We designed a retro-isomer and seven circularized “β-tile” peptide analogs of a typical rabbit α-defensin, NP-1. The analogs retained defensin-like architecture after the characteristic end-to-end, Cys5-Cys31 (C I:C VI), α-defensin disulfide bond was replaced by a backbone peptide bond. The retro-isomer of NP-1 was as active as the parent compound, suggesting that overall topology and amphipathicity governed its antimicrobial activity. A β-tile design with or without a single cross-bracing disulfide bond sufficed for antimicrobial activity, and some of the analogs retained activity against Escherichia coli and Salmonella typhimurium in NaCl concentrations that rendered NP-1 inactive. The new molecules had clustered positive charges resembling those in protegrins and tachyplesins, but were less cytotoxic. Such simplified α-defensin analogs minimize problems encountered during the oxidative folding of three-disulfide defensins. In addition, they are readily accessible to a novel thia zip cyclization procedure applicable to large unprotected peptide precursors of 31 amino acids in aqueous solutions. Collectively, these findings provide new and improved methodology to create salt-insensitive defensin-like peptides for application against bacterial diseases.

Cationic antimicrobial peptides constitute a host defense mechanism that is used by insects, plants, and animals (including humans) to combat infection (1–5). Recent evidence suggests that such peptides may constitute a major defense against microbes on mucosal surfaces (6–10). Most antimicrobial peptides have structures that are predominantly either α-helical or β-sheet. Structure-function studies of α-helical antimicrobial peptides have received considerable attention, partly owing to their facile chemical synthesis (5). Less is known about antimicrobial β-sheet peptides that, like defensins, are challenging to synthesize chemically.

Defensins range in size from 3 to 5 kDa and contain three or four intramolecular disulfide bonds (1–5). They have been grouped into several structural classes, based on their cystine pairings, structures and homologies. These include the α- and β-defensins of vertebrates and the plant/insect defensins. α-Defensins were first identified in granules of leukocytes (1), and were later found in small intestinal Paneth cells (4). β-Defensins are expressed by epithelial cells, by some glands, and (in some species) by leukocytes (6–10). Insect/plant defensins are larger than α- and β-defensins and have an α-helical structural component absent from vertebrate defensins (11).

Collectively, defensins exhibit antimicrobial activity toward many bacteria, yeast, fungi, and enveloped viruses (1–5). Certain defensins also exert chemotactic, mitogenic, corticostatic, and cytotoxic effects (12–14). The antibacterial activity of α- and β-defensins is greatly affected by the ambient concentration of NaCl, and links between local salt-dependent inactivation of defensins and the chronic pulmonary infections in patients with cystic fibrosis have been proposed (13, 14).

In their monomeric forms, all α- and β-defensins contain three anti-parallel β-strands that are cross-braced by three or four disulfide bonds (15, 16). The folded structures of α- and β-defensins are very similar, despite differences in disulfide pairing. α-Defensins contain three disulfide bonds and are circularly permuted by an end-to-end, Cys5-Cys31 (C I:C VI), disulfide bond (Fig. 1). Recently, four end-to-end peptide bond-circularized peptides with 29–31 amino acid residues were isolated from plants (17–20). These molecules (kalata, cyclophycotride, and circulins A and B) also contain three disulfide bonds and are the largest cyclic peptides yet found in nature. The circulins provide precedents and incentives for designing α-defensin analogs with end-to-end cyclic structures (Fig. 1). In the discussion that follows, we will call such analogs “β-tile peptides.” This paper describes the design, synthesis, and antimicrobial activity of β-tile peptides based on the α-defensin, rabbit NP-1. These novel defensin analogs exhibited promising antimicrobial profiles, and were significantly less salt-sensitive than NP-1.

