Studies on Peptides. CLXVIII.[1,2] Syntheses of Three Peptides Isolated from Horseshoe Crab Hemocytes, Tachyplesin I, Tachyplesin II, and Polyphemusin I

Kenichi AKAJI,[a] Nobutaka FUJII,[a] Fuminori TOKUNAGA,[b] Toshiyuki MIYATA,[b] Sadaaki IWANAGA,[b] and Haruaki YAJIMA*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto 606, Japan and Department of Biology, Faculty of Science, Kyushu University,[b] Fukuoka 812, Japan. *Received March 25, 1989*

Tachyplesin I, a 17-residue peptide amide with two disulfide bridges isolated from an acid extract of horseshoe crab hemocyte debris, was synthesized by the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase method followed by two steps of deprotection and subsequent air-oxidation. The thioanisole-mediated deprotection with 1 M trifluoromethanesulfonic acid was first employed to cleave the peptide amide from the resin and, at the same time, to deprotect the side chain protecting groups employed, except for the S-Acm (acetamidomethyl) group. The 4 Acm groups attached were next cleaved by silver trifluoromethanesulfonate. In addition, two related peptides, tachyplesin II and polyphemusin I, were similarly synthesized. Synthetic tachyplesin I inhibited the lipopolysaccharide-mediated activation of clotting factor C to the same extent as did the corresponding natural peptide. The relative potencies of synthetic tachyplesin II and synthetic polyphemusin I with respect to natural tachyplesin I (taken as 1) were 2.1 and 0.61, respectively.

Keywords tachyplesin I synthesis; tachyplesin II synthesis; polyphemusin I synthesis; Fmoc-based solid phase synthesis; amide peptide synthesis; thioanisole-mediated deprotection; trifluoromethanesulfonic acid; trimethylsilyltrifluoromethanesulfonate; trimethylsilyl bromide

In 1988, Iwanaga *et al.*[3] elucidated the structure of a novel 17-residue peptide amide possessing two disulfide bonds isolated from an acid extract of horseshoe crab (*Tachypleus tridentatus*) hemocyte debris (Fig. 1). This peptide, named tachyplesin I (T-I), forms a complex with bacterial lipopolysaccharide (LPS) and significantly inhibits the LPS-mediated activation of factor C, the initiation factor in the Limulus clotting cascade.[4] It also inhibits the growth of gram-negative and -positive bacteria. Following the structural elucidation of tachyplesin I, two related peptide amides, tachyplesin II (17-residue peptide) and polyphemusin I (18-residue peptide), were also isolated from the hemocytes of *Tachypleus tridentatus* and American horseshoe crab, *Limulus polyphemus*, respectively, and their structures were elucidated by the same group of investigators.[5] Tachyplesin II (T-II) and polyphemusin I (P-I) seem to have similar biological activities to those of tachyplesin I, since the amino acid sequences of these two peptide amides show very high homology with that of tachyplesin I (Fig. 1). In order to obtain sufficient material to examine their biological effects on bacteria and interactions with bacterial LPS, we undertook the solid-phase syntheses of these three peptide amides.

In order to construct each peptide chain, the Fmoc-based solid-phase method,[6] rather than the Boc-based solid phase method,[7] was employed, since each peptide contains an acid-labile Trp residue.[8] Fmoc-amino acid derivatives bearing protecting groups based on *tert*-butyl alcohol were employed, together with Arg(Mtr)[9] and Cys(Acm).[10] As the starting resin, 4-methylbenzhydrylamine (MBHA) resin[11] or 2,4′-dimethoxybenzhydrylamine (DMBHA) resin[12] was employed and the cleavage yields of the peptide amides from each resin were compared.

For the synthesis of T-I, the first C-terminal residue, Fmoc–Arg(Mtr)–OH, was loaded on the MBHA resin by the DIPCDI procedure[13] with the aid of HOBt.[14] The combination of piperidine treatment[15] and DIPCDI plus HOBt procedure served to elongate the peptide chain manually, but in principle, according to the automated program proposed by Sheppard *et al.*[6] The resin became negative to the ninhydrin test[16] after single coupling at every condensation step. The amino acid composition of the protected (T-I)–resin thus assembled was in fairly good agreement with that predicted by theory after acid hydrolysis with 12 N HCl–phenol–AcOH = 2 : 1 : 1.[17] Starting with the DMBHA resin, protected T-II and P-I were similarly prepared.

