

Effect of Chain Length of Cationic Model Peptides on Antibacterial Activity

Takuro Niidome,† Noboru Matsuyama, Makoto Kunihara, Tomomitsu Hatakeyama,* and Haruhiko Aoyagi

Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852-8521

Received September 24, 2004; E-mail: tanido@net.nagasaki-u.ac.jp

Cationic model peptides containing simple repeats, H-(LARL)$_3$-(LRAL)$_n$-NH$_2$ ($n = 0$, 1, 2, and 3), were synthesized, and their antibacterial and hemolytic activities were investigated. Antibacterial activity decreased with an increase in the chain length of the peptides, whereas the reverse situation was found for hemolytic activity. As a result of measuring the bacterial membrane permeabilization, it was indicated that the complicated surface membrane structure of bacteria prevented the penetration of the long and rigid peptides into the inner membrane portion. Another peptide, H-(LARL)$_3$-PRAL-(LRAL)$_2$-NH$_2$, which has a kink in the middle portion of the molecule, showed some antibacterial activity in spite of its long peptide chain.

Among various kinds of compounds produced by higher organisms, antibacterial peptides are one of the interesting research targets.[1] Antibacterial peptides, which interact with bacterial cell membrane, generally have 15–40 amino acid residues, and have exhibited their biological activities by membrane perturbation.[2] Such peptides are expected to be used as medical supplies, because they quickly lyse bacteria to avoid the appearance of resistant bacteria. Peptides that interact with a phospholipid membrane often have an amphiphilic and α-helical structure.[3] Therefore, many amphiphilic antibacterial peptides have been designed, and their interactions with lipid and cell membranes were examined in various ways.[1]

We previously found that a cationic peptide, Ac-(LARL)$_3$-NHCH$_3$ (4_3), caused a perturbation of anionic lipid membrane, and had strong antibacterial activity against gram-positive bacteria.[4] To investigate effect of the chain length and the hydrophobicity of the peptides on their interaction with lipid and cell membranes, some model peptides, including Ac-(LARL)$_3$-(LRAL)$_3$-NHCH$_3$ (4_6), were designed. Peptide 4_6 has a double-chain length of 4_3, and the alanine and arginine residues are exchanged in the C-terminal half portion in order to hold the cationic arginine residues on one lateral side of the α-helix. Peptide 4_6 unexpectedly showed no antibacterial activity, although it had stronger hemolytic activity than 4_3.[4b] Furthermore, H-(LARL)$_3$-(LRAL)$_3$-OH (decap-4_6), which is also amphiphilic and α-helical, had weak antibacterial activity.[5] Decap-4_6 was able to form cation-selective ion channels with several levels of conductances on a planar DiphytPC bilayer.[6] On the other hand, H-(LARL)$_3$-(LRAL)$_2$-OH (decap-4_5) had no antibacterial activity and showed erratic conductances. These finding suggested that short peptides, such as 4_3, have quite different properties from long peptides, such as 4_6 and decap-4_6. However, there has been no systematic study on the effect of the chain lengths of peptides on their behaviors against lipid membranes.

To define the boundary of the molecular size of the peptides to show antibacterial activity, and clarify why long peptides, such as 4_6 and decap-4_6, lose or weaken the activity, we designed four model peptides with different chain lengths ($4_3'$, $4_4'$, $4_5'$, and $4_6'$), and a proline-containing peptide (P-$4_6'$). Their amino acid sequences are shown in Table 1. The N- and C-terminal protecting groups of 4_6 are not always necessary, because decap-4_6 has antibacterial activity. However, natural antibacterial peptides, e.g., magainin 2, often have a protected C-terminal. The C-terminal amidation of an antibacterial peptide, pleurocidin, was found to increase the activity.[7] Therefore, an N-unprotected and C-terminal amide-protected peptide was selected in this study. The properties and biological activities of the peptides were examined.

