensin. Previously described defensins from various species share substantial amino acid sequence homology (see Lehrer et al., *supra*, 1991), and theta defensins also are likely to be relatively highly conserved. As disclosed herein, theta defensins 30 of rhesus macaque and human are very similar (see Example V). Accordingly, a DNA library, which can be a genomic library or a cDNA library, prepared from cells of any mammal, for example, from leukocytes, can be screened

using degenerate oligonucleotide probes to obtain a nucleic acid molecule encoding a theta defensin.

The skilled artisan will recognize that, in order to effectively screen a DNA library using oligonucleotide probes based on SEQ ID NO:1, the oligonucleotides should reflect a relatively conserved portion of the encoded peptide and should comprise the least degenerate codons. Thus, for screening a human nucleic acid library, for example, the artisan will recognize that oligonucleotide probes preferably are prepared based on a region of the monkey theta defensin sequence that likely is conserved among species, for example, a probe based on Arg-4 to Arg-9 or Arg-4 to Cys-12 of SEQ ID NO:1 (numbering according to Figure 4A). Hybridization conditions such as those described, for example, in Example V can be used to obtain nucleic acid molecules encoding theta defensins from other species.

Oligonucleotide probes can be used to screen a DNA library using hybridization methods, including the polymerase chain reaction. Hybridization conditions are selected based, for example, on the length and nucleotide composition of the probes (or PCR primers) and can be determined empirically or estimated using formulas for calculating such conditions (see, for example, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989)).

Thus, the invention further provides oligonucleotide sequences comprising a portion of the coding sequence of a theta defensin, particularly of SEQ ID NO:1.

As used herein, the phrase "moderately stringent hybridization" refers to conditions that permit a target nucleic acid to bind a complementary nucleic acid. The hybridized nucleic acids will generally have at least about 60% identity, at least about 75% identity, at least about 85% identity, or at least about 90% identity. Moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 42°C.

The phrase "high stringency hybridization" refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C, for example, if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein. High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C.

The phrase "low stringency hybridization" refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 22°C, followed by washing in 1X SSPE, 0.2% SDS, at 37°C. Denhart's solution contains 1% Ficoll™, 1% polyvinylpyrrolidone, and 1% bovine serum albumin (BSA). 20X SSPE (sodium chloride, sodium phosphate, ethylene diamide tetraacetic acid (EDTA)) contains 3M sodium chloride, 0.2M sodium phosphate, and 0.025 M (EDTA). Other suitable moderate stringency and high stringency hybridization buffers and conditions are well known to those of skill in the art and are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory

Manual, 2nd ed., Cold Spring Harbor Press, Plainview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)). Nucleic acids encoding polypeptides
5 hybridize under moderately stringent or high stringency conditions to substantially the entire sequence, or substantial portions, for example, typically at least 15-30 nucleotides of the invention nucleic acid sequences.

10 A nucleic acid molecule encoding a precursor theta defensin or analog thereof can be cloned into an appropriate vector, particularly an expression vector, and the encoded peptide can be expressed in a host cell or using an *in vitro* transcription/translation reaction,
15 thereby providing a means to obtain large amounts of the theta defensin. Thus, the invention provides vectors containing a nucleic acid molecule encoding a theta defensin precursor, as well as host cells that can maintain the vectors and, if desired, allow expression of
20 the theta defensin encoded by the nucleic acid molecule contained in the vector. Vector and host cell systems are well known in the art and commercially available.

The invention also provides antibodies that specifically bind a theta defensin. As used herein, the
25 term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. With regard to an anti-theta defensin antibody of the invention, the term "antigen" means a native or synthesized theta defensin,
30 including a peptide portion of the theta defensin, that can, but need not, be cyclized or contain intrachain crosslinks. An anti-theta defensin antibody, or antigen binding fragment of such an antibody, is characterized by

having specific binding activity for a theta defensin or a peptide portion thereof of at least about $1 \times 10^5 \text{ M}^{-1}$. Thus, Fab, F(ab')₂, Fd and Fv fragments of an anti-theta defensin antibody, which retain specific binding activity for a theta defensin, are included within the definition of an antibody.

In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989).

These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989) ; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

Anti-theta defensin antibodies specific for theta defensin have been generated by conjugating acyclic theta defensin, which was oxidized but not cyclized, to ovalbumin (see Example IV). Additional anti-theta

defensin antibodies can be raised using a theta defensin immunogen such as an isolated theta defensin having the amino acid sequence of SEQ ID NO:1, which can be prepared from natural sources or produced recombinantly, or a
5 peptide portion of the theta defensin. A non-immunogenic theta defensin peptide or portion thereof can be made immunogenic by coupling the hapten to a carrier molecule such bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as
10 a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane (*supra*, 1988).

An anti-theta defensin antibody is useful, for
15 example, for determining the presence or level of a theta defensin in a tissue sample, which can be a lysate or a histological section, or for cloning a nucleic acid molecule encoding a theta defensin from an appropriate expression library. An anti-theta defensin antibody also
20 can be used to substantially purify theta defensin from a sample, for example, following expression of the theta defensin from a recombinant nucleic acid molecule. In addition, an anti-theta defensin antibody raised against a linear form of the theta defensin or against a peptide
25 portion of the theta defensin can be used to screen an expression library, for example, a lambda gt11 library, to identify a clone containing a cDNA encoding the theta defensin.

A theta defensin peptide or an
30 anti-theta defensin antibody can be labeled so as to be detectable using methods well known in the art (Hermanson, "Bioconjugate Techniques" (Academic Press 1996); Harlow

and Lane, 1988; chap. 9). For example, the peptide or antibody can be labeled with various detectable moieties including a radiolabel, an enzyme, biotin or a fluorochrome. Reagents for labeling a peptide or antibody can be included in a kit containing the peptide or antibody or can be purchased separately from a commercial source. Thus, the invention further provides a kit, which contains a theta defensin or an anti-theta defensin antibody or both. Such a kit also can contain a reaction cocktail that provides the proper conditions for performing an assay, for example, an ELISA or other immunoassay for determining the level of expression of a theta defensin in a sample, and can contain control samples that contain known amounts of a theta defensin and, if desired, a second antibody specific for the anti-theta defensin antibody. Where the kit is to be used for an immunoassay, it can include a simple method for detecting the presence or amount of a theta defensin in a sample that is bound to the antibody.

Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art. In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (Harlow and Lane, *supra*, 1988). Essentially, spleen cells from a mouse immunized, for example, with theta defensin having the amino acid sequence of SEQ ID NO:1 can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labeled theta defensin to identify clones that secrete anti-theta defensin monoclonal antibodies. Hybridomas expressing anti-theta defensin monoclonal antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of

the antibodies, which are useful, for example, for preparing standardized kits as described above. Similarly, a recombinant phage that expresses, for example, a single chain anti-theta defensin antibody also provides a monoclonal antibody that can used for preparing standardized kits.

A theta defensin or analog thereof having antimicrobial activity can be applied to an environment capable of sustaining the survival or growth of a microorganism or to an environment at risk of supporting such survival or growth, thus providing a means for reducing or inhibiting microbial growth or survival. Accordingly, the invention relates to methods of using a theta defensin or a theta defensin analog to reduce or inhibit microbial growth by contacting an environment capable of sustaining microbial growth or survival with the antimicrobial peptide.

As used herein, reference to "an environment capable of sustaining survival or growth of a microorganism" means a gaseous, liquid or solid material, including a living organism, in or upon which a microorganism can live or propagate. In view of the broad range of environments that allow the survival or growth of microorganisms as diverse, for example, as viruses, bacteria, fungi, protozoans and helminths, and further in view of the disclosed effectiveness of a theta defensin against a broad spectrum of such microorganisms, the range of such environments that can be treated using a method of the invention necessarily is broad and includes, for example, a tissue or bodily fluid of an organism such as a human; a liquid such as water or an aqueous solution such as contact lens solution or eyewash solution; a food such as a food crop, a food

product or a food extract; and an object such as the surface of an instrument used, for example, to prepare food or to perform surgery; and a gas such as that used for anesthetization in preparation for surgery.

5 A method of the invention encompasses administering to the environment an effective amount of a theta defensin or analog thereof such that the antimicrobial peptide can contact a microorganism in the environment, thereby reducing or inhibiting the ability
10 of the microorganism to grow or survive. A theta defensin can be used in a variety of procedures for reducing or inhibiting the survival or growth of microorganisms, including the microbicidal inhibition of survival of a microorganism as well as the microbistatic
15 inhibition of growth. As such, a theta defensin can be used, for example, as a therapeutic agent, a food preservative, a disinfectant or a medicament.

A cyclic theta defensin can be particularly useful as a therapeutic agent for treating a patient
20 suffering from a bacterial, viral, fungal or other infection due to a microorganism susceptible to the antimicrobial activity of the theta defensin, since a cyclic theta defensin is particularly resistant to the activity of endogenous proteases and peptidases. The
25 resistance of a theta defensin or analog thereof is due, in part, to the cyclization of the peptide, such that it lacks a free amino terminus and a free carboxyl terminus. Thus, the invention provides methods of treating an individual suffering from a pathology caused, at least in
30 part, by microbial infection, by administering a theta defensin to the individual under conditions that allow the theta defensin to contact the infecting microorganisms, thereby reducing or inhibiting the

survival or growth of the microorganism and alleviating the severity of the infection.

For use as a therapeutic agent, the theta defensin can be formulated with a pharmaceutically acceptable carrier to produce a pharmaceutical composition, which can be administered to the individual, which can be a human or other mammal. A pharmaceutically acceptable carrier can be, for example, water, sodium phosphate buffer, phosphate buffered saline, normal saline or Ringer's solution or other physiologically buffered saline, or other solvent or vehicle such as a glycol, glycerol, an oil such as olive oil or an injectable organic ester.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or increase the absorption of the theta defensin. Such physiologically acceptable compounds include, for example, carbohydrates such as glucose, sucrose or dextrans; antioxidants such as ascorbic acid or glutathione; chelating agents such as EDTA, which disrupts microbial membranes; divalent metal ions such as calcium or magnesium; low molecular weight proteins; or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition.

A pharmaceutical composition containing a theta defensin can be administered to an individual by various routes, including by intravenous, subcutaneous, intramuscular, intrathecal or intraperitoneal injection;

orally, as an aerosol spray; or by intubation. If desired, the theta defensin can be incorporated into a liposome, a non-liposome lipid complex, or other polymer matrix, which further can have incorporated therein, for example, a second drug useful for treating the individual. Use, for example, of an antimicrobial indolicidin peptide incorporated into liposomes has been demonstrated to have antifungal activity *in vivo* (Ahmad et al., Biochem. Biophys. Acta 1237:109-114 (1995)).

10 Liposomes, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, 15 Boca Raton FL, 1984)).

The skilled artisan will select a particular route and method of administration based, for example, on the location of a microorganism in a subject, the particular characteristics of the microorganism, and the specific theta defensin or theta defensin analog that is administered.

Food and food products also can be treated with a theta defensin for the purpose of preserving the food or eliminating or preventing infection by microorganisms. For example, shellfish and poultry products routinely harbor enteric pathogenic microorganisms. The growth or survival of such microorganisms can be reduced or inhibited by contacting the product with the theta defensin. Food crops such as fruits, vegetables and grains can be treated with a theta defensin in order to reduce or inhibit post-harvest spoilage caused by microorganisms, for example, by administering the analog topically using an aerosolized form of the analog. In addition, transgenic plants or animals useful in the food

industry can be produced by introducing a nucleic acid molecule encoding a precursor of a theta defensin into the germline cells of such organisms. Methods for producing transgenic plants and animals are well known and routine in the art. Stable transgenic expression as well as transient transgene expression can be used (see, for example, the GENEWARE system; Biosource Technologies; Vacaville CA).

A theta defensin also can be used as a disinfectant to reduce or inhibit the survival or growth of microorganisms on an object or in a solution. A theta defensin can be used to treat essentially any object or solution that can sustain microbial growth, where the survival or growth of the microorganisms is undesirable. In particular, an object or solution that comes into contact with a mammal such as a human, for example, baby wipes, diapers, band-aids, towelettes, make-up products and eyewash and contact lens solutions can be treated with a theta defensin or analog thereof. In such methods, the theta defensin can be applied topically to the object or can be added to the solution or can be in an aerosolized form in a gas.

In order to exhibit antimicrobial activity in an environment, an effective amount of a theta defensin is administered to the environment. As used herein, the term "effective amount" refers to the amount of a theta defensin that reduces or inhibits the survival or growth of a microorganism in an environment. In particular, an effective amount of a theta defensin produces only minimal effects against the environment, although the level of an acceptable deleterious effect is weighed against the benefit caused by the antimicrobial effect.

A theta defensin or analog thereof can be administered to a subject such as a human systemically at a dose ranging from 1 to 100 mg/kg body weight, for example, at a dose of about 10 to 80 mg/kg, particularly about 10 to 50 mg/kg. A theta defensin also can be incorporated into liposomes, if desired, in which case the total amount administered to a subject generally can be reduced. Furthermore, a theta defensin can be administered orally to a subject at a dose ranging from about 1 to 100 mg/kg body weight, for example at a dose of about 10 to 200 mg/kg, in particular about 20 to 100 mg/kg. In addition, a theta defensin can be administered topically to an environment, which can be a human subject, or can be placed in a solution, at a concentration of about 0.1 to 10 mg/ml, for example, at a concentration of about 0.5 to 5 mg/ml. Although theta defensins generally are effective in microgram per ml amounts, an effective amount for administration to a particular environment will depend, in part, on the environment. For example, when administered to a mammal such as a human, a theta defensin, in addition to having antimicrobial activity, can have an undesirable side effect. The skilled artisan will recognize that the level of such side effects must be considered in prescribing a treatment and must be monitored during the treatment period, and will adjust the amount of the theta defensin that is administered accordingly.

An effective amount of a theta defensin also will vary depending, for example, on the characteristics of the target microorganism, the extent of prior infection or growth and the specific theta defensin or analog thereof that is administered. In addition, an effective amount depends on the form in which the theta defensin is administered. For example, incorporation of

another antimicrobial peptide, indolicidin, into liposomes allowed administration of a higher amount of the peptide than "free" indolicidin, without producing unacceptable side effects, such that fungal infection in 5 mice could be cured (Ahmad et al., *supra*, 1995).

The invention additionally provides a method of reducing or inhibiting growth or survival of a microorganism in an individual by administering a molecule, wherein the molecule increases expression of a 10 theta defensin. Theta defensins are polypeptides expressed in leukocytes of mammals, in particular primates, including humans. Thus, theta defensins function as part of the endogenous defense system for a mammal to combat microbial infections. Since theta 15 defensins are expressed in mammals, methods to increase expression of theta defensin in the organism can be used to reduce or inhibit microbial growth in the organism. Using the genomic clones described herein, one skilled in the art can readily determine regulatory molecules that 20 can alter transcription of a theta defensin gene and screen for those molecules that effect an increase in theta defensin expression. Cytokines, for example, monocyte chemoattractant protein 1 (MCP-1), interleukin 8 (IL8) or other cytokines, that activate granulocytes can 25 be tested for stimulatory activity of theta defensin expression. Cytokines, or other compounds, can be screened for stimulatory activity. Compounds having stimulatory activity can be used to increase expression of a theta defensin in an organism to reduce or inhibit 30 growth or survival of a microorganism in an individual.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I**PREPARATION AND CHARACTERIZATION OF THETA DEFENSIN**

This example provides methods for purifying and characterizing a cyclic theta defensin.

5 Native theta defensin was purified from *Rhesus* macaque peripheral leukocytes. Briefly, leukocytes were obtained from anticoagulated whole blood of adult rhesus macaques after erythrocytes were depleted by dextran sedimentation. The cell pellet (6×10^6 cells; 91%
10 neutrophils, 5% mononuclear cells, 4% eosinophils) was snap frozen, suspended in 0.5 ml ice cold 30% acetic acid and stirred on melting ice for 18 h. The suspension was clarified by centrifugation at 4°C, the supernatant was lyophilized, and then dissolved in 0.5 ml methanol-water
15 (80:20). After 6-8 h of stirring at 8°C, the sample was clarified by centrifugation and the supernatant was lyophilized. The dry powder was dissolved in 0.5 ml 5% acetic acid prior to RP-HPLC.

Rhesus theta defensin-1 (RTD-1) was isolated
20 during studies to characterize defensins of rhesus macaque neutrophils. Peripheral blood neutrophils (> 90% PMN) were subjected to sequential acetic acid and water/methanol extraction steps as described above, and the extract was fractionated by reversed phase HPLC
25 (Figure 1A). An α -defensin-enriched extract of 6×10^6 leukocytes (91 % PMNs) was fractionated by RP-HPLC on a 0.46 x 25 cm C-18 column equilibrated in 0.1% aqueous trifluoroacetic acid (TFA) and developed with a linear acetonitrile gradient. RTD-1 eluted in the arrow-marked
30 peak.

Chromatographic peaks eluting between 20 and 50 minutes were purified to homogeneity. Analytical RP-HPLC of purified RTD-1 is shown in Figure 1B. The purity of RTD-1 was assessed by RP-HPLC of RTD-1 obtained from the peak (arrow) in Figure 1A on an analytical C-18 column developed with acetonitrile at 0.5% per min. Acid-urea polyacrylamide gel electrophoresis (PAGE) was also used to analyze purified peptides. Samples of 30% acetic acid extract (2×10^6 cell equivalents), methanol/water extracted phase (1×10^7 cell equivalents) and 1 μ g of RTD-1 were resolved on a 12.5% acid-urea polyacrylamide gel and stained with formalin-Coomassie blue. Acid-urea PAGE showed the purification of RTD-1 and confirmed the purity RTD-1.

The purified chromatographic peaks were screened for antibacterial activity against *Escherichia coli* ML35 and *Staphylococcus aureus* 502a. Briefly, antibacterial activity was screened with an agar diffusion assay using lyophilized samples of HPLC fractions dissolved in 5 μ l of 0.01% acetic acid as described by Lehrer et al., J. Immunol. Methods 137:167-173 (1991)). RTD-1 was found to have the greatest activity of any of the peptides isolated.

Microbicidal peptides were characterized by amino acid analysis (ACCUTAG; Waters; Milford MA) and automated Edman degradation. Sequence analysis was performed by automated Edman degradation with on-line PTH amino acid analysis. Seven of the eight active peptides were found to be α -defensins that were similar to previously characterized human peptides. RTD-1 (arrow in Figure 1A), was relatively abundant, as indicated by HPLC and acid-urea PAGE, and possessed the greatest antibacterial activity of any of the peptides isolated.

The yield of RTD-1 was approximately 100 µg per 10⁹ neutrophils.

Amino acid analysis revealed that RTD-1 contained 18 amino acids: 1 Thr, 1 Val, 1 Leu, 1 Phe, 1
5 Ile, 2 Gly, 5 Arg, and 6 Cys. RTD-1 was also analyzed by mass spectroscopy, performed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) on a PerSeptive Biosystems Voyager RP mass spectrometer (PerSeptive; Framingham MA). Samples (1-10 pmol) were
10 dissolved in water-acetonitrile (1:1) containing 0.1% TFA. MALDI-TOF mass spectroscopy analysis of the native peptide (2082.0) and S-pyridylethylated peptide (2720.3) (Henschen, Advanced Methods in Protein
Microsequence Analysis, Wittmann-Liebold et al., eds.,
15 Springer-Verlag, Berlin, p. 244 (1996)) demonstrated that the six cysteines exist as three intramolecular disulfides.

Attempts to sequence RTD-1 failed, indicating blockage of the amino terminus. Therefore, the primary
20 structure of RTD-1 was determined by sequencing overlapping chymotryptic and tryptic fragments. Briefly, RTD-1 disulfides were reduced with dithiothreitol (DTT) and alkylated with 4-vinyl pyridine so that cysteine was analyzed as the S-pyridylethyl derivative.
25 S-pyridylethylated peptide (2 nmol) was digested at 37°C for 10 min with 0.4 µg TPCK trypsin or TLCK α-chymotrypsin in 50 µl 1% ammonium bicarbonate, pH 8.0. Peptide fragments were purified by C-18 RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS, and
30 automated sequencing. Figure 2A shows the amino acid sequence of the peptide chain as determined by Edman sequencing and MALDI-TOF MS of purified fragments produced by partial acid hydrolysis (methanol/HCl) and

digestion with trypsin (T) and chymotrypsin (CT). The sequence analysis revealed that the peptide is entirely cyclized through peptide bonds (see Figure 2B). The cyclization of the backbone accounts for the 18 atomic mass number (a.m.u.) difference between the measured mass (2082.0 obtained; 2081.7 calculated) of RTD-1 and the theoretical mass of a linear peptide (2099.7) of the same composition.

The disulfide structure of RTD-1 was determined by characterizing protease digestion fragments produced by sequential incubation of native peptide with trypsin and thermolysin. Briefly, 2.5 nmol of RTD-1 was digested at 37°C for 16 h with 0.5 µg TPCK trypsin in 50 µl of 0.1 M pyridine acetate, pH 6.4. The digest, when fractionated by RP-HPLC, gave one predominant peak. Analysis by MALDI-TOF MS demonstrated that trypsin cleavage occurred at all five arginines, releasing a 17-residue, four stranded oligopeptide connected by three disulfides (see Figure 3). 50 pmol of the tryptic 17-residue oligopeptide was digested with 10 ng of thermolysin in 5 µl of 0.1% TFA, adjusted to pH 7 with 0.1 M ammonium bicarbonate supplemented with 10 mM CaCl₂, for 2 h at 37°C. To the reaction mixture was added 5 µl 0.1% TFA-acetonitrile (1:1). One µl aliquots were analyzed by MALDI-TOF MS as described above. Alternatively, about 3 nmol of the 17-mer were digested with thermolysin under similar conditions, and the thermolytic fragments were isolated by HPLC. MALDI-TOF MS analysis of individual peaks confirmed the fragment pattern obtained by analysis of the unfractionated digestion mixture.