Cleavage rates of peptide amides from the benzhydrylamine-type resins vary depending on the nature of the C-terminal amino acid attached, as well as substituents on the benzhydrylamine moiety.[11,18] In addition, protected T-I, T-II, and P-I possess 5 or 6 Arg(Mtr) residues respectively and it has been claimed[19] that occasionally this Mtr group could not be cleaved satisfactorily by the standard deprotecting reagent, TFA-thioanisole.[20] Thus, we decided to examine the applications of more effective deprotecting reagents, *i.e.*, TMSBr,[21] TMSOTf,[22] and TFMSA.[23] As model experiments, the protected (T-I)–MBHA resin and the protected (T-II)–DMBHA resin were each treated with a 1 M solution of each deprotecting reagent mentioned above in TFA at 4 °C or 25 °C. Thioanisole and two additional scavengers, *m*-cresol and EDT, were used. Each deprotected peptide amide (S-Acm peptide) was partially purified by gel-filtration on Sephadex G-10 and the cleavage yields were compared. As shown in Table I, none of the three reagents gave a satisfactory yield (less than 25%), when the treatment was performed at 4 °C. However, at elevated temperature, 25 °C, both 1 M

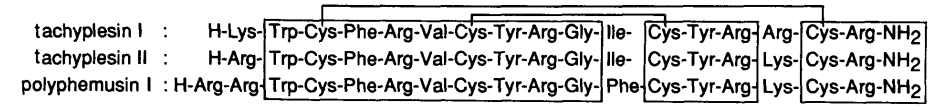

Fig. 1. Structures of Tachyplesin I, Tachyplesin II, and Polyphemusin I

Table I. Cleavage Yield from Methylbenzhydrylamine (MBHA) Resin and Dimethoxybenzhydrylamine (DMBHA) Resin

No.	Reagent	Temperature (°C)	Time (h)	Tachyplesin I[a] (%)	Tachyplesin II[b] (%)
1	1 M TMSBr	4	3	<10	<10
2	1 M TMSOTf	4	3	20	<10
3	1 M TFMSA	4	3	25	<10
4	1 M TMSOTf	25	2	87	87
5	1 M TFMSA	25	1.5	98	94

a) Synthesized on MBHA resin. *b*) Synthesized on DMBHA resin.

Table II. Amino Acid Ratios in an LAP Digest of Synthetic Peptide and the Cys(Acm) Intermediates

	Cys(Acm) peptide			Synthetic peptide		
	T-I	T-II	P-I	T-I	T-II	P-I
Gly	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
Cys	4.03(4)[a]	3.99(4)[a]	3.86(4)[a]	1.73(2)[b]	1.76(2)[b]	1.59(2)[b]
Val	0.95(1)	0.89(1)	0.86(1)	0.99(1)	0.98(1)	0.98(1)
Ile	0.97(1)	0.98(1)		1.00(1)	1.01(1)	
Tyr	1.88(2)	1.92(2)	1.88(2)	1.92(2)	1.90(2)	1.79(2)
Phe	0.99(1)	0.94(1)	1.85(2)	1.15(1)	0.98(1)	1.80(2)
Lys	0.91(1)	1.03(1)	1.02(1)	1.03(1)	0.98(1)	0.95(1)
Trp	0.99(1)	0.82(1)	0.74(1)	0.89(1)	0.85(1)	0.79(1)
Arg	4.76(5)	4.64(5)	5.25(6)	4.95(5)	4.89(5)	5.35(6)
Recov. (%)	63	64	77	84	76	92

Numbers in parentheses are theoretical. *a*) Determined as Cys(Acm). *b*) Determined as cystine.

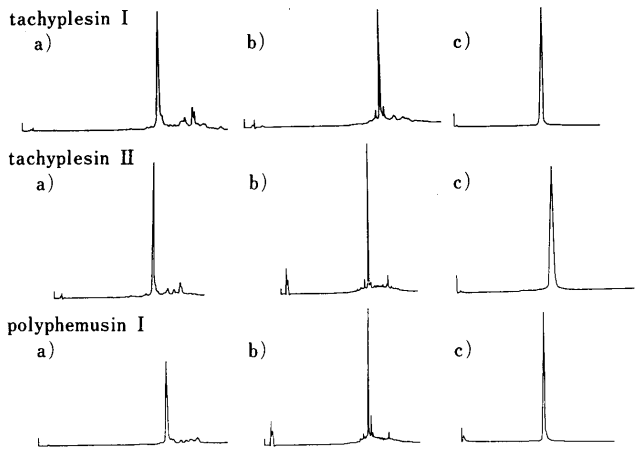

Fig. 2. Analytical HPLC of Synthetic Peptides

a) Cys(Acm)-peptide, b) crude air-oxidized sample, c) mixture of the synthetic sample and the natural peptide.

