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Design of Non-Cysteine-Containing Antimicrobial β -Hairpins: Structure–Activity Relationship Studies with Linear Protegrin-1 Analogues[†]

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ABSTRACT: Protegrins are short, cationic peptides that display potent, broad-spectrum antimicrobial activity. PG-1, the first of the five natural analogues discovered, forms a rigid antiparallel two-stranded β -sheet that is stabilized by two disulfide bonds. The two strands of the sheet are linked by a short two-residue loop segment. Removal of the disulfide bridges (e.g., in Cys \rightarrow Ala analogues) is known to cause marked loss of antimicrobial activity. We have used basic principles of β -hairpin design to develop linear analogues of PG-1 that lack cysteine but nevertheless display PG-1-like activity. Our most potent reengineered molecules contain three essential design features: (i) the four cysteine residues of PG-1 are replaced by residues that have high propensity for β -strand conformation, (ii) D-proline is placed at the $i + 1$ position of the reverse turn to promote a type II' β -turn, and (iii) amino functionality is incorporated at the γ -carbon of the D-proline residue to mimic the charge distribution of the natural β -hairpin. Structural studies revealed that the antimicrobial potency of the non-disulfide-bonded peptides can be correlated to the stability of the β -hairpin conformations they adopt in aqueous solution. The presence of 150 mM NaCl was found to have little effect on the antimicrobial activity of PG-1, but one of our linear analogues loses some potency under these high salt conditions. Despite this discrepancy in salt sensitivity, NMR and CD data indicate that neither PG-1 nor our linear analogue experiences a significant decrease in β -hairpin conformational stability in the presence of 150 mM NaCl. Thus, salt inactivation is not due to destabilization of the β -hairpin conformation. Furthermore, our results show that β -sheet design principles can be used to replace conformation-stabilizing disulfide bridges with noncovalent conformation-stabilizing features.

Protegrins are short (16–18 residue), cationic peptides that display potent antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria (1–5). These peptides were originally isolated from porcine leukocytes, and five natural analogues have been identified to date (PG-1 through PG-5; Figure 1). Protegrins resemble other cationic antimicrobial peptides such as horseshoe crab tachyplesins and mammalian defensins. Characteristic features of protegrin sequences include a high content of arginine, an amidated C-terminus, and four conserved cysteines at positions 6, 8, 13, and 15 that are disulfide-bonded in the arrangement Cys₆–Cys₁₅ and Cys₈–Cys₁₃. The solution structure of synthetic PG-1 was reported independently by two groups in 1996 (6, 7). The peptide was found to adopt a rigid two-stranded β -sheet that is stabilized by two disulfide bonds. The two strands of the sheet are linked by a short loop that cannot be easily categorized into any of the canonical β -turn types. The basic residues are concentrated at the turn region and at the two termini. A hydrophobic cluster involving the

side chains of Leu₅, Tyr₇, Phe₁₂, Val₁₄, and Val₁₆ projects to one side of the hairpin backbone, endowing the structure with an amphiphilic character. Although PG-1 was found to be monomeric in aqueous solution (6), NMR studies in the presence of perdeuterated phospholipids revealed several NOEs that suggest that the peptide dimerizes in a head-to-tail fashion in membrane environments (8). These findings support the hypothesis that protegrins function by forming oligomeric pores in bacterial membranes, similar to the mechanism postulated for human defensin HNP-3 (9).

Protegrins have received attention as potential therapeutic agents for disorders such as cystic fibrosis because their antibacterial activity is insensitive to high salt concentrations (10), in contrast to the cationic sheet-forming antimicrobial peptides secreted by the endothelial cells of the human lung (β -defensins) (11). The potency of protegrin sequence variants appears to be highly dependent on the position and number of disulfide bonds stabilizing the β -hairpin conformation. Removal of both disulfide bridges, either by substitution of cysteine with alanine (10, 12) or by use of acetamidomethyl-protected cysteine (13), results in substantial reduction of activity. However, Tam et al. reported that activity of non-disulfide-bonded analogues could be restored by cyclization of the peptide backbone (14). In studies with variants containing a single disulfide bridge (one pair of cysteine residues replaced with alanine), Harwig et al. found that a “bullet” analogue (disulfide bond distal to the turn)

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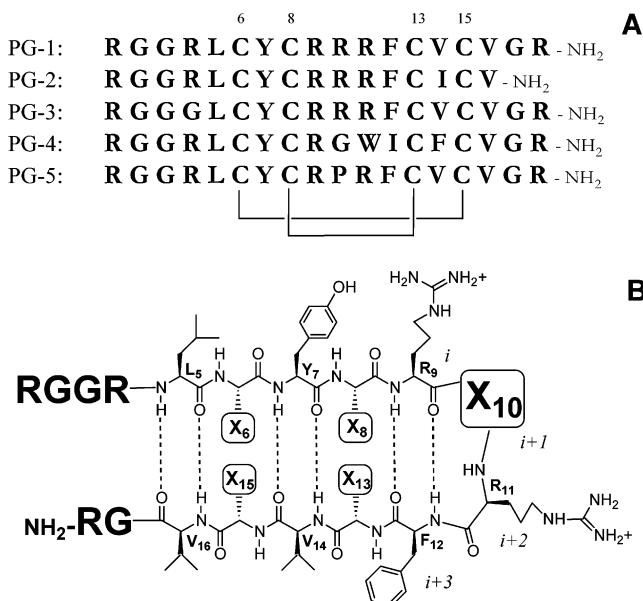
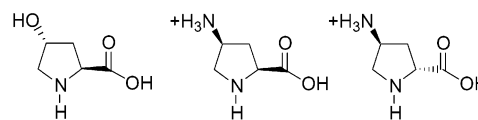


