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Bidirectional Tandem Pseudoproline Ligations of Proline-Rich Helical Peptides

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Abstract: We have developed a bidirectional ligation strategy for preparing proline-rich peptides that couples three unprotected segments in tandem to form two pseudoproline bonds (thia- or oxaproline) without the need for a protection scheme. Ligation in the C→N direction exploits the regioselectivity of an amino terminal (NT)-Cys in forming a thiaproline bond over an NT-Ser or NT-Thr peptide in forming an oxaproline bond with a peptide that bears a carboxyl terminal (CT)-glycoaldehyde ester. Thus, successive ligations of three unprotected segments in a predetermined order formed a thiaproline and then an oxaproline bond. However, ligation through the N→C direction is flexible. An NT-Cys, NT-Ser, or NT-Thr segment bearing a CT-glycerol ester as a masked CT-glycoaldehyde was used to form a pseudoproline bond with another CT-glycoaldehyde ester segment. Oxidative activation of the glycerol ester product to a CT-glycoaldehyde ester effected another round of pseudoproline ligation with an NT-Ser, NT-Thr, or NT-Cys segment. This sequential process could be extended for ligating three or more segments. Optimized conditions for this bidirectional strategy were applied successfully to syntheses of five analogues of a proline-rich helical antimicrobial peptide, the 59-residue bactenecin 7 (Bac 7), using three segments containing 24, 14, and 21 amino acids, respectively. CD spectra showed that Bac 7 and its analogues displayed typical polyproline II helical structures in phosphate buffers. Furthermore, the ψPro-containing analogues exhibited antibacterial activity similar to Bac 7.

Introduction

Proline-rich sequences are often found as multiple tandem repeats in proteins [1−4] and antimicrobial peptides.[5−8] Structurally, proline-rich peptides or proteins are known to display helical structures.[9] Collagen, the most abundant structural protein in humans and animals, consists of three peptide chains that form an extended triple helix.[10] In each peptide chain, the tripeptide sequence Gly-X-Y, where X and Y are often proline or 4-hydroxyproline (Hyp), repeats about 300 times. Another type of proline-rich sequence is found in cationic antimicrobial peptides including RP39, mucin glycoproteins, and the bactenecin family.[5−8] Two bactenecins, Bac 5 and Bac 7 with approximate molecular masses of 5 and 7 kD, respectively, are characterized by a high content of proline (>45%) and arginine (>20%) residues. Bac 5 contains 43 amino acid residues with a repeated sequence of Arg-Pro-Pro separated by single

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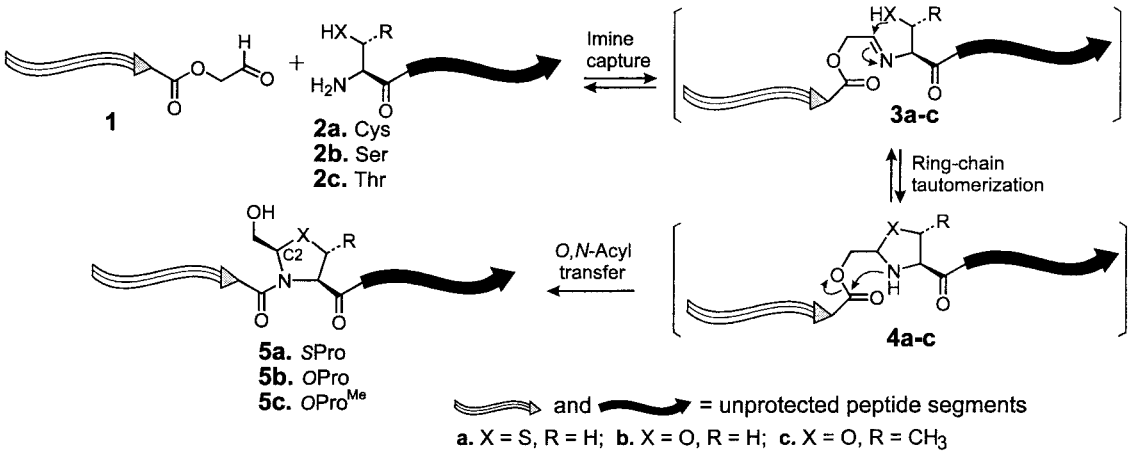

Figure 1. Orthogonal pseudoproline ligation of a CT-glycoaldehyde segment **1** with an NT-Cys, NT-Ser, or NT-Thr segment **2a**−**c** to form a pseudoproline bond (ψPro) **5a**, thiaproline (*S*Pro); **5b**, oxaproline (*O*Pro); or **5c**, 5-methyloxaproline (*O*Pro^{Me}). The pseudoprolines with a hydroxymethyl moiety at C2 are *R*-epimers.

hydrophobic residues.[11] CD and 2D NMR studies indicate that Bac 5 displays a poly-L-proline II helical structure, and represents a new structural category [12,13] that differs from the α-helix and β-sheet structures of antimicrobial peptides.[14] Bac 7, a 59-residue peptide, contains the three tandem repeats of a tetradecamer including several Pro-Arg-Pro triplets alternating with single apolar residues.[6] However, the structure of Bac 7 has not been studied.[15]

In addition to its repeated sequence, Pro is also involved in several types of reverse turns including types I, II, IV, and VIII.[16−19] In globular and membrane proteins, Pro often occurs as a kink in the middle of an α-helix.[20,21] The *cis*−*trans* isomerization of an Xaa-Pro bond (Xaa = any amino acid) has been implicated in critical roles in protein folding [22] and signal transduction.[23]

These peculiar effects of Pro have led to the development of various proline analogues and pseudoprolines (ψPro) that are intended to produce Pro-like reverse turns,[24,25] to give better control of the *cis*−*trans* ratios,[26−30] and to prepare various forms of polyproline helices.[31] A popular method of ψPro synthesis is based on cyclic condensations of Cys, Ser, Thr, Asn, and Trp with aldehydes or ketones to form pre-made ψPro units as building blocks in stepwise or convergent synthesis.[32−35] Another method employs acid- or metal-catalyzed condensations on small protected peptides.[33c,36] However, these methods are generally not suitable for preparing large ψPro-containing peptides because pseudoprolines, particularly oxaprolines, are unstable to the conditions of acidic deprotection or cleavage steps in conventional synthetic strategies.[33] To overcome this limitation, bioincorporation of thiaproline into a protein such as annexin V through aminoacyl-tRNA synthetases has been developed.[37] However, attempts to incorporate oxaproline or selenaproline into annexin V were unsuccessful.