EXPERIMENTAL PROCEDURES

Solvents, all of HPLC grade, were obtained from VWR Scientific Co. and used without further purification. Boc amino acids and 4-methylbenzhydrylamine (MBHA) resin were purchased from Bachem Biosciences (Torrance, CA). Other chemicals were obtained from Aldrich. Analytical reverse phase-HPLC was performed on a Shimadzu system with a 0.46 × 25-cm C18, Vydac column, using a flow rate of 1 ml/min with a linear gradient of buffer B (60% acetonitrile in H2O, 0.04% trifluoroacetic acid) in buffer A (5% acetonitrile in H2O, 0.045% trifluoroacetic acid).
CH$_3$OH, and DCM and drying DCM (3:1, v/v) for 2 h. After thorough washing with DMF, DCM, and DMF, and then treated with a mixture of 1 eq each of cysteine (MALDI-MS).

The reaction mixture was shaken at room temperature until on-resin ninhydrin testing indicated that free amino sites were absent. The resins were washed in order with DMF, DCM, CH$_3$OH, and DCM and drying in vacuo, the mercaptopropionyl MBHA resin 4 was obtained in quantitative yield.

Boc amino acid (4 eq) preactivated with BOP (4 eq) and DIEA (6 eq) for 5 min was added to the suspension of mercaptopropionyl MBHA resin (1 eq) in DMF (10 ml/g resin). The mixture was shaken at room temperature for 2 h, and free thiol groups were monitored by Ellman’s 5 reagent (23). The resins were washed in order with DMF, DCM, CH$_3$OH, and DCM and used for stepwise peptide synthesis.

**Synthesis of Peptide Thioesters**—All thioesters were prepared by solid-phase synthesis (24, 25) starting from Boc amino acid thioester resin 5. Solid phase synthesis was carried out on a peptide synthesizer 430A (Applied Biosystems Inc., Foster City, CA) using conventional Boc chemistry and DCC/HOBt coupling protocol. The side chain protecting groups of the Boc amino acids were as follows: Arg (Tos), Asp (OcHex), Cys (4-MeBzl and Acm), Glu (OcHex), His (Dnp), Lys (ClZ), Ser (Bzl), and Thr (Bzl). After peptide chain elongation, the resin was treated with a high HF procedure (HF/anisole, 90:10, v/v). After the HF evaporated, the residual solid was washed with ether. The crude peptide thioesters were extracted with 50% acetic acid, purified by preparative HPLC and lyophilized. Alternatively, the crude peptide thioesters were extracted into 8 μl urea and these urea solutions were used for “one-pot” cyclization-oxidation.

**General Procedure for Thio-Zip Cyclization**—In condition A (“physiological”), the peptide thioester (10 mM) was dissolved in 0.2 M phosphate-citric acid buffer (pH 7.5) containing 3 eq of TCEP. The reaction was monitored by HPLC until completion. In condition B (“denaturing”), the peptide thioesters were dissolved in 8 μl urea containing 0.1 M Tris-HCl at pH 7.2. 10 eq each of triis(2-carboxyethyl)phosphine and mercaptopropionic acid were added to prevent undesired by-product formation. The 8 μl urea solution was sequentially dialyzed in a desalting urea solution that ultimately contained 100% water. The completion of the thio-thioester exchange cyclization was monitored by HPLC.

**General Procedure for Two-step Disulfide Formation**—Step 1 was oxidation with Me$_2$SO. After completion of cyclization, oxidation of free SH to form the disulfide bond was achieved by adding 10–20% Me$_2$SO to the peptide solution. The reaction was monitored by analytical HPLC. At the completion of oxidation, the solution was used directly for the next step. Step 2 was concomitant removal of S-acetyl-amido group and formation of disulfide bond(s). The urea peptide solution was adjusted to pH 3 by adding acetic acid. The solution was flushed with nitrogen for 10 min, and then iodine/methanol (10 mM) was added dropwise to the solution until a brown color persisted. The reaction mixture was maintained in a nitrogen atmosphere in a darkened vessel for 45 min, then cooled in an ice-bath and quenched with 1 M ascorbic acid. The peptide was purified on preparative HPLC.