Cleavage by trypsin generated a major product that was purified by HPLC, the mass of which was determined to be 1998.1. Comparison of the mass and amino acid analysis of this peptide revealed that it was produced by cleavage at the carboxyl side of all 5 arginines, thus generating a 17-residue oligopeptide composed of 4 substituent chains linked by three disulfides (calculated mass = 1997.5) (Figure 3). To distinguish between the 8 possible disulfide pairings in the 17-mer, the oligopeptide was digested with thermolysin and the resulting fragments were analyzed by MALDI-TOF MS as described above. MS analysis (Figure 3; calculated values in parentheses) of the digest or of HPLC-purified fragments disclosed thermolytic cleavage at Cys-14/Ile-15 and at Cys-5/Leu-6 (arrows), producing four major thermolytic fragments, indicated as Th-1 to Th-4 in Figure 3. The masses of all fragments were consistent with the disulfide assignments shown in Figure 3.

The m/z values of the thermolysin fragments were consistent with only one cystine motif, which is shown in Figure 3, revealing that the cyclic chain is stabilized by 3 disulfides in a picket fence-like array that stabilizes two hypothetical β -strands connected by turns at both ends (see Figure 4). Schematically, RTD-1 resembles the Greek letter theta (Figure 4), hence the selection of "theta" defensin to describe this molecular motif.

RTD-1 is the first example of a macrocyclic peptide or protein in animals. It is highly cationic, possessing a net charge of +5 at pH 7 (calculated pI >12), and its dense cystine motif in RTD-1 is distinct from that determined for α or β defensins (Tang and Selsted, J. Biol. Chem. 268:6649-6653 (1993)). The

cyclic structure of RTD-1 reveals that primate cells possess a post-translational processing pathway capable of producing a head-to-tail ligated peptide chain. Analogous macrocyclic peptides have been isolated from plants of the Rubiaceae family and, like RTD-1, these molecules possess three intramolecular disulfides (Derua et al., Biochem. Biophys. Res. Commun. 228:632-638 (1996)). Two of these peptides are reported to have antiviral activity against HIV-1 (Gustafson et al., J. Amer. Chem. Soc. 116:9337 (1994)). The plant peptides differ from RTD-1 in their size (29-31 amino acids) and their cystine motif, which is characterized by "overlapping" disulfides (see Figure 4). Thus far, the genes encoding these plant peptides have not been characterized, nor have mechanisms been proposed for the formation of the cyclic backbone. The only other known macrocyclic peptides are cysteine-free peptides. One, AS-48, is a plasmid-encoded peptide expressed by *Enterococcus faecalis* (Galvez et al., Antimicrob. Agents Chemother. 33:437 (1989)). The second is J25, a microcin from *E. coli* (Blond et al., Eur. J. Biochem. 259:747-755 (1999)).

Searches for amino acid sequence similarity to RTD-1 were carried out using all 18 possible linearized peptides as query sequences (Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997)). Taking into consideration the linear cysteine spacing and disulfide connectivities of RTD-1, the most similar protein sequence identified was that of the porcine antimicrobial peptide protegrin 3 (PG-3) (see Figure 4). Protegrins are 17-18 amino acid, di-disulfide containing peptides that are members of the cathelicidin family of antimicrobial peptides (Zanetti et al., FEBS Lett. 374:1-5 (1995)). Cathelicidins share a high degree of sequence similarity

in the prepro-regions of their precursors, but the
carboxyl termini, containing the antimicrobial peptide
segments, vary markedly. Like protegrins, RTD-1 is
predicted to be predominantly composed of two disulfide
5 stabilized β strands connected by turns.

A model of RTD-1 was constructed by energy
minimization of the covalent structure. Briefly, the
RTD-1 backbone and disulfides were constructed using the
Insight II program. Energy minimization was used to
10 allow the structure to relax *in vacuo*, and the molecule
was then placed into a 25.0 Å radius sphere of water.
With the peptide fixed, water molecules were first energy
minimized, and the energy of the entire complex was then
minimized. Molecular dynamics simulations were then
15 carried out at 300 K. After 5 psec, the total energy did
not show fluctuations greater than 183 atom units, and
the structure appeared stable. Further energy
minimization resulted in the peptide structure shown in
Figure 4. The consistent valence force field (cvff) was
20 used in all molecular mechanics and molecular dynamics
calculations. Figure 5 shows the coordinates used to
generate the molecular model shown in Figure 4.

As shown in Figure 4, RTD-1 is remarkably
similar to the solution structure of protegrin 1. This
25 similarity suggested the possibility that RTD-1 is a
member of the cathelicidin family. However, subsequent
studies demonstrated that RTD-1 is not a cathelicidin,
but rather the product of two α -defensin-related genes
(see Example V).

These results demonstrate that theta defensin isolated from macaque neutrophils, RTD-1, is a macrocyclic peptide linked head-to-tail and containing three intramolecular disulfide bonds.

5

EXAMPLE II**SOLID PHASE SYNTHESIS OF THETA DEFENSIN**

This example describes chemical synthesis of theta defensin.

A synthetic version of RTD-1 was produced by
10 solid phase synthesis. Inspection of the theta defensin disulfide motif suggested that assembly of a linear 18-mer in which Gly¹ was placed at the amino terminus (see Figure 4) would both facilitate disulfide-bond formation and proximate positioning of the amino and carboxyl
15 termini for cyclization. A linear version of RTD-1 was assembled using Fmoc chemistry, cleaved, deprotected, and the reduced peptide was purified by RP-HPLC at pH 2.1. A schematic of the synthesis is shown in Figure 6A.

The linear peptide chain of the monkey peptide
20 was assembled on PEG-PS resin at 0.2 mmol scale on a Millipore 9050 Plus continuous-flow peptide synthesizer (Millipore; Bedford MA). Fmoc-chemistry was utilized and the following protecting groups were employed: Arg(Pbf) (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl);
25 Cys(Trt) (trityl or triphenylmethyl); and Thr(tBu) (*tert*-butyl). All amino acids except cysteine were coupled by
O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N,N*-diisopropylethylamine (HATU/DIEA)
30 activation. Cysteine was coupled as the preformed pentafluorophenyl ester. Ile, Leu and Thr were double-

coupled. The protecting groups were removed and the peptide was cleaved from the resin by 5 hour treatment with 100 ml of Reagent-K, containing TFA-phenol-water-thioanisole-1,2-ethanedithiol (82.5:5:5:5:2.5), at room temperature with agitation. The crude peptide was separated from the resin by glass fiber filtration. The resin was rinsed consecutively with 5 ml of Reagent-K, 50 ml of 30% acetic acid/water and 50 ml of methylene chloride, and the washes were added to the initial filtrate. After vigorous mixing and phase-separation, the peptide-containing aqueous phase was extracted twice with methylene chloride (2 x 50 ml) and lyophilized, giving 225 mg (54 % yield) of crude product obtained as a white powder.

15 A 25 mg sample of lyophilized crude material was dissolved in 6 M guanidine HCl, 0.2 M Tris-HCl, 0.2 mM EDTA, and reduced with an excess of dithiothreitol at 50°C for 4 hrs under nitrogen. The reaction mixture was acidified by addition of acetic acid to a 5% v/v final concentration, and the reduced product was isolated by RP-HPLC purification on a C-18 column using 0.1% TFA/acetonitrile-water solvent system. The reduced peptide was diluted to 0.1 mg/ml in 0.1% acetic acid, and the pH was adjusted to 7.5 with ammonium hydroxide.

20 Oxidation was carried out by stirring in an open vessel at room temperature for 18 hr, after which time the solution was found to be negative for free sulfhydryls based on a negative reaction with Ellman's reagent, and HPLC analysis showed quantitative conversion of the reduced peptide to the oxidized form.

The oxidized peptide was purified by RP-HPLC on a semi-preparative C-18 column using a 0.1% HCl/acetonitrile-water solvent system yielding 9.0 mg of

peptide. MALDI-TOF MS measurements were consistent with the acyclic form of RTD-1 shown in Figure 6A. The peptide was air oxidized, repurified by HPLC, and converted to the hydrochloride salt by RP-HPLC in 5 solvents containing 0.1% HCl as described above.

The oxidized peptide was then cyclized by activating the carboxyl group (Figure 6A). The oxidized acyclic synthetic peptide was cyclized to form an amide bond between the amino group of Gly-1 and the carboxyl 10 group of Arg-18. After 3.0 mg of acyclic oxidized RTD-1 was repeatedly lyophilized to remove volatile components, cyclization was carried out in 3.0 ml of dimethylsulfoxide containing 60 and 20 equivalents of ethylenediaminecarbodiimide and 1-hydroxybenzotriazole 15 (HOBt), respectively, for 18 hours at room temperature. The resulting solution was lyophilized and purified by RP-HPLC.

The cyclization reaction mixture gave a major peak that coeluted with natural RTD-1. MALDI-TOF mass 20 spectroscopy demonstrated that the product (1.7 mg, 56.6% yield) had the expected mass of the cyclic peptide. In addition, the material in this peak coeluted with natural RTD-1 on analytical RP-HPLC, co-electrophoresed with natural RTD-1 on acid-urea PAGE, and had identical CD 25 spectra in water, methanol, and 10 mM sodium phosphate, pH 7.4.

The cyclized peptide, generated by formation of a peptide bond between Gly¹ and Arg¹⁸, was formed with a yield of 56%. Analysis of the disulfide pattern of 30 cyclized synthetic RTD-1 was carried out as described for the natural peptide, and confirmed that the cysteines were correctly paired. The equivalence of natural and

synthetic RTD-1 was confirmed by MALDI-TOF MS, analytical RP-HPLC (Figure 6B), which showed co-elution of synthetic and natural RTD-1, and acid-urea PAGE, which showed comigration of synthetic and natural RTD-1. Circular dichroism spectroscopy was also performed on synthetic and natural RTD-1 (Figure 6C). Circular dichroic spectra of synthetic and natural RTD-1 were determined in water, 10 mM sodium phosphate buffer, and methanol at a peptide concentration of 111 µg/ml (53.3 µM). CD spectroscopy confirmed the equivalence of the synthetic and natural RTD-1.

These results indicate that theta defensin can be chemically synthesized in a form equivalent to natural RTD-1.

15

EXAMPLE III

ANTIMICROBIAL ACTIVITY OF THETA DEFENSIN

This example demonstrates that theta defensin exhibits broad spectrum antimicrobial activity.

Agar diffusion assays and microbicidal suspension assays were used to examine the activity of theta defensin against *Staphylococcus aureus* 502A, *Escherichia coli* ML35, *Listeria monocytogenes*, and *Cryptococcus neoformans*. For agar diffusion assays, theta defensin activity was determined at concentrations 10, 30, 100 or 300 µg/ml in agar plates seeded with 1×10^6 colony forming units of each microorganism. theta defensin demonstrated a dose dependent increase in the zone of inhibition for each of the microorganisms examined (see Figure 7).

The *in vitro* antimicrobial properties of RTD-1 were further evaluated in microbicidal assays against a panel of bacterial and fungal test organisms. Increasing concentrations of natural and synthetic RTD-1 were
5 incubated with *Staphylococcus aureus* 502a for 2 h at 37°C in 10 mM PIPES, pH 7.4 (Figure 8). Killing was quantified by colony counts. As shown in Figure 8, nearly complete killing (99 to 99.99%) of this organism was achieved at peptide concentrations of 2-4 µg/ml of
10 natural and synthetic RTD-1, and both preparations reduced colony counts to below the level of detection at peptide concentrations \geq 4 µg/ml.

Additional antimicrobial assays were conducted on other microbial organisms. Figure 9 shows
15 microbicidal activity of RTD-1. In Figure 9A, *S. aureus* 502a was incubated with increasing concentrations of natural or synthetic peptide for 30 min at 37°C in 10 mM PIPES, pH 7.4, containing 5 mM glucose. Killing was quantified by colony counts. In Figures 9B to 9D, each
20 test organism was incubated for 2 hr with RTD-1, as in Figure 9A, at the peptide concentrations indicated. The limit of detection (1 colony per plate) was equal to 1×10^3 colony forming units in the incubation mixture. The results shown in Figure 9 demonstrate that the synthetic
25 RTD-1 killed gram positive bacteria (*S. aureus*, *L. monocytogenes*), gram negative bacteria (*E. coli* ML 35, *S. typhimurium*), and fungi (*C. albicans* and *C. neoformans*) at similar peptide concentrations.

Several previous studies have demonstrated that
30 *in vitro* defensin-mediated microbicidal activity is antagonized by increased ionic strength (Bals et al., Infect. Immun. 66:1225 (1998); Valore et al., J. Clin. Invest. 101:1633 (1998); Goldman et al., Cell 88:553

(1997); Smith et al., Cell 85:229 (1996)). It has been proposed that salt sensitivity of airway defensins underlies the susceptibility of cystic fibrosis patients to pulmonary infections. The effect of ionic strength on RTD-1 bactericidal activity was tested in a killing assay against *S. aureus* 502a. Killing of *S. aureus* 502a was assessed after a 2 h incubation as in Figure 9A, with 10 $\mu\text{g/ml}$ of natural or synthetic RTD-1 supplemented with increasing concentrations of NaCl (Figure 9E). NaCl concentrations as high as 150 mM had little effect on the staphylocidal activity of natural or synthetic RTD-1 (Figure 9E). These results indicate that RTD-1 is clearly distinguished from the salt-mediated inhibition of α or β defensins.

An acyclic version of theta defensin was also tested for antimicrobial activity. As shown in Figure 10, *S. aureus* was incubated with increasing concentrations of acyclic RTD-1 with (solid circles) or without (open circles) 130 mM NaCl. Killing activity was quantified by colony counts after 18 hrs. In contrast to the cyclic form of theta defensin, the acyclic form exhibits lower activity in the presence of NaCl (Figure 10). The cyclic form is about three times more active than the acyclic form of theta defensin in both gram positive bacteria (*Staphylococcus*) and gram negative bacteria (*E. coli*). These results demonstrate that an acyclic form of theta defensin has antimicrobial activity.

The microbicidal activity of RTD-1 was further characterized. The microbicidal activity in the presence of physiological salt concentrations was tested against various microorganisms in various buffers. As shown in Table 1, RTD-1 was potently microbicidal against a wide

microbial spectrum in the presence of physiological concentration of sodium chloride. Furthermore, the killing activity was observed in various buffer compositions.

Table I. Microbicidal Activities of RTD-1 in Physiologic Sodium Chloride

RTD-1 Concen- tration	Microorganism (1-2 x 10 ⁶ CFU/ml)	Incubation Time and Temperature	Buffer composition	%killed
10 mg/ml	Staphylococcus aureus 502a	2h, 37 C	10 mM PIPES, pH 7.4, 5 mM glucose, 150 mM NaCl	>99
10 mg/ml	Staphylococcus aureus 502a	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>99.99
10 mg/ml	Salmonella typhimurium, PhoP ⁻	2h, 37 C	10 mM PIPES, pH 7.4, 5 mM glucose, 150 mM NaCl	>99.99
10 mg/ml	Salmonella typhimurium, PhoP ⁻	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>99.99
10 mg/ml	Listeria monocytogenes EDG	2h, 37 C	10 mM PIPES, pH 7.4, 5 mM glucose, 150 mM NaCl	>99.99
10 mg/ml	Listeria monocytogenes EDG	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>99.99
10 mg/ml	Candida albicans	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>98
10 mg/ml	Escherichia coli ML35	2h, 37 C	10 mM PIPES, pH 7.4, 5 mM glucose, 150 mM NaCl	>99
10 mg/ml	Escherichia coli ML 35	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>99.99
10 mg/ml	Cryptococcus neoformans 271a	2h, 37 C	10 mM Tris-HCl, pH 7.4, 150 mM NaCl	>99.99
10 mg/ml	Cryptococcus neoformans 271a	2h, 37 C	10 mM PIPES, pH 7.4, 5 mM glucose, 150 mM NaCl	>99.99

RTD-1 antimicrobial activity was also characterized in the presence of serum. As shown in Table 2, RTD-1 was potently microbicidal against Gram positive and Gram negative bacteria in the presence of 5 serum. Control incubations under the conditions shown in Table 2 but lacking RTD-1 were completely inactive against *S. aureus* and *E. coli*.

Table II. Microbicidal Activities of RTD-1 in Human AB Serum

RTD-1 Concentration	Microorganism (1-2 x 10 ⁶ CFU/ml)	Incubation Time and Temperature	Buffer composition	%killed
25 mg/ml	Staphylococcus aureus 502a	2h, 37 C	10 mM Tris-HCl, pH 7.4, 50% human AB serum	>99*
25 mg/ml	Escherichia coli ML35	2h, 37 C	10 mM Tris-HCl, pH 7.4, 25% human AB serum	>99*

* control incubation mixtures lacking RTD-1 were completely inactive against S. aureus and E. coli.

MES: Check with Tim on stability of E. coli in 25% human serum

These results demonstrate that theta defensin, both synthetic and natural, has wide antimicrobial activity against gram positive bacteria, gram negative bacteria and fungi.

5

EXAMPLE IV**IMMUNOLOCALIZATION OF RTD-1 IN RHESUS LEUKOCYTES**

This example describes the generation of anti-RTD-1 antibody and determination of the localization of RTD-1 in rhesus leukocytes.

10

Anti-RTD-1 antibody was produced by immunizing New Zealand white rabbits with an immunogen composed of the oxidized, open chain version of the peptide (see Figure 6A) conjugated to ovalbumin. Briefly, immunogen was prepared by conjugating 1.2 mg acyclic RTD-1 (Figure 15 6A) with 1.2 mg ovalbumin in 2.4 ml of 0.1 M sodium phosphate, pH 7.4, containing 0.1% glutaraldehyde. The mixture was stirred for 18 h at room temperature, quenched with 0.3 M glycine and the mixture was dialyzed in 500 molecular weight cut off tubing against water and 20 lyophilized. Two New Zealand white rabbits were immunized with the conjugate. The antisera from both rabbits had a titer of greater than 1:2500 as determined by competitive ELISA using RTD-1 conjugated to goat gamma globulin as the target antigen.

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Dot blot analysis demonstrated that anti-RTD-1 antiserum reacted with natural and synthetic RTD-1, and the oxidized acyclic version of RTD-1. The anti-RTD-1 antibody did not recognize any of the previously characterized α -defensins (HNP 1-4) expressed by human 30 leukocytes nor any of the rhesus leukocyte α -defensins.

To determine the immunolocalization of RTD-1 in rhesus macaque leukocytes and determine which leukocytic lineages express RTD-1, cytospin preparations of peripheral blood buffy coat cells, fixed with 4% paraformaldehyde, were incubated with 1:100 rabbit anti-RTD-1 antiserum and developed with biotinylated goat anti-rabbit IgG. The fixed cells were washed and incubated with avidin/biotin/glucose oxidase complex, which was subsequently visualized with nitroblue tetrazolium. Cells were counterstained with Nuclear Fast Red. For a negative control, buffy coat cells were incubated with anti-RTD-1 antiserum that was preabsorbed with synthetic acyclic RTD-1 (1 mg per ml antiserum).

Immunostaining of buffy coat leukocytes demonstrated strong, punctate staining in neutrophil cytoplasm, similar to immunolocalization studies of neutrophil α -defensins, which are stored in azurophil granules. Though staining less strongly than neutrophils, monocytes were also immunopositive, while lymphocytes and eosinophils were negative. These results demonstrate the presence of RTD-1 in the two major phagocytic cells of the blood.

EXAMPLE V

THETA DEFENSIN IS THE PRODUCT OF TWO INDEPENDENT GENES

ENCODING DISTINCT PORTIONS OF THETA DEFENSIN

This example describes the cloning of two distinct theta defensin genes from macaques, each gene encoding a specific portion of theta defensin.

In order to understand the transcriptional and translational pathways involved in the production of cyclic RTD-1, the corresponding cDNA was cloned. The

finding that RTD-1 is expressed in myeloid cells suggested that its mRNA would be abundant in bone marrow cells. Using rhesus macaque bone marrow mRNA as template, 3' rapid amplification of cDNA ends (RACE) was carried out using degenerate primers corresponding to different 6 or 7 amino acid sequences in the RTD-1 backbone. Polymerase chain reaction (PCR) products were subcloned and sequenced, revealing that portions of the RTD-1 mature peptide sequence were amplified using the degenerate primer corresponding to GVCRCIC (SEQ ID NO:34). The 3' RACE products were then used to probe a rhesus macaque bone marrow cDNA library. Fifteen positive clones were isolated and sequenced, disclosing two very similar cDNAs termed RTD1a and RTD1b.

Figure 11 shows the full length cDNAs of RTD1a (SEQ ID NO:13) and RTD1b (SEQ ID NO:15) and the corresponding deduced amino acid sequences (SEQ ID NOS:14 and 16, respectively). Full length cDNA sequences are shown with the deduced amino acid sequences. Underlined amino acids are found in RTD-1, and superscript numbers correspond to the residue numbering of RTD-1 shown in Figure 2B. ATG of the initiation methionines are in bold, as are the polyadenylation sites at the 3' ends of the sequences (Figure 11).

At the DNA level, both clones showed a high degree of identity, 90.8% and 91.2% for RTD1a and RTD1b, respectively, to regions of a human defensin-related pseudogene, GI501091, GenBank accession number U10267. One of the stop codons in this human sequence corresponds exactly to the position of the stop codon in the RTD-1 sequences (Figure 11).

At the amino acid level, the RTD-1 precursors were most similar to HNP-4, one of the four known human myeloid α -defensins (Figure 12). α -defensins are antimicrobial peptides expressed at high levels in neutrophils, in Paneth cells of the small intestine, and in a number of other specialized epithelia. Although the RTD-1 and α -defensin sequences and disulfide structures are quite different (Figure 12), the RTD1a and RTD1b mRNAs encode polypeptides that are very similar in sequence to myeloid α -defensin precursors (43% identity). However, RTD 1a and 1b appear to be truncated α -defensins, as stop codons are present in the coding sequences about half way through the open reading frame corresponding to the mature α -defensin peptides (Figure 13).

Inspection of the RTD1a and RTD1b cDNAs revealed that they each encode 76 amino acid prepropeptides in which are contained 9 of the 18 residues in the mature RTD-1 peptide. From RTD1a, amino acids 65 to 73 correspond to RTD-1 residues 13 to 18 and 1 to 3. In RTD1b, the same residues 65 to 73 in the precursor correspond to RTD-1 amino acids 4 to 12 (Figures 12 and 13). A tripeptide at the carboxyl end of each precursor is removed prior to a pair of ligation events necessary for peptide cyclization.