In pursuing a different strategy to form various pseudoproline bonds, we have developed methods for orthogonal ligations using unprotected peptide segments to form thiaproline or oxaproline bonds.[38,39] Both methods employ a CT-glycoaldehyde peptide segment **1** to couple an NT-dinucleophile segment such as NT-Cys **2a**, NT-Ser **2b**, or NT-Thr **2c**, first through an imine **3a**−**c** to form a thiazolidine or oxazolidine ester intermediate **4a**−**c**, and then an *O*,*N*-acyl migration to afford a thiaproline bond *S*Pro **5a** or oxaproline bond *O*Pro **5b** or *O*Pro^{Me}

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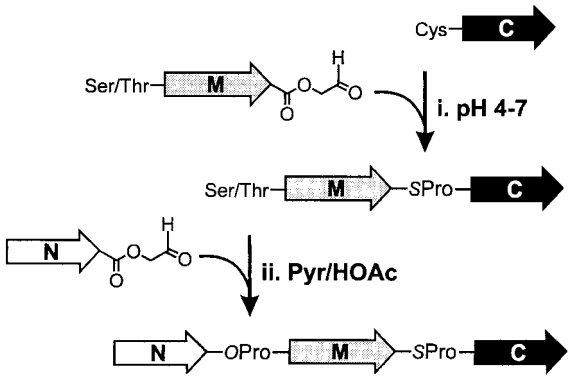

Figure 2. C→N approach of three-segment tandem ligation. (**i**) thiaproline ligation in aqueous buffers. (**ii**) oxaproline ligation in pyridine−acetic mixtures. N = Asp-Ser-Phe-Gly-OCH_2CHO **9a**; M = Ser-Leu-Ile-Leu-Asn-Gly-OCH_2CHO **10a**; C = Cys-Phe-Lys-Ile-OH **12a**.

5c (Figure 1). The pseudoproline formed at the ligation site is a novel proline mimetic that retains the amide peptide backbone but contains an additional C2-hydroxymethyl moiety. The newly created stereocenter C2 at the ψPro ring is an *R*-epimer due to the $A^{1,3}$-effect of chiral C^{α} at the NT-Cys **2a**, NT-Ser **2b**, or NT-Thr **2c** peptide.[39,40]

Thiaproline ligation can be distinguished from oxazolidine ligation through the use of different solvents and reaction conditions. Thiaproline ligation between CT-glycoaldehyde **1** and NT-Cys **2a** proceeds efficiently in both aqueous conditions and nonaqueous pyridine−acetic acid mixtures. In contrast, oxaproline ligation with NT-Ser **2b** or NT-Thr **2c** peptides is not observed in aqueous solutions and requires the use of nearly anhydrous conditions such as pyridine−acetic acid mixtures.[39,41] Even under such nonaqueous conditions, thiaproline ligation is >1000-fold faster than oxaproline ligation. This type of solvent-driven regioselectivity could be exploited for a tandem ligation strategy, in which three or more unprotected peptide segments are coupled without a protection scheme (Figure 2). Since a ψPro has a *cis* enhancement on the Xaa-ψPro bond,[30,39] a tandem ligation strategy would also enable the study of the conformational effects of these two novel pseudoprolines in polyproline-helical peptides such as Bac 7.

In this paper, we describe the development of a bidirectional tandem pseudoproline ligation strategy for the synthesis of polyproline helical peptide analogues of Bac 7 through thiaproline or oxaproline bonds. Furthermore, we have also determined the conformational and biological effects of pseudoproline replacements in proline-rich helical peptides.

Results and Discussion

C→N Three-Segment Tandem Ligation. The strategy for the C→N tandem ligation of three unprotected peptide segments by a thiaproline and then an oxaproline ligation is shown in Figure 2. For convenience, these segments are designated in the N→C direction as an amino (N) segment bearing a CT-glycoaldehyde, a middle (M) segment carrying both a CT-glycoaldehyde ester and an NT-Ser or NT-Thr, and a carboxyl (C) segment bearing an NT-Cys.

Model studies were employed to optimize the tandem ligation conditions (data not shown). A two-stage thiaproline ligation of the C-segment (Cys-Phe-Lys-Ile-OH) and M-segment (Ser-Leu-Ile-Leu-Asn-Gly-OCH_2CHO **10a**) was found to be optimal in two-stage aqueous buffers first at pH 5.3 for 10 h, and then at pH 6.5 for 20 h. At pH 5.3, HPLC monitoring showed two major intermediates as the *R,S*-epimers of thiazolidine-ester which were converted via an *O,N*-acyl shift at pH 6.6 after 20 h in 86% yield to a single amide product, an *S*Pro-containing MC-segment (Ser-Leu-Ile-Leu-Asn-Gly-**SPro**-Phe-Lys-Ile-OH). Adjusting the ligation condition to pH 6.6 at the second stage accelerated the *O,N*-acyl rearrangement without observable side reactions due to the thiazolidine-ester hydrolysis or random intramolecular acyl transfer reaction that occurs at pH > 6.6. Furthermore, under these conditions, inter-or intramolecular oxaproline ligation of M-segment **10a** forming oligomers or cyclic peptides, respectively, was not observed.

The MC segment product was purified by HPLC and then subjected to an oxaproline ligation with the N-segment (Asp-Ser-Phe-Gly-OCH_2CHO **9a)** in a pyridine−acetic acid mixture (1:1, mol/mol). The oxaproline ligation was completed in 35 h to afford in 78% yield the three-segment ligated NMC product (Asp-Ser-Phe-Gly-***O*Pro**-Leu-Ile-Leu-Asn-Gly-**SPro**-Phe-Lys-Ile-OH) with two pseudoprolines. No oxazolidine-ester intermediates were observed. It should be noted that the three-segment tandem ligation scheme was simplified by the absence of a protection or deprotection step between each ligation.