FIGURE 1: (A) Sequence alignment of the five natural protegrin homologues originally isolated from porcine leukocytes. Positions of the four conserved cysteines and the arrangement of disulfide bonds are indicated. (B) General schematic of designed peptides in intended β -hairpin conformation. The desired hydrogen-bonding pattern is shown as dotted lines. The four non-hydrogen-bonded (NHB) sites that were varied are indicated (X₆, X₈, X₁₃, and X₁₅). Residues involved in the 2:2 reverse turn are annotated with the nomenclature of Sibanda et al. (39), and position 10 (which was varied) is also indicated.

had comparable activity to PG-1, whereas a “kite” analogue (disulfide bond proximal to the turn) was less active (10). Furthermore, PG-1 and the bullet peptide maintained activity in the presence of 100 mM NaCl, but the kite analogue did not. Overall, these findings suggest that activity and salt sensitivity are closely related to the stability of the protegrin β -hairpin conformation. Recently, a comprehensive structure–activity relationship study was conducted on a small library of sequence analogues in an attempt to develop related peptides with enhanced antimicrobial potency (12). Several promising analogues were identified, one of which (IB-367) is currently in phase III clinical trials as an aerosol treatment for oral mucositis and phase I trials as a preventative agent against airway colonization by pathogens in cystic fibrosis patients (15, 16).

Previous efforts in our laboratory have been directed toward design and structural characterization of stable, monomeric, autonomously-folding β -hairpins (17–23). We have used these model systems to investigate the forces that contribute to β -hairpin conformational stability (24–26). Our general strategy in the development of stable β -hairpins involves (i) careful choice of residues to promote stabilizing interactions between adjacent strands, (ii) consideration of intrinsic conformational preferences of residues, and (iii) incorporation of an unnatural D-proline residue in the turn region to promote formation of a type I' or type II' β -turn. The present study began with an effort to use the understanding acquired in previous work to design protegrin analogues that do not contain disulfide bonds but are nevertheless active. The resulting peptides were then the subject of structure–activity relationship (SAR) studies to identify the features that are important for antimicrobial activity.

Chart 1: Chemical Structures of Modified Proline Residues^a



^a γ -(S)-Amino-L-proline (middle) was synthesized with appropriate protecting groups (omitted here for clarity) from enantiomerically pure *trans*-hydroxy-L-proline (left). A complete description of the synthetic procedure may be found in the Supporting Information. The isomer γ -(S)-amino-D-proline (right) was obtained by a similar protocol from *cis*-hydroxy-D-proline.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. All peptides were synthesized using standard Fmoc chemistry with HBTU activation on an Applied Biosystems Model 432A Synergy automated peptide synthesizer. An aminomethyl-function-alized (AM) Rink amide polystyrene resin (Applied Biosystems) was employed. *N*- α -Fmoc amino acids (with orthogonal side chain protecting groups) were purchased from Novabiochem. After all residues were coupled, simultaneous cleavage and side chain deprotection were achieved by treatment with a cocktail containing 83.5% TFA, 10% 1,2-ethanedithiol, 5% H₂O, and 2.5% thioanisole for 4–5 h. Crude peptides were then precipitated with cold diethyl ether, dissolved in acetonitrile/water, and lyophilized. Peptides containing disulfide bonds were oxidized either by bubbling air through a 0.1 mg/mL solution of the crude peptide in H₂O for 3–7 days or by treatment of the dilute peptide solution with a large molar excess of oxidized glutathione (GSSG). Oxidation progress was monitored by analytical RP-HPLC (Vydac silica C4 column, 5 μ m, 250 mm \times 4 mm) and matrix-assisted laser desorption/ionization time-of-flight spectrometry (MALDI-TOF; measurements performed on a Bruker REFLEX II instrument). The absence of free thiols by Ellman's test (27) indicated that no unoxidized material was present in the final purified peptides. All peptides were purified by RP-HPLC on Vydac C4 silica columns (semi-preparative 5 μ m, 250 mm \times 10 mm, or preparative 8 μ m, 250 mm \times 25 mm) using a nonlinear gradient of water/acetonitrile containing 0.1% TFA. Peptide identity was confirmed with MALDI-TOF, and homogeneity was evaluated by analytical RP-HPLC.

Synthesis of Aminoproline. The procedure for synthesis of γ -(S)-amino-L-proline with appropriate protecting group strategy from *trans*-hydroxy-L-proline (Chart 1) may be found in the Supporting Information. An analogous protocol was used to prepare γ -(S)-amino-D-proline from *cis*-hydroxy-D-proline with comparable yields (enantiomerically pure starting materials purchased from Aldrich). In both syntheses, several of the intermediates were characterized by ¹H NMR at 250 or 300 MHz. Molecular weights of the final products observed by electrospray–ionization mass spectrometry (ESI-MS) were consistent with the expected masses. After the development of this route in our laboratory, a similar synthesis was reported (28).