Table III. Biological Effect of Synthetic Peptides

Peptide	ED_{50} (μg)[a]	
	Natural	Synthetic
Tachyplesin I	3.0	3.1
Tachyplesin II	N.D.	1.5
Polyphemusin I	N.D.	5.1

a) Amount necessary to inhibit 2.5 pg LPS-mediated activation of factor C in Limulus clotting cascade. N.D. = not determined.

TMSOTf/TFA and 1 M TFMSA/TFA gave better yields than before (87% and 98%, respectively). No significant difference between the MBHA resin and the DMBHA resin support was noted.

Thus, in the preparative experiments, 1 M TFMSA–thioanisole/TFA was employed to cleave each peptide amide from the corresponding resin and at the same time to remove all protecting groups employed except for the Acm group from Cys. Each treated peptide was purified by gel-filtration on Sephadex G-10. At this stage, each partially purified Cys(Acm)-peptide amide was found to possess amino acids in ratios fairly consistent with those predicted by theory after leucine aminopeptidase (LAP) digestion (Table II) and exhibited a sharp main peak on high performance liquid chromatography (HPLC) (Fig. 2a). Next, each gel-filtered Cys(Acm)-peptide amide was treated with silver trifluoromethanesulfonate (AgOTf)[24] in the presence of anisole to remove the 4 Acm groups from the Cys residues. After treatment with DTT, followed by gel-filtration on Sephadex G-10, each reduced peptide amide was subjected to air-oxidation at pH 7.5 to establish the disulfide bonds. The progress of the reaction was examined by HPLC. After disappearance of the peak corresponding to the starting reduced peptide, a sharp symmetrical peak emerged without any sizable side peaks due to the formation of disulfide isomers or polymers in each case (Fig. 2b). Each oxidized peptide was adsorbed on Diaion HP-20 and then eluted with 60% CH_3CN in 1 N AcOH. Finally, each crude oxidized peptide was purified by HPLC on an Asahipak ODP-90 preparative column using a gradient of CH_3CN in 0.1% TFA. The yields of these peptides calculated from the protected peptide resins were 24—27%.

The purity of each peptide thus obtained was examined by amino acid analysis after LAP digestion, analytical HPLC, and sequencing. Each synthetic peptide gave the amino acids in the ratios predicted by theory (Table II) and exhibited a sharp single peak on analytical HPLC (Asahipak ODP-50 column). The purity of each synthetic peptide was evaluated as more than 97% by analytical HPLC and from the cumulative preview on sequencing. Each synthetic peptide had a retention time identical with that of the corresponding natural peptide on analytical HPLC (Fig. 2c). In addition, each synthetic peptide was proved to be a monomer by fast atom bombardment-mass spectrometry (FAB-MS).

The inhibitory effect of each synthetic peptide on the LPS-mediated activation of clotting factor C in the Limulus clotting cascade was compared with that of natural tachyplesin I in the known assay system[25] (Table III). The amount of synthetic tachyplesin I necessary for 50% inhibition (ED_{50}) of 2.5 pg LPS-mediated activation of factor C was nearly the same as that of the natural peptide. In terms of ED_{50}, synthetic tachyplesin II was 2.1 times more active than natural tachyplesin I, but synthetic polyphemusin I was 1.6 times less active.

Thus, the structure of tachyplesin I, tachyplesin II, and polyphemusin I are confirmed by the syntheses described above. In these syntheses, 1 M TFMSA–thioanisole/TFA served as the best reagent to deprotect and cleave the desired peptide amides from the MBHA resin and DMBHA resin support, when the reaction was performed at 25 °C. The results indicated that deprotecting reagents

TABLE IV. Manual Schedule for the Fmoc-Based Solid Phase Synthesis

Manipulation	Reagent	Solvent	Time/repeat
Deprotection	20% piperidine/DMF	DMF	5 min × 3
Washing		DMF	1 min × 6
Coupling	Fmoc-A.A–OH (2.5 eq) DIPCDI + HOBt	DMF	2 h × 1
Washing		DMF	1 min × 4

A.A = amino acid.

play an important role in Fmoc-based solid-phase syntheses of peptide amides, depending mostly on the nature of the C-terminal amino acid attached to the resin, compared to solid-phase syntheses of the peptide with the free carboxyl-end.