Syntheses of unprotected C-segments **12a**−**d** were straightforward using conventional Boc chemistry on a commercially available resin **11**, but N- and M-segments required the use of an acetal resin **6** (Figure 3).[42,43] N-segments **9a** and **9b** were synthesized using Fmoc chemistry on the cyclic acetal resin **6** as carboxyl terminal (CT)-1,2-diol precursors **7a** and **7b** which were then quantitatively converted to the corresponding aldehyde by sodium periodate in aqueous buffers at pH 4. However, syntheses of M-segments **10a**−**c** or any N-segments with NT-

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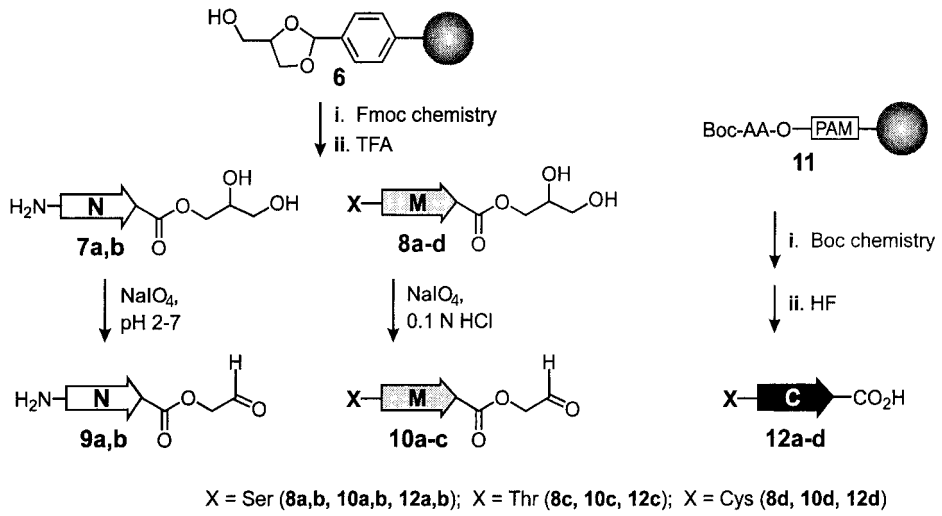

Figure 3. Syntheses of unprotected N-, M-, and C-peptide segments. N-segments **9a**,**b** and M-segments **8d** and **10a**−**c** were synthesized on cyclic acetal resin **6**. The C-segments **12a**−**d**, were synthesized using the PAM resin **11**.

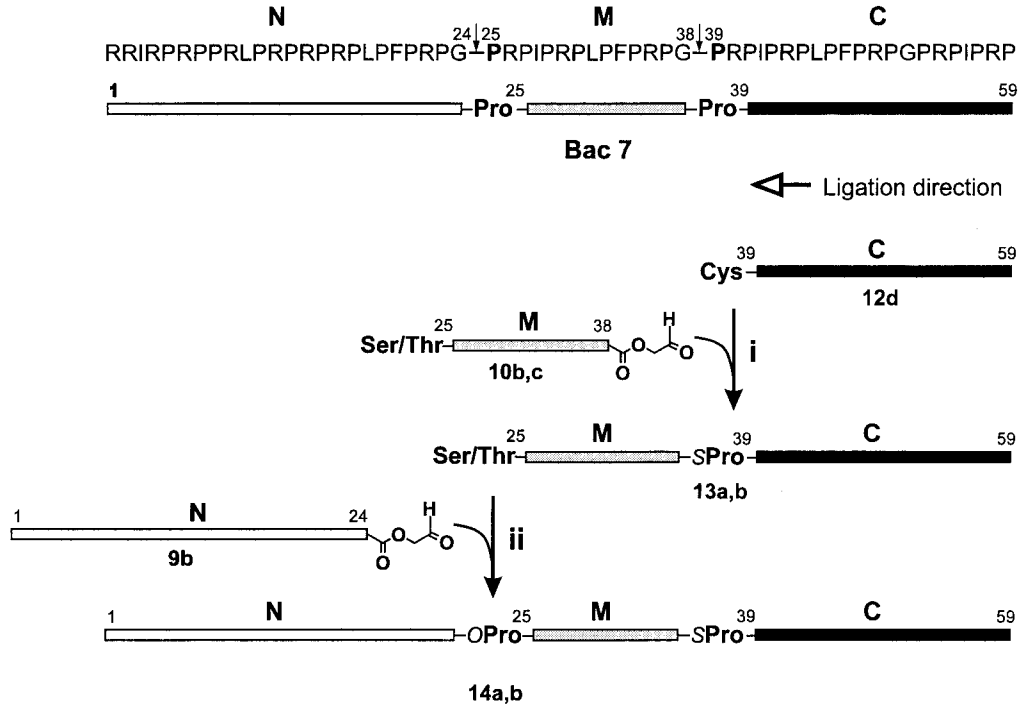

Figure 4. C→N three-segment tandem ligation of **Bac 7**, affording two analogues [*O*Pro 25, *S*Pro 39] Bac 7 **14a** and [*O*Pro^{Me} 25, *S*Pro 39] Bac 7 **14b.** (**i**) thiaproline ligation in aqueous buffers at pH 5.2 for 10 h, then at pH 6.6 for 20 h. (**ii**) oxaproline ligation in pyridine−acetic acid mixture (1:1, mol/mol) for 35 h. Here, *O*Pro stands for *O*Pro and *O*Pro^{Me}.

Ser and NT-Thr required selective oxidation because the N-terminal 1,2 amino alcohols of NT-Ser or NT-Thr are susceptible to periodate oxidation, particularly under neutral to basic pH conditions.[44] At pH 6.6, the major product (68.8%) was oxidation at the NT-1,2-amino alcohol of NT-Ser **8a**. However, under strongly acidic conditions at pH < 2, selective oxidation of CT-1,2-diol was achieved due to protonation of the NT-amine group. The desired product **10a** was obtained in 86.3% yield in 0.1 N HCl solutions. Similarly, **10b** and **10c** were obtained from **8b** and **8c** in 84.6 and 90.9% yields, respectively.

The optimized conditions based on model peptide studies were applied to the C→N syntheses of two Bac 7 analogues, [*O*Pro25, *S*Pro39] Bac 7 **14a** and [*O*Pro^{Me}25, *S*Pro39] Bac 7 **14b**, by three N-, M-, and C segments consisting of 24, 14, and 21 amino acids, respectively. Their ligation sites were selected at Pro25 and Pro39 where the Gly-Pro sequence offers the least steric hindrance (Figure 4). Thiaproline ligation between the C-segment of NT-Cys **12d**, the M-segment with CT-glycoaldehyde and NT-Ser **10b** or NT-Thr **10c** was performed in the optimized two-stage aqueous conditions. Two intermediates as the *R,S*-epimers of thiazolidine-esters were observed at the pH 5.3 stage, which were converted via an *O,N*-acyl shift to a single amide product of NT-Ser **13a** or NT-Thr **13b** at the second stage of pH 6.2 (Figure 5). Subsequent oxaproline ligation in pyridine−acetic acid mixtures (1:1, mol/mol) between CT-glycoaldehyde of N-segment **9** and NT-Ser of **13a** or NT-Thr of **13b** completed the syntheses of **14a** and **14b**. HPLC monitoring showed that the reaction was clean and predominantly gave a single product **14a** or **14b** as well as the unreacted