Antimicrobial Assays. Inhibitory activity of peptides was tested against four organisms: *Escherichia coli* JM109 (29), *Bacillus subtilis* BR151 (30), penicillin-resistant *Staphylococcus aureus* 1206 (31), and vancomycin-resistant *Enterococcus faecium* A634 (32). Minimal inhibitory concentrations (MICs) were determined using standard microdilution tech-

Table 1: Peptides Synthesized and Tested for Antimicrobial Activity^a

peptide	sequence									charge	[MH] ⁺ _{calcd} /[MH] ⁺ _{obs}
natural sequence											
PG-1	RGGRRL	C	Y	C	RRRF	C	V	C	VGR	+7	2155.1/2155.2
D-proline-containing analogues											
1	RGGRRL	T	Y	T	RpRF	T	V	T	VGR	+6	2092.2/2092.5
2	RGGRRL	V	Y	T	RpRF	T	V	I	VGR	+6	2102.3/2102.7
3	RGGRRL	F	Y	V	RpRF	I	V	T	VGR	+6	2148.3/2148.7
4	RGGRRL	W	Y	W	RpRF	W	V	W	VGR	+6	2432.3/2432.4
5	RGGRRL	W	Y	T	RpRF	W	V	W	VGR	+6	2347.3/2437.9
6	RGGRRL	W	Y	T	RpRF	T	V	W	VGR	+6	2262.3/2262.3
peptides containing γ -(S)-aminoproline derivatives											
7	RGGRRL	T	Y	T	Rp*RF	T	V	T	VGR	+7	2108.2/2107.5
8	RGGRRL	W	Y	W	Rp*RF	W	V	W	VGR	+7	2448.4/2448.2
9	rggrl	t	y	t	rP*rf	t	v	t	vgr	+7	2108.2/2107.5
single disulfide bond-containing analogues											
10	RGGRRL	C	Y	T	RpRF	T	V	C	VGR	+6	2094.1/2094.6
11	RGGRRL	C	Y	T	RRRF	T	V	C	VGR	+7	2153.2/2153.5
control peptides											
12	RGGRRL	T	Y	T	RPRF	T	V	T	VGR	+6	2092.2/2092.6
13	RGGRRL	A	Y	A	RpRF	A	V	A	VGR	+6	1972.2/1972.0
14	RGGRRL	A	Y	A	Rp*RF	A	V	A	VGR	+7	1988.2/1987.7

^a All peptides were synthesized as C-terminal amides. Lower case letters correspond to amino acids with D-chirality. Proline residues containing amino functionalization at the γ -carbon are annotated with an asterisk. Net charges were calculated for peptides at neutral pH. Experimentally observed masses were obtained from MALDI-TOF.

niques from two independent experiments performed in duplicate. Stock solutions were prepared by dissolving the purified peptides in water to 2 mg/mL (based on dry weight of peptides as TFA salts, assuming one molecule of TFA per cationic residue). A 2-fold dilution series of each peptide was then prepared from these stock solutions in Difco brain heart infusion (BHI) broth media in sterile 96-well Falcon microtiter plates. To each well was added 50 μ L of a 10⁶ cfu/mL bacterial suspension in BHI (total well volume 100 μ L), and the microtiter plates were incubated for 6 h at 37 °C. For high salt studies, the dilution series was prepared in broth media containing 300 mM NaCl, which was diluted another 2-fold upon addition of the bacterial suspension to give a final salt concentration of 150 mM. The MIC was defined as the lowest concentration required for complete inhibition of growth as monitored by OD₅₉₀. The determined MIC values are accurate to a factor of 2 (i.e., only differences in activity greater than 2-fold are significant).

Nuclear Magnetic Resonance. Samples for ¹H NMR analysis were prepared by dissolving the purified peptides to 1–3 mM in 9:1 H₂O/D₂O containing 40 mM CD₃COOD/CD₃COONa, pH 3.8 (uncorrected), with and without 150 mM NaCl. A sample for **1** was also prepared in pure D₂O under the same buffering conditions without salt. All spectra were recorded on a Varian INOVA 600 MHz spectrometer at 24 or 4 °C, as required to resolve spectral overlap. Water signal suppression was achieved either by the WATERGATE method (33) or by selective presaturation. TOCSY (34), ROESY (35), and NOESY (36) data were acquired with a 7000 Hz spectral window, a 1 s recycle delay, and mix times of 80, 200, and 200 ms, respectively. Typical two-dimensional data sets consisted of 500–600 free induction decay (FID) increments of 16–24 transients each (corresponding to 1938–3874 data points in f_2). Data were processed in Varian VNMR software and referenced to internal 2-(trimethylsilyl)-1-propanesulfonic acid. Spin system assignments were achieved by standard methods (37).

Circular Dichroism. Measurements were performed on an AVIV Model 202SF spectrometer with 5 s averaging times

and 1 nm step size. Spectra were acquired in aqueous 40 mM Tris, pH 7.2, with or without 150 mM NaCl at 24 °C. Peptide concentrations were 0.05–0.1 mM as determined by UV absorbance at 275.5 nm (38). Samples were analyzed in a quartz cuvette with 1 mm path length. Spectra were corrected for baseline absorbance and normalized for path length, number of amide chromophores, and concentration.

RESULTS

Design of Analogues. PG-1 and 14 analogues were synthesized and tested for inhibitory activity (see Table 1). A generalized diagram of the protegrin β -hairpin structure is found in Figure 1B; the sites of variation are highlighted in this diagram (residues 6, 8, 10, 13, and 15). The strands of the β -hairpin are connected by a tight two-residue loop, and this structure can be categorized as a 2:2 β -hairpin in the nomenclature of Sibanda et al. (39). The two amino acids involved in this type of β -hairpin loop and the two flanking residues comprise a β -turn (residues i to $i + 3$ in Figure 1B). β -Turns are classified by the ϕ and ψ torsion angles of the central two residues ($i + 1$ and $i + 2$) (40–43). In the natural protegrin structure (6), the dihedral angles of Arg₁₀ and Arg₁₁ do not match either of the most common reverse turns found in 2:2 β -hairpins (type I' or type II') or any of the other defined reverse turn types.