Experimental

Fmoc-amino acid derivatives were purchased from Kokusan Chemical Works, Ltd. Leucine aminopeptidase (Lot. 42F-8035) and MBHA resin were purchased from Sigma and Peptide Institute, Inc., respectively. DMBHA resin was prepared from amino-methylated polystyrene resin as reported before.[26] HPLC was conducted with a Waters 204 compact model. Sequence analysis was carried out by using an Applied Biosystems 470A gas-phase protein sequencer equipped with a 120A PTH analyzer.

Solid-Phase Synthesis Solid-phase synthesis was carried out manually according to the principle of Sheppard *et al.*[6] (Table IV). The MBHA resin (400 mg, amine content 0.15 mmol) was used for the synthesis of tachyplesin I. The DMBHA resin (270 mg each, amine content 0.20 mmol) was used for the syntheses of tachyplesin II and polyphemusin I. The following Fmoc amino acids and derivatives (2.5 eq each) were condensed by the use of DIPCDI (2.5 eq) in the presence of HOBt (2.5 eq); *i.e.*, Gly, Val, Ile, Phe, Trp, Cys(Acm), Tyr(tBu), Lys(Boc), and Arg(Mtr). Every condensation reaction was continued until the resin became negative to the Kaiser test. After assembling the respective amino acids, each protected peptide resin was hydrolyzed in 12 N HCl–phenol–AcOH = 2 : 1 : 1. Amino acid ratios in each hydrolysate (numbers in parentheses are theoretical): [(T-I)–resin] Gly 1.00 (1), Cys N.D., Val 0.88 (1), Ile 0.58 (1), Tyr 1.93 (2), Phe 0.92 (1), Lys 0.85 (1), Trp N.D., Arg 5.47 (5); [(T-II)–resin] Gly 1.00 (1), Cys N.D., Val 0.87 (1), Ile 0.56 (1), Tyr 1.80 (2), Phe 0.87 (1), Lys 0.98 (1), Trp N.D., Arg 4.52 (5); and [(P-I)–resin] Gly 1.00 (1), Cys N.D., Val 0.88 (1), Tyr 1.78 (2), Phe 1.83 (2), Lys 0.99 (1), Trp N.D., Arg 5.23 (6).

Preliminary Experiment for Deprotection of the Protected Peptide Resins The protected (T-I)–MBHA resin (50 mg) and the protected (T-II)–DMBHA resin (50 mg) were treated with a 1 M solution of one of three reagents (TMSBr, TMSOTf, and TFMSA) in TFA (3 ml) in the presence of 1 M thioanisole and two additional scavengers, *m*-cresol (100 eq) and EDT (300 eq), under the reaction conditions (4 °C or 25 °C for 1.5—3 h) shown in Table I. Each resin was removed by filtration and washed twice with TFA (1 ml). The filtrate and the washing were combined and ice-chilled dry ether (100 ml) was added to precipitate each product. After centrifugation, the ether was removed by decantation and the residue was dissolved in H_2O (3 ml). The solution was applied to a column of Sephadex G-10 (2.7 × 50 cm), which was eluted with 4 N AcOH. The eluate was monitored by ultraviolet (UV) absorption measurement at 280 nm. The fractions corresponding to each front main peak (6 ml each) were combined and the solvent was removed by lyophilization to give a powder. Each yield calculated from the protected resin is listed in Table I.

Preparation of Tachyplesin I after 1 M TFMSA–Thioanisole/TFA Treatment The protected (T-I)–MBHA resin (500 mg, 70 μmol) was treated with 1 M TFMSA–thioanisole/TFA (20 ml) in the presence of *m*-cresol (0.49 ml, 100 eq) and EDT (1.27 ml, 300 eq) at 25 °C for 1.5 h. The product, isolated as stated above, was dissolved in H_2O (8 ml) and applied to a column of Sephadex G-10 (3.7 × 50 cm), which was eluted with 4 N AcOH. The eluate was monitored by UV absorption measurement at 280 nm and the fractions corresponding to the front main peak (tube Nos. 26—45, 8 ml each) were combined. The solvent was removed by lyophilization to give a powder; 203 mg (isolation yield 98%). Amino acid ratios in a LAP digest are listed in Table II. The gradient elution pattern on an Asahipak ODP-50 HPLC column (4.6 × 150 mm) with CH_3CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min is shown in Fig. 2a.