The RTD1.1 and RTD1.2 genomic sequences were determined, confirming that the corresponding cDNAs for RTD1a and RTD1b, respectively, derive from distinct transcriptional units (Figure 13). The 3 exon, 2 intron gene structure and organization are very similar to that of the myeloid α -defensins characterized in humans, rabbits, and guinea pigs.

Expression of RTD-1 mRNA was analyzed by northern blotting of RNA from selected rhesus tissues using a random prime labeled PCR product containing nucleotides 200 to 231 in RTD1a and 195 to 326 in RTD1b.

5 The DNA probe for specific hybridization to RTD1a and RTD1b is shown in Figure 14. Hybridization was performed at 42°C overnight in 5x SSPE (20x SSPE is 3M NaCl, 0.2M phosphate, pH 7.4, 0.025M ethylenediaminetetraacetic acid (EDTA); 4x Denhardt's (50x Denhardt's is 1% Ficoll

10 1% polyvinylpyrrolidone, 1% bovine serum albumin (BSA)); 4.8% sodium dodecyl sulfate (SDS); and 40% formamide. The blots were washed at 42°C, followed by washing at 50°C with 0.5x SSC (20x SSC is 3M NaCl, 0.3M sodium citrate, pH 7.0) and 2% SDS. These probes were shown to be

15 specific for RTD-1 by Southern slot blot analysis, as they did not hybridize to plasmids containing known rhesus myeloid defensin cDNAs in Southern Blots, but they hybridized strongly to plasmids containing the RTD1a and RTD1b cDNAs.

20 Various tissues were analyzed for expression of RTD-1 mRNA, including lymph node, stomach, thyroid, jejunum, liver, adrenal, thymus, kidney, lung, pancreas, ovary, colonic mucosa, trachea, spleen, bone marrow, skeletal muscle, brain, and testis. RTD-1 mRNA was

25 detected only in bone marrow. The hybridizing signal was 0.54 kb, consistent with the size of the cDNA.

Human theta defensin cDNA was also isolated. The human theta defensin cDNA was amplified from human bone marrow cDNA using primers deduced from RTD1a and

30 RTD1b. Figure 15 shows the human theta defensin cDNA sequence (SEQ ID NO:28) and the deduced amino acid sequence (SEQ ID NO:29). The human theta defensin peptide region corresponds to amino acid residues 65 to

73 in the precursor (SEQ ID NO:18).

To confirm that RTD-1 is in fact produced by the ligation of RTD-1a and RTD-1b gene products, transfection experiments were conducted using the human promyelocytic cell line HL-60. Since synthesis of azurophil granule contents occurs through the promyelocyte stage, it was likely that the cellular machinery for synthesis and processing RTD-1 would exist in this cell line.

Cells were transfected with pcDNA3.1 (Invitrogen; San Diego CA) constructs containing the RTD1a and RTD1b coding sequences downstream of the CMV immediate early promoter. Stable transfectants and control HL-60 cells were immunostained with anti-RTD-1 antibody (see Example IV). Cells transfected with vectors containing the RTD1a and RTD1b cDNAs were strongly immunopositive. Non-transfected cells stained with anti-RTD-1 anti-serum were immunonegative, as were transfected cells stained with preimmune serum. These data confirm the relationship between RTD-1 peptide and the two cDNAs and indicate that transfected HL-60 cells can be useful for studying the processing pathway leading to the final cyclic structure.

These results demonstrate that RTD-1 peptide is the product of two genes, RTD-1a and RTD-1b, which are expressed and processed to form the RTD-1 theta defensin.

EXAMPLE VI

IDENTIFICATION OF RTD HOMODIMERS

This example describes the identification of RTD homodimers in addition to RTD-1.

Each of the two RTD-1 precursors, RTD1a and RTD1b, contribute 9-amino acids from their carboxyl terminal regions to produce the 18-amino acid mature, cyclic peptide. Since the two 9-amino acid segments differ slightly, RTD-1 can be considered to be a cyclized heterodimer. It was possible that the 9-amino acid peptides could be combined to form homodimeric products.

Cyclic homodimers of RTD1a and RTD1b, termed RTD-2 and RTD-3, respectively, were isolated from Rhesus macaque leukocytes. RTD-2 and RTD-3 were purified as described for RTD-1 (see Example I) and characterized by amino acid analysis and mass spectroscopy. The peptides were shown to be identical to synthetic versions of the respective cyclized molecules precisely as described for the analysis of RTD-1 (Example I). The sequences and disulfide bonds of RTD-2 and RTD-3 are shown in Figure 16 and compared to RTD-1. RTD-1 has a net charge of +5, RTD-2 has a net charge of +4, and RTD-3 has a net charge of +6.

These results demonstrate that at least three forms of RTD exist, the heterodimer RTD-1 and the homodimers RTD-2 and RTD-3.

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Although the invention has been described with reference to the examples provided above, it should be

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understood that the claims are to be given a purposive construction considering the specification as a whole.

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 ctgcacgctc aggcagaggc acgtcaggca agagctgatg aagctgccgc ccagcagcag 1560
 cctggagcag atgatcaggg aatggctcat tcctttacac ggctgaaaa cggcgtcttt 1620

ccgctttcag gtgagacagg ccggcatgca gagctacagg gtctagaggg atggatggga 1680
 gacagagtcg ggaatcgagt ctcagtggtc catgtcacct agatggcttc atttagcatc 1740
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 ttctgtctat aaagatttga ggcagctctg cctggagagt aaccattctt ttattccctt 1860
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 tcaagatatg tctgtgaaat tcaagtaggt ttaagacatg aagagacagt ctgactagtt 1980
 ctttctggat tcaaacaagt catcttcatt acacggagaa tatttgactg tatctataca 2040
 accgtttcta agagtagaga caagcctaag agtgcattca ggtgtttgtg tttgatgggg 2100
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 ggttgactct gccggatctg ctgccgctga gttccagaa tcaagaaaaa tacgctcaga 2460
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<211> 132

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
Construct

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ccactttcag agtcagcgaa aggcttgagg tgcatttgca cacgaggatt ctgccgtttg 120

ttataatgtc ac 132

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 <223> Description of Artificial Sequence: Synthetic
 Construct

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 ccgctttcag agtcagcag aggcttgagg tgcctttgca gacgaggagt ttgccaaactg 120

ttataaaggc gt 132

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 <213> Homo sapiens

<220>
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 1 5 10

gcc ctg cac gct cag gca gag gca cgt cag gca aga gct gat gaa gct 96
 Ala Leu His Ala Gln Ala Glu Ala Arg Gln Ala Arg Ala Asp Glu Ala
 15 20 25 30

gcc gcc cag cag cag cct gga gca gat gat cag gga atg gct cat tcc 144
 Ala Ala Gln Gln Gln Pro Gly Ala Asp Asp Gln Gly Met Ala His Ser
 35 40 45

ttt aca tgg cct gaa aac gcc gct ctt cca ctt tca gag tca gcg aaa 192
 Phe Thr Trp Pro Glu Asn Ala Ala Leu Pro Leu Ser Glu Ser Ala Lys
 50 55 60

ggc ttg agg tgc att tgc aca cga gga ttc tgc cgt atg tta taa 237
 Gly Leu Arg Cys Ile Cys Thr Arg Gly Phe Cys Arg Met Leu
 65 70 75

cgtcgc 243

<210> 29

<211> 76

<212> PRT

<213> Homo sapiens

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Met Arg Thr Phe Ala Leu Leu Thr Ala Met Leu Leu Leu Val Ala Leu
 1 5 10 15

His Ala Gln Ala Glu Ala Arg Gln Ala Arg Ala Asp Glu Ala Ala Ala
 20 25 30

Gln Gln Gln Pro Gly Ala Asp Asp Gln Gly Met Ala His Ser Phe Thr
 35 40 45

Trp Pro Glu Asn Ala Ala Leu Pro Leu Ser Glu Ser Ala Lys Gly Leu
 50 55 60

Arg Cys Ile Cys Thr Arg Gly Phe Cys Arg Met Leu
 65 70 75

<210> 30

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<223> Description of Artificial Sequence: Synthetic
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ggtgaaagtc tcagtcgctt tccgaactcc acgtaaactgt gtgctcctaa gacggcaaac 120

aatattacag tg 132

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 <223> Description of Artificial Sequence: Synthetic
 Construct

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 aatatttccg ca 132

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Thr Arg

<210> 33
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 <213> Homo sapiens

<400> 33
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Arg Arg

<210> 34
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<220>

<223> Description of Artificial Sequence: synthetic
primer

<400> 34

Gly Val Cys Arg Cys Ile Cys

1

5

WHAT IS CLAIMED IS:

1. A theta defensin peptide, wherein said theta defensin peptide is a cationic, arginine-rich cyclic peptide having each amino acid linked by a peptide bond and having intrachain crosslinks, said intrachain crosslinks formed between two amino acids, wherein said theta defensin peptide lacks a free amino or carboxyl terminus, and wherein said theta defensin peptide possesses antimicrobial activity, having the amino acid sequence:

Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8,

wherein:

Xaa1 independently is an aliphatic amino acid;

Xaa2 is an aromatic amino acid;

Xaa3 is Cys or Trp;

Xaa4 independently is Arg or Lys;

Xaa5 is Cys or Trp;

Xaa6 is Cys or Trp;

Xaa7 is Thr or Ser; and

Xaa8 is Arg or Lys,

wherein an intrachain crosslink is formed between:

Xaa3 at position 3 and Xaa3 at position 16;

Xaa5 at position 5 and Xaa5 at position 14; and

Xaa6 at position 7 and Xaa6 at position 12.

2. The theta defensin peptide of claim 1, having the amino acid sequence:

Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-

Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8,

wherein:

Xaa1 independently is Gly, Ile, Leu, Val or Ala;

Xaa2 is Phe, Trp or Tyr;

Xaa3 is Cys or Trp;

Xaa4 independently is Arg or Lys;

Xaa5 is Cys or Trp;

Xaa6 is Cys or Trp;

Xaa7 is Thr or Ser; and

Xaa8 is Arg or Lys.

3. The theta defensin peptide of claim 1 or 2, wherein Xaa1 is linked through a peptide bond to Xaa8.

4. The theta defensin peptide of claim 1, wherein said intrachain crosslink is a disulfide crosslink.

5. The theta defensin peptide of claim 1, wherein said intrachain crosslink is a di-tryptophan crosslink.

6. The theta defensin peptide of claim 1, wherein said intrachain crosslink is a lanthionine crosslink.

7. The theta defensin peptide of claim 2 or 3, having the amino acid sequence:

Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1).

8. The theta defensin peptide of claim 7, comprising three disulfide crosslinks consisting of

Xaa3 at position 3 and Xaa3 at position 16;

Xaa5 at position 5 and Xaa5 at position 14; and

Xaa6 at position 7 and Xaa6 at position 12.

9. A theta defensin peptide, wherein said theta defensin peptide is a cationic, arginine-rich cyclic peptide having each amino acid linked by a peptide bond and having intrachain crosslinks, said intrachain crosslinks formed between two amino acids, wherein said theta defensin peptide lacks a free amino or carboxyl terminus, and wherein said theta defensin peptide possesses antimicrobial activity, comprising the amino acid sequence

Arg-Cys-Ile-Cys-Thr-Arg-Gly-Phe-Cys (SEQ ID NO:18) or
Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys (SEQ ID NO:20),

wherein an intrachain crosslink is formed between:

Cys at position 3 and Cys at position 16;
Cys at position 5 and Cys at position 14; and
Cys at position 7 and Cys at position 12.

10. The theta defensin peptide of claim 9, having the amino acid sequence:

Gly-Phe-Cys-Arg-Cys-Ile-Cys-Thr-Arg-Gly-Phe-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:32).

11. The theta defensin peptide of claim 9, having the amino acid sequence:

Gly-Val-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Leu-Cys-Arg-Arg (SEQ ID NO:33).

12. The theta defensin peptide of claim 10 or 11, wherein the Gly at position 1 is linked through a peptide bond to the Arg at position 18.

13. The theta defensin peptide of claim 9, wherein a disulfide bond is formed between:

Cys at position 3 and Cys at position 16;
Cys at position 5 and Cys at position 14; and
Cys at position 7 and Cys at position 12.

14. A theta defensin peptide, wherein said theta defensin peptide is a cationic, arginine-rich cyclic peptide having each amino acid linked by a peptide bond and having intrachain crosslinks, said intrachain crosslinks formed between two amino acids, wherein said theta defensin peptide lacks a free amino or carboxyl terminus, and wherein said theta defensin peptide possesses antimicrobial activity, having the amino acid sequence:

Xaa1-Xaa2-Xaa9-Xaa4-Xaa10-Xaa1-Xaa11-Xaa4-Xaa4-Xaa1-Xaa1-Xaa12-
Xaa4-Xaa13-Xaa1-Xaa14-Xaa7-Xaa8,

wherein:

Xaa1 independently is an aliphatic amino acid;

Xaa2 is an aromatic amino acid;

Xaa4 independently is Arg or Lys;

Xaa7 is Thr or Ser;

Xaa8 is Arg or Lys;

Xaa9 is Glu, Asp, Lys or Ser;

Xaa10 is Glu, Asp, Lys or Ser;

Xaa11 is Glu, Asp, Lys or Ser;

Xaa12 is Glu, Asp, Lys or Ser;

Xaa13 is Glu, Asp, Lys or Ser;

Xaa14 is Glu, Asp, Lys or Ser wherein an intrachain crosslink is formed between:

Xaa9 and Xaa14;

Xaa10 and Xaa13; and
Xaa11 and Xaa12.

15. The theta defensin peptide of claim 14, wherein said crosslink is selected from the group consisting of lactam and lactone.

16. The theta defensin peptide of any one of claims 1-15, said theta defensin peptide having antimicrobial activity against a microorganism selected from the group consisting of a gram positive bacterium, a gram negative bacterium, a yeast and a fungus.

17. The theta defensin peptide of claim 16, wherein said microorganism is selected from the group consisting of *Staphylococcus* sp., *Listeria* sp., *Escherichia* sp., *Salmonella* sp., *Candida* sp., and *Cryptococcus* sp.

18. The theta defensin peptide of claim 17, wherein said microorganism is selected from the group consisting of *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Candida albicans*, and *Cryptococcus neoformans*.

19. The theta defensin peptide of any one of claims 1-15, said theta defensin peptide having antimicrobial activity against a protozoan.

20. The theta defensin peptide of claim 19, wherein said protozoan is selected from the group consisting of *Giardia* sp. and *Acanthamoeba* sp.

21. The theta defensin peptide of any one of claims 1-15, said theta defensin peptide having antimicrobial activity against a virus.

22. The theta defensin peptide of claim 21, wherein said virus is human immunodeficiency virus-1.

23. A precursor of the theta defensin peptide of any one of claims 1-22, wherein said precursor is a linear peptide comprising the amino acid sequence:

Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8,

wherein:

Xaa1 independently is an aliphatic amino acid;

Xaa2 is an aromatic amino acid;

Xaa3 is Cys or Trp;

Xaa4 independently is Arg or Lys;

Xaa5 is Cys or Trp;

Xaa6 is Cys or Trp;

Xaa7 is Thr or Ser; and

Xaa8 is Arg or Lys,

wherein said precursor can be cyclized to form the mature cyclic theta defensin peptide by exposing the precursor peptide to the appropriate conditions for effecting formation of the intrapeptide crosslinks,

wherein an intrachain crosslink is formed between:

Xaa3 at position 3 and Xaa3 at position 16;

Xaa5 at position 5 and Xaa5 at position 14; and

Xaa6 at position 7 and Xaa6 at position 12.

24. A pharmaceutical composition, comprising the theta defensin peptide of any one of claims 1-22 and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, which is associated with a liposome.

26. The pharmaceutical composition of claim 24, which is associated with a non-liposome lipid complex.

27. An antibody that specifically binds the theta defensin peptide of any one of claims 1-22.

28. The antibody of claim 27, wherein said theta defensin peptide has the amino acid sequence:

Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1).

29. The antibody of claim 27, which is a monoclonal antibody.

30. An isolated nucleic acid molecule encoding a theta defensin peptide of any one of claims 1-22 or a precursor of the theta defensin peptide of claim 23.

31. The nucleic acid molecule of claim 30, said theta defensin peptide comprising the amino acid sequence:

Xaa1-Xaa2-Xaa3-Xaa4-Xaa5-Xaa1-Xaa6-Xaa4-Xaa4-Xaa1-Xaa1-Xaa6-Xaa4-Xaa5-Xaa1-Xaa3-Xaa7-Xaa8,

wherein:

Xaa1 independently is Gly, Ile, Leu, Val or Ala;

Xaa2 is Phe, Trp or Tyr;
Xaa3 is Cys or Trp;
Xaa4 independently is Arg or Lys;
Xaa5 is Cys or Trp;
Xaa6 is Cys or Trp;
Xaa7 is Thr or Ser; and
Xaa8 is Arg or Lys,
or a nucleic acid molecule complementary thereto.

32. The nucleic acid molecule of claim 31, wherein said theta defensin peptide has the amino acid sequence:

Gly-Phe-Cys-Arg-Cys-Leu-Cys-Arg-Arg-Gly-Val-Cys-Arg-Cys-Ile-Cys-Thr-Arg (SEQ ID NO:1).

33. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1a nucleotide sequence referenced as SEQ ID NO:17.

34. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1b nucleotide sequence referenced as SEQ ID NO:19.

35. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1a nucleotide sequence referenced as SEQ ID NO:13.

36. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1b nucleotide sequence referenced as SEQ ID NO:15.

37. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1a nucleotide sequence referenced as SEQ ID NO:24.

38. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the RTD1b nucleotide sequence referenced as SEQ ID NO:25.

39. The nucleic acid molecule of claim 30, said nucleic acid molecule comprising the human theta defensin nucleotide sequence referenced as SEQ ID NO:28.

40. A vector encoding a theta defensin peptide, said vector comprising an expression element operationally linked to a nucleotide sequence encoding a theta defensin peptide, said nucleotide sequence comprising the nucleic acid molecule of claim 30.

41. A method of reducing or inhibiting growth or survival of a microorganism in vitro in an environment capable of sustaining the growth or survival of the microorganism, comprising administering an effective amount of a theta defensin peptide of any one of claims 1-22 to said environment, thereby reducing or inhibiting the growth or survival of the microorganism.

42. The method of claim 41, which has antimicrobial activity against a microorganism selected from the group consisting of a gram positive bacterium, a gram negative bacterium, a yeast and a fungus.

43. The method of claim 42, wherein said microorganism is selected from the group consisting of *Staphylococcus* sp., *Listeria* sp., *Escherichia* sp., *Salmonella* sp., *Candida* sp., and *Cryptococcus* sp.

44. The method of claim 43, wherein said microorganism is selected from the group consisting of *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella*

typhimurium, *Candida albicans*, and *Cryptococcus neoformans*.

45. The method of claim 41, which has antimicrobial activity against a protozoan.

46. The method of claim 45, wherein said protozoan is selected from the group consisting of *Giardia* sp. and *Acanthamoeba* sp.

47. The method of claim 41, which has antimicrobial activity against a virus.

48. The method of claim 47, wherein said virus is human immunodeficiency virus-1.

49. The method of claim 41, wherein said environment is a food or food product.

50. The method of claim 41, wherein said environment is a solution.

51. The method of claim 50, wherein said solution is a contact lens solution.

52. The method of claim 50, wherein said solution is an eye wash solution.

53. The method of claim 41, wherein said environment is an inanimate object comprising a surface.

54. A use of an effective amount of a theta defensin peptide of any one of claims 1-22 for the preparation of a pharmaceutical composition for reducing or inhibiting growth or survival of a microorganism in a mammal.

55. The use of claim 54, wherein said use is topical.

56. The use of claim 54, wherein said use is by injection.
57. The use of claim 54, wherein said use is oral.
58. A method of preparing the cyclic peptide of any one of claims 1 to 22 comprising,
- (a) synthesizing a linear peptide of an amino acid sequence corresponding to the amino acid sequence of a theta defensin peptide of any one of claims 1 to 22,
 - (b) forming one or more crosslink bonds within said linear peptide, and
 - (c) cyclizing said peptide by linking the carboxyl and amino termini to form a cyclic peptide.
59. The method of claim 58, wherein said crosslink is selected from the group consisting of disulfide, lanthionine, lactam and lactone.
60. The method of claim 58, wherein the cysteine residues used in said linear peptide are in a pre-formed activated ester form.
61. The method of claim 59, wherein the carboxyl terminus and amino terminus of said linear peptide are each approximately the same number of amino acids from the nearest cysteine.
62. The method of claim 61, wherein said disulfide bonds are formed by oxidation.
63. The method of claim 62, wherein said cyclizing is done with ethylenediaminecarbodiimide and N-hydroxybenzotriazole in a solvent.
64. The method of claim 63, where approximately 60

equivalents of ethylenediaminecarbodiimide and approximately 20 equivalents of N-hydroxybenzotriazole are used.

65. The method of claim 64, where the dimethylsulfoxide is the solvent.

66. The method of claim 58, wherein said cyclized peptide is resistant to exo-peptidases.

67. An in vitro method of expressing a theta defensin peptide, comprising

(a) administering the vector of claim 40 to a cell; and
(b) expressing said encoded theta defensin peptide,
wherein said peptide forms a theta defensin peptide.

68. The method of claim 67, wherein said vector encodes a second theta defensin peptide.

69. The method of claim 67, wherein a second vector encoding a second theta defensin peptide is administered to said cell.

70. A use of an effective amount of a theta defensin peptide of any one of claims 1-22 for reducing or inhibiting growth or survival of a microorganism in a mammal.

71. A use of an effective amount of a theta defensin peptide of any one of claims 1-22 in the manufacture of a medicament for reducing or inhibiting growth or survival of a microorganism in a mammal.

72. The use of claim 70 or 71, wherein said use is topical.

73. The use of claim 70 or 71, wherein said use is by

injection.