In our initial designs, the four cysteines that occupy the non-hydrogen-bonded (NHB) sites of the natural β -hairpin core were replaced with branched residues that have high intrinsic preference for β -sheet conformation (peptides **1** and **2**) (41, 44–46). In addition, D-proline was incorporated at the $i + 1$ position of the reverse turn. It has been reported by our and other laboratories that D-proline and L-alanine at the $i + 1$ and $i + 2$ positions, respectively, of model systems promote β -hairpin formation by inducing a type II' β -turn (17, 18, 47). We hypothesized that we could maintain this geometric preference with L-arginine at $i + 2$ in place of L-alanine, since arginine is not branched at the β -carbon. Previous studies on protegrin analogues have shown that

positive charge in this loop position is essential for activity (**12**). Subsequent analogues in our series are similar in design to **1** and **2**, with substitution of various aromatic residues at the NHB positions (peptides **3–6**). One analogue (**4**) was inspired by a recently discovered “tryptophan zipper” β -hairpin motif (**48**). Our motivation for including aromatic groups in the NHB positions was twofold: (i) to introduce potential stabilizing interactions between hydrophobic groups on adjacent strands and (ii) to increase the net lipophilicity of the β -hairpin core.

To mimic more closely the charge distribution of the natural β -hairpin, we incorporated a cationic proline derivative [γ -(*S*)-aminoproline; see Chart 1] into the turn region of two non-cysteine-containing analogues (**7** and **8**). In addition, a stereoisomer of **7** was synthesized in which the chirality of all backbone stereocenters is reversed (**9**; because the amino groups on the proline rings have identical configuration in **7** and **9**, these peptides are not enantiomers). Two single disulfide bond-containing versions were generated in which positions 8 and 13 are occupied by threonine and the *i* + 1 position of the turn by either D-proline (**10**) or L-arginine (**11**). These peptides are analogous to the bullet variants studied by Harwig et al. (**10**) and were designed to gauge the relative importance of rigidity and charge at the turn in the context of a single disulfide.

Three control peptides were generated in order to evaluate our design principles. Substitution of L-proline for D-proline at the *i* + 1 position abolishes β -hairpin formation in several designed peptides because of incompatibility of the L-Pro-containing loop with the intrinsic right-handed twist of the β -strands (**17–19**, **21**). To determine whether the activity of our D-proline-containing peptides is a consequence of β -hairpin conformation rather than simple sequence similarity, a diastereomer of **1** was synthesized in which D-proline was replaced by L-proline (**12**). To examine whether choice of residues at the NHB positions is important for activity, we synthesized two peptides containing alanine in place of cysteine with either D-proline (**13**) or γ -(*S*)-amino-D-proline (**14**) at the turn. While these peptides contain turn-promoting D-proline residues, they are not expected to adopt stable β -hairpin structures in solution because alanine has poor intrinsic preference for β -sheet conformation (**41**, **44–46**). Furthermore, the small methyl side chains of alanine preclude the possibility of β -hairpin-stabilizing interstrand hydrophobic interactions.

Peptide Synthesis and Purification. Crude peptides were obtained in good overall yield from solid-phase synthesis. Difficulties in handling of tryptophan-containing peptides arose because of adventitious oxidation of the indole moiety. This degradation was minimized by storage of lyophilized peptides under inert atmosphere. Generally, signs of indole oxidation (coloration) were observed if tryptophan-containing peptides were left in solution for more than 48 h. For all peptides, the masses observed experimentally by MALDI-TOF were in good agreement with the theoretical masses (Table 1). The homogeneities of all purified peptides were adequate for biological assays (>98%) as evaluated by analytical RP-HPLC.

Antimicrobial Activity. Results of assays with four bacterial species (Table 2) show that PG-1 analogues lacking disulfide cross-links can nevertheless display significant antibacterial activity (especially **1**, **7**, and **9**). Initial peptide designs (**1**

Table 2: Antimicrobial Activity of Peptides^a

peptide	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. faecium</i>
(A) Minimum Inhibitory Concentrations Determined in BHI Broth ($\mu\text{g mL}^{-1}$)				
PG-1	6.3	3.1	12.5	3.1
1	6.3	25	50	12.5–25
2	12.5	25	200	12.5
3	12.5–25	12.5	>200	12.5
4	>50 ^b	12.5	>200	12.5
5	12.5	12.5	200	12.5
6	6.3	25	>200	25
7	3.1	12.5	25	12.5
8	50	12.5	200	12.5
9	3.1	6.3	12.5	6.3
10	6.3	3.1	12.5	3.1
11	6.3	3.1	12.5	6.3
12	100	200	>200	>200
13	50	200	>200	>200
14	12.5	100	>200	>200
(B) Minimum Inhibitory Concentrations Determined in BHI Broth + 150 mM NaCl ($\mu\text{g mL}^{-1}$)				
PG-1	6.3	3.1	25	6.3
1	12.5	50	>200	100
7	6.3	25	100	50
9	12.5	25	25	25–50
10	6.3	6.3	25	6.3

^a Minimum inhibitory concentrations (MICs) determined from two independent experiments, each performed in duplicate. The 2-fold serial dilution series for each peptide were prepared in 96-well microtiter plates, and 50 μL of a 10^6 cfu/mL bacterial suspension was added. The microtiter plates were then incubated for 6 h at 37 °C. The MIC was defined as the lowest peptide concentration required for complete inhibition as monitored by OD₅₉₀. ^bAccurate MIC determination was not possible due to formation of peptide aggregates above 50 $\mu\text{g mL}^{-1}$.