Results and Discussion

Peptide Design. We have synthesized 4_3, 4_6, decap-4_6, and decap-4_5, and examined their properties and biological activities. Among them, 4_3 had strong antibacterial activity, whereas the activity of decap-4_6 was weak, and 4_6 and decap-4_5 had no activity. Although 4_6 interacted with the lipid bilayer more weakly than 4_3, its hemolytic activity was stronger than that of 4_3.[4,5] Decap-4_6 was able to form ion channels in an artificial lipid membrane, but neither 4_3 nor decap-4_5 did it.[5] In this study, cationic model peptides containing simple repeats, H-(LARL)$_3$-(LRAL)$_n$-NH$_2$ ($4_3'$, $n = 0$; $4_4'$, $n = 1$; $4_5'$, $n = 2$; $4_6'$, $n = 3$) were designed. Antibacterial peptides with more than 20 amino acid residues often contain a proline or glycine residue as a kink maker in the middle portion of the molecules.[3,8] The finding that 4_6 has a rigid structure and no antibacterial activity[4b] suggested that the introduction of a proline residue into 4_6 would promise to show the activity. Therefore, H-(LARL)$_3$-PRAL-(LRAL)$_2$-NH$_2$ (P-$4_6'$) was also designed. After synthesis of the peptides and purification of the

† Present address: Department of Chemistry and Biochemistry, Faculty of Engineering, Kyushu University, Higashi-ku, Fukuoka 812-8581

Published on the web March 11, 2005; DOI 10.1246/bcsj.78.473

Table 1. Amino Acid Sequences and Analytical Data of the Peptides

Peptide	Amino acid sequence[a]	MALDI-TOF-MS		Amino acid analysis[b]			
		Found	Calcd ($[M + H]^+$)				
$4_3'$	H-(LARL)$_3$-NH$_2$	1378.5	1378.8 ($C_{63}H_{121}N_{22}O_{12}$)	A 3.00 (3)	L 6.55 (6)	R 2.88 (3)	
$4_4'$	H-(LARL)$_3$-LRAL-NH$_2$	1832.0	1832.4 ($C_{84}H_{160}N_{29}O_{16}$)	A 4.00 (4)	L 7.86 (8)	R 3.82 (4)	
$4_5'$	H-(LARL)$_3$-(LRAL)$_2$-NH$_2$	2285.7	2286.0 ($C_{105}H_{199}N_{36}O_{20}$)	A 5.00 (5)	L 10.89 (10)	R 4.78 (5)	
$4_6'$	H-(LARL)$_3$-(LRAL)$_3$-NH$_2$	2739.3	2739.6 ($C_{126}H_{238}N_{43}O_{24}$)	A 6.00 (6)	L 13.31 (12)	R 6.11 (6)	
P-$4_6'$	H-(LARL)$_3$-PRAL-(LRAL)$_2$-NH$_2$	2724.0	2723.6 ($C_{125}H_{234}N_{43}O_{24}$)	A 6.00 (6)	L 11.38 (11)	R 5.78 (6)	P 0.92 (1)

a) L, leucine; A, alanine; R, arginine; P, proline. b) Hydrolysis was performed in 6 M HCl for 24 h at 110 °C. Numbers in parenthesis are theoretical nembers of amino acid residues in the peptides.

Table 2. α-Helix Contents and Biological Activities of the Peptides

Peptide	α-Helix content/%[a]				MIC/μM[e]				Hemolytic activity/%[j]
	Buffer[b]	98% TFE	PC[c]	PC/PG[d]	*S.a.*[f]	*B.s.*[g]	*E.c.*[h]	*P.a.*[i]	
$4_3'$	12	38	33	40	2	2	8	32	0
$4_4'$	34	44	53	66	4	4	32	64	33
$4_5'$	70	71	86	90	>64	>64	>64	>64	53
$4_6'$	76	73	91	96	>64	>64	>64	>64	70
P-$4_6'$	25	38	44	46	8	16	>64	>64	74

a) [Peptide] = 20 μM; 25 °C. b) 20 mM Tris–HCl buffer (pH 7.4). c) [DOPC] = 1 mM. d) [DOPC/DOPG (3:1)] = 1 mM. e) Liquid based assay; TSB medium (pH 7.4); inoculum size, 10^4 cells mL^{-1}. f) *S. aureus* NBRC 12732. g) *B. subtilis* NBRC 3134. h) *E. coli* NBRC 12734. i) *P. aureginosa* NBRC 12582. j) Percentage of hemolysis at a peptide concentration of 0.3 μM.

crude products by gel filtration, followed by RP-HPLC, identification of the final products was done by MALDI-TOF-MS and amino acid analysis. The results were satisfactory (Table 1).