**Characterization**—Peptides were hydrolyzed with 6 N HCl at 110 °C for 24 h, and amino acid analysis was performed to confirm the composition of the desired peptides. Analytical HPLC for all peptides was performed on a Vydac C$_8$ column (250 × 4.6 mm) with a linear gradient of 0–85% buffer B in buffer A (see above) at a flow rate of 1.0 ml/min. All synthetic peptides were characterized by MALDI-MS. CD spectra were recorded at room temperature on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a 1-mm path length quartz cell and a peptide concentration of 10$^{-4}$ M in water.

**Syntheses of Cyclic Defensin 2a and 2g**—Starting from Boc-Leu-thiophosphinyl-MBHA resin, the peptide thioester was assembled using a standard DCC/HOBt coupling protocol on Applied Biosystems 430A synthesizer. After chain elongation, the resin was treated with thiopehnol/DMF (1:9 v/v) solution to remove Dnp protecting group from His. The resin then was treated with high HF procedure and the crude peptide thioester (M$_r$: calculated, 4,034.9; found, 4,034.0) was extracted into 150 ml of 8 μl urea. To the urea solution was added 10 eq of TCEP and 50 eq of thiophosphoric acid. The solution was dialyzed in 2,000 ml of descending concentrations of 8 to 0 μl urea, 0.1 μl Tris-HCl at pH 7.5 to complete the thio zip cyclization in 24 h. To form the disulfides, Me$_2$SO (10% in volume) was added to the diazolyzed cyclic peptide (M$_r$: calculated, 3929.8; found, 3929.0) solution. The solution was stirred and the oxidative folding was monitored by HPLC. After 24 h, the oxidation was completed to give cyclic peptide with Cys$^3$–Cys$^6$ (C I:C VI) disulfide and Cys$^5$–Cys$^{10}$ (C II:C V) disulfide and Cys$^{15}$–Cys$^{20}$ (C III:C IV) disulfide Analogs 2b and 2e—A procedure described in the general protocol for the synthesis of peptide thioesters, thio zip cyclization and Me$_2$SO oxidation (first step of two step disulfide formation) worked well for the synthesis of 2b and 2e to give the desired peptides (M$_r$: calculated, 3693.4; found, 3694.2; Mf: calculated, 3693.4; found, 3692.4).

**Synthesis of 10-Thio- and 10-Sulfonylethyl cDefensin Analogs 2d and 2e**—2d was obtained by “one-pot” cyclization procedure after cleavage from the thioester resin (M$_r$: calculated, 3650.4; found, 3650.3). To an ice-cold solution of 2.5 mg of cNP 2d in 250 μl of 85% formic acid and 250 μl of freshly prepared, ice-cooled peroxycyric acid (26) was added. After cooling the reaction solution in an ice bath for 1 h, 5 ml of water was added and the solution was lyophilized to give 10-sulfonyl-cNP. The product was purified from HPLC, giving 2.1 mg of 2e with a yield of 85% (M$_r$: calculated, 3698.4; found, 3698.2).

**Synthesis of Dimerized cNP 2f**—2.5 mg of cNP 2d was dissolved in 2 ml of 0.2 M phosphate buffer (pH 7.2). To this solution, 0.4 ml of Me$_2$SO was added. The solution was stirred at room temperature for 24 h. The dimerized product 2f was isolated by HPLC in yield 50% (M$_r$: calculated, 7298.8; found, 7297.7).