and **2**) had good activity against all strains except *S. aureus*, although potency of **1** was substantially decreased in the presence of 150 mM NaCl, which contrasts with the retention of PG-1 activity under high salt conditions. Incorporation of an aromatic residue (Phe) into one of the NHB positions and rearrangement of hydrophobic branched residues to create a potential hydrophobic cluster (**3**) had no effect on potency. Introduction of tryptophan into the core of the hairpin resulted in a change in the activity spectrum, depending on the number of tryptophans and their position. Peptide **4** displayed significant inhibitory activity against *B. subtilis* and *E. faecium* but not against *E. coli* or *S. aureus*. Peptides **5** and **6**, which contain three and two tryptophans, respectively, displayed activity comparable to that of **1** and **2**.

The activity of **7** [γ -(*S*)-amino-D-proline in the turn] was not significantly different from that of **1** (D-proline in turn). This result suggests that there is little or no enhancement of activity upon incorporation of an additional positive charge at the turn region. Similarly, replacement of D-Pro by the cationic analogue in the turn region of the tetratryptophan sequence resulted in little change in potency (**4** vs **8**). Peptide **7** displayed activity very similar to that of its pseudoenantiomer **9**, which is consistent with previous studies that have shown that antimicrobial peptide activity often does not depend on absolute configuration (**49**, **50**). Peptide **9** displays activity comparable to that of PG-1 against all four organisms. As expected, introduction of a single disulfide bond distal to the reverse turn [**10** and **11**; analogous to the bullet analogue of Harwig et al. (**10**)] restored antimicrobial potency to levels comparable to those observed for PG-1 (**10** vs **1**).

There appears to be little difference between the disulfide-bonded analogues containing D-proline (**10**) and arginine (**11**) at the $i + 1$ position of the reverse turn.

To evaluate our design principles, we examined several peptides that were not expected to be active. Previously, we have demonstrated that incorporation of L-proline at the $i + 1$ position of the reverse turn abolishes β -hairpin formation due to incompatibilities with the intrinsic right-handed twist of β -strands (17–19, 21). Peptide **12**, the L-proline diastereomer of **1**, is expected to be disordered in solution. As anticipated, **12** displays little or no antimicrobial activity against *B. subtilis*, *S. aureus*, and *E. faecium* and only moderate activity against *E. coli*. To assess whether choice of residues at the NHB positions is important for activity, we prepared peptides **13** and **14**, in which the four threonine residues at NHB positions of **1** and **7**, respectively, have been replaced with alanine. Alanine shows high intrinsic preference for helical or turn conformation and low propensity for β -sheet formation (41, 44–46). Furthermore, the small methyl side chain of alanine renders the residue incapable of forming favorable interstrand interactions that are important in stabilization of β -hairpin conformations. Peptides **13** and **14** should therefore adopt little or no β -hairpin structure in solution. These analogues are moderately active toward *E. coli* and inactive toward the other three bacteria, which suggests that β -hairpin formation is important for antibacterial activity.

As expected, PG-1 retained activity against all four bacteria in the presence of 150 mM NaCl (10, 14). In contrast, peptide **1** showed a marked decrease in activity against *S. aureus* and *E. faecium* in high salt concentrations. Peptide **7** showed activity similar to that of **1** in high salt (observed differences were within 2-fold uncertainty); **9** was generally similar but displayed enhanced potency against *S. aureus* in comparison to **1**. None of the linear peptides maintained an equally potent activity spectrum in the presence of 150 mM NaCl in comparison to that observed in the absence of salt. Incorporation of a single disulfide bridge distal to the turn (peptide **10**) was sufficient to restore salt-insensitive antibacterial potency, consistent with previously reported observations with single disulfide bond-containing analogues (10).

Structural Analysis by NMR. We selected **1**, **13**, and synthetic PG-1 for conformational analysis in an attempt to correlate structure with activity. PG-1 has previously been shown to form a highly stable, rigid β -hairpin (6, 7). Peptide **1** displays somewhat reduced activity in comparison to PG-1, and we desired to investigate if this attenuated activity results from diminished β -hairpin population. Furthermore, potency of **1** is substantially reduced in the presence of 150 mM NaCl, and study of **1** therefore allows us to determine whether salt effects on activity result from salt-induced structural changes. Peptide **13** shows poor activity even in the absence of salt and allows us to determine whether activity in the absence of salt correlates with extent of β -hairpin formation.

High-resolution ^1H NMR data were acquired for **1**, **13**, and synthetic PG-1 in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ and 40 mM $\text{CD}_3\text{COOD}/\text{CD}_3\text{COONa}$, pH 3.8. Complete assignments were achieved using standard methods (37), and chemical shift values for PG-1 at 24 °C were found to be similar to previously published data acquired at 20 °C (6). Only one set of resonances was observed in the TOCSY spectrum for PG-

1, confirming that the oxidation procedure resulted in formation of intramolecular disulfide bonds in a single arrangement. Fahner et al. reported multiple conformations in their spectroscopic characterization of natural PG-1 (isolated from porcine leukocytes) but not of synthetic PG-1 (6). In our analysis, we detected no traces of a second conformation for PG-1 in aqueous solution. However, at 24 °C, a minor conformation was observed in the two-dimensional spectra of **1** and **13**, which we attribute to rotamers about the tertiary amide of D-proline-10 in slow exchange. Coalescence of the two sets of signals was observed at temperatures above ~ 60 °C for both peptides. We conclude that data acquisition was performed under nonaggregating conditions because 1:10 dilution of NMR samples resulted in identical one-dimensional spectra for all three peptides. It seems highly unlikely that these peptides would aggregate below this concentration range (0.1–0.3 mM) because of their high charge density (+5 to +6 charges per peptide at pH 3.8). Previously, PG-1 was determined to be monomeric at low millimolar concentrations in aqueous buffer by pulsed-field gradient diffusion experiments (6).