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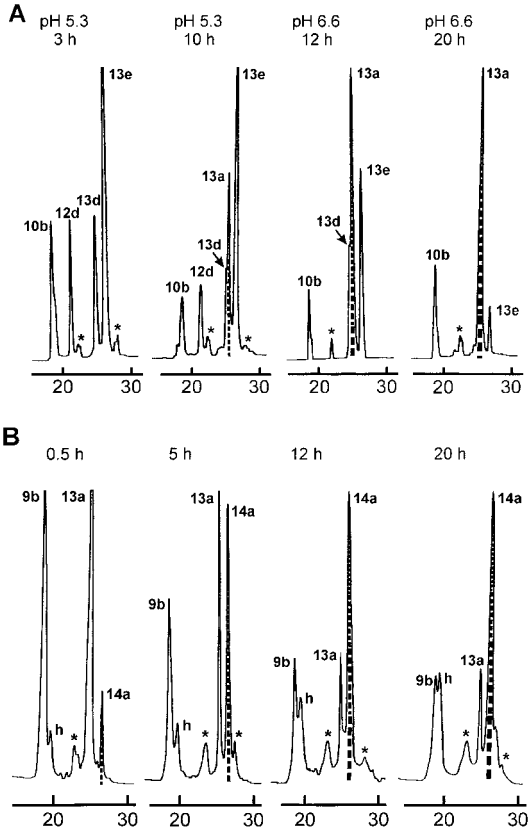

Figure 5. HPLC profiles showing the process of C→N three-segment tandem ligation of **10b**, **12d**, and **9b** to the Bac7 analogue **14a**. (A) Thiaproline ligation between C-segment **12d** and M-segment **10b** in aqueous buffers, first at pH 5.2 for 10 h to form thiazolidine esters **13d**,**e**; then after *O*,*N*-acyl transfer at pH 6.6 for 20 h to form the amide bond product **13a** (hatched peak). (B) Oxaproline ligation of **13a** with an N-segment **9b** in pyridine−acetic acid (1:1, mol/mol) to form **14a** (labled). *, impurities from pyridine. **h**, hydrolysis product of **10b** to carboxylic acid.

starting materials and hydrolyzed N-segment **9b** as observable byproducts (Figure 5). The results of this synthesis of the Bac 7 analogues are summarized in Table 1.

N→C Three-Segment Sequential Ligation. To avoid the selective oxidation needed in the C→N approach, an N→C three-segment ligation strategy was developed for syntheses of three Bac 7 analogues, [*S*Pro25, *O*Pro39] Bac 7 **17a**, [*S*Pro25, *O*Pro^{Me}39] Bac 7 **17b** and [*S*Pro25, *S*Pro39] Bac 7 **17c** (Figure 6). For comparison, the same ligation sites as those in the C→N approach were used, but the ligation sequence was reversed, starting with an N-segment (Figure 6).

Syntheses of N-segment **9b** bearing a CT-glycoaldehyde, M-segment **8d** bearing an NT-Cys and a CT-glycerol ester, and the C-segments bearing an NT-Ser **12b**, NT-Thr **12c**, and NT-Cys **12d** are shown in Figure 3.[43] Similar to the C→N approach, the thiaproline ligation between N-segment **9b** and M-segment **8d** was performed in two-stage aqueous buffers, in which the 1,2-diol moiety of CT-glycerol ester was not affected. The *O*,*N*-acyl rearranged amide product **15** of thiaproline ligation was obtained in 92.5% yield. Oxidative activation of the peptide glycerol ester **15** by $NaIO_4$ in aqueous buffers at pH 4 converted the resulting peptide to a glycoaldehyde ester **16**. Oxaproline or thiaproline ligation between **16** and three C-segments, NT-Ser **12b**, NT-Thr **12c**, and NT-Cys **12d**, afforded the final products **17a**, **17b**, or **17c**, respectively. The ligation courses in this N→C approach as monitored by HPLC (data not shown) were similar to those in the C→N approach (Figure 5) and gave the desired product as a major peak. The results are summarized in Table 1.

Table 1. Summary of Strategy and Yield in the Syntheses of Five Bac 7 Analogues through C→N and N→C Three-Segment Tandem Ligation

ligation sequence		ligation site*	product	yield (%)
a. C→N approach				
M (14aa) + **C** (21aa)	**10b + 12d**	25 **SPro** 59	**13a**	92.6
	10c + 12d	25 **SPro** 59	**13b**	93.1
N (24aa) + **MC** (35aa)	**9b + 13a**	1 **OPro** SPro 59	**14a**	80.7
	9b + 13b	1 **OPro^{Me}** SPro 59	**14b**	77.6
b. N→C approach				
N (24aa) + **M** (14aa)	**9b + 8d**	1 **SPro** 38	**15**	92.5
C (21aa) + **NM** (38aa)	**12b + 16**	1 SPro **OPro** 59	**17a**	78.2
	12c + 16	1 SPro **OPro^{Me}** 59	**17b**	76.4
	12d + 16	1 SPro **SPro** 59	**17c**	85.0

*Ligation sites shown in bold.

The N→C approach does not require a predetermined order as in the C→N approach because the C terminus of the M-segment exists as a CT-glycerol ester. The thiaproline or oxaproline ligation of the M-segment with the N-segment can be carried out specifically in aqueous buffers or in pyridine−acetic acid mixtures. Under these conditions, the CT-glycerol ester is stable, and an oxidative conversion of the CT-glycerol ester to a CT-glycoaldehyde is required to effect the subsequent pseudoproline ligation. Thus, this approach could be expanded to more than three segments by an iterative process to provide a convenient method for the syntheses of peptide and proteins containing proline-rich repeated sequences.[1,2]

Confirmation of Xaa-ψPro Bond. The ligation sites, *O*Pro25 and *S*Pro39 of **14a**,**b** as well as *S*Pro25 and *O*Pro39 of **17a**−**c**, were confirmed amide bonds by FT-IR which showed the absence of the ester peaks in the region from 1710 to 1780 cm^{-1}. The amide linkage was further confirmed by attempted aminolysis with 1.0 M H_2NOH at pH 9.1 and hydrolysis with 0.1 M LiOH for 10 h. Under these conditions, the susceptible unrearranged ester bond would yield two cleaved segments, but no aminolysis or hydrolysis product was detected by HPLC. Furthermore, aldehydes have been known to form heterocycles with an Arg side chain.[45] On the basis of MS analysis, no evidence of Arg modification by the glycoaldehyde moiety was found.