**Two-stage Radial Diffusion Assays**—In the first stage, the target bacteria (typically 1–4 × 10$^9$ colony-forming units/ml) were uniformly dispersed in a thin 10-ml gel underlay that contained 1% agarose, 10 mU buffer ± 100 mM NaCl, and 0.3 mg/ml trypticase soy broth. After punching a series of 2.2-mm diameter wells in the underlay, 5-μl samples containing a dilution series of the peptides of interest were introduced. After allowing 3 h for the peptides to diffuse radially into the gel, a nutrient-rich overlay (6% trypticase soy agar) was poured to allow viable bacteria in the underlay to form visible microcolonies. Typically, plates were read after an overnight incubation, although for rapid growers, such as Escherichia coli and Pseudomonas aeruginosa, the plates could be read within 6 h. The presence of a clear, colony-free zone around the well indicated antimicrobial activity. The assay yields quantitative and reliable minimal inhibitory concentration (MIC) data for purified peptides when their concentration is known and a dilution series is applied. The calculations, correlations between radial diffusion, colony count, and microbrolution dilution assays, etc., were described by Steinberg et al. and Turner et al. (27–29).
Microbroth Dilution Assays—The assays were performed according to National Committee for Clinical Laboratory Standards recommended procedures. Because the principal component in Mueller-Hinton broth (MHB) is derived from casein, a polyanionic milk protein, we modified the NCCLS procedure slightly by subjecting the MHB to anion exchange chromatography prior to use (28). Refining the MHB eliminates its propensity to complex and even precipitate polycationic peptides such as defensins. Refined MHB contains essentially the same concentrations of the various amino acids as standard MHB and supports microbial growth well. An alternative procedure to prevent precipitation and complex formation in MHB was recently published (27). The results of microbroth dilution and radial diffusion assays were similar.

RESULTS

Disulfide-to-lactam Backbone Design

Our principle for designing β-tile peptides was based on a disulfide-to-lactam backbone replacement strategy that results in a closed-chain structures. This was possible because NP-1 is already circularly permuted owing to its end-to-end disulfide bond arrangement (Fig. 1). In all circularized analog designs, cystine pair (Cys⑩,30, C10C VI) of NP-1 was replaced with Gly residues while the NH2 and COOH termini were circularized as a peptide bond. The newly formed ends contained a cluster of highly charged positive charges that mimic protegrins. In addition, the two remaining disulfide bonds were incrementally deleted to create different one-disulfide and no-disulfide analogs. By not altering the size and the amphipathic β-stranded scaffolding of NP-1, we expected to retain their ability to oligomerize and to reach the hydrophobic threshold for membranolytic activity.

Analogs Selected for Structure-Activity Relationships

Seven NP-1 analogs, 2a–2g, were prepared (Fig. 2). Peptide 2a was a circularized version of the open-chain NP-1 1, with two Gly replacing the Cys ⑩,30 (C10C VI) SS pair. Peptides 2b and 2c were cyclic analogs, each containing one SS pair (Cys⑩,20 in 2b and Cys⑩,30 in 2c). Peptides 2d and 2e were cyclic analogs without SS pairs. At position 10, peptide 2d contained a single free thiol and peptide 2e had a negatively charged sulfonate. Peptide 2f was a covalent dimer of 2d formed through SS bond formation at Cys⑩. Finally, peptide 2g was a retro-isomer of Peptide 2a.

These cyclic analogs allowed us to test the validity of the β-tile designed peptide, e.g. 2a, the structural rigidity of a β-tile peptide with a single SS pair, e.g. 2b, 2c; without any SS pair, 2d-2e; and the topology with a retro-sequence in 2g containing a reversed amino acid sequence of 2a. In a circular permutation, the retro-isomer 2g contains the amide backbone in a reverse direction but retains the overall topology and the same charge clusters as 2a (30–32). The retro-isomer could also test whether a circularized β-tile structure is similar to NP-1 and support our hypothesis that the triple β-strands serve as scaffolding with the charges clustered on the ends. Finally, a covalent dimer 2f was formed with an intermolecular SS pair at Cys⑩ located at the extended loop but not at the predicted

FIG. 2. A, amino acid sequences of NP-1 1, circularized cNP-1 2a and its cyclic retro isomer 2g; B, three groups of cyclic analogs of cNP-1 2b–2f. Open circles indicate unchanged amino acid residues with respect to NP-1. Cys to Gly replacements are shown in shaded circles. Disulfide linkages are shown by connecting lines. Arrow indicates the NH2 to COOH direction of peptide bonds that link the NH2 and COOH termini of NP-1.