Deviation of α -proton chemical shifts from tabulated random coil values is frequently used to obtain insight on peptide secondary structure formation (51, 52). The α -protons of residues that adopt β -sheet conformation are generally shifted downfield relative to residues in a random coil state, while α -proton chemical shifts of residues in α -helices or turns are shifted upfield relative to random coil. Previous work in our laboratory has shown that these α -proton chemical shift measurements may be used to quantify β -hairpin populations (53). The deviation of observed α -proton chemical shifts from tabulated random coil values for the core regions (Leu₅ to Val₁₆) of PG-1, **1**, and **13** are shown in Figure 2. Data for residues Leu₅–Arg₉ and Phe₁₂–Val₁₆ of synthetic PG-1 suggest high population of the β -hairpin conformation, as evidenced by the large positive deviation of α -proton chemical shift from random coil values. Peptide **1** also appears to have a significant β -sheet population. The four threonine residues of **1** show significantly smaller deviations than the corresponding cysteine residues of PG-1, but the α -proton chemical shift data for other residues are comparable for PG-1 and **1**. For both PG-1 and **1**, the relatively small $\Delta\delta\text{H}_{\alpha\text{H}}$ for Val₁₄ may be attributable to ring current effects from Tyr₇, which would lie directly across from Val₁₄ in the predicted β -sheet structure (see Figure 1). In contrast, the α -proton chemical shift data suggest that **13** is not structured under these conditions. Similarly, NMR data for **12** suggest that the diastereomer of **1** containing L-proline in place of D-proline at position 10 is also unstructured (not shown).

Additional evidence of β -hairpin formation by peptide **1** was observed in the form of long-range NOEs (Figure 3). A medium-intensity NOE was observed between the α -protons of Thr₆ and Thr₁₅. Cross-strand NOEs were also detected between the aromatic protons of Tyr₇ and the aliphatic protons of Val₁₄ and Val₁₆ (additional interstrand NOEs, e.g., between α -protons of Thr₈ and Thr₁₃, may have been present but could not be unambiguously assigned because of spectral overlap). Intrastrand NOEs were observed between the side chains of Tyr₇ and Arg₉ and between Phe₁₂ and Val₁₄ (not shown). These data show that the hydrophobic cluster consisting of Leu₅, Tyr₇, Phe₁₂, Val₁₄, and Val₁₆ of PG-1 is