CD Measurement. CD measurements were performed in 20 mM Tris–HCl buffer (pH 7.4) (1 M = 1 mol dm^{-3}), TFE, and Tris–HCl buffer (pH 7.4) in the presence of DOPC or DOPC/DOPG (3:1) vesicles to examine the secondary structure of the peptides and their properties under aqueous and non-aqueous conditions (Table 2). In Tris–HCl buffer (pH 7.4), short peptides, $4_3'$ and $4_4'$, had low α-helicities, while long peptides, $4_5'$ and $4_6'$, showed high α-helicities. P-$4_6'$, which is long, but has a kink in the middle portion of the peptide chain, showed a low α-helix content of 25%. TFE, an α-helix formation-enhancing solvent, induced an increase in the α-helicities of the short peptides and P-$4_6'$, but gave little effect on $4_5'$ and $4_6'$. It is likely that $4_5'$ and $4_6'$ inherently have a rigid conformation in analogy with 4_6,[4] and decap-4_5 and decap-4_6.[5] In the presence of neutral phospholipid, DOPC, vesicles, α-helix contents of the peptides other than $4_3'$ were slightly higher than those in TFE. The result that $4_3'$ showed low α-helicity in DOPC vesicles may due to the weak interaction of $4_3'$ with DOPC bilayer. High α-helix contents of 90% or more were observed for $4_5'$ and $4_6'$ in the presence of anionic phospholipid, DOPC/DOPG (3:1), vesicles. It is noteworthy that P-$4_6'$, which corresponds to a dimer of 4_3, showed a similar α-helicity to $4_3'$, suggesting that P-$4_6'$ probably contains two $4_3'$-like conformations in the anionic phospholipid bilayer.

Antibacterial and Hemolytic Activities. The antibacterial activities of the peptides were examined by the serial solution dilution method using two gram-positive bacteria (*Staphycoccus aureus* and *Bacillus subtilis*) and two gram-negative bacteria (*Eschelichia coli* and *Pseudomonus aureginosa*), according to the literature.[9] The MICs of the peptides are listed in Table 2. Short peptide $4_3'$ had strong activity of 2 μM against gram-positive bacteria, and moderate activity against gram-negative bacteria while long peptides $4_5'$ and $4_6'$ showed no activity at 64 μM. Since the antibacterial activity of $4_4'$ was intermediate between the activities of the short and long peptides, it is clear that the chain length of the peptides is correlated with the antibacterial activity. The rigid and long peptide chains of $4_5'$ and $4_6'$ may be disadvantageous, when such peptides pass through the complicated surface membrane[10] to reach the inner phospholipid bilayer of bacteria. P-$4_6'$ gave subtle results: moderate antibacterial activity against gram-positive bacteria, but no activity against gram-negative bacteria. It is likely that P-$4_6'$ cannot easily pass through the outer non-lipid layer of gram-negative bacteria due to its long chain length, although P-$4_6'$ has no straight chain structure.

The cytotoxicity of antibacterial peptides is undesirable for medical use.[11] Therefore, the hemolytic activity, one of cytotoxicity evaluations, was measured using fresh rabbit red blood cells according to the literature.[7] The result is shown in Fig. 1 and Table 2. The hemolytic activities of the peptides increased along with an increase in the chain length. Long peptides are advantageous for interacting with red blood cell membranes, because they have larger hydrophobic regions than the short peptides, and red blood cells have neutral phospholipids in the outer side of the membrane. P-$4_6'$ exhibited the strongest hemolytic activity. The long and flexible structure of P-$4_6'$ may enhance its interaction with cell membranes.