74. The use of claim 70 or 71, wherein said use is oral.

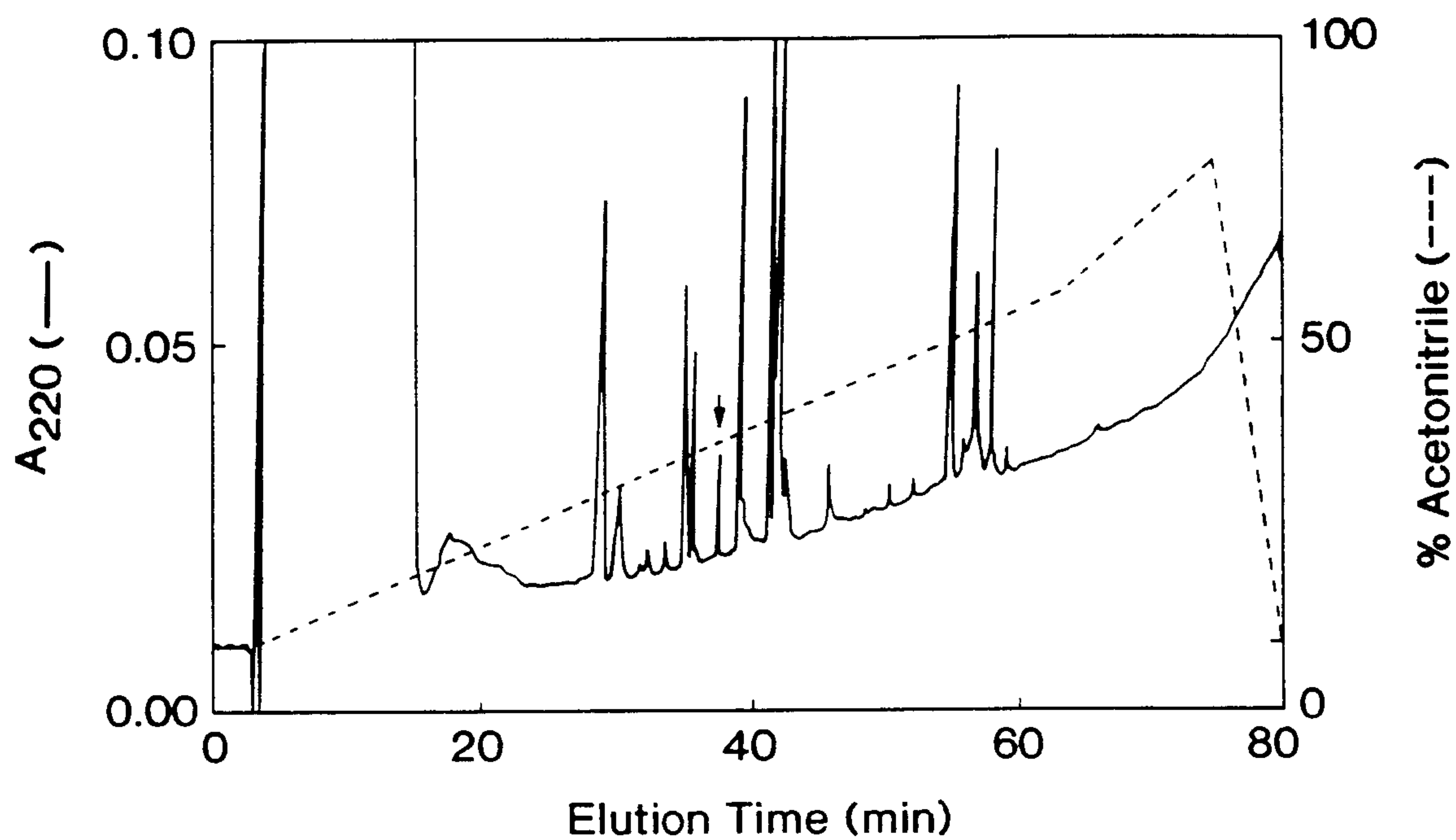


FIG. 1A

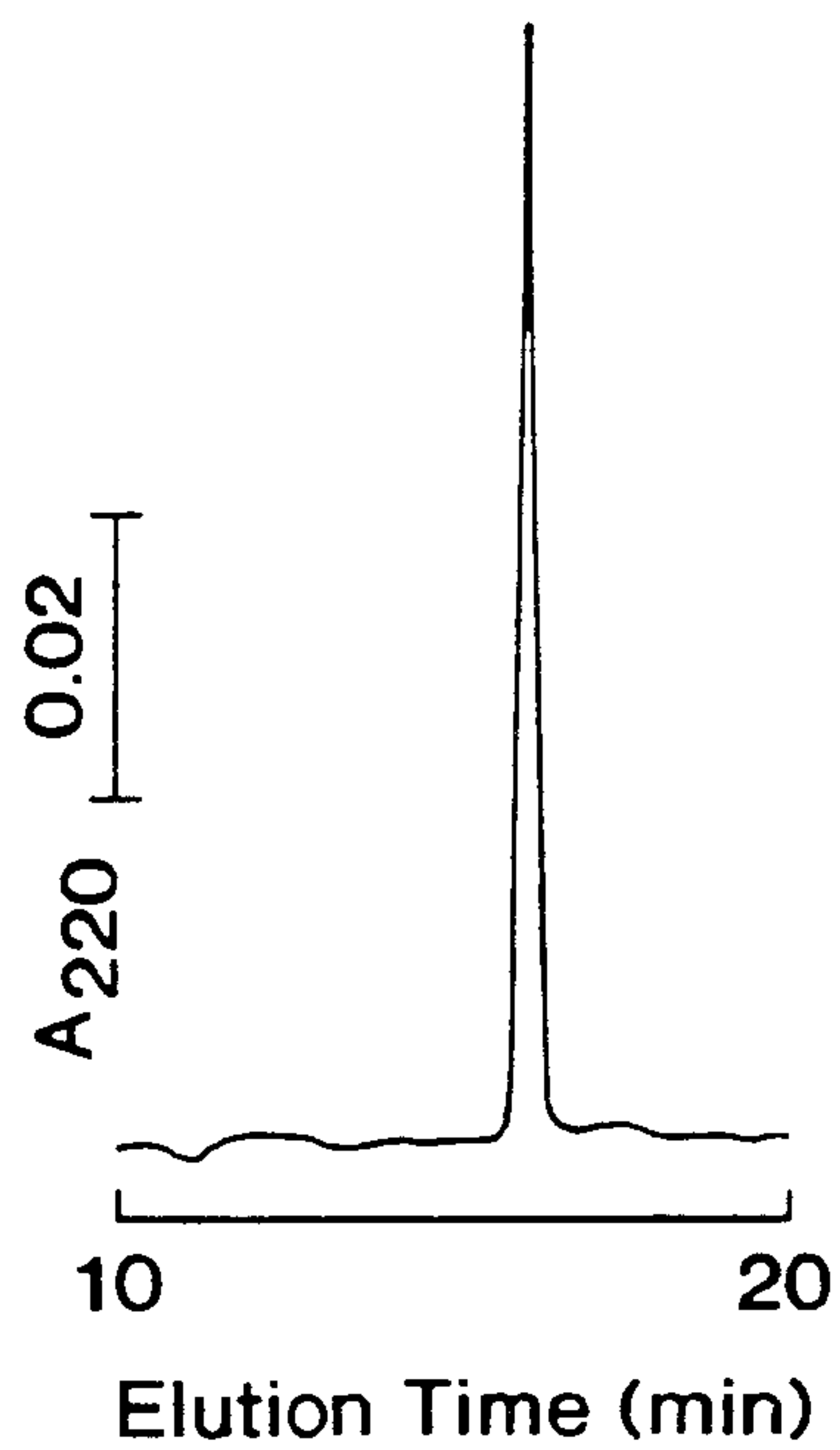


FIG. 1B

PEPTIDE	SEQUENCE	MASS (m/z)
T-2	G ¹ -F-C(R)	586.6 (586.7)
CT-1	C-R-C-L	704.5 (703.9)
T-3	C-L-C-R	704.6 (703.8)
CT-2	C-R-R-G-V-C	903.0 (903.1)
T-4	R-G-V-C-R	694.3 (694.8)
CT-3	R-C-I-C-T-R ¹⁸ -G ¹ (F)	1164.5 (1165.7)
T-1	C-I C-T-R	805.4 (805.0)
MeOH/HCl	T-R ¹⁸ -G ¹ -F-C-R-C-L-C-R-R-G-V-C-R-C-I-C	not analyzed

FIG. 2A

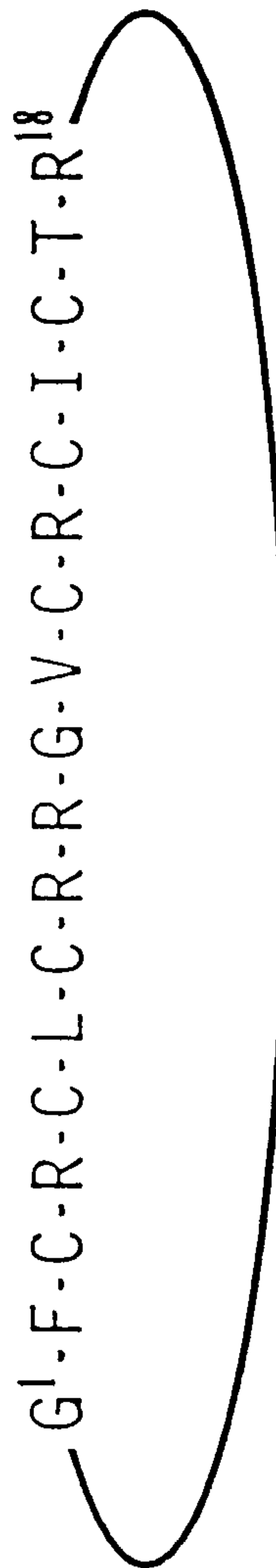


FIG. 2B

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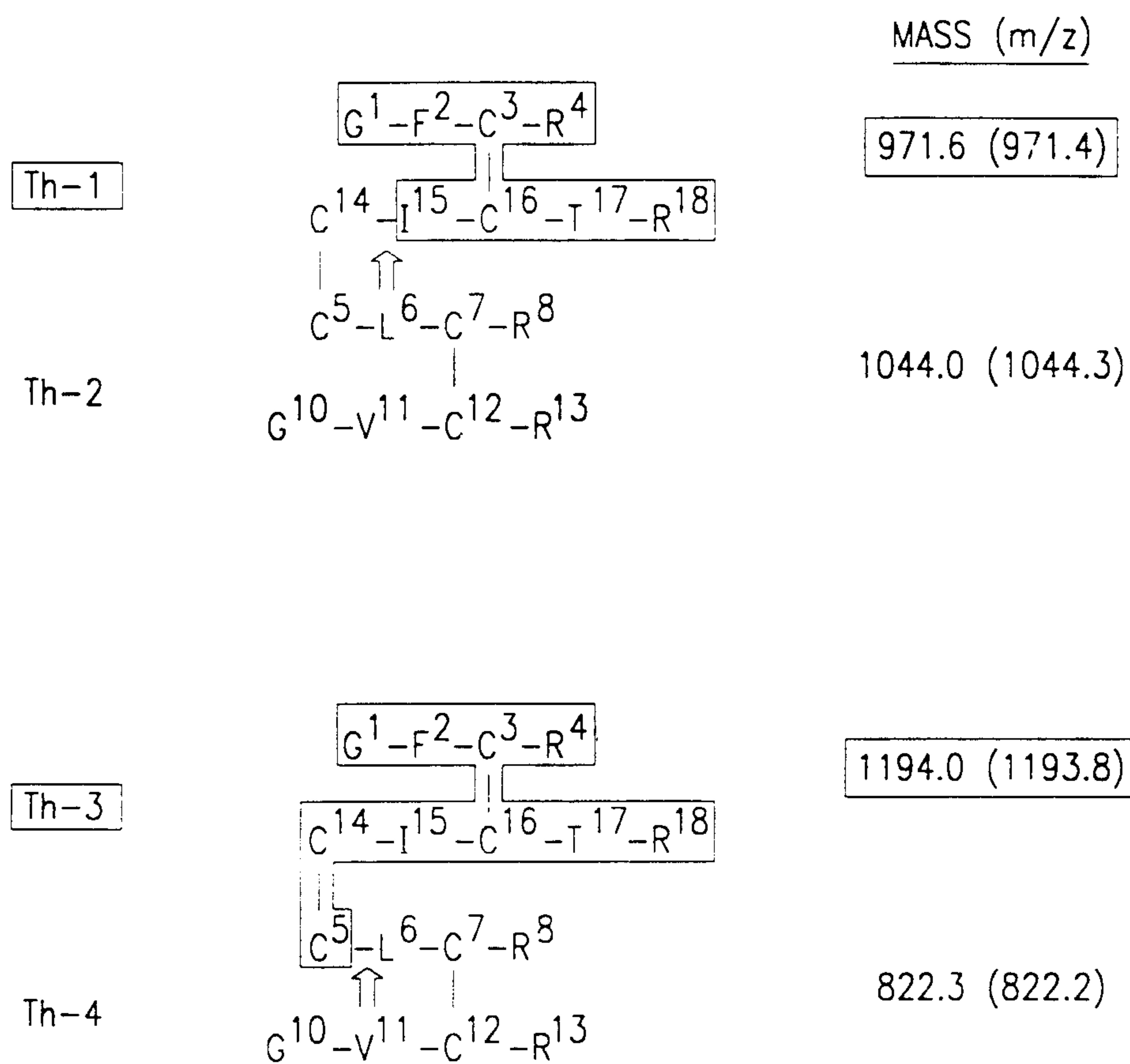
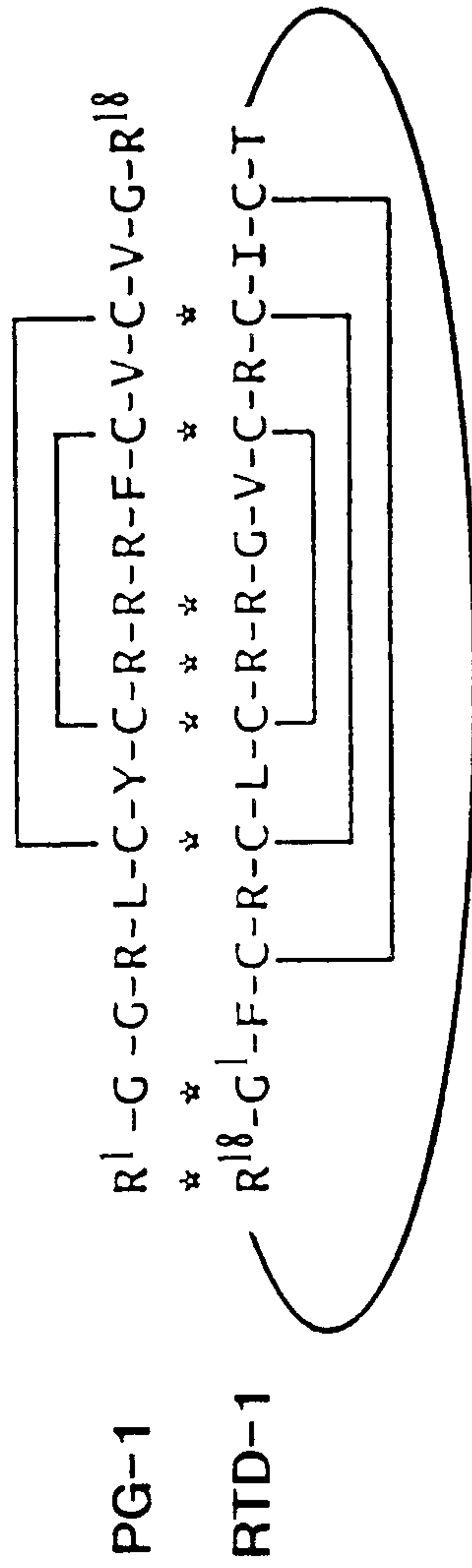
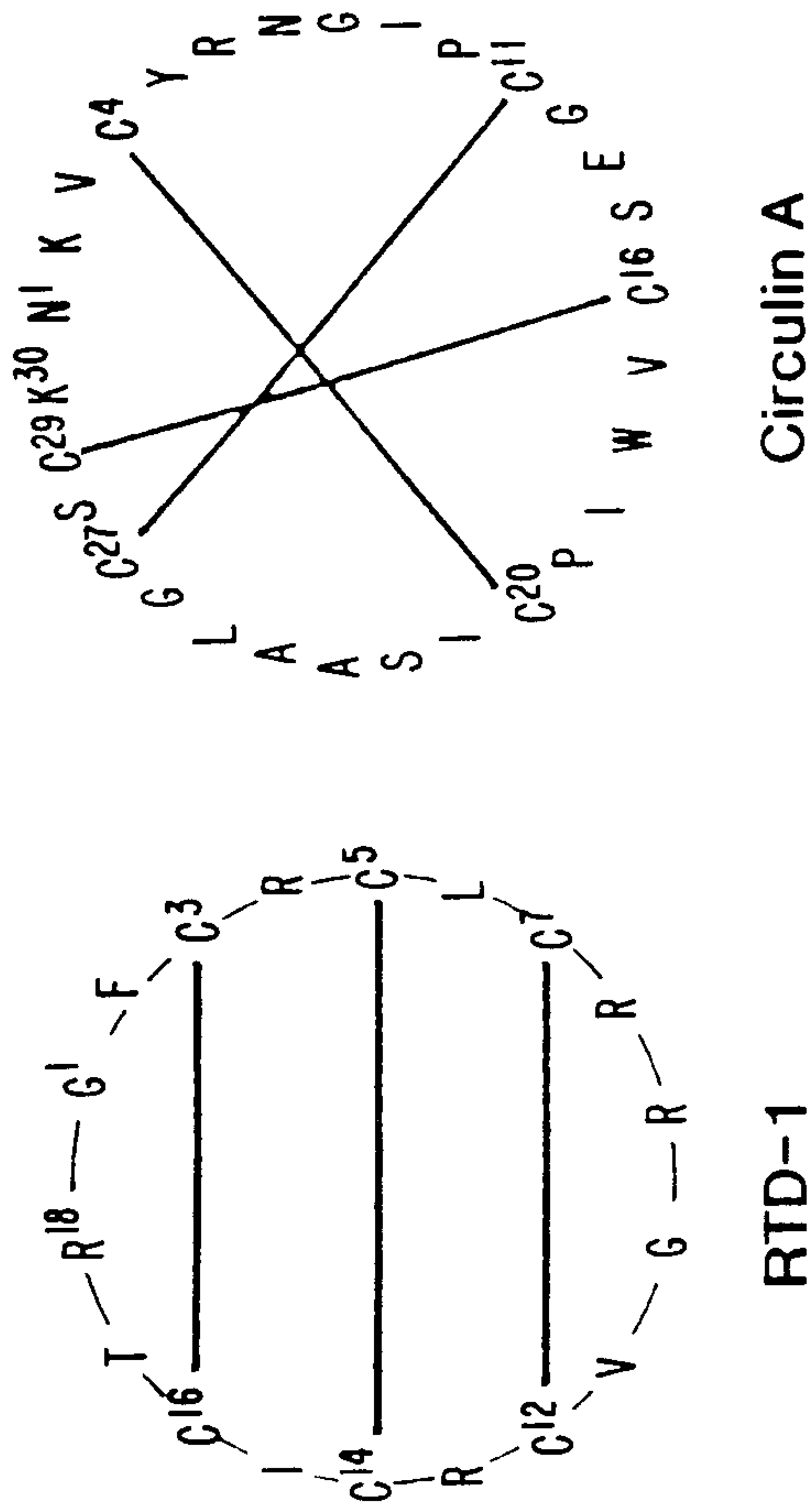
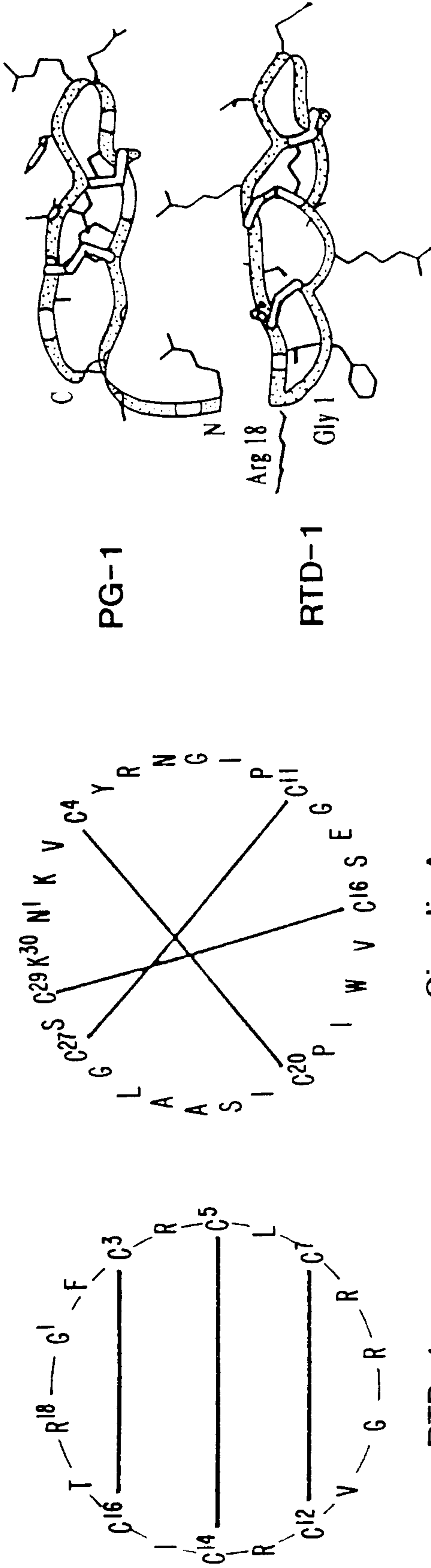