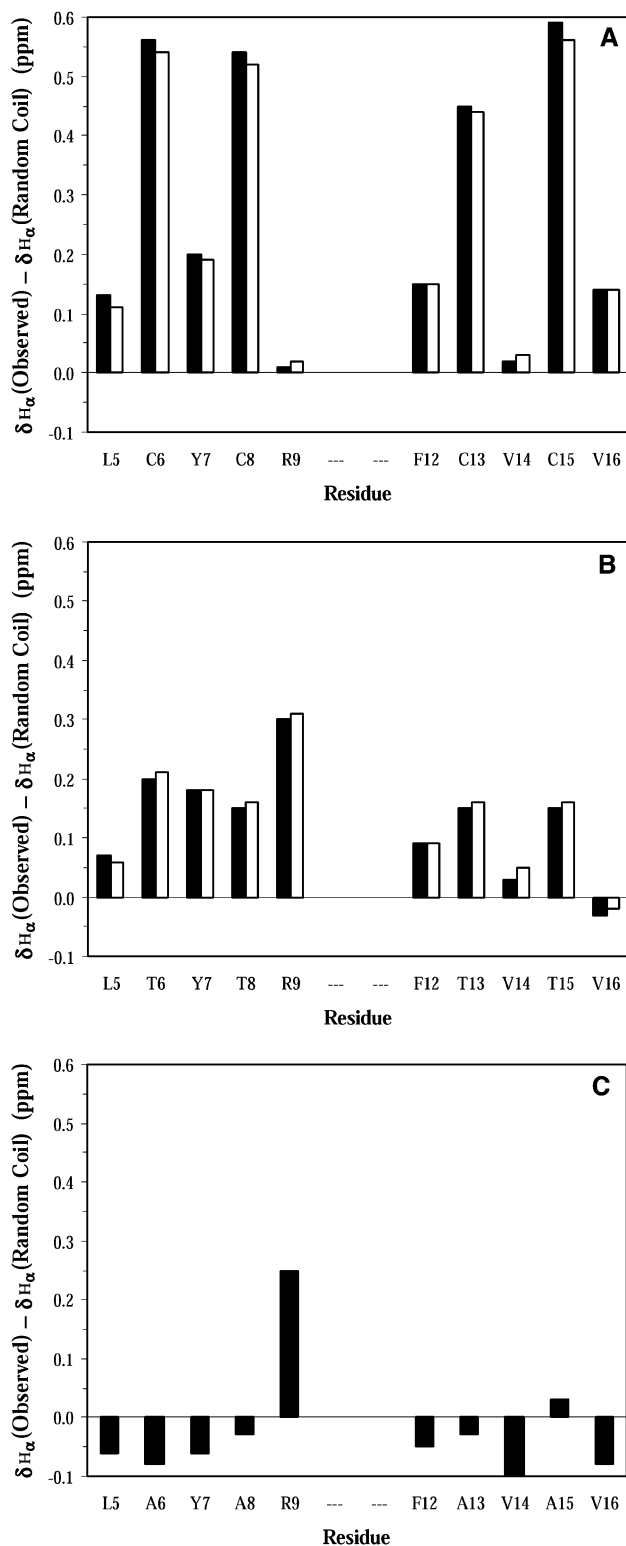


FIGURE 2: Deviation of observed α -proton chemical shifts from tabulated random coil values for the hairpin core region at 24 °C for (A) synthetic PG-1, (B) peptide **1**, and (C) peptide **13**. Residues involved in the turn (positions 10 and 11) are not plotted. Data were acquired in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ containing 40 mM $\text{CD}_3\text{COOD}/\text{CD}_3\text{-COONa}$, pH 3.8 (filled bars), and in the presence of 150 mM NaCl (open bars) for PG-1 and peptide **1**. Large positive deviations ($\Delta\delta_{\text{H}\alpha} \geq 0.1$ ppm) for PG-1 and **1** imply high β -sheet content. The similarity of $\Delta\delta_{\text{H}\alpha}$ values for PG-1 and **1** in the presence and absence of 150 mM NaCl suggests no significant change in stability of secondary structures in high salt concentrations. Chemical shift values for residues in the random coil state were taken from ref 37.

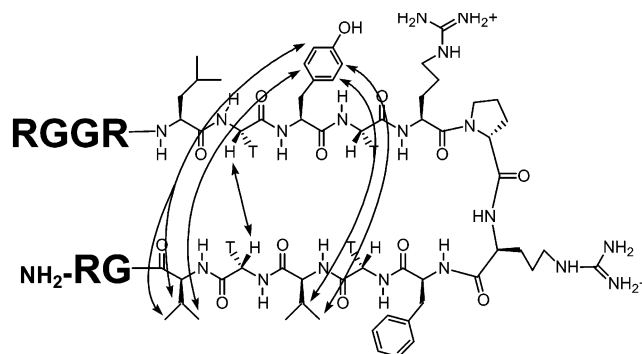


FIGURE 3: Selected cross-strand NOEs observed for peptide **1** at 4 °C. Side chains of residues at NHB positions are omitted for clarity. Several additional long-range signals were observed but could not be unambiguously assigned due to spectral overlap; these are not included. Buffering conditions as in Figure 2.

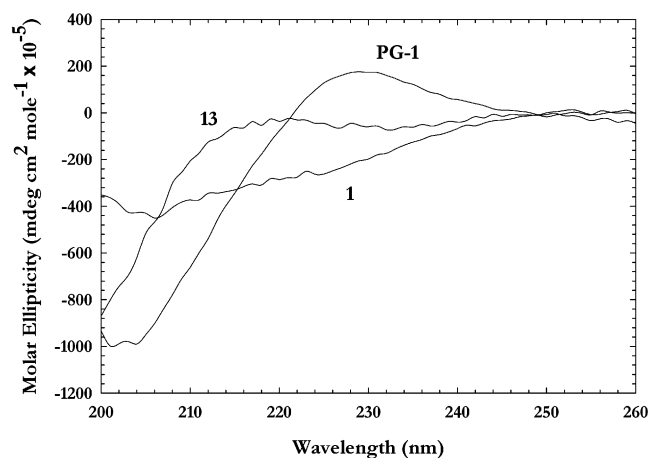


FIGURE 4: Circular dichroism spectra of synthetic PG-1, peptide **1**, and peptide **13** at 24 °C in aqueous 40 mM Tris, pH 7.2. Spectra for PG-1 and **1** were unchanged in the presence of 150 mM NaCl (not shown).

maintained in linear variant **1**. When ^1H NMR spectra for peptide **1** and PG-1 were acquired in the presence of 150 mM NaCl, very few changes in α -proton chemical shift were observed (Figure 2). This behavior implies that there is little or no decrease in stability of the β -hairpin conformation in the presence of high salt concentration. Several of the interstrand NOEs present in the absence of salt between the side chains of Tyr₇ and Val₁₄ and between Tyr₇ and Val₁₆ were also observed for **1** in 150 mM NaCl (data not shown).

Overall, the NMR data indicate that PG-1 is well-structured, **1** is moderately structured, and **13** is poorly structured in aqueous buffered solutions. Furthermore, while addition of 150 mM NaCl has a pronounced effect on the antibacterial activity of **1**, this high salt concentration does not seem to alter the β -hairpin population of **1**.

Circular Dichroism. Circular dichroism spectra for PG-1, **1**, and **13** in aqueous 40 mM Tris, pH 7.2, are shown in Figure 4. A maximum at 225–230 nm and a minimum at ~ 207 nm were observed for PG-1, similar to previously reported results (14). Spectra for peptides **1** and **13** display no distinctive extrema in aqueous solution. In the presence of 150 mM NaCl, no change was observed in the CD spectrum for PG-1 or **1** (data not shown). These results are consistent with our analysis by NMR, indicating that neither PG-1 nor **1** is conformationally altered by high salt concentrations.