CD Characterization. To investigate the influence of pseudoproline replacement by *O*Pro, *O*Pro^{Me}, and *S*Pro on the structure of Bac7, CD spectroscopy was employed to characterize the Bac 7 structure and those of its eight analogues containing one or two ψPro bonds (Figure 7). **14a**,**b** and **17a**−**c** prepared by C→N and N→C three-segment ligation strategies comprise two ψPro bonds, whereas [*O*Pro25] Bac7 **18a**, [*O*Pro^{Me}25] Bac 7 **18b**, and [*S*Pro25] Bac 7 **18c** prepared by two-segment pseudoproline ligation [43] contain a single ψPro bond in each peptide. For each peptide, the CD spectrum was determined in 0.02−0.2 mM sodium phosphate buffers at pH 7.2.

As shown in Figure 7, the CD spectra of Bac 7 and its eight analogues were consistent with typical polyproline helix II structures, exhibiting strong broad negative $\pi-\pi^*$ bands at

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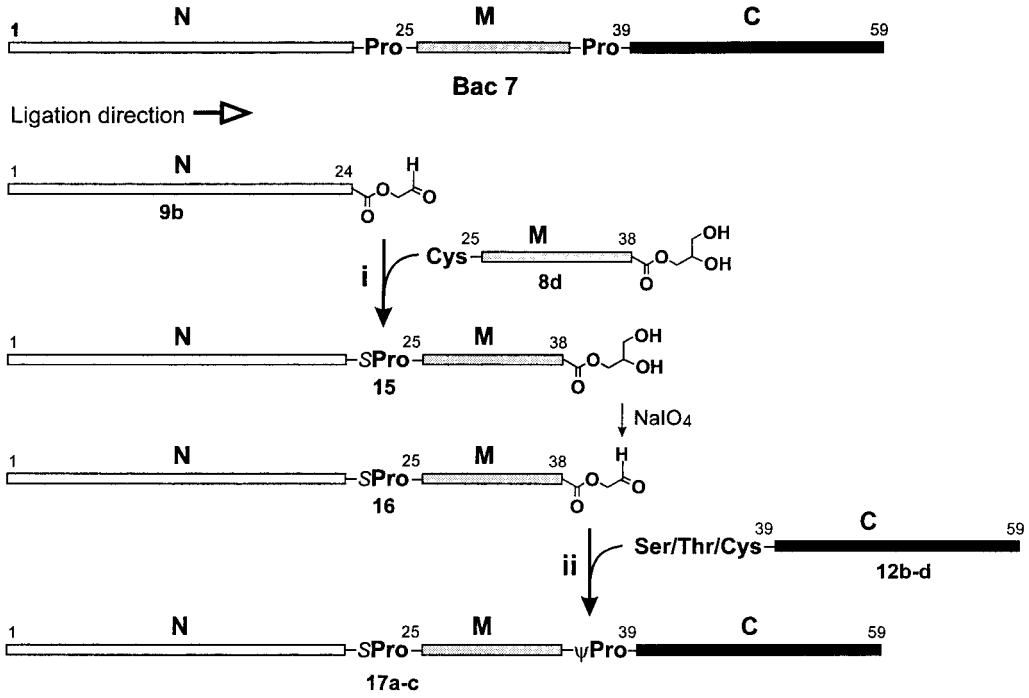

Figure 6. Synthesis of Bac 7 through an N→C three-segment sequential ligation strategy affording three analogues **17a**−**c.** Conditions for **i** and **ii** are similar to those in Figure 4. ψPro standing for *O*Pro, *O*Pro^{Me}, and *S*Pro; for structures, see Figure 1.

∼206 nm.[46−48] These results indicated that these peptides could form stable polyproline helical structures in aqueous buffers. Furthermore, dilution experiments did not show significant changes in CD spectra of Bac 7 and its eight analogues at concentrations from 0.02 to 0.2 mM (data not shown), suggesting these analogues largely exist in monomeric forms.

The replacement of ψPro caused a shift in the maximal negative $\pi-\pi^*$ band to shorter wavelength (blue shift). Compared with the maximal negative band of Bac 7 at 209 nm, different blue shifts were observed in its analogues. For example, a blue shift from 209 to 203 nm was observed for Bac analogue **17a** (Figure 7A). This blue shift is likely caused by the presence of a minor population of *cis* conformations (poly-L-proline I helix).[12,13] Proline has a significant propensity to impart the *cis* isomer in peptides and proteins due to the low free energy between the *cis* and *trans* isomers of an Xaa-Pro bond (Xaa = any amino acid).[49,50] We have previously observed that pseudoprolines enhance *cis* conformation with 40−67% *cis* isomers of Xaa-ψPro bonds, in the order of *O*Pro > *O*Pro^{Me} >SPro.[39] This order is consistent with the trends of the blue shifts: [*O*Pro25] Bac7 **18a** (4.6 nm) > [*O*Pro^{Me}25] Bac 7 **18b** (1.5 nm) > [*S*Pro25] Bac 7 **18c** (0.6 nm). The *cis* isomer caused a structural conversion from polyproline helix II to I. Similar results were also observed by Mutter et al.[51] in C2-disubstitued oxaproline-containing peptides.

The *cis* effects on the polyproline helical structures were further confirmed by the Bac 7 analogues **17a**−**c** with two ψPro substitutions. The blue shifts were [*O*Pro25, *S*Pro39] Bac 7 **14a** (6 nm) > [*O*Pro25] Bac7 **18a** (4.6 nm), [*O*Pro^{Me}25, *S*Pro39] Bac 7 **14b** (3.5 nm) > [*O*Pro^{Me}25] Bac 7 **18b** (1.5 nm) and [*S*Pro25, *S*Pro39] Bac 7 **17c** (0.9 nm) > [*S*Pro25] Bac 7 **18c** (0.6 nm). In addition, the substitution at position 25 is a little more sensitive than that at position 39, so that the blue shifts were [*O*Pro25, *S*Pro39] Bac 7 **14a** (6 nm) > [*S*Pro25, *O*Pro39] Bac 7 **17a** (5 nm) and [*O*Pro^{Me}25, *S*Pro39] Bac 7 **14b** (3.5 nm) > [*S*Pro25, *O*Pro^{Me}39] Bac 7 **17b** (2.5 nm).