Membrane Permeability. The outer membrane of *E. coli*, a typical gram-negative bacteria, contains lipopolysaccharide as a main component,[12] which plays an important role in membrane translocation. On the other hand, the inner membrane

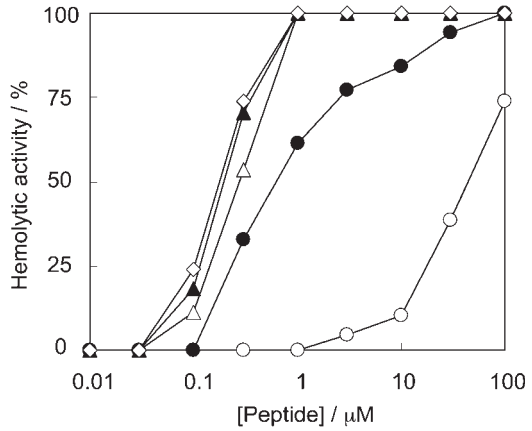

Fig. 1. Profiles of hemolysis as a function of the peptide concentration for rabbit blood cells. Peptide $4_3'$ (open circle), $4_4'$ (closed circle), $4_5'$ (open triangle), $4_6'$ (closed triangle), and P-$4_6'$ (open diamond).

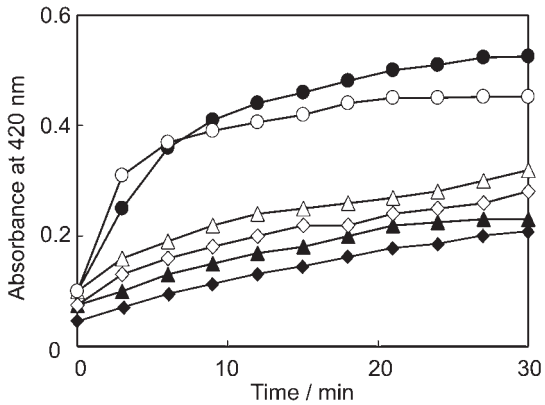

Fig. 2. Effects of the peptides on the permeabilization of inner membrane of *E. coli.* [Peptide] = 32 μM, [ONPG] = 50 μM. Peptide $4_3'$ (open circle), $4_4'$ (closed circle), $4_5'$ (open triangle), $4_6'$ (closed triangle), P-$4_6'$ (open diamond), and control (closed diamond).

has phosphatidylethanolamine and phosphatidylglycerol as the main neutral and anionic components, respectively. The membrane permeabilization of the peptides was evaluated by measuring the β-galactosidase activity using *o*-nitrophenyl β-galactopyranoside (ONPG) as a substrate according to the literature.[13] The absorbance at 420 nm of *p*-nitrophenol, produced by enzymatic hydrolysis, was measured over a period of 30 min. The result is shown in Fig. 2. The absorbance gradually increased with the passage of time, and two types of curves were observed. Peptides $4_3'$ and $4_4'$ caused a rapid increase of permeabilization at on early time, and then a slow increase, whereas $4_5'$, $4_6'$, and P-$4_6'$ showed slow curves on the whole. The membrane permeabilization of the peptides is roughly consistent with their antibacterial activity.

In conclusion, the antibacterial activity of the peptides decreases with an increase in their chain length. This is due to a difficulty of passage of the long and rigid peptides through the non-lipid surface layer of bacteria, because these peptides were able to easily interact with a neutral blood cell membrane and cause membrane perturbation. Peptide P-$4_6'$ showed low α-helicity and some antibacterial activity against gram-positive bacteria, whereas its hemolytic and permeabilization activities were similar to those of $4_6'$. The proline residue in biologically active peptides is sometimes important for flexibility of the molecules, or the retention of active conformations. For example, alanine → proline substitution in 4_3 caused a decrease in the antibacterial activity.[14] An analog of antibacterial pardaxin with an alanine residue instead of a proline residue at position 13 was found to be less cytotoxic than pardaxin.[15] Furthermore, we recently found that the substitution of a proline residue at position 15 by alanine in maculatin 1.1, an antibacterial peptide, caused a loss of the activity against gram-negative bacteria,[16] indicating that the hinge structure formed by the proline residue plays a key role in the biological activity. The findings in this study that the rigid and long structure of the amphiphilic peptides is disadvantageous for antibacterial activity, and that P-$4_6'$ has a unique character, would be helpful information for designing biologically active peptides.

Experimental

Peptide Synthesis. Peptides were synthesized with Fmoc-amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink Amide resin) on a Shimadzu PSSM-8 peptide synthesizer. The crude products were purified by gel chromatography on a Sephadex G-10 column (10% AcOH) followed by RP-HPLC on a YMC-Pack ODS column (0–100% acetonitrile/0.1% TFE). The purified peptides were identified by amino acid analysis and MALDI-TOF-MS (Table 1).