DISCUSSION

Design of Non-Cysteine-Containing Antimicrobial β -Hairpins. Here we have reported the first PG-1 analogues that lack cysteine but nevertheless display significant antibacterial activity. Previous studies on protegrin variants have shown that antibacterial activity is highly dependent on β -hairpin stabilization by disulfide bonds or backbone cyclization (10, 12–14). The design features of one of our most potent analogues (**7**) include the use of threonine in place of all four cysteine residues [threonine displays high intrinsic preference for β -strand conformation (41, 44–46)], incorporation of D-proline at the $i + 1$ position of the β -turn to promote β -hairpin folding, and functionalization of the γ -carbon of the proline with an amino group to mimic the charge distribution of PG-1. An isomer of **7** in which all of the backbone stereocenters are reversed (**9**) displayed activity comparable to that of PG-1 against all four organisms tested. Peptides comprised of D-amino acids (D-peptides) have increased stability in the presence of proteases and are therefore frequently explored as potential drug candidates.

Assessment of Design Features. Choice of residues at the NHB positions appears to be important, as **13** and **14** (which contain alanine at each of the four sites) display poor activity despite the reverse turn promoted by D-proline. From these results we conclude that use of sheet-promoting residues at the NHB sites is necessary (but not sufficient) for the activity of linear analogues. Furthermore, inactivity of **14** shows that while overall charge is important (12, 14), simply mimicking the charge distribution of the protegrin-1 molecule does not compensate for a poorly structured β -hairpin. Although the threonine residues at NHB sites in **1** cannot be replaced by alanine residues without drastic consequences, only modest changes in activity result from replacement of some or all threonine residues with hydrophobic alternatives (**1** vs **2–6** and **7** vs **8**). Previous studies have shown that maintaining the hydrophobic cluster formed by residues in HB positions, Leu₅, Tyr₇, Phe₁₂, Val₁₄, and Val₁₆, is important for activity (12); therefore, we did not alter these residues. In the solution structure of PG-1 this cluster projects to one side of the plane of the β -hairpin (6, 7). Our observations demonstrate that hydrophobic clusters engineered onto the opposite face of the β -hairpin have little effect on antimicrobial activity.

Peptide **12** was synthesized to examine the importance of the enforced reverse turn in our linear analogues. Surveys of turn conformations found in natural proteins have shown that the most common β -turns are the type I and II turns but that the turns found in 2:2 β -hairpins are predominantly type I' and II' turns (42). This correlation between the normally rare type I' and II' β -turns and β -hairpins appears to arise because strands in β -sheets have an intrinsic right-handed twist (54), and the geometries of the common type I and II turns are not compatible with this intrinsic β -strand twist (42). The conformational constraints of the pyrrolidine ring of D-proline lock the ϕ torsional angle at 63°, which matches the ϕ angle found in residues at the $i + 1$ position type I' and II' turns. In contrast, L-proline induces type I and II turn formation. In model systems, β -hairpin formation is promoted if D-proline is incorporated at the $i + 1$ position of the reverse turn but discouraged if this residue is replaced with L-proline (17–19, 21). Peptide **12** is a diastereomer of **1** in which L-proline occupies position 10. As expected, **12** shows poor

antimicrobial activity, which suggests that an appropriate local conformational propensity in the loop segment, in addition to sheet-stabilizing residues at the NHB positions, is important for activity. From these results, it is clear that no single design feature is dominant in the non-cysteine-containing variants. Active linear analogues require several features including strand-promoting residues at the NHB positions, a stabilized reverse turn with appropriate conformation, and a charge distribution similar to that of PG-1.

We generated two variants containing a single disulfide bond (**10** and **11**). Peptide **10** contains the hairpin-promoting D-proline at position 10, while **11** contains arginine at this position. Both of these analogues display potency comparable to that of PG-1, confirming that activity is highly dependent on stability of the β -hairpin conformation. These observations also demonstrate that net charge and turn rigidity have a smaller effect on the antimicrobial activity of disulfide-stabilized β -hairpins than on linear β -hairpins. Peptide **10**, like PG-1, also displayed no loss in activity in the presence of 150 mM NaCl. PG-5 is a natural homologue of PG-1 that contains L-proline at the $i + 1$ position of its β -turn (Figure 1A) but displays potent antimicrobial activity equal to that of PG-1 (55). In PG-5, the incompatibility of the type I or II β -turn with β -hairpin structure is superseded by the two disulfide bonds that lock the peptide into a rigid conformation. We have shown with **10** and **11** that modifications in β -turn propensity at position 10 result in no change in activity if the structure is stabilized by a disulfide bond. Thus, substitution of D-proline for L-proline in the loop region of PG-5 is unlikely to alter its potency.

Structural Correlations. Conformational analysis of selected peptides suggests that a propensity for β -hairpin folding is necessary but not sufficient for antimicrobial activity. The necessity for β -hairpin formation is indicated by the poor activity of **12–14**. In **12**, β -hairpin formation is precluded by L-proline in the turn segment, and in **13** and **14**, β -hairpin formation is discouraged by the four alanine residues. In all three of these peptides the hydrophobic cluster residues are present (Leu₅, Tyr₇, Phe₁₂, Val₁₄, and Val₁₆), and there is a PG-1-like distribution of positive charges. Our results show that the ability to adopt a β -hairpin conformation in solution does not guarantee high activity because **1** retains the β -hairpin conformation but loses antibacterial activity in the presence of 150 mM NaCl. High salt concentration adversely affects the activity of many antimicrobial peptides (11), and there has been speculation that salt can disrupt the folding of these peptides. The behavior we report from **1** shows that the salt effect, at least in this case, does not correlate with a change in folding in solution (it is possible that salt influences folding at membrane surfaces).

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SUPPORTING INFORMATION AVAILABLE

Procedure for the synthesis of γ -(S)-amino-L-proline with appropriate protecting group strategy from *trans*-hydroxy-L-proline. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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