Cyclic Defensins

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dimeric face of α-defensins. Such an analog was intended as a probe for sites of oligomerization other than the dimeric site revealed by crystal structure (33).

Orthogonal Cyclization for the Synthesis of β-Tile Peptides

Recently, our laboratory developed an orthogonal cyclization method for ligating free peptides as end-to-end cyclic peptides in aqueous solutions (34–39). Such an orthogonal cyclization is accomplished without protecting groups or activation steps. More importantly, it can distinguish the reactivity of an α-amine from the ε-amines of Lys in the peptide sequence to impart orthogonality or chemoselectivity to the reaction. Fig. 3 illustrates the mechanism of the orthogonal cyclization. It proceeds first through a capture of two segments to give either a branched O- or S-ester intermediate, followed by a downhill, entropic-driven O or S to N-acyl transfer to form the amide bond. The acyl transfer reaction mimics the final step of spontaneous intein splicing through intramolecular acyl transfer (40, 41). To arrive at the O- or S-ester intermediate, we have developed methods of prior capture through thiazolidine (34–36), thioester (37, 38), disulfide (39), and acylimidazole (42), yielding thia proline, Cys, His, or Met at the ligation site. These methods have also been further developed for intramolecular ligation to form circularized peptides and proteins. Recently, we have incorporated a ligation method of an Xaa-Cys bond into a novel strategy (43) for the synthesis of circularized peptide. This new method was applied to the synthesis of circularized rabbit NP-1 analogs 2a–2g and consisted of the following steps.

Solid Phase Stepwise Synthesis and HF Cleavage from Resin to Give a Free, Linear Peptide Thioester Precursor—The synthesis started with a resin support containing a detachable thioester linker esterified to the COOH-terminal amino acid 5 and ended with a Cys at the amino terminus 6. Thus, the circularization involved an Xaa-Cys bond (Xaa = any amino acid) and required assembly of a Cys at the NH2 terminus of the sequence. Because a β-tile peptide is circularly permuted, a non-hindered amino acid is usually selected for the COOH terminus among all the Xaa-Cys pairs in the NP-1 sequence. The sequence was then assembled via Boc chemistry to give a fully protected peptide attached onto the resin. The thioester linkage was then detached from the resin supports by acid cleavage (HF) to remove all protecting groups. The crude peptide was circularized off-resin by the orthogonal cyclization method. In analogs 2a and 2g, a pair of Cys was intentionally blocked by Acm group 6, which is stable to the HF cleavage step.

Circularization through a “Thia Zip” Reaction in Aqueous Conditions—The circularization of the free peptide thioester was performed at about pH 7.5 under a reducing condition by using a large excess of a small thiol- and water-soluble trialkyl phosphine. This process was greatly accelerated in a Cys-rich peptide through the thia zip reaction involving a series of thiol-assisted intramolecular rearrangements to achieve the circularization. In a thia zip reaction, a thiolactone is formed by sulfur-assisted intramolecular rearrangements to achieve the circularization. In such a scheme, one of the SS pairs is blocked by an Acm group, while the other is blocked with a methylbenzyl protecting group, which was deprotected during the HF step to release the peptide from the resin support. The first step involved the S-Acm-mediated oxidation to form the first SS pair. The second step involved the concomitant removal and oxidation of the S-Acm protecting group by I2, in acidic conditions, to form the remaining disulfide bond.

Disulfide Formation by Me2SO (44). Formation of the SS pairs to give 9 was performed directly after the circularization by adding Me2SO (10% in volume) to the aqueous solution. This procedure worked well for those compounds containing one or two cross-bracing SS bonds such as CNP-1 2a and analogs 2b, 2c, and 2g (Fig. 3). For 2a and 2g, the intermediate 9 was purified by HPLC for the next oxidation step.