CD Measurement. DOPC and DOPC/DOPG (3:1), small unilamellar vesidles, were prepared for CD measurements. After phospholipid (7.3 mg) was dissolved in $CHCl_3$/MeOH (3:1, 2 mL), the mixture was dried by a stream of N_2 gas. The dried lipid was hydrated in 20 mM Tris–HCl buffer (pH 7.4, 10 mL) using a Branson bath-type sonicator. The suspension was sonicated for 10 min at 50 °C using a Taitec Ultrasonic Processor VP-ST. The vesicles were left for 30 min at 25 °C before measurements. The lipid concentration was 1 mM. CD spectra were recorded on a JASCO J-720 W spectrometer with a thermostatted cell holder using a quartz cell of 1.0 mm path length. The peptides were dissolved in 20 mM Tris–HCl buffer (pH 7.4), TFE, and the buffer in the presence of 1 mM phospholipid vesicles. The peptide concentration was 20 μM. Measurements were done at 25 °C. The helix contents were calculated according to a method of Scholtz et al.,[17] and the mean residue ellipticity is given in deg·dm^2·mol^{-1}.

Antibacterial and Hemolytic Assays. The serial solution dilution method was used to determine the MIC values, as described by Yoshida et al.[9] The cell suspension was dissolved in a tryptic soy broth (TSB) medium (pH 7.4) to 10^4 cells mL^{-1}. Several concentrations of the peptide solution were placed in test tubes, made up to 20 μL with the medium, and a cell suspension (180 μL) was added. After incubation for 24 h at 37 °C, the absorbance at 620 nm was measured. The hemolytic activity was assayed according to a procedure of Yoshida et al.[9] Phosphate-buffered saline (PBS) (pH 7.5, 3 mL) was added to fresh rabbit blood (1 mL). The mixture was centrifuged at 2000 rpm for 3 min, and the precipitates were collected. After being washed with PBS three times, the precipitates were suspended in PBS (2 mL). PBS (1 mL) was added to the rabbit erythrocyte solution (5 μL), followed by the peptides. The mixture was incubated for 20 min at 37 °C, and then centrifuged. The supernatant was monitored at 430 nm using a Hitachi U-200 spectrophotometer. To measure the absorbances of 100%

and 0% activities, 10% Triton X-100 (10 μL) and water (10 μL) instead of peptide were respectively added to the erythrocyte solution.

Inner Membrane Permeability. A determination of the inner membrane permeability was performed by measuring the β-galactosidase activity using ONPG as a substrate according to a procedure by Pellegrini et al.[13] A suspension of *E. coli* (10^4 cells mL^{-1}, 880 μL) was added to a solution of 2.5 mM ONPG in a TSB medium (pH 7.4, 20 μL). After 15 min of incubation, a 320 μM peptide solution (100 μL) was added. The inner membrane permeabilization was monitored by measuring the rate of *o*-nitrophenol production at 420 nm every 1 min. An equivalent volume of water was used instead of the peptide solution in the control assay.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology. We thank Prof. N. Fujii of Kinki University and Prof. Y. Shimohigashi of Kyushu University for carrying out MALDI-TOF-MS.

References

1 a) Y. Shai, *Biopolymers (Pept. Sci.)*, **66**, 236 (2002). b) A. Tossi, L. Sandri, and A. Giangaspero, *Biopolymers (Pept. Sci.)*, **37**, 4 (2000). c) T. Niidome and H. Aoyagi, *Curr. Top. Pept. Protein Res.*, **3**, 79 (1999). d) G. Saberwal and R. Nagaraj, *Biochim. Biophys. Acta*, **1197**, 109 (1994).

2 a) T. Tachi, R. F. Epand, R. M. Epand, and K. Matsuzaki, *Biochemistry*, **41**, 10723 (2002). b) M. Dathe, T. Wieprecht, H. Nikolenko, L. Handel, W. L. Maloy, D. L. MacDonald, H. Beyerman, and M. Binert, *FEBS Lett.*, **403**, 208 (1997). c) T. Kiyota, S. Lee, and G. Sugihara, *Biochemistry*, **35**, 13196 (1996). d) M. Hugosson, D. Andreu, H. G. Boman, and E. Glaser, *Eur. J. Biochem.*, **223**, 1027 (1994).