Antibacterial Activity. To examine the effects of ψPro replacements on antimicrobial activity, Bac 7 and five analogues **14a**,**b** and **17a**−**c**, derived from the bidirectional tandem ligation strategy, were assayed against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria as well as a fungal strain (*Candida kefyr*) using the radial diffusion method.[52] The results (Table 2) showed that **14a**,**b** and **17a**−**c** displayed activities similar to that of Bac 7 with minimal inhibition concentrations (MIC) at 0.2−0.4 μM.

Conclusions

Two-segment ligation strategies by thiaproline,[38] oxaproline,[39] cysteine,[53,54] glycine,[55] methionine,[56] and histidine[57] bonds have been developed for peptide and protein syntheses. This repertoire of orthogonal ligation methods specific for different NT-amino acids of peptide segments permits the logical development of a tandem ligation scheme that can successively ligate multiple

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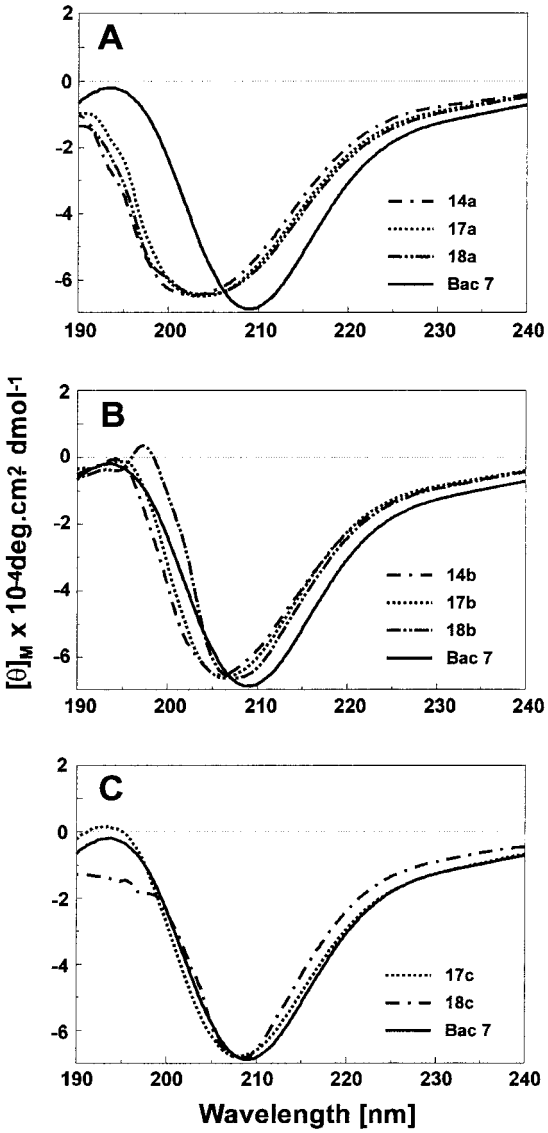

Figure 7. Comparison of CD spectra of Bac 7 and eight Bac 7 analogues in phosphate buffers at pH 7.2. (A) Bac 7 with [*O*Pro 25, *S*Pro 39] Bac 7 **14a**, [*S*Pro 25, *O*Pro 39] Bac 7 **17a** and [*O*Pro 25] Bac 7 **18a.** (B) Bac 7 with [*O*Pro^{Me} 25, *S*Pro 39] Bac 7 **14b**, [*S*Pro 25, *O*Pro^{Me} 39] Bac 7 **17b** and [*O*Pro^{Me} 25] Bac 7 **18b.** (C) Bac 7 with [*S*Pro 25, *S*Pro 39] Bac 7 **17c** and [*S*Pro 25] Bac 7 **18c**.

peptide segments without the need for protection or deprotection. The bidirectional strategy developed in this study provides an example, validating the usefulness of orthogonal ligation. Five Bac 7 analogues **14a**,**b** and **17a**−**c** with different pseudoproline replacements are obtained in good yields and purities. CD studies reveal that Bac 7 adopted polyproline II helical structures in neutral aqueous solutions, whereas the replacements of the *cis*-enhanced *S*Pro and *O*Pro in Bac 7 analogues **14a**,**b** and **17a**−**c** result in a minor population of polyproline I structures in their predominant polyproline II forms. Nevertheless, the antimicrobial activities of these ψPro analogues are similar to that of Bac 7.

A potential application of the bidirectional strategy is to provide various protein analogues with different ψPro combinations at the ligation sites for studies of protein folding pathways and structure−function relations. Furthermore, the versatility of a bidirectional ligation strategy makes it possible for combinatorial synthesis of proteins with unprotected segments from synthetic or recombinant source. Additionally, the tandem ligation strategy can be used for protein conjugation and peptide dendrimer synthesis.[58,59]

Table 2. Comparison of Antimicrobial Activity of Bac 7 and Five ψPro Analogues **14a**,**b** and **17a**−**c** from Three-Segment Ligation

	position of ψPro		MIC (μM)[a]		
peptide	25	39	E. coli	S. aureus	C. kefyr
Bac 7	Pro	Pro	0.24	0.23	0.21
14a	*O*Pro	*S*Pro	0.28	0.35	0.22
14b	*O*Pro^{Me}	*S*Pro	0.22	0.37	0.20
17a	*S*Pro	*O*Pro	0.26	0.20	0.24
17b	*S*Pro	*O*Pro^{Me}	0.21	0.28	0.22
17c	*S*Pro	*S*Pro	0.22	0.31	0.40

[a] MIC, minimal inhibition concentration, was obtained by radial diffusion assay with underlay gel containing 1% agarose in 10 mM phosphate buffer.

Experimental Section

Abbreviations. Standard abbreviations are used for the amino acids and protecting groups (IUPAC-IUB Commission for Biochemical Nomenclature, *J. Biol. Chem*., **1985**, *260*, 14). Other abbreviations are as follows: CD, circular dichroism; DIEA, diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-MS; matrix assisted laser desorption ionization mass spectrometry; *O*Pro, 2-hydroxymethyloxaproline; *O*Pro^{Me}, 2-hydroxymethyl-5-methyloxaproline; Pyr, pyridine; RP-HPLC, reversed phase high performance liquid chromatography; *S*Pro, 2-hydroxymethylthiaproline; TFA, trifluoroacetic acid.