FIG. 3



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ATOM	1	N	ARG	1	4.445	1.973	1.040	0.00	0.00	N
ATOM	2	CA	ARG	1	5.522	1.436	0.144	0.00	0.00	C
ATOM	3	C	ARG	1	4.975	0.520	-1.001	0.00	0.00	C
ATOM	4	C	ARG	1	5.013	0.909	-2.171	0.00	0.00	O
ATOM	5	CB	ARG	1	6.757	0.901	0.940	0.00	0.00	C
ATOM	6	CG	ARG	1	8.115	0.817	0.196	0.00	0.00	C
ATOM	7	CD	ARG	1	8.247	-0.348	-0.805	0.00	0.00	C
ATOM	8	NE	ARG	1	9.635	-0.394	-1.339	0.00	0.00	N1+
ATOM	9	CZ	ARG	1	10.094	-1.285	-2.220	0.00	0.00	C
ATOM	10	NH1	ARG	1	9.363	-2.248	-2.726	0.00	0.00	N
ATOM	11	NH2	ARG	1	11.336	-1.194	-2.598	1.00	0.00	N
ATOM	12	H	ARG	1	4.004	2.884	0.861	0.00	0.00	H
ATOM	13	HA	ARG	1	5.896	2.332	-0.389	0.00	0.00	H
ATOM	14	1HB	ARG	1	6.913	1.571	1.809	0.00	0.00	H
ATOM	15	2HB	ARG	1	6.517	-0.076	1.403	0.00	0.00	H
ATOM	16	1HG	ARG	1	8.325	1.782	-0.307	0.00	0.00	H
ATOM	17	2HG	ARG	1	8.908	0.718	0.964	0.00	0.00	H
ATOM	18	1HD	ARG	1	7.985	-1.303	-0.303	0.00	0.00	H
ATOM	19	2HD	ARG	1	7.523	-0.218	-1.635	0.00	0.00	H
ATOM	20	HE	ARG	1	10.329	0.298	-1.044	1.00	0.00	H
ATOM	21	1HH1	ARG	1	8.398	-2.263	-2.391	0.00	0.00	H
ATOM	22	2HH1	ARG	1	9.794	-2.889	-3.396	0.00	0.00	H
ATOM	23	1HH2	ARG	1	11.891	-0.439	-2.191	0.00	0.00	H
ATOM	24	2HH2	ARG	1	11.669	-1.886	-3.272	0.00	0.00	H
ATOM	25	N	GLY	2	4.471	-0.678	-0.668	0.00	0.00	C
ATOM	26	CA	GLY	2	3.645	-1.487	-1.607	0.00	0.00	C
ATOM	27	C	GLY	2	2.571	-2.369	-0.935	0.00	0.00	O
ATOM	28	O	GLY	2	2.483	-3.558	-1.244	0.00	0.00	H
ATOM	29	H	GLY	2	4.420	-0.799	0.356	0.00	0.00	H
ATOM	30	1HA	GLY	2	3.133	-0.847	-2.352	0.00	0.00	H
ATOM	31	2HA	GLY	2	4.311	-2.140	-2.202	0.00	0.00	N
ATOM	32	N	PHE	3	1.744	-1.787	-0.048	1.00	0.00	C
ATOM	33	CA	PHE	3	0.707	-2.540	0.716	1.00	0.00	C
ATOM	34	C	PHE	3	-0.574	-1.652	0.805	1.00	0.00	O
ATOM	35	O	PHE	3	-0.719	-0.840	1.725	1.00	0.00	C
ATOM	36	CB	PHE	3	1.236	-2.949	2.125	1.00	0.00	C
ATOM	37	CG	PHE	3	2.397	-3.960	2.159	1.00	0.00	C
ATOM	38	CD1	PHE	3	3.705	-3.524	2.398	1.00	0.00	C
ATOM	39	CD2	PHE	3	2.159	-5.321	1.934	1.00	0.00	C
ATOM	40	CE1	PHE	3	4.760	-4.433	2.407	1.00	0.00	C
ATOM	41	CE2	PHE	3	3.215	-6.230	1.945	1.00	0.00	C
ATOM	42	CZ	PHE	3	4.514	-5.786	2.179	1.00	0.00	C
ATOM	43	H	PHE	3	1.994	-0.817	0.174	1.00	0.00	H
ATOM	44	HA	PHE	3	0.434	-3.475	0.183	1.00	0.00	H
ATOM	45	1HB	PHE	3	1.516	-2.038	2.686	1.00	0.00	H
ATOM	46	2HB	PHE	3	0.399	-3.371	2.714	1.00	0.00	H
ATOM	47	HD1	PHE	3	3.909	-2.478	2.569	1.00	0.00	H
ATOM	48	HD2	PHE	3	1.157	-5.676	1.741	1.00	0.00	H
ATOM	49	HE1	PHE	3	5.768	-4.090	2.587	1.00	0.00	H
ATOM	50	HE2	PHE	3	3.027	-7.279	1.768	1.00	0.00	H

FIG. 5A

SUBSTITUTE SHEET (RULE 26)

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ATOM	51	HZ	PHE	3	5.333	-6.491	2.183	1.00	0.00	H
ATOM	52	N	CYS	4	-1.485	-1.783	-0.178	1.00	0.00	N
ATOM	53	CA	CYS	4	-2.676	-0.902	-0.303	1.00	0.00	C
ATOM	54	C	CYS	4	-3.883	-1.384	0.565	1.00	0.00	C
ATOM	55	O	CYS	4	-4.495	-2.417	0.278	1.00	0.00	O
ATOM	56	CB	CYS	4	-3.015	-0.813	-1.807	1.00	0.00	V
ATOM	57	SG	CYS	4	-1.735	0.014	-2.797	1.00	0.00	D
ATOM	58	H	CYS	4	-1.244	-2.461	-0.908	1.00	0.00	H
ATOM	59	HA	CYS	4	-2.421	0.128	0.002	1.00	0.00	H
ATOM	60	HB	CYS	4	-3.217	-1.814	-2.236	1.00	0.00	H
ATOM	61	2HB	CYS	4	-3.953	-0.243	-1.946	1.00	0.00	H
ATOM	62	N	ARG	5	-4.225	-0.622	1.622	1.00	0.00	N
ATOM	63	CA	ARG	5	-5.375	-0.934	2.515	1.00	0.00	C
ATOM	64	C	ARG	5	-6.403	0.241	2.508	1.00	0.00	C
ATOM	65	O	ARG	5	-6.065	1.381	2.846	1.00	0.00	O
ATOM	66	CB	ARG	5	-4.827	-1.238	3.937	1.00	0.00	C
ATOM	67	CG	ARG	5	-5.877	-1.865	4.888	1.00	0.00	C
ATOM	68	CD	ARG	5	-5.313	-2.174	6.285	1.00	0.00	C
ATOM	69	NE	ARG	5	-6.356	-2.883	7.076	1.00	0.00	N1+
ATOM	70	CZ	ARG	5	-6.158	-3.497	8.243	1.00	0.00	C
ATOM	71	NH1	ARG	5	-5.016	-3.482	8.886	1.00	0.00	N
ATOM	72	NH2	ARG	5	-7.153	-4.148	8.774	1.00	0.00	N
ATOM	73	H	ARG	5	-3.631	0.210	1.776	1.00	0.00	H
ATOM	74	HA	ARG	5	-5.883	-1.856	2.164	1.00	0.00	H
ATOM	75	1HB	ARG	5	-3.968	-1.934	3.853	1.00	0.00	H
ATOM	76	2HB	ARG	5	-4.404	-0.313	4.380	1.00	0.00	H
ATOM	77	1HG	ARG	5	-6.752	-1.190	4.979	1.00	0.00	H
ATOM	78	2HG	ARG	5	-6.263	-2.796	4.425	1.00	0.00	H
ATOM	79	1HD	ARG	5	-4.398	-2.793	6.184	1.00	0.00	H
ATOM	80	2HD	ARG	5	-5.007	-1.233	6.787	1.00	0.00	H
ATOM	81	HE	ARG	5	-7.304	-2.991	6.704	1.00	0.00	H
ATOM	82	1HH1	ARG	5	-4.945	-3.988	9.771	1.00	0.00	H
ATOM	83	2HH1	ARG	5	-4.279	-2.952	8.417	1.00	0.00	H
ATOM	84	1HH2	ARG	5	-8.031	-4.162	8.252	1.00	0.00	H
ATOM	85	2HH2	ARG	5	-6.978	-4.625	9.661	1.00	0.00	H
ATOM	86	N	CYS	6	-7.669	-0.051	2.153	0.00	0.00	H
ATOM	87	CA	CYS	6	-8.750	0.970	2.109	0.00	0.00	C
ATOM	88	C	CYS	6	-9.798	0.729	3.238	0.00	0.00	C
ATOM	89	O	CYS	6	-10.685	-0.120	3.112	0.00	0.00	O
ATOM	90	CB	CYS	6	-9.307	1.004	0.667	0.00	0.00	C
ATOM	91	SG	CYS	6	-9.911	2.662	0.297	0.00	0.00	S
ATOM	92	H	CYS	6	-7.825	-1.007	1.819	0.00	0.00	H
ATOM	93	HA	CYS	6	-8.328	1.972	2.283	0.00	0.00	H
ATOM	94	1HB	CYS	6	-8.529	0.778	-0.088	0.00	0.00	H
ATOM	95	2HB	CYS	6	-10.109	0.258	0.513	0.00	0.00	H
ATOM	96	N	LEU	7	-9.654	1.449	4.368	1.00	0.00	N
ATOM	97	CA	LEU	7	-10.388	1.150	5.633	1.00	0.00	C
ATOM	98	C	LEU	7	-11.434	2.249	6.009	1.00	0.00	C
ATOM	99	O	LEU	7	-11.151	3.452	5.967	1.00	0.00	O
ATOM	100	CB	LEU	7	-9.374	0.760	6.757	1.00	0.00	C

FIG. 5B

SUBSTITUTE SHEET (RULE 26)

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ATOM	101	CG	LEU	7	-8.386	1.757	7.435	1.00	0.00	C
ATOM	102	CD1	LEU	7	-7.508	2.568	6.467	1.00	0.00	C
ATOM	103	CD2	LEU	7	-9.054	2.697	8.452	1.00	0.00	C
ATOM	104	H	LEU	7	-8.903	2.151	4.337	1.00	0.00	H
ATOM	105	HA	LEU	7	-10.966	0.215	5.476	1.00	0.00	H
ATOM	106	1HB	LEU	7	-9.954	0.273	7.565	1.00	0.00	H
ATOM	107	2HB	LEU	7	-8.762	-0.073	6.359	1.00	0.00	H
ATOM	108	HG	LEU	7	-7.689	1.128	8.025	1.00	0.00	H
ATOM	109	1HD1	LEU	7	-6.711	3.120	6.998	1.00	0.00	H
ATOM	110	2HD1	LEU	7	-7.007	1.923	5.722	1.00	0.00	H
ATOM	111	3HD1	LEU	7	-8.097	3.320	5.909	1.00	0.00	H
ATOM	112	1HD2	LEU	7	-8.302	3.262	9.032	1.00	0.00	H
ATOM	113	2HD2	LEU	7	-9.705	3.447	7.967	1.00	0.00	H
ATOM	114	3HD2	LEU	7	-9.676	2.141	9.177	1.00	0.00	H
ATOM	115	N	CYS	8	-12.667	1.827	6.355	0.00	0.00	N
ATOM	116	CA	CYS	8	-13.782	2.747	6.706	0.00	0.00	C
ATOM	117	C	CYS	8	-13.756	3.194	8.202	0.00	0.00	C
ATOM	118	O	CYS	8	-13.835	2.372	9.120	0.00	0.00	O
ATOM	119	CB	CYS	8	-15.112	2.052	6.334	0.00	0.00	C
ATOM	120	SG	CYS	8	-15.536	2.183	4.573	0.00	0.00	S
ATOM	121	H	CYS	8	-12.799	0.811	6.359	0.00	0.00	H
ATOM	122	HA	CYS	8	-13.732	3.653	6.080	0.00	0.00	H
ATOM	123	1HB	CYS	8	-15.135	0.991	6.649	0.00	0.00	H
ATOM	124	2HB	CYS	8	-15.953	2.528	6.873	0.00	0.00	H
ATOM	125	N	ARG	9	-13.685	4.516	8.431	0.00	0.00	N
ATOM	126	CA	ARG	9	-13.874	5.123	9.780	0.00	0.00	C
ATOM	127	C	ARG	9	-15.143	6.030	9.740	0.00	0.00	C
ATOM	128	O	ARG	9	-15.171	7.045	9.036	0.00	0.00	O
ATOM	129	CB	ARG	9	-12.598	5.905	10.199	0.00	0.00	C
ATOM	130	CG	ARG	9	-11.403	5.004	10.601	0.00	0.00	C
ATOM	131	CD	ARG	9	-10.168	5.811	11.037	0.00	0.00	C
ATOM	132	NE	ARG	9	-9.107	4.873	11.498	0.00	0.00	N1+
ATOM	133	CZ	ARG	9	-7.962	5.228	12.082	0.00	0.00	C
ATOM	134	NH1	ARG	9	-7.612	6.472	12.300	0.00	0.00	N
ATOM	135	NH2	ARG	9	-7.145	4.287	12.460	0.00	0.00	N
ATOM	136	H	ARG	9	-13.622	5.093	7.583	0.00	0.00	H
ATOM	137	HA	ARG	9	-14.035	4.337	10.547	0.00	0.00	H
ATOM	138	1HB	ARG	9	-12.303	6.603	9.389	0.00	0.00	H
ATOM	139	2HB	ARG	9	-12.846	6.559	11.059	0.00	0.00	H
ATOM	140	1HG	ARG	9	-11.723	4.325	11.418	0.00	0.00	H
ATOM	141	2HG	ARG	9	-11.146	4.340	9.752	0.00	0.00	H
ATOM	142	1HD	ARG	9	-9.805	6.431	10.192	0.00	0.00	H
ATOM	143	2HD	ARG	9	-10.454	6.513	11.848	0.00	0.00	H
ATOM	144	HE	ARG	9	-9.235	3.861	11.406	0.00	0.00	H
ATOM	145	1HH1	ARG	9	-8.298	7.161	11.984	0.00	0.00	H
ATOM	146	2HH1	ARG	9	-6.722	6.657	12.767	0.00	0.00	H
ATOM	147	1HH2	ARG	9	-7.438	3.323	12.293	0.00	0.00	H
ATOM	148	2HH2	ARG	9	-6.278	4.578	12.915	0.00	0.00	H
ATOM	149	N	ARG	10	-16.215	5.637	10.462	0.00	0.00	N
ATOM	150	CA	ARG	10	-17.558	6.306	10.411	0.00	0.00	C

FIG. 5C

SUBSTITUTE SHEET (RULE 26)

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ATOM	151	C	ARG	10	-18.386	6.033	9.106	0.00	0.00	C
ATOM	152	O	ARG	10	-19.548	5.622	9.208	0.00	0.00	O
ATOM	153	CB	ARG	10	-17.530	7.803	10.875	0.00	0.00	C
ATOM	154	CG	ARG	10	-18.722	8.328	11.730	0.00	0.00	C
ATOM	155	CD	ARG	10	-19.968	8.899	11.012	0.00	0.00	C
ATOM	156	NE	ARG	10	-20.779	7.815	10.397	0.00	0.00	N1+
ATOM	157	CZ	ARG	10	-22.072	7.865	10.088	0.00	0.00	C
ATOM	158	NH1	ARG	10	-22.840	8.904	10.304	0.00	0.00	N
ATOM	159	NH2	ARG	10	-22.596	6.811	9.533	0.00	0.00	N
ATOM	160	H	ARG	10	-16.078	4.751	10.960	0.00	0.00	H
ATOM	161	HA	ARG	10	-18.128	5.773	11.197	0.00	0.00	H
ATOM	162	1HB	ARG	10	-16.626	7.944	11.500	0.00	0.00	H
ATOM	163	2HB	ARG	10	-17.343	8.470	10.010	0.00	0.00	H
ATOM	164	1HG	ARG	10	-19.024	7.571	12.481	0.00	0.00	H
ATOM	165	2HG	ARG	10	-18.324	9.154	12.353	0.00	0.00	H
ATOM	166	1HD	ARG	10	-20.560	9.461	11.764	0.00	0.00	H
ATOM	167	2HD	ARG	10	-19.665	9.645	10.248	0.00	0.00	H
ATOM	168	HE	ARG	10	-20.325	6.941	10.080	1.00	0.00	H
ATOM	169	1HH1	ARG	10	-22.362	9.705	10.720	0.00	0.00	H
ATOM	170	2HH1	ARG	10	-23.822	8.848	10.030	0.00	0.00	H
ATOM	171	1HH2	ARG	10	-21.958	6.023	9.378	0.00	0.00	H
ATOM	172	2HH2	ARG	10	-23.590	6.840	9.301	0.00	0.00	H
ATOM	173	N	GLY	11	-17.826	6.288	7.910	1.00	0.00	N
ATOM	174	CA	GLY	11	-18.511	6.016	6.617	1.00	0.00	C
ATOM	175	C	GLY	11	-17.565	5.667	5.451	1.00	0.00	C
ATOM	176	O	GLY	11	-17.541	4.520	5.003	1.00	0.00	O
ATOM	177	H	GLY	11	-16.840	6.585	7.986	1.00	0.00	H
ATOM	178	1HA	GLY	11	-19.226	5.175	6.720	1.00	0.00	H
ATOM	179	2HA	GLY	11	-19.136	6.884	6.335	1.00	0.00	H
ATOM	180	N	VAL	12	-16.817	6.655	4.932	1.00	0.00	N
ATOM	181	CA	VAL	12	-15.952	6.477	3.722	1.00	0.00	C
ATOM	182	C	VAL	12	-14.651	5.637	3.988	1.00	0.00	C
ATOM	183	O	VAL	12	-13.950	5.844	4.985	1.00	0.00	O
ATOM	184	CB	VAL	12	-15.688	7.880	3.064	1.00	0.00	C
ATOM	185	CG1	VAL	12	-14.756	8.829	3.857	1.00	0.00	C
ATOM	186	CG2	VAL	12	-15.155	7.773	1.617	1.00	0.00	C
ATOM	187	H	VAL	12	-16.891	7.553	5.419	1.00	0.00	H
ATOM	188	HA	VAL	12	-16.566	5.916	2.987	1.00	0.00	H
ATOM	189	HB	VAL	12	-16.669	8.392	2.979	1.00	0.00	H
ATOM	190	1HG1	VAL	12	-13.729	8.426	3.491	1.00	0.00	H
ATOM	191	2HG1	VAL	12	-14.677	9.824	3.382	1.00	0.00	H
ATOM	192	3HG1	VAL	12	-15.118	8.997	4.887	1.00	0.00	H
ATOM	193	1HG2	VAL	12	-14.141	7.330	1.573	1.00	0.00	H
ATOM	194	2HG2	VAL	12	-15.809	7.151	0.978	1.00	0.00	H
ATOM	195	3HG3	VAL	12	-15.093	8.764	1.127	1.00	0.00	N
ATOM	196	N	CYS	13	-14.317	4.713	3.063	0.00	0.00	N
ATOM	197	CA	CYS	13	-13.052	3.937	3.118	0.00	0.00	C
ATOM	198	C	CYS	13	-11.828	4.755	2.598	0.00	0.00	C
ATOM	199	O	CYS	13	-11.730	5.076	1.409	0.00	0.00	O
ATOM	200	CB	CYS	13	-13.246	2.600	2.374	0.00	0.00	C

FIG. 5D

SUBSTITUTE SHEET (RULE 26)

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ATOM	201	SG	CYS	13	-14.168	1.373	3.345	0.00	0.00	S
ATOM	202	H	CYS	13	-14.951	4.647	2.261	0.00	0.00	H
ATOM	203	HA	CYS	13	-12.854	3.655	4.159	0.00	0.00	H
ATOM	204	1HB	CYS	13	-13.735	2.743	1.392	0.00	0.00	H
ATOM	205	2HB	CYS	13	-12.268	2.139	2.143	0.00	0.00	H
ATOM	206	N	ARG	14	-10.889	5.085	3.504	1.00	0.00	N
ATOM	207	CA	ARG	14	-9.666	5.853	3.152	1.00	0.00	C
ATOM	208	C	ARG	14	-8.532	4.905	2.651	1.00	0.00	C
ATOM	209	O	ARG	14	-7.977	4.113	3.420	1.00	0.00	O
ATOM	210	CB	ARG	14	-9.264	6.723	4.379	1.00	0.00	C
ATOM	211	CG	ARG	14	-7.945	7.536	4.266	1.00	0.00	C
ATOM	212	CD	ARG	14	-7.860	8.519	3.077	1.00	0.00	C
ATOM	213	NE	ARG	14	-6.479	9.065	2.938	1.00	0.00	N1+
ATOM	214	CZ	ARG	14	-6.046	10.234	3.413	1.00	0.00	C
ATOM	215	NH1	ARG	14	-6.786	11.050	4.122	1.00	0.00	N
ATOM	216	NH2	ARG	14	-4.818	10.584	3.160	1.00	0.00	N
ATOM	217	H	ARG	14	-11.032	4.677	4.441	1.00	0.00	H
ATOM	218	HA	ARG	14	-9.924	6.571	2.346	1.00	0.00	H
ATOM	219	1HB	ARG	14	-10.097	7.412	4.620	1.00	0.00	H
ATOM	220	2HB	ARG	14	-9.181	6.068	5.270	1.00	0.00	H
ATOM	221	1HG	ARG	14	-7.777	8.084	5.213	1.00	0.00	H
ATOM	222	2HG	ARG	14	-7.104	6.818	4.213	1.00	0.00	H
ATOM	223	1HD	ARG	14	-8.098	7.987	2.134	1.00	0.00	H
ATOM	224	2HD	ARG	14	-8.640	9.304	3.144	1.00	0.00	H
ATOM	225	HE	ARG	14	-5.774	8.543	2.406	1.00	0.00	H
ATOM	226	1HH1	ARG	14	-6.381	11.933	4.437	1.00	0.00	H
ATOM	227	2HH1	ARG	14	-7.735	10.711	4.288	1.00	0.00	H
ATOM	228	1HH2	ARG	14	-4.259	9.941	2.597	1.00	0.00	H
ATOM	229	2HH2	ARG	14	-4.506	11.486	3.525	1.00	0.00	H
ATOM	230	N	CYS	15	-8.170	5.034	1.363	0.00	0.00	N
ATOM	231	CA	CYS	15	-7.079	4.245	0.743	0.00	0.00	C
ATOM	232	C	CYS	15	-5.655	4.759	1.143	0.00	0.00	C
ATOM	233	O	CYS	15	-5.259	5.883	0.814	0.00	0.00	O
ATOM	234	CB	CYS	15	-7.334	4.257	-0.778	0.00	0.00	C
ATOM	235	SG	CYS	15	-8.884	3.429	-1.256	0.00	0.00	S
ATOM	236	H	CYS	15	-8.792	5.616	0.794	0.00	0.00	H
ATOM	237	HA	CYS	15	-7.164	3.186	1.042	0.00	0.00	H
ATOM	238	1HB	CYS	15	-7.333	5.288	-1.182	0.00	0.00	H
ATOM	239	2HB	CYS	15	-6.511	3.736	-1.303	0.00	0.00	H
ATOM	240	N	ILE	16	-4.902	3.917	1.872	1.00	0.00	N
ATOM	241	CA	ILE	16	-3.499	4.208	2.301	1.00	0.00	C
ATOM	242	C	ILE	16	-2.563	3.058	1.802	1.00	0.00	C
ATOM	243	O	ILE	16	-2.787	1.881	2.109	1.00	0.00	O
ATOM	244	CB	ILE	16	-3.362	4.466	3.848	1.00	0.00	C
ATOM	245	CG1	ILE	16	-3.997	3.381	4.774	1.00	0.00	C
ATOM	246	CG2	ILE	16	-3.880	5.877	4.225	1.00	0.00	C
ATOM	247	CD1	ILE	16	-3.464	3.357	6.218	1.00	0.00	C
ATOM	248	H	ILE	16	-5.380	3.056	2.182	1.00	0.00	H
ATOM	249	HA	ILE	16	-3.153	5.136	1.802	1.00	0.00	H
ATOM	250	HB	ILE	16	-2.274	4.477	4.065	1.00	0.00	H