Two-step Oxidation to Provide Regioselectivity in SS Bond Formation (45). For compounds containing two SS pairs, an orthogonal SS-forming strategy was also used to confirm the disulfide pairing of 2a and 2g by a two-step SS formation scheme. In such a scheme, one of the SS pairs is blocked by an Acm group, while the other is blocked with a methylbenzyl protecting group, which was deprotected during the HF step to release the peptide from the resin support. The first step involved Me2SO-mediated oxidation to form the first SS pair. The second step involved the concomitant removal and oxidation of the S-Acm protecting group by I2, in acidic conditions, to form the remaining disulfide bond.

Purification and Characterization—The peptides were purified by C18- or C8-reverse phase HPLC. The identity of synthetic products was confirmed by amino acid and mass spectrometry analysis. The CD spectra of all analogs showed a negative band with minimum at 217 nm, the characteristic of β-sheet structures (data not shown). The cyclic amide bond and disulfide pairings of 2a and 2g was confirmed by enzymatic digestion with trypsin and chymotrypsin to yield appropriate segments linking either the NH2 to COOH termini or the cystine pairs (21). The digested segments were then purified by HPLC and their identity determined by mass spectrometric analysis. Independently, the disulfide pairing was also confirmed by a chemoselective route of synthesis in which the disulfide pairings were orthogonally protected to produce the desired disulfide connectivity.
In general, defensins manifest broad spectrum antimicrobial activity when tested in media containing low concentrations of NaCl. Under these low salt conditions, NP-1 killed three Gram-negative bacteria (E. coli, Salmonella typhimurium, Klebsiella pneumoniae, and P. aeruginosa) with a MIC of 1.5–2.6 μg/ml. Also under these conditions, the MICs of NP-1 for Listeria monocytogenes (a Gram-positive bacterium) and Candida albicans (a yeast-like fungus) were 2.1 and 5.4 μg/ml, respectively. However, when these tests were performed in the presence of 100 mM NaCl, NP-1 lost its ability to kill E. coli, K. pneumoniae, S. typhimurium, and C. albicans (MIC > 500 μg/ml), retaining only its activity against L. monocytogenes and P. aeruginosa.

### Table I

| Antimicrobial activity | Defensins |
|------------------------|-----------|
| E. coli                | 1 | 2a | 2b | 2c | 2d | 2e | 2f | 2g |
| H-salt                 | >500 | 16.1 | 4.9 | 5.7 | 10.6 | 11.3 | 3.3 | 6.2 |
| L-salt                 | 1.5 | 1.6 | 1.6 | 1.6 | 1.8 | 0.8 | 1.9 | 0.6 |
| S. typhimurium         | >500 | >500 | 47.9 | 18.5 | 19.1 | 13.7 | 25.1 | 2.9 |
| H-salt                 | 2.6 | 4.3 | 2.0 | 2.5 | NT | NT | NT | NT |
| L-salt                 | 5.9 | 6.0 | 2.6 | 2.6 | 2.9 | 2.0 | 3.2 | 1.4 |
| P. aeruginosa          | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 |
| H-salt                 | 2.6 | 4.8 | 2.5 | 2.3 | 13.4 | 17.1 | 4.9 | 18.7 |
| L-salt                 | 5.9 | 6.0 | 2.6 | 2.6 | 2.9 | 2.0 | 3.2 | 1.4 |
| K. pneumoniae          | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 |
| H-salt                 | 2.3 | 5.3 | 2.5 | 5.8 | 3.2 | 2.9 | 3.7 | 2.5 |
| L-salt                 | 5.4 | 9.0 | 19.9 | 21.7 | >158 | >158 | >158 | >158 |
| L. monocytogenes       | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 |
| H-salt                 | 5.4 | 19.0 | 19.9 | 21.7 | >158 | >158 | >158 | >158 |
| L-salt                 | 5.4 | 19.0 | 19.9 | 21.7 | >158 | >158 | >158 | >158 |