General. Analytical HPLC was run on a Shimadzu 10A system using a Vydac C18 column (4.6 × 250 mm, 5 μm) with a flow rate of 1.0 mL/min, monitored at 225 nm. Preparative HPLC was performed on Waters 600 equipment with a Vydac C18 column (22 × 250 mm). All HPLC was carried out with a reversed phase linear gradient of buffer A, 0.05% TFA in H_2O, and buffer B, 60% CH_3CN in H_2O with 0.04% TFA. Mass spectra were obtained by MALDI-TOF method on a PerSeptive Biosystems Voyager Elite 2 instrument. FT-IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer with the sample filmed on CaF_2 surface.

Peptide Segments Syntheses. a. N- and M-segments. The peptide chain assembly was accomplished on the acetal resin **6** using Fmoc/tBu strategy (Figure 3).[43] The tethering of the first amino acid to the acetal resin **6** was achieved using 4 equiv of $(Fmoc\text{-}Gly)_2O$ and a catalytic amount of DMAP and HOBt in anhydrous DMF at RT for 12 h. Stepwise synthesis used HBTU/HOBt [60] as coupling agents and 20% piperidine in DMF as deprotecting agents. In each synthesis cycle, 2.5 equiv of amino acid was used. Final cleavage of peptides from the resin was performed with TFA−glycerol−anisole−thioanisole (90:4:3:3, 40 mL/g resin) for 3 h. Preparative HPLC gave the purified peptide glycerol esters **7a**,**b**, **8a**−**d** (60−75% yields based on the substitution of resin **6**). **8d** was used directly as an M-segment in the N→C strategy without a periodate oxidation step. **7a**,**b** was completely converted to N-segment **9a**,**b** with 3 equiv of $NaIO_4$ in aqueous buffers at pH 2 to 7 for 15−30 min. **8a**−**c** were converted to M-segment **10a**−**c** by selective oxidation with 2 equiv of $NaIO_4$ in 0.1 N HCl solution for 5−15 min. The analysis data for these segments are shown below. **7a**, Asp-Ser-Phe- Gly-$OCH_2CH(OH)CH_2OH$, 78.8% yield from **6**, t_R = 13.2 min, HRMS *m*/*z* 499.2034 ($M + H^+$, $C_{21}H_{31}N_4O_{10}$ requires 499.2040); **7b**, Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu-Pro-Arg-Pro-Arg-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-$OCH_2CH(OH)CH_2OH$, 74.4% yield based on the acetal resin **6**, t_R = 17.4 min, MALDI-MS *m*/*z* 3014.2 ($M + H^+$, 3012.6 calcd for $C_{136}H_{231}N_{51}O_{27}$). **9a**, Asp-Ser-Phe- Gly-OCH_2CHO, 94.2% yield from **7a**, t_R = 12.6 min,

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HRMS *m*/*z* 467.1788 (M + H$^+$, $C_{20}H_{27}N_4O_9$ requires 467.1778); **9b**, Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu-Pro-Arg-Pro-Arg-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-OCH$_2$CHO, 94.4% yield from **7b**, t_R = 17.4 min, MALDI-MS *m*/*z* 2980.2 (M + H$^+$, 2980.6 calcd for $C_{135}H_{227}N_{51}O_{26}$); **8a**, Ser-Leu-Ile-Leu-Asn-Gly-OCH$_2$CH(OH)CH$_2$OH, 75.8% from **6**, t_R = 12.4 min (20−60% B), HRMS *m*/*z* 690.4031 (M + H$^+$, $C_{30}H_{56}N_7O_{11}$ requires 690.4037); **8b**, Ser-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-OCH$_2$CH(OH)CH$_2$OH, 77.1% yield based on the acetal resin **6**, t_R = 19.4 min (20−60% B), MALDI-MS *m*/*z* 1661.4 (M + H$^+$, 1661.0 calcd for $C_{77}H_{125}N_{23}O_{18}$); **8c**, Thr-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Gly-OCH$_2$CH(OH)-CH$_2$OH, 81.7% yield based on the acetal resin **6**, t_R = 19.8 min, MALDI-MS *m*/*z* 1675.8 (M + H$^+$, 1675.1 calcd for $C_{78}H_{127}N_{23}O_{18}$); **8d**, Cys-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Gly-OCH$_2$CH(OH)CH$_2$OH, 71.5% yield based on the acetal resin **6**, t_R = 20.7 min, MALDI-MS *m*/*z* 1678.5 (M + H$^+$, 1677.1 calcd for $C_{77}H_{125}N_{23}O_{17}S$); **10a**, Ser-Leu-Ile-Leu-Asn-Gly-OCH$_2$CHO, 86.3% from **8a**, t_R = 12.8 min (20−60% B), HRMS *m*/*z* 658.3781 (M + H$^+$, $C_{29}H_{52}N_7O_{10}$ requires 658.3775); **10b**, Ser-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-OCH$_2$CHO, 84.3% from **8b**, t_R = 19.4 min (20−60% B), MALDI-MS *m*/*z* 1630.9 (M + H$^+$, 1628.9 calcd for $C_{76}H_{121}N_{23}O_{17}$); **10c**, Thr-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Gly-OCH$_2$CH(OH)CH$_2$OH, 90.1% from **8c**, t_R = 19.8 min, MALDI-MS *m*/*z* 1644.1 (M + H$^+$, 1642.9 calcd for $C_{77}H_{123}N_{23}O_{17}$).

b. C-segments. The C-segment peptides **12a**−**d** were synthesized on PAM resin using Boc/Bzl and HBTU/HOBt strategy.[60] The peptides were cleaved from the resin by anhydrous HF-anisole (95:5, v/v) and then purified by HPLC. The amino acid analysis and MS gave the desired results. **12a**, Cys-Phe-Lys-Ile-OH, t_R = 13.2 min, HRMS *m*/*z* 510.2733 (M + H$^+$, $C_{24}H_{40}N_5O_5S$ requires 510.2750); **12b**, Ser-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 22.4 min (20−60 B%), MALDI-MS *m*/*z* 2402.11 (M + H$^+$, 2400.9 calcd for $C_{112}H_{182}N_{36}O_{23}$); **12c**, Thr-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 22.5 min, MALDI-MS *m*/*z* 2416.4 (M + H$^+$, 2414.9 calcd for $C_{113}H_{184}N_{36}O_{23}$); **12d**, Cys-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 23.3 min, MALDI-MS *m*/*z* 2419.1 (M + H$^+$, 2416.9 calcd for $C_{112}H_{182}N_{36}O_{22}S$).