FIG. 5E

SUBSTITUTE SHEET (RULE 26)

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ATOM	251	1HG1	ILE	16	-5.099	3.490	4.779	1.00	0.00	H
ATOM	252	2HG1	ILE	16	-3.827	2.376	4.343	1.00	0.00	H
ATOM	253	1HG2	ILE	16	-3.687	6.125	5.285	1.00	0.00	H
ATOM	254	2HG2	ILE	16	-3.394	6.668	3.624	1.00	0.00	H
ATOM	255	3HG2	ILE	16	-4.969	5.964	4.060	1.00	0.00	H
ATOM	256	1HD1	ILE	16	-3.945	2.557	6.809	1.00	0.00	H
ATOM	257	2HD1	ILE	16	-2.373	3.171	6.247	1.00	0.00	H
ATOM	258	3HD1	ILE	16	-3.652	4.308	6.749	1.00	0.00	H
ATOM	259	N	CYS	17	-1.511	3.396	1.030	1.00	0.00	N
ATOM	260	CA	CYS	17	-0.568	2.392	0.470	1.00	0.00	C
ATOM	261	C	CYS	17	0.877	2.602	1.011	1.00	0.00	C
ATOM	262	O	CYS	17	1.608	3.490	0.562	1.00	0.00	O
ATOM	263	CB	CYS	17	-0.695	2.398	-1.069	1.00	0.00	C
ATOM	264	SG	CYS	17	-0.233	0.772	-1.695	1.00	0.00	S
ATOM	265	H	CYS	17	-1.430	4.391	0.798	1.00	0.00	H
ATOM	266	HA	CYS	17	-0.878	1.380	0.777	1.00	0.00	H
ATOM	267	1HB	CYS	17	-1.734	2.590	-1.399	1.00	0.00	H
ATOM	268	2HB	CYS	17	-0.072	3.185	-1.536	1.00	0.00	H
ATOM	269	N	THRC	18	1.286	1.785	2.001	1.00	0.00	N
ATOM	270	CA	THRC	18	2.596	1.951	2.701	1.00	0.00	C
ATOM	271	C	THRC	18	3.762	1.236	1.943	1.00	0.00	C
ATOM	272	OXT	THRC	18	4.027	0.047	2.147	1.00	0.00	O
ATOM	273	CB	THRC	18	2.448	1.596	4.214	1.00	0.00	C
ATOM	274	OG1	THRC	18	3.682	1.831	4.877	1.00	0.00	O
ATOM	275	CG2	THRC	18	2.017	0.167	4.594	1.00	0.00	C
ATOM	276	HN	THRC	18	0.597	1.079	2.289	1.00	0.00	H
ATOM	277	HA	THRC	18	2.845	3.033	2.723	1.00	0.00	H
ATOM	278	HB	THRC	18	1.702	2.293	4.648	1.00	0.00	H
ATOM	279	HG1	THRC	18	4.200	1.031	4.751	1.00	0.00	H
ATOM	280	1HG2	THRC	18	1.934	0.057	5.692	1.00	0.00	H
ATOM	281	2HG2	THRC	18	1.027	-0.090	4.175	1.00	0.00	H
ATOM	282	3HG2	THRC	18	2.733	-0.599	4.244	1.00	0.00	H
TER										

FIG. 5F

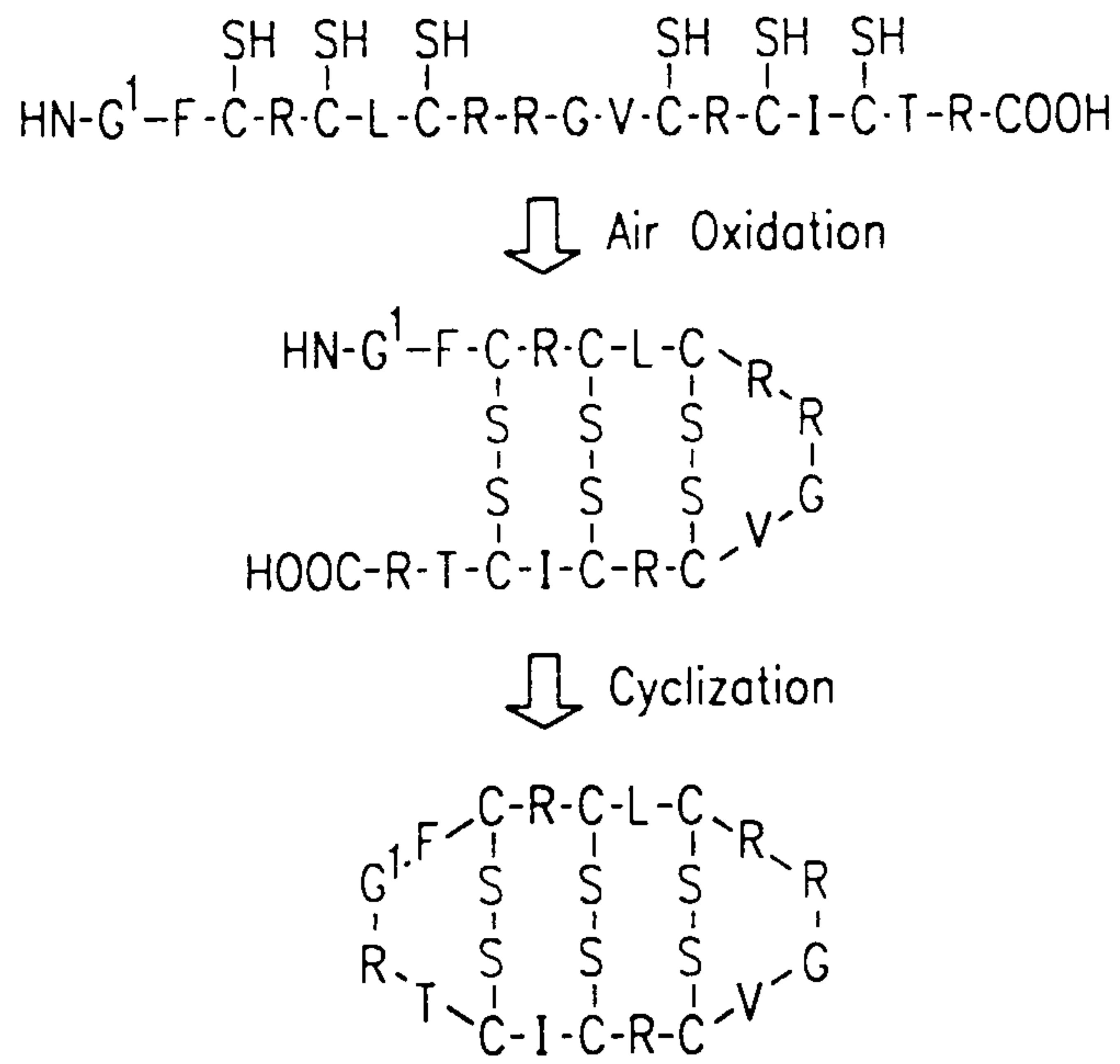


FIG. 6A

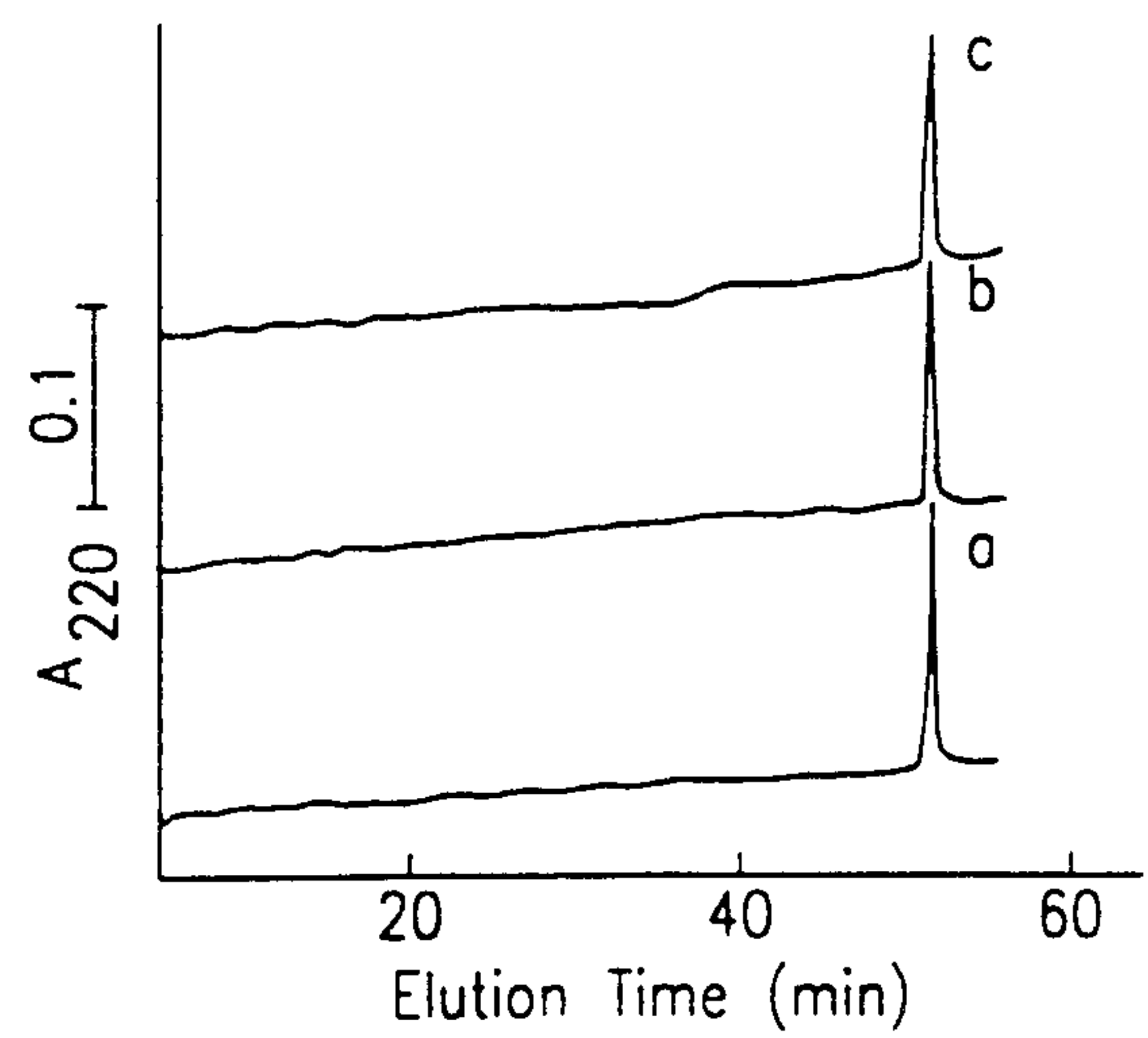


FIG. 6B

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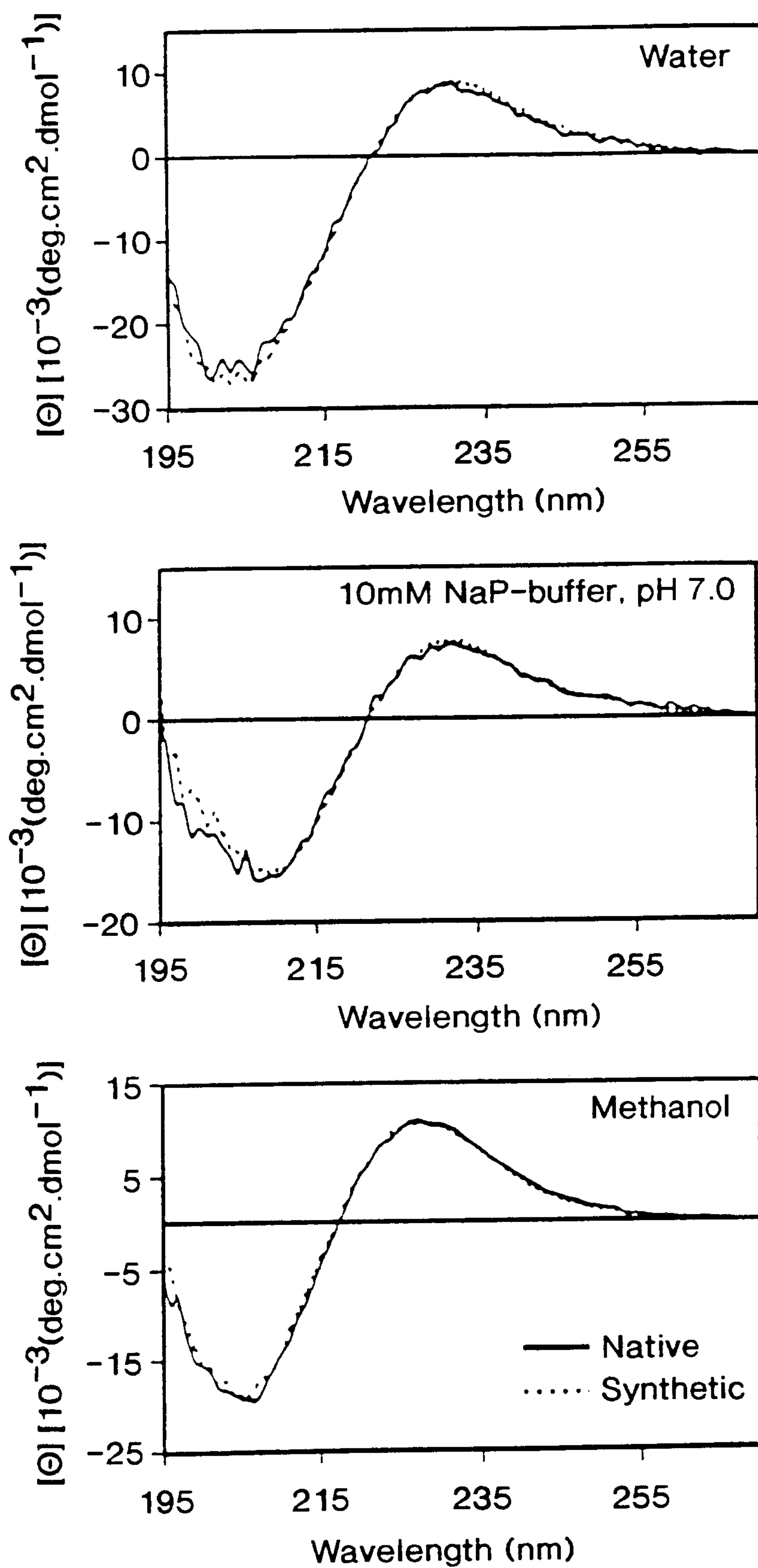


FIG. 6C

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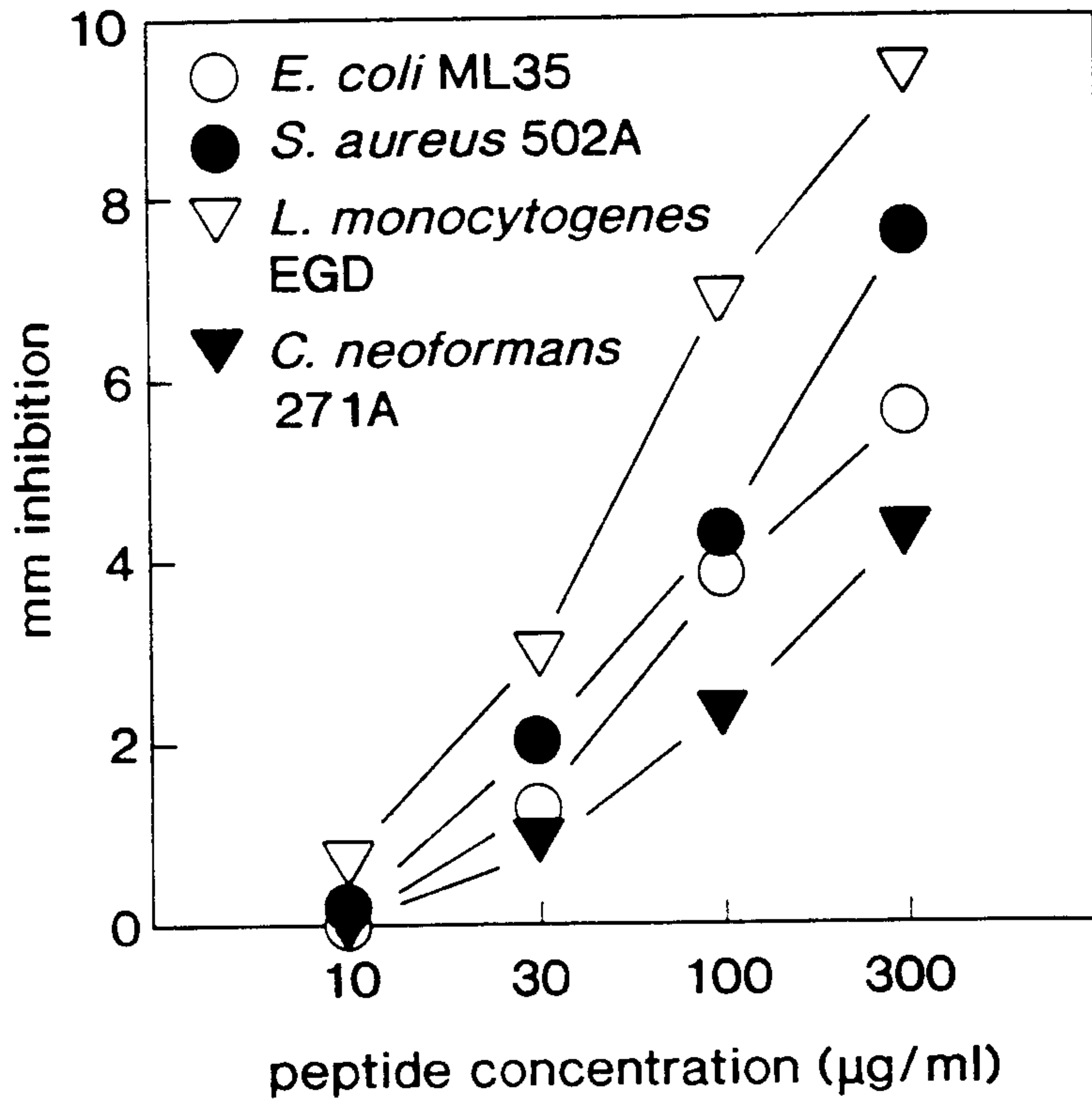