3 a) M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5449 (1987). b) A. M. Cole, P. Weis, and G. Diamond, *J. Biol. Chem.*, **272**, 12008 (1997). c) D. Rapaport, S. Nir, and Y. Shai, *Biochemistry*, **33**, 12615 (1994). d) T. Yoshimura, Y. Goto, and S. Aimoto, *Biochemistry*, **31**, 6119 (1992).

4 a) S. Lee, H. Mihara, H. Aoyagi, T. Kato, N. Izumiya, and N. Yamasaki, *Biochim. Biophys. Acta*, **862**, 211 (1986). b) N. Ohmori, T. Niidome, T. Hatakeyama, H. Mihara, and H. Aoyagi, *J. Pept. Res.*, **51**, 103 (1998).

5 Y. Agawa, S. Lee, S. Ono, H. Aoyagi, M. Ohno, T. Taniguchi, K. Anzai, and Y. Kirino, *J. Biol. Chem.*, **266**, 20218 (1991).

6 Abbreviations used are as follows: CD, circular dichroism; Diphyt PC, diphytanoyl-3-phosphatidylcholine; DOPC, dioleoyl-3-phosphatidylcholine; DOPG, dioleoyl-3-phosphatidylglycerol; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; ONPG, *o*-nitrophenyl β-galactopyranoside; PBS, phosphate buffered saline; RP-HPLC, reversed phase high-performance liquid chromatography; TFE, 2,2,2-trifluoroethanol; TSB, tryptic soy broth.

7 Y. Fukuoka, Y. Matsushita, S. Furukawa, T. Niidome, T. Hatakeyama, and H. Aoyagi, *Bull. Chem. Soc. Jpn.*, **76**, 1857 (2003).

8 a) J. Y. Lee, A. Boman, C. X. Scun, M. Andersson, M. Jornvall, V. Mutt, and H. G. Boman, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9159 (1989). b) A. Mor, H.-V. Nguyen, A. Delfour, D. Nigliore-Samour, and P. Nicolas, *Biochemistry*, **30**, 8824 (1991). c) T. Ganz, M. E. Selsted, D. Szklarek, S. S. Harwing, K. Daher, D. F. Bainton, and R. I. Lehrer, *J. Clin. Invest.*, **76**, 1427 (1985).

9 K. Yoshida, Y. Mukai, T. Niidome, C. Takashi, Y. Tokunaga, T. Hatakeyama, and H. Aoyagi, *J. Pept. Res.*, **57**, 119 (2001).

10 a) B. Lutenberg and L. VanAlphen, *Biochim. Biophys. Acta*, **737**, 51 (1983). b) H. Nikaido and M. Vaara, *Microbial. Rev.*, **49**, 1 (1985).

11 a) D. L. Lee and R. S. Hodges, *Biopolymers (Pept. Sci.)*, **71**, 28 (2003). b) J. P. Tam, Y.-A. Lu, and J.-L. Yang, *Eur. J. Biochem.*, **269**, 923 (2002).

12 H. G. Labischinski, G. Barnickel, H. Bradaczek, D. Naumann, E. T. Rietchel, and P. Giesbrecht, *J. Bacteriol.*, **162**, 9 (1985).

13 A. Pellegrini, C. Dettling, U. Thomas, and P. Hunziker, *Biochim. Biophys. Acta*, **1526**, 131 (2001).

14 S. Lee, N. G. Park, T. Kato, H. Aoyagi, and T. Kato, *Chem. Lett.*, **1989**, 599.

15 a) Y. Shai, D. Bach, and A. Yanovsky, *J. Biol. Chem.*, **265**, 20202 (1990). b) C. J. Barrow, K. Nakanishi, and K. Tachibana, *Biochim. Biophys. Acta*, **1112**, 235 (1992).

16 T. Niidome, K. Kobayashi, H. Arakawa, T. Hatakeyama, and H. Aoyagi, *J. Pept. Sci.*, **10**, 414 (2004).

17 J. M. Scholtz, H. Quian, E. J. York, J. M. Stewart, and R. L. Baldwin, *Biopolymers*, **31**, 1463 (1991).