C→N Three-Segment Tandem Ligation of Bac 7. The C→N strategy consisted of two orthogonal pseudoproline ligations. The thiaproline ligation between C-segment **12d** (12.1 mg, 5.0 μmol) and M-segment **10b** (8.9 mg, 5.5 μmol) was carried out initially at 20 °C in 1 mL of phosphate buffer at pH 5.3 for 10 h. The *R*,*S*-epimers of thiazolidine ester intermediates were observed as two major products, which were converted to a single amide bond product **13a** by adjusting the pH to 6.6 with 1 M NaHCO$_3$. The conversion was completed at pH 6.6 for 20 h to yield **13a** in 92.6% yield (Figure 5A, Table 1). Similarly, the amide product **13b** of thiaproline ligation between **12d** (12.1 mg, 5.0 μmol) and **10c** (9.1 mg, 5.5 μmol) was obtained in 93.1% yield. The following oxaproline ligations between N-segment **9b** (4.2 mg, 2.2 μmol) and the thiaproline ligation products **13a** (8.1 mg, 2.0 μmol) and **13b** (8.1 mg, 2.0 μmol) were performed in 0.5 mL of pyridine−acetic acid mixture (1:1, mol/mol) for 35 h at 20 °C, respectively, giving two Bac 7 analogues **14a** and **14b** in 80.7 and 77.6% yields, respectively (Figure 5B, Table 1). The ligation products were confirmed by chemical analysis and MS. **13a**, t_R = 24.5 min (20−50% B in 30 min), MALDI-MS *m*/*z* 4026.8 (M + H$^+$, 4026.9 calcd for $C_{188}H_{300}N_{59}O_{38}S$); **13b**, t_R = 24.8 min, MALDI-MS *m*/*z* 4040.7 (M + H$^+$, 4040.9 calcd for $C_{189}H_{302}N_{59}O_{38}S$); **14a**, t_R = 25.7 min (20−55% B in 30 min), MALDI-MS *m*/*z* 6986.7 (M + H$^+$, 6988.5 calcd for $C_{323}H_{524}N_{110}O_{63}S$); **14b**, t_R = 26.2 min, MALDI-MS *m*/*z* 6999.0 (M + H$^+$, 7002.5 calcd for $C_{324}H_{526}N_{110}O_{63}S$).

N→C Three-Segment Tandem Ligation of Bac 7. The N→C strategy started with a thiaproline ligation between N-segment **9b** (13.2 mg, 4.4 μmol) and M-segment **8d** (6.7 mg, 4.0 μmol) that was carried out at 20 °C in 1 mL of phosphate buffer, first at pH 5.3 for 10 h and then at pH 6.6 for 20 h. The amide bond product **15** with a CT-glycerol ester was obtained in 92.6% yield and was completely converted to peptide aldehyde ester **16** by 3 equiv of NaIO$_4$ at pH 4. The subsequent pseudoproline ligations between **16** (6.4 mg, 1.4 μmol) and C-segments **12b** (2.9 mg, 1.2 μmol), **12c** (2.9 mg, 1.2 μmol) and **12d** (3.0 mg, 1.3 μmol) were performed in 1 mL of pyridine−acetic acid mixture (1:1, mol/mol) at 20 °C for 35 h, giving three Bac 7 analogues **17a**, **17b,** and **17c** in 74.2, 76.4, and 81.0% yields, respectively (Table 1). The ligation products were confirmed by chemical analysis and MS. **15**, t_R = 22.6 min (25−55% B in 30 min), MALDI-MS *m*/*z* 4640.5 (M + H$^+$, 4638.6 calcd for $C_{212}H_{349}N_{74}O_{42}S$); **16**, t_R = 23.1 min, MALDI-MS *m*/*z* 4608.7 (M + H$^+$, 4606.6 calcd for $C_{211}H_{525}N_{345}O_{41}S$); **17a**, t_R = 25.7 min, MALDI-MS *m*/*z* 6987.9 (M + H$^+$, 6988.5 calcd for $C_{323}H_{524}N_{110}O_{63}S$); **17b**, t_R = 26.1 min, MALDI-MS *m*/*z* 7026.1 (M + Na$^+$, 7024.5 calcd for $C_{324}H_{525}N_{110}O_{63}SNa$). **17c**, t_R = 26.6 min, MALDI-MS *m*/*z* 7029.9 (M + Na$^+$, 7026.6 calcd for $C_{323}H_{523}N_{110}O_{62}SNa$).

Circular dichroism (CD) Spectroscopy. CD measurements of Bac 7 and its eight analogues **14a**,**b**, **17a**−**c**, and **18a**−**c** were carried out on a Jasco 720 spectropolarimeter connected to a temperature controller and an IBM computer. Each peptide was dissolved in phosphate buffers at pH 7.2 in a concentration of 0.02−0.2 mM. CD spectra were recorded at 20 °C in a quartz cell with 0.1 mm path length, using a 2.0 nm bandwidth, a sensitivity of 50 mdeg, a time constant of 4 s, and a 50 nm/min scanning speed with 0.2 nm resolution.

Antimicrobial Assays. Gram-negative *E. coli ATCC 25922* and Gram-positive *S. aureus 29213* bacteria, as well as the fungal strain *C. kefyr ATCC 37095* were used for antimicrobial assays. The strains were incubated in trypticase soy broth (TSB) and used for experiments less than 4 weeks after being taken from stock.

A sensitive and reproducible two-stage radial diffusion assay method developed by Lehrer et al.[52] was employed. Briefly, a (1−4) × 10^6 cfu/ml aliquot of a test organism was mixed with 10 mL of molten underlay gel solution and poured into 10 × 10-cm Petri dishes to form a uniform layer. The gel solution contained 10 mM sodium phosphate buffer, 0.03% TSB, and 0.02% Tween 20. In high-salt assay conditions, the gel solution contained 100 mM NaCl, whereas no NaCl was used in the low-salt assay. After solidification, gel wells with 3-mm diameters were made by a template in an evenly spaced array. An aliquot of 5 μL of a serial half-log dilution of testing peptides **14a**,**b** and **17a**−**c** at seven concentrations was added to each well after removing the gel plugs. The dishes were incubated at 37 °C for 3 h to allow test peptides to diffuse into the underlay gels. Gels were overlaid with 10 mL of 1% agarose in 6% of TSB (w/v). After incubation at 37 °C for 16−24 h, the diameter of the clear zone surrounding the wells (colony-free) was measured under the microscope. Antimicrobial activities were expressed in units (0.1 mm = 1 U), and the MICs were determined from the *x* intercepts of the dose−response curves. The low-salt results of activities are summarized in Table 2.

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