FIG. 7

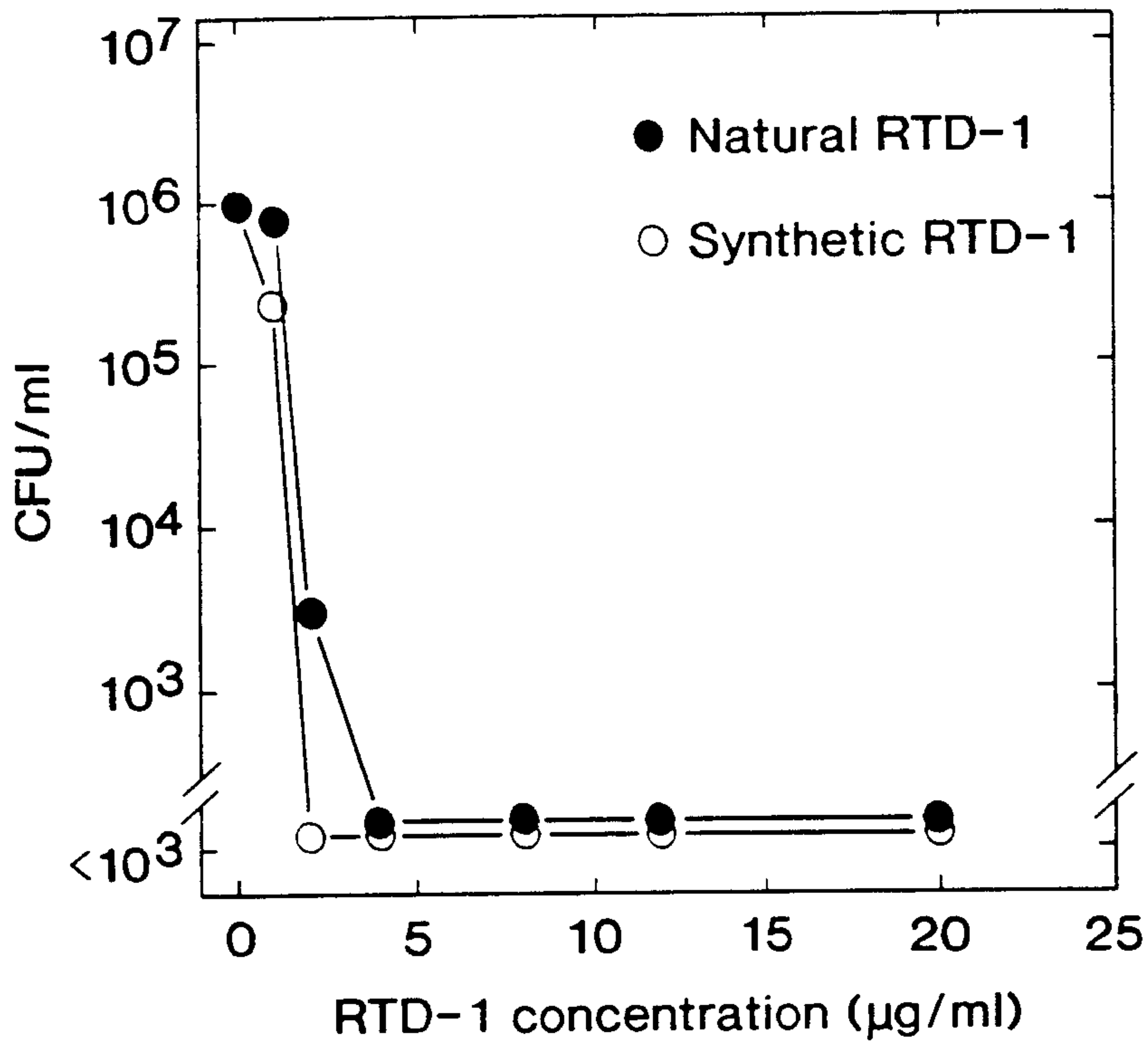


FIG. 8

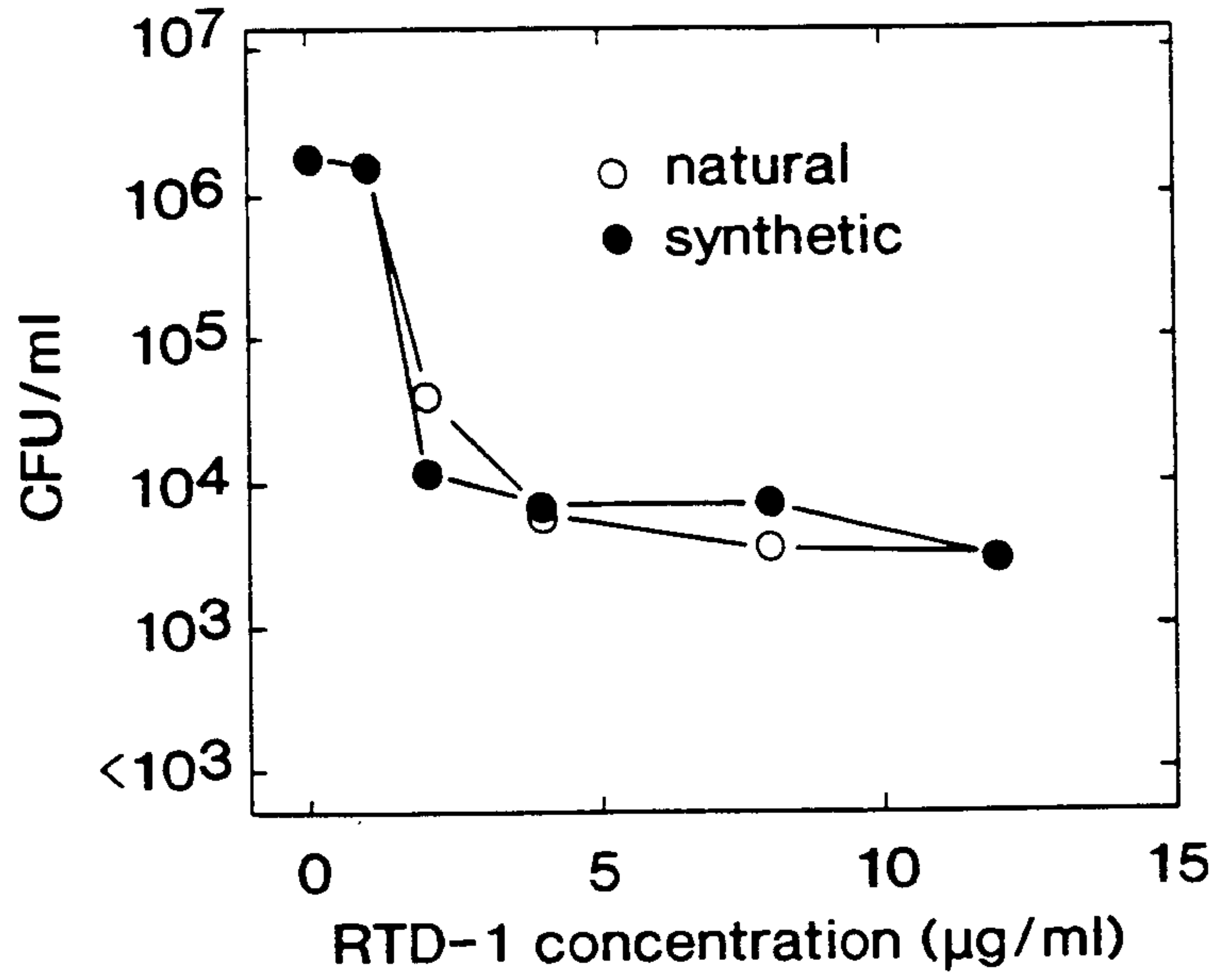


FIG. 9A

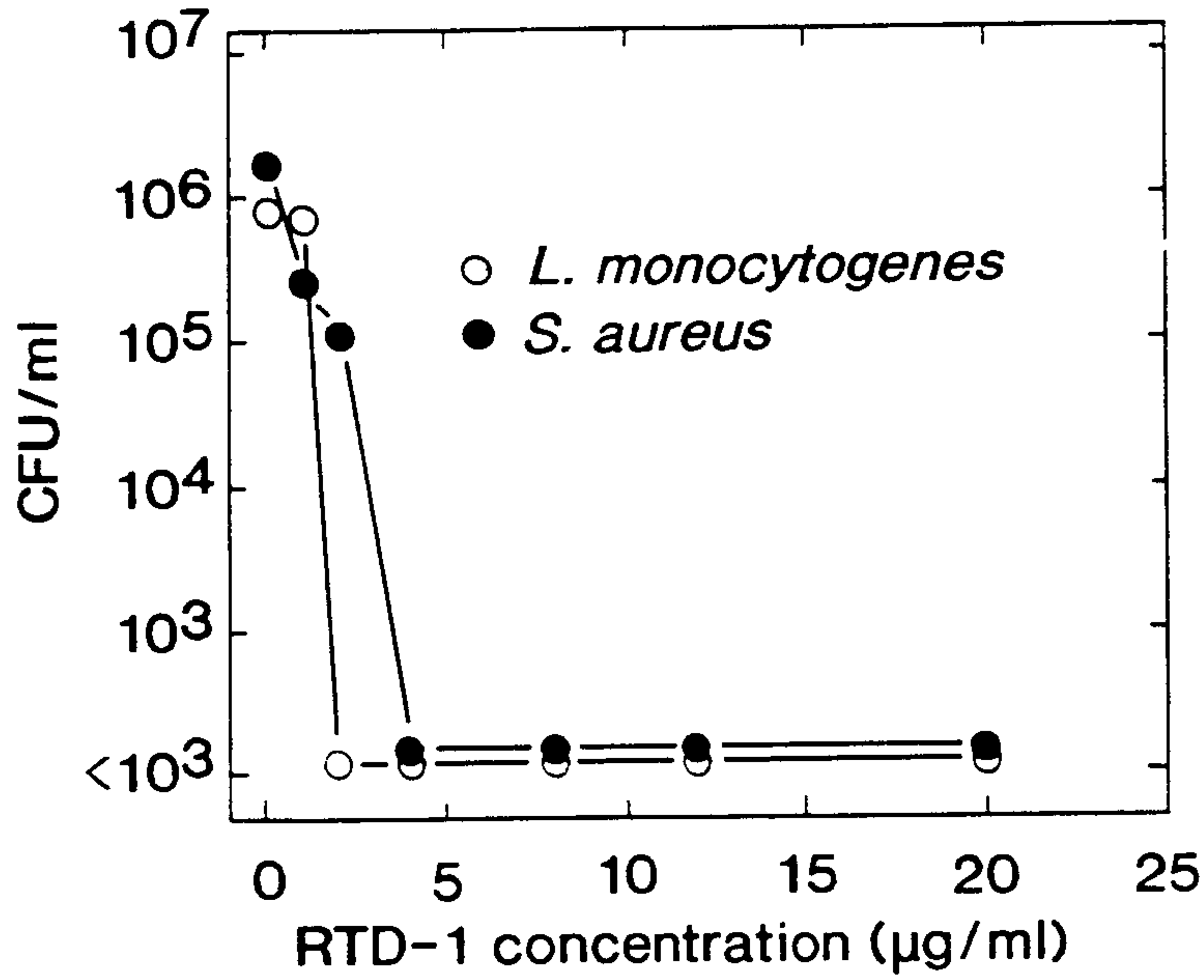


FIG. 9B

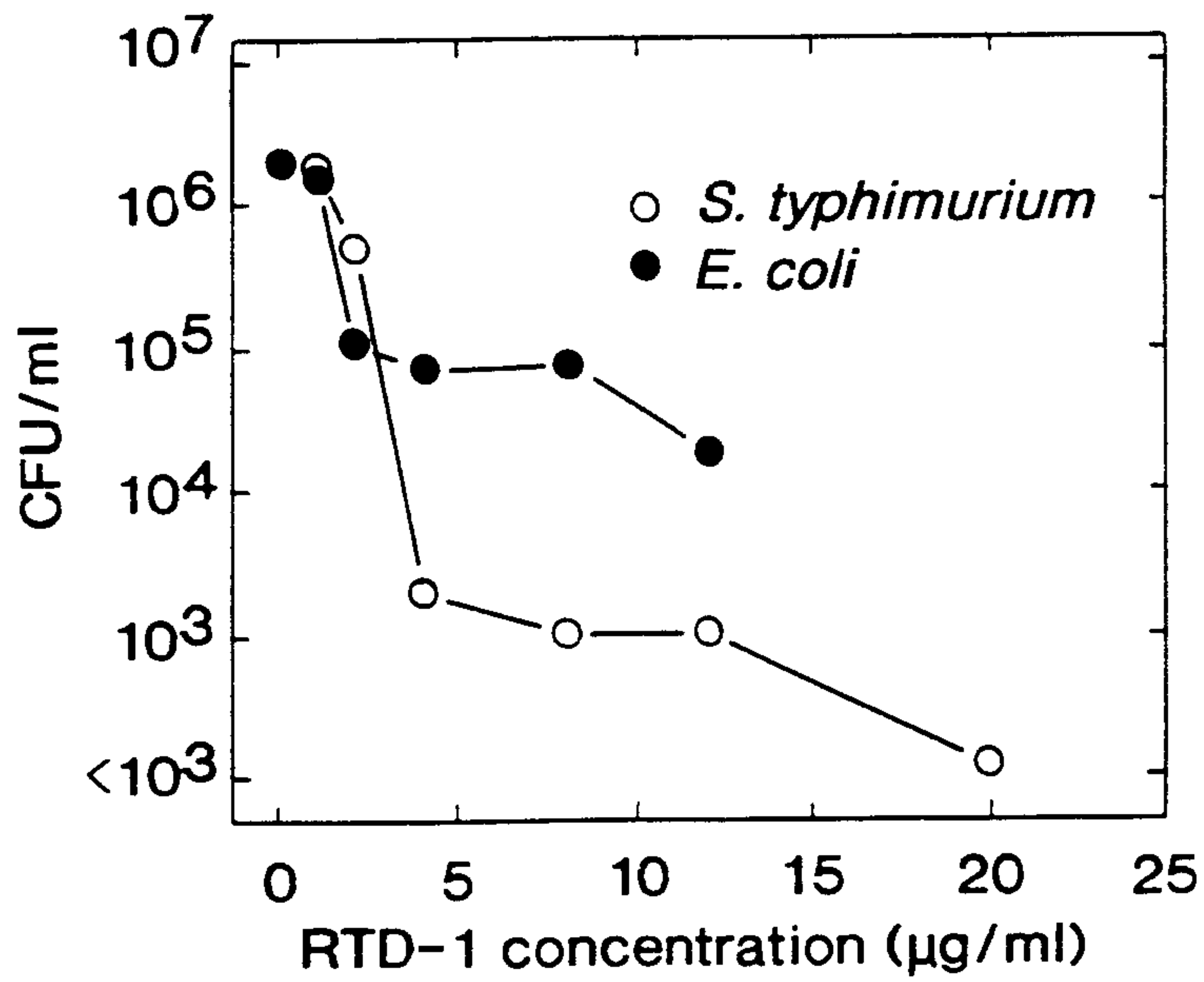


FIG. 9C

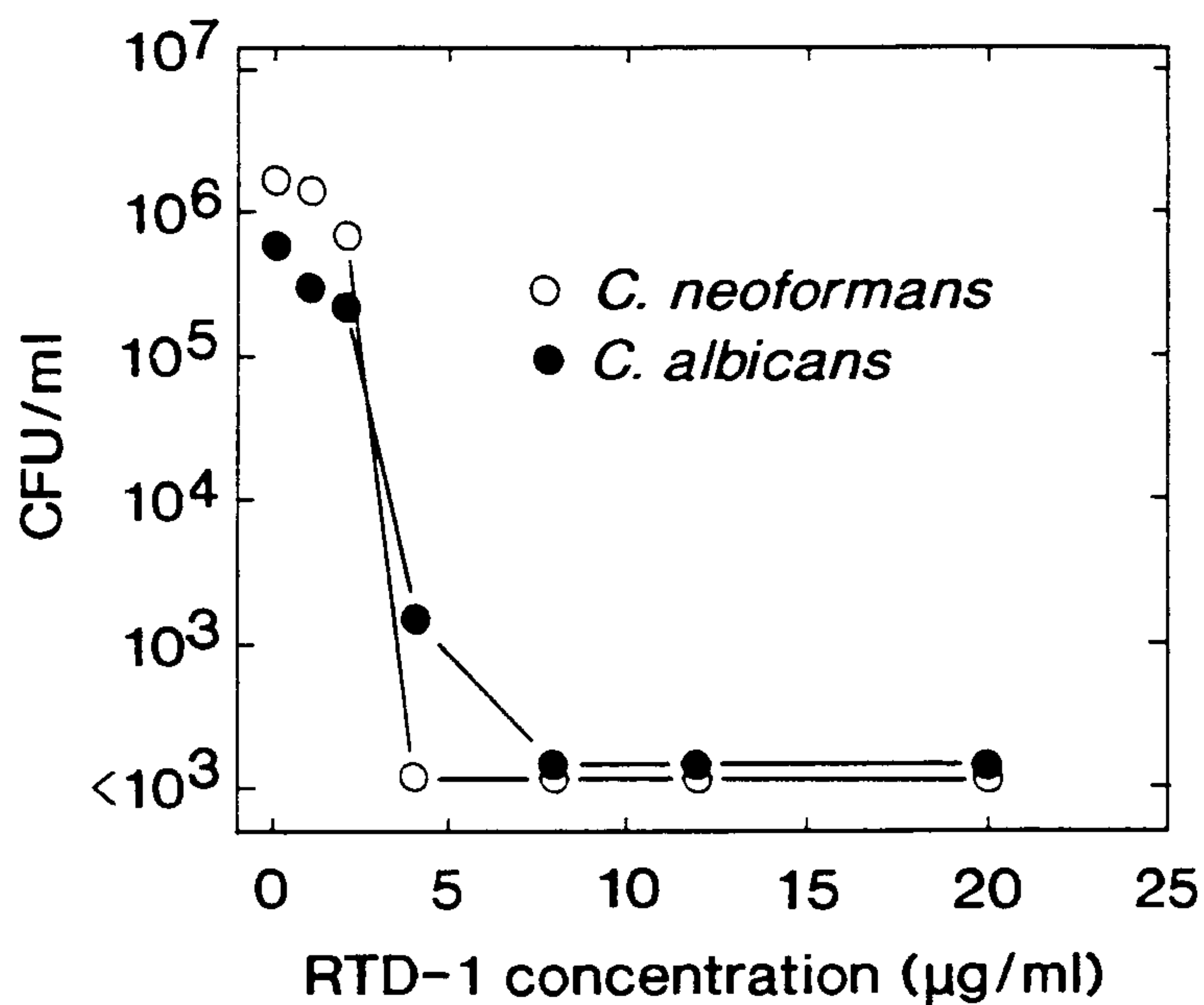


FIG. 9D

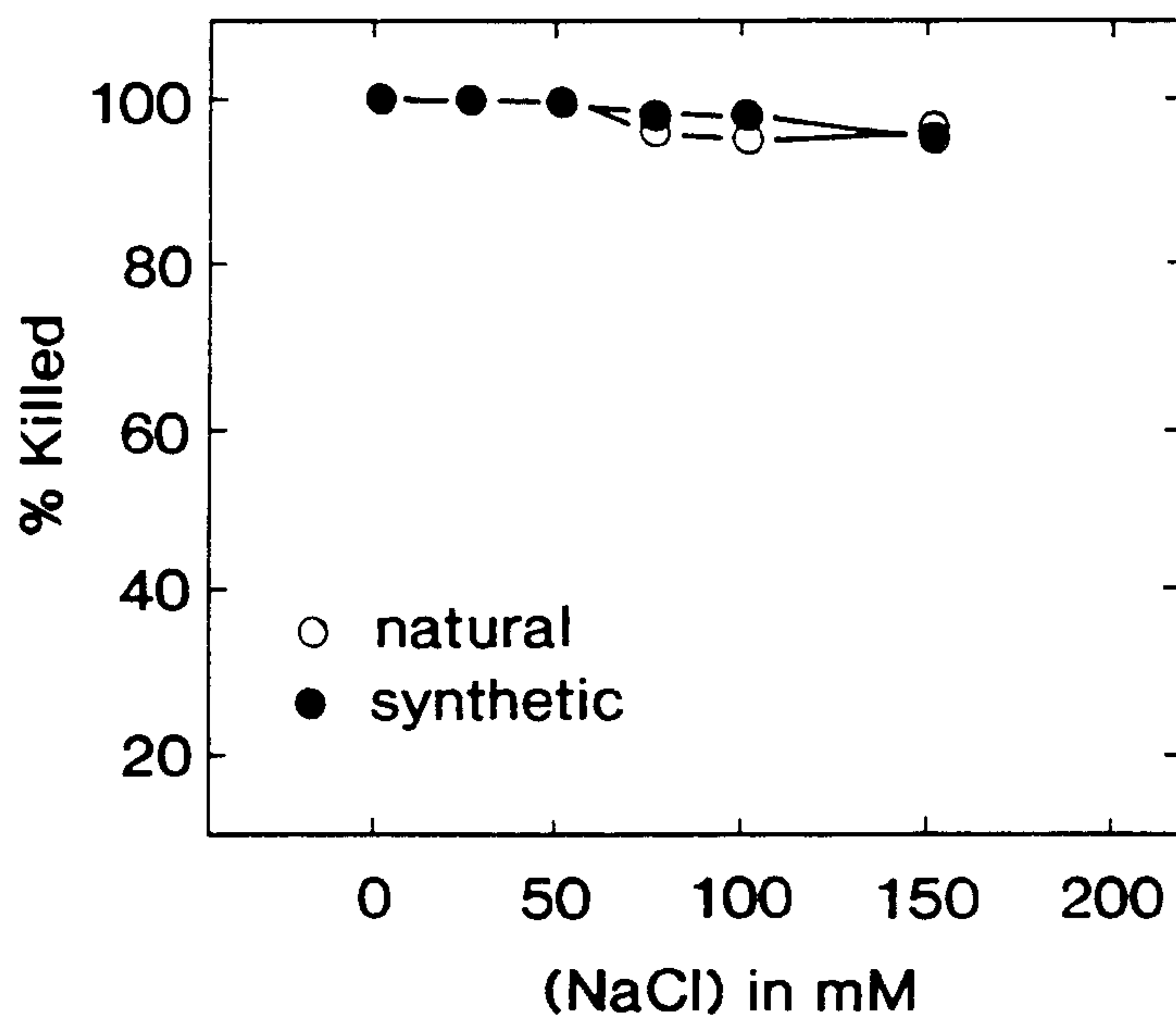


FIG. 9E

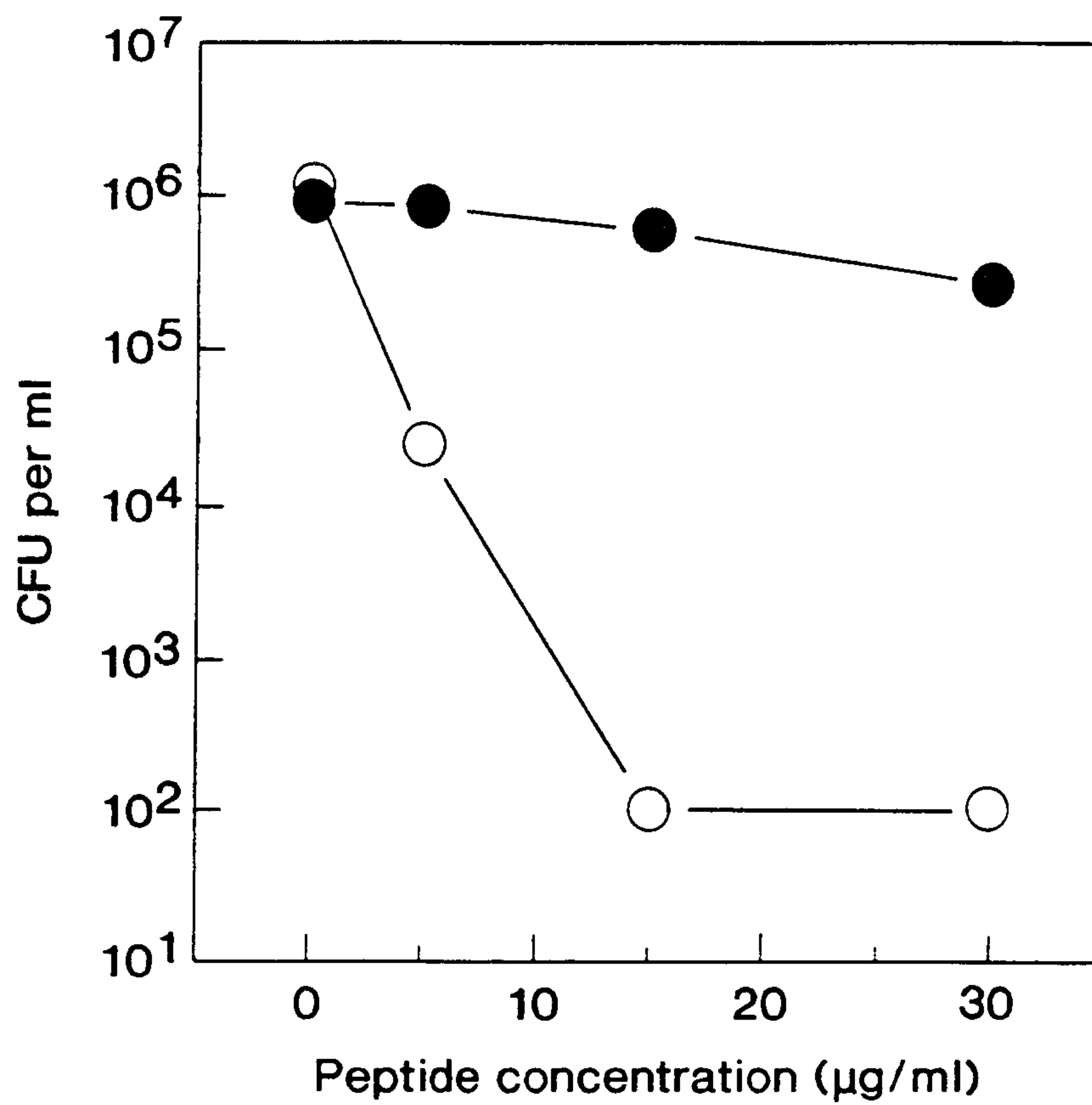


FIG. 10

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RTD1a

GACGGCTGCTGTTGCTACAGGAGACCCAGGACAGAGGACTGCTGTCTGCACTCTCTCTTC 60

ACTCTGCCTAACTTGAGGATCTGTCACTCCAGCCATGAGGACCTTCGCCCTCCTCACCGC 120
M R T F A L L T A

CATGCTTCTCCTGGTGGCCCTGCACGCTCAGGCAGAGGCACGTCAGGCAAGAGCTGATGA 180
M L L L V A L H A Q A E A R Q A R A D E

AGCTGCCGCCAGCAGCAGCCTGGAACAGATGATCAGGGAATGGCTCATTCTTTACATG 240
A A A Q Q Q P G T D D Q G M A H S F T W

GCCTGAAAACGCCGCTCTTCCACTTTCAGAGTCAGCGAAAGGCTTGAGGTGCATTTGCAC 300
P E N A A L P L S E S A K G L R¹³ C¹⁴ I¹⁵ C¹⁶ T¹⁷

ACGAGGATTCTGCCGTTTGTATAATGTCACCTTGGGTCTGCGCTTTTCGTGGTTGACT 360
R¹⁸ G¹ F² C³ R L L stop

CCACCGGATCTGCTGCCGCTGAGCTTCCAGAATCAAGAAAATATGCTCAGAAGTTACTT 420

TGAGAGTTAAAAGAAATTCTTGCTACTGCTGTACCTTCTCCTCAGTTTCCTTTTCTCATC 480

CCAAATAAATACCTTATCGC 500

RTD1b

GACCGCTGCTCTTGCTACAGGAGACCCGGGACAGAGGACTGCTGTCTGCCCTCTCTCTTC 60

ACTCTGCCTAACTTGAGGATCTGCCAGCCATGAGGACCTTCGCCCTCCTCACCGCCATGC 120
M R T F A L L T A M L

TTCTCCTGGTGGCCCTGCACGCTCAGGCAGAGGCACGTCAGGCAAGAGCTGATGAAGCTG 180
L L V A L H A Q A E A R Q A R A D E A A

CCGCCAGCAGCAGCCTGGAGCAGATGATCAGGGAATGGCTCATTCTTTACACGGCCTG 240
A Q Q Q P G A D D Q G M A H S F T R P E

AAAACGCCGCTCTTCCGCTTTCAGAGTCAGCGAGAGGCTTGAGGTGCCTTTGCAGACGAG 300
N A A L P L S E S A R G L R⁴ C⁵ L⁶ C⁷ R⁸ R⁹ G¹⁰

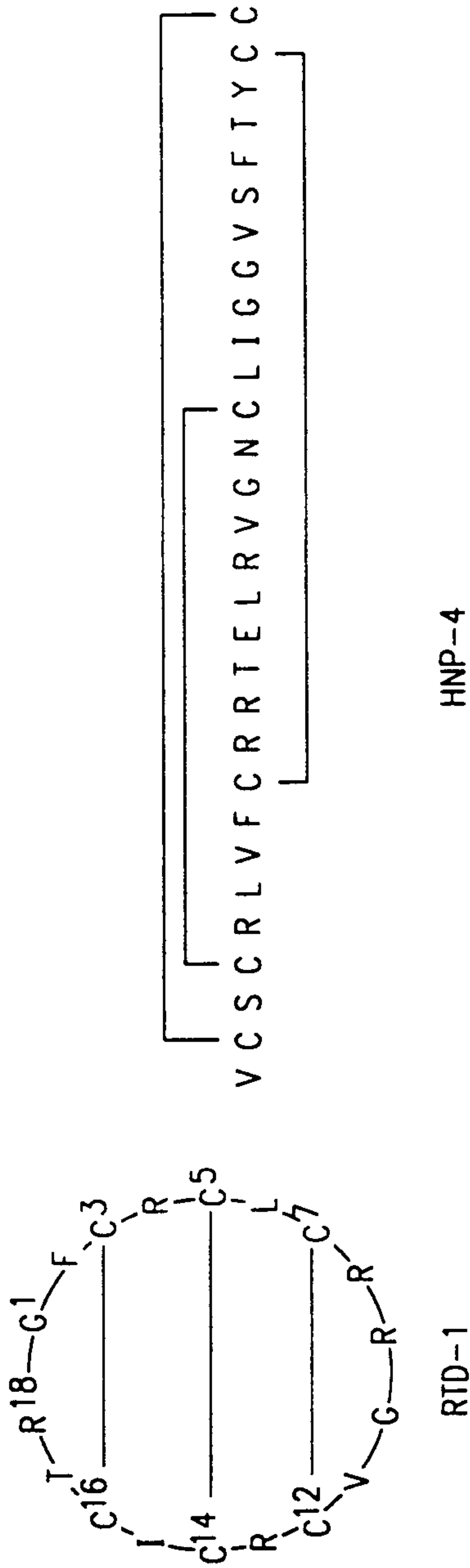
GAGTTTGCCAACTGTTATAAAGGCGTTTGGGGTCTGCGCTTTTCGTGGTTGACTCTGCC 360
v¹¹ c¹² Q L L stop

GGATCTGCTGCCGCTGAGCTTCCAGAATCAAGAAAATACGCTCAGAAGTTACTTTGAGA 420

GTTGAAAGAAATTCCTGTTACTCCTGTACCTTGTCTCAATTTCTTTTCTCATCCCAA 480

TAAATACCTTCTCGC 495

FIG. 11



HNP-4

FIG. 12A

Signal	10	20	30	40	50	60	70	80	90
RTD1a	MRTFALLTAMLLV	ALHAQA	EARQARADEAA	QQPGTDDQGM	AHSFTWPENAAL	PLSESAKGLR	RCICTRGFCRLL	CHLGSCAFGR	LHRICC
RTD1b	A.....	R.....	R.....	RCLCRRGVCQLL	RR.....
HNP-4	..II...A.I.-	...QVR.GPL...	...G...PG.E.R.PE..	DIST..A.DKSS..	QV.G.TR.MV.S.RLV...	RTELRV.N.LIG.VSE	TY..TRVD		

FIG. 12B

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RTD1.1

GACGGCTGCTGTTGCTACAGGAGACCCAGGACAGAGGACTGCTGTCTGCACTCTCTCTTC	60
ACTCTGCCTAACTTGAGGATCTgtaagtaacacaaaacttaaactttcctgtcgaggttt	120
gaacattgaagctgtgcccctaactctgacctgtgactcctgggcccaccccagagagacct	180
agtgggtgaatcccctgctgtgcatttctgtctgaacctctgggggctgctgggagcatt	240
ggctaccagctcaattaatagagaaactcaaggaatttccttctaattacatgtgtccta	300
cttgacacatccaacagagacaacaatagctccttaaaacacccttttgtttgagagaa	360
gccaatccagatcctcggcctgtttttcaatcagggtatttgttatttactattgagttg	420
tttgactgccttatgtatttagatatttacccttctaccacttaggatttgcaactatc	480
gtctttcattttctgggttgcctttttcactcagttgattatttgtttggttttttg	540
cgtgcagatgctttagaggctcagtgccagccccacttgccctttttccatttattgctg	600
tgtctttgggtgcatagcaaagatatcattaccaacatcaatgtcaaagcgtcatcttca	660
tatattcctctcgtcgttttatggtttcaggctctatgtttgggtcttcaatccatttgag	720
ttgatttggtgtatatagatatgataaggccacatgtatcaaacatcaaatecctaagggtgc	780
agacagagatatataccattttaactcttattcacatctctatagagctggaaacaaattt	840
ttggctgtagatgaactttttaccctcgatatgtcagtggtcatttcacctatcatatgat	900
agggtcattgttctcttcacactggccccacaggaggctactcacccatgccttcggg	960
agtgtggtcaagcccttgatgcctccaataaatgactccttacttgataggaaatcatac	1020
ctgctgccagagtgtagacctacagagagtagtagggccatctgcaggaagagacatttg	1080
tcgcctgacctcattgaataaaaatcactgctgttatcctttgctagaagagttaaaagta	1140
aatatttcgtaaagtgagaaacaggaatcctcatcatcctcatcaaaccagcacaga	1200
cactaaacatagagattcaaactagagtgaagctgggagaccaaaaagaagaaaacatgg	1260
acattgagaccaatgggatcccacacaatctccagtgaaatgcacacctcctctctctga	1320
gaaggttcaaggtttctgtctctgagcctcctctctgcagacatagaaatccagactaa	1380
ctcctctctcccgacttgctcctcctgctctgcctcttccagGTCCTCCAGCCATGAG	1440
GACCTTCGCCCTCCTCACCGCCATGCTTCTCCTGGTGGCCCTGCACGCTCAGGCAGAGGC	1500
ACGTCAGGCAAGAGCTGATGAAGCTGCCGCCAGCAGCAGCCTGGAACAGATGATCAGGG	1560
AATGGCTCATTCTTTACATGGCCTGAAAACGCCGCTCTTCCACTTTCAGgtgagacagg	1620
ccggcatgcagagctgcagggtctagagggatggatgggagacagagtcgggaatcgagt	1680
ctcagtggtccttgctcacctagatggcttcatttagcatctctgggccttggttttctca	1740
tctataaattgaatacagaaccaaataaatctagcaggtttctgtctataaagacttgag	1800
gcagctctgcctggagagtaaccattcttttattcctttacttcttaacgatcctttca	1860
ctttagaaaatcaataaaaattaaaaataagacttgaatcaacatagtctgtgaaatt	1920
cagtaggtttaagatatgaagaaacagctctgctagttctttctggattcaaacaagtcat	1980
cttcattacatggataatatttgactgtatctatacaaccgtttctaaagagtagagacaa	2040
gcctaagagtgcggttcagggtgtgtgtctgatggggcagaagcacaataatgaaagcaaat	2100