### Table II

| Cytotoxicity (μg/ml) | 1 | 2a | 2b | 2c |
|----------------------|---|----|----|----|
| Cytotoxicity (μg/ml) | 35.6 | 395.4 | 264.1 | >400 |
| Hemolysis (μg/ml)   | 1898 | 1830 | 3188 | 4791 |

**Fig. 4.** Comparison of amphipathic β-strand and the charged turn of the long β-strand membrane-active proteins (LALF, LBP, and BPI), two protegrins (PG-1 and -3), tachyplesin (TP-1), NH₂-terminal region of α-defensins (hNP-1 and rNP-1), naturally occurring cyclic theta defensin (RTD-1), and newly created end (RRVV) of circularized defensin analog (cNP-1).
(49). All seven β-tile analogs retained the antimicrobial activity of NP-1. Moreover, the analogs killed E. coli and S. typhimurium when tested under high salt conditions wherein NP-1 was considerably less effective. The β-tile peptides were non-hemolytic and showed decreased cytotoxicity for human ME-180 cells, relative to NP-1.

Most antimicrobial peptides disrupt microbial cell membranes and kill bacteria quickly: properties that could forestall the emergence of bacterial resistance. Microbial killing by α-defensins has been attributed to the formation of multimeric mote salt insensitivity in antimicrobial peptides. Circularization per misformed disulfide bonds, and aggregation (56). End-to-end sulfide bonds, their synthesis can be subject to poor solubility, clustering hypothesis include the dimeric models after the unprotected peptide is released from the resin essentially eliminates the oxidative folding problem, leading to mis-energetically favorable because of the great distance between remaining cross-bracing disulfide bonds is essential for activity when a macrocyclic structure is already in place. Simplification of β-tile peptides such as 2b–2e with one disulfide bond effect-ively eliminates the oxidative folding problem, leading to mis-formed disulfide-containing defensins.

Cycling linear peptides 30 or more amino acids long is not energetically favorable because of the great distance between the amino and carboxyl termini. Moreover, multiple tiers of protecting groups are generally required to achieve synthesis of end-to-end structures by conventional methods. The orthogonal cyclization of β-tile peptides brings efficiency and simplicity to the synthesis. Using this chemistry, free linear precursors can be immediately circularized in aqueous solution at around pH 7.5. Since the subsequent thia zig cyclication step proceeds through smaller intermediates, entropic barriers are reduced and complete circularization can be achieved within 12 h. After adding Me$_2$SO to the crude peptide mixtures to enhance disulfide oxidation (44), purified disulfide-bonded β-tile peptides can be obtained in excellent yield.

With orthogonal cyclization, chemical syntheses occurring after the unprotected peptide is released from the resin essentially provides β-tile peptides, 2b–2e, in a “one-pot” reaction that proceeds immediately to end-to-end cyclization and oxidation. Alternatively, on-resin cyclization can be done with peptides freed of protecting groups while still attached to the resin support (21). Such improvements minimize handling, increase synthetic efficiency, and could facilitate the approach of a combinatorial library for structure-function analysis of defensins.

Finally, the orthogonal cyclization method and β-tile design is likely to prove useful for preparing synthetic analogs of other disulfide-rich bioactive molecules, which share structural character-istics with defensins but act by binding to specific proteins or receptors.

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Addendum—While this manuscript was undergoing revision, Tang et al. (57) reported the discovery of an 18-residue cyclic tri-disulfide anti-biotic peptide termed as rhoes theta defensin-1 (RTD-1) from leukocytes of rhesus monkeys (Fig. 4). The biosynthesis of this cyclic peptide involves G$	extsuperscript{3}$-head-to-tail linkage of two α-defensin nonapeptides. Interes-tingly, this RTD-1 can be considered as a truncated and cyclic α-defen-sin. Its considerable antimicrobial activity in salt concentrations and cluster of positive charge at its reverse turn are similar to our designed synthetic β-tile peptide analogs described in this report.

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