gagaataagtctcaaatcctgtatgaccagcactgctctgtgtatttattcttaatgact	2160
gaagttggtcatgctaccggccctaatgcagccgacatcactcattagctagcacatgac	2220
ttctccaggattccctttgccaccactgctgacctctgatccatttaacgatgctctct	2280
ctgtgttcccagAGTCAGCGAAAGGCTTGAGGTGCATTTGCACACGAGGATTCTGCCGTT	2340
TGTTATAATGTCACCTTGGGTCTGCGCTTTTCGTGGTTGACTCCACCGGATCTGCTGCC	2400
GCTGAGCTTCCAGAATCAAGAAAAATATGCTCAGAAGTTACTTTGAGAGTTAAAAGAAAT	2460
TCTTGCTACTGCTGTACCTTCTCCTCAGTTTCTTTTCTCATCCCAAATAAATACCTTCT	2520
CGC	2523

FIG. 13A

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RTD1.2

GACCGCTGCTCTTGCTACAGGAGACCCGGGACAGAGGACTGCTGTCTGCCCTCTCTCTTC	60
ACTCTGCCTAACTTGAGGATCTgtaagtaacacaaaacttaaactttcctgtcgaggttt	120
gaacattgaagctgtgacccaatctgacctgtgactcctgggccaccccagagggacct	180
agtgggtgaatcccctgctgtgcatttctgtctgaacctctgggggctgctgggagcatt	240
ggctaccagctcaattaatagagaaaactcaagaaatttccttctacttacacgtgtccta	300
cttgacacgtccaacagagacaacaatagctccttaaaacacccttttatttgagagaa	360
gccgatcctgctcctcggcctatTTTTcaatcaggttatttcttatttgctactgagttg	420
tttgattgccttatgcatttagatgttcaccctttctaccacttagggtttgcaactatt	480
gtctttcattttctgagttgctttttcactcagttgattatttatttggttggtttggttt	540
tttgacgtgcatttgcttttagaggctcagtgcagccccacttgtctcttttcccgtttatt	600
gcctgtgtctttgggtgcatagcaaagatatcattaccaacatcaatgtcaaagcattat	660
cttcatatgttcctctcgtcgttttacggtttcaggactatgtttggggtcttcaatccatt	720
ttgagttggtttggtgaaatagatatgataaagaccacatgtatcaaacatcaaactctaa	780
ggaggagtagatataaccatttttcttcttattcattatctctatagagctgga	840
aatgaatttttcagtgtagatgaaattttgaccttgatatcactgtgttcatttcaccta	900
tcgcatgatagggtcattgtcctcttcacattggccccctacaggaggctacacacctcat	960
gccttcatgagagtgatcatgcctatgatgcctgcaacaaatcactcttcacttgacagg	1020
aaattcatgcctgctgccagagtgtagacctatagagagtcgtggggccatctgaaggaa	1080
aggagacatttgatcctgaacttactgaacaaagcactgctgttatcctttggtagaac	1140
agtaaaaagtaaatatgtaatgaagtgagaaacaggagaaagatgccaggttcctcatct	1200
tcaccatcctctccatcagcacagacactaaacatagagattcaaactagagtgaaagct	1260
gggagagcaaaaagaagaaaacatggacattgagaccaatgggatcccatacaatctccag	1320
tgaaatgcacagctcctctctctgagaagggtcaagatttcctgtctctgagccttctct	1380
ctgcagacatagaaatccagactaactcctctctcccgaacttgtctgctcctgctcttcc	1440
tcctccagGCCAGCCATGAGGACCTTCGCCCTCCTCACCGCCATGCTTCTCCTGGTGGCC	1500
CTGCACGCTCAGGCAGAGGCACGTCAGGCAAGAGCTGATGAAGCTGCCGCCAGCAGCAG	1560
CCTGGAGCAGATGATCAGGGAATGGCTCATTCTTTACACGGCCTGAAAACGCCGCTCTT	1620
CCGCTTTCAGgtgagacaggccggcatgcagagctacagggtctagagggatggatggga	1680
gacagagtcgggaatcgagttctcagtggtccatgtcacctagatggcttcatttagcatc	1740
tctgggccttggttttctcatctataaattgaaatagagagccaaagaagtctaacaggtt	1800
ttctgtctataaagatttgaggcagctctgcctggagagtaaccattcttttattccctt	1860
acttcccttaatgatcctttcacttttagagaatcaataaaattaaaaataaaaacttgaaa	1920
tcaagatatgtctgtgaaattcaagtaggtttaagacatgaagagacagctctgactagtt	1980
ctttctggattcaacaagtcatcttcattacacgggagaatatttgactgtatctataca	2040
accgtttctaaagagtagagacaagcctaagagtgcatcagggtgtttgtgtttgatgggg	2100
cacaggcacaanaaatgagagcaaatgagaataagttctcaaactcctgtgtgaccagcacta	2160
ctctgtgtattttattcctactgactgaggttggtcatgctaccggcccgaatgcagctga	2220
catccctcatttagctagcacatgacttctccaggattccctttgtcactcactgcagacc	2280
ttctgatccatttatgatgctttctctgtgtccccagAGTCAGCGAGAGGCTTGAGGTGC	2340
CTTTGCAGACGAGGAGTTTGCCAACCTGTTATAAAGGCGTTTGGGGTCTGCGCTTTTCGT	2400
GGTTGACTCTGCCGGATCTGCTGCCGCTGAGCTTCCAGAATCAAGAAAAATACGCTCAGA	2460
AGTTACTTTGAGAGTTGAAAGAAATTCCTGTTACTCCTGTACCTTGTCTCAATTTCTTT	2520
TTCTCATCCCAAATAAATACTTCTCGC	2548

FIG. 13B

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CCTGGAACAGATGATCAGGGAATGGCTCATTCTTTACATGGCCTGAAAACGCCGCTCTT 60
GGACCTTGTCTACTAGTCCCTTACCGAGTAAGGAAATGTACCGGACTTTTGCGGCGAGAA

CCACTTTCAGAGTCAGCGAAAGGCTTGAGGTGCATTTGCACACGAGGATTCTGCCGTTTG 120
GGTGAAAGTCTCAGTCGCTTCCGAACTCCACGTAAACGTGTGCTCCTAAGACGGCAAAC

TTATAATGTCAC 132
AATATTACAGTG

FIG. 14A

CCTGGAGCAGATGATCAGGGAATGGCTCATTCTTTACACGGCCTGAAAACGCCGCTCTT 60
GGACCTCGTCTACTAGTCCCTTACCGAGTAAGGAAATGTGCCGGACTTTTGCGGCGAGAA

CCGCTTTCAGAGTCAGCGAGAGGCTTGAGGTGCCTTTGCAGACGAGGAGTTTGCCAACTG 120
GGCGAAAGTCTCAGTCGCTCTCCGAACTCCACGGAAACGTCTGCTCCTCAAACGGTTGAC

TTATAAAGGCGT 132
AATATTTCCGCA

FIG. 14B

CCAGCCATGAGGACCTTCGCCCTCCCTCACCCGCCATGCTTCTCCTGGTGGCCCTGCACGGCT 60
 M R T F A L L I, T A M L L L, V A L H A
 CAGGCAGAGGCACGTCAGGCAAGAGCTGATGAAGCTGCCGCCCCAGCAGCAGCCTGGAGCA 120
 Q A E A R Q A R A D E A A Q Q Q P G A
 GATGATCAGGGAATGGCTCATTCCTTTACATGGCCTGAAACGCCCTCTTCCACTTCA 180
 D D Q G M A H S F T W P E N A A L P L S
 GAGTCAGCGAAAGGCTTGAGGTGCATTTGCACACGAGGATTCGCCCGTATGTTATAACGT 240
 E S A K G L R C I C T R G G F C R M L end
 CGC 243

FIG. 15

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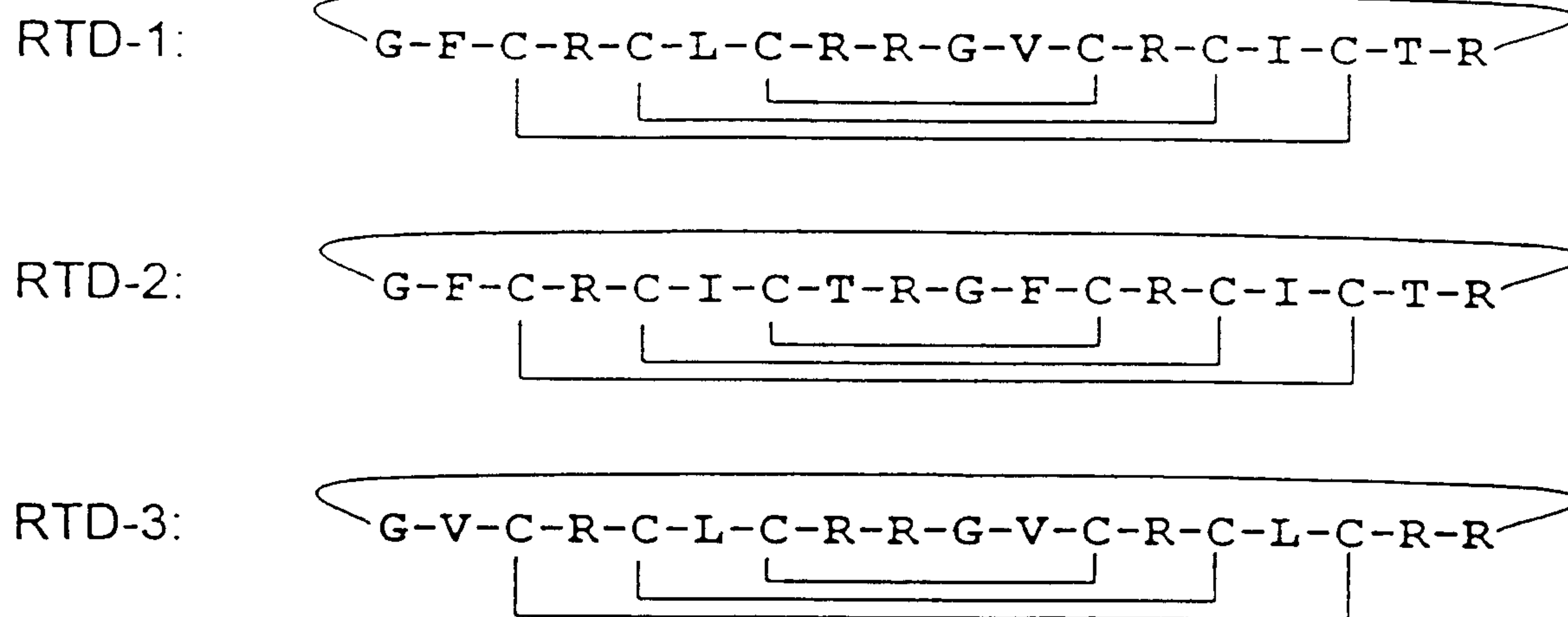


FIG. 16