The Cys(Acm)-peptide (200 mg, 67 μmol) thus obtained was treated with AgOTf (690 mg, 40 eq) in TFA (20 ml) in the presence of anisole (200 μl) at 4 °C for 1 h. Ice-chilled dry ether (200 ml) was added to the reaction mixture and the resulting powder was collected by centrifugation. After being washed twice with ice-chilled dry ether (20 ml), the product was dissolved in 4 N AcOH (10 ml) and DTT (830 mg, 80 eq) was added. The mixture was stirred at room temperature overnight and then centrifuged. The supernatant was applied to a column of Sephadex G-10 (3.7 × 50 cm), which was eluted with 4 N AcOH. The fractions corresponding to the front main peak (tube Nos. 25—48, 8 ml each, monitored by UV absorption measurement at 280 nm) were combined. The pH of the solution was adjusted to 7.5 with concentrated NH_4OH and the total volume of the solution was brought to 2 l with H_2O (peptide concentration 0.1 mg/ml). The solution was kept standing at 4 °C. Periodically, an aliquot (250 μl) was examined by analytical HPLC on an Asahipak ODP-50 column (4.6 × 150 mm), which was eluted with a gradient of CH_3CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min. The eluate was monitored by UV absorption measurement at 280 nm. After 5 d, when the starting material had disappeared on HPLC, the pH of the solution was adjusted to 6 with 4 N AcOH and Diaion HP-20 resin (*ca.* 10 g) was added. The mixture was stirred for 1 h, then the resin was collected by filtration and washed twice with H_2O (200 ml). The peptide was eluted from the resin with 60% CH_3CN in 1 N AcOH. After the removal of CH_3CN by evaporation at room temperature, the remaining solvent was removed by lyophilization to give a fluffy powder; yield 151 mg (84%). Its gradient elution pattern on an Asahipak ODP-50 HPLC column (4.6 × 150 mm) with CH_3CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min is shown in Fig. 2b.

Finally, the crude air-oxidized peptide was purified by HPLC. A sample (10 mg in 500 μl of H_2O) was applied to an Asahipak ODP-90 column (21.5 × 300 mm), which was eluted with a gradient of CH_3CN (20—40%, 40 min) in aqueous 0.1% TFA at the flow rate of 7 ml/min. The eluate corresponding to the main peak (retention time 22.0 min, detected by UV absorption measurement at 280 nm) was collected and the solvent was removed by lyophilization to give a white fluffy powder. The rest of the sample was similarly purified; yield 48 mg (26%, calculated from the protected peptide resin). A mixture of the synthetic sample and the natural peptide gave a single peak (retention time 18.0 min) on a Chemcosorb 7-ODS-H HPLC column (2.1 × 150 mm) eluted with a gradient of 80% CH_3CN in 0.1 M TEA (27—50%, 40 min) in aqueous 0.1 M TEA at the flow rate of 0.4 ml/min (Fig. 2c). The predicted sequence of synthetic tachyplesin I was confirmed by sequencing analysis, in which the cumulative preview was less than 3%. Amino acid ratios in a LAP digest of the synthetic peptide are listed in Table II. FAB-MS *m/z*: 2265.1 $(M+H)^+$ (Calcd 2264.7).

Preparation of Tachyplesin II and Polyphemusin I after 1 M TFMSA–Thioanisole/TFA Treatment Tachyplesin II and polyphemusin I were similarly prepared. The protected (T-II)–DMBHA resin (200 mg, 34 μmol) and the protected (P-I)–DMBHA resin (200 mg, 32 μmol) were treated with 1 M TFMSA–thioanisole/TFA (10 ml) in the presence of *m*-cresol (100 eq) and EDT (300 eq) at 25 °C for 1.5 h, respectively. Each Cys(Acm)-peptide was partially purified by gel-filtration on Sephadex G-10 (3.7 × 50 cm) as stated above: yield; 95 mg (94%) for the tachyplesin II derivative and 96 mg (93%) for the polyphemusin I derivative. Amino acid ratios of the LAP digests are listed in Table II. The gradient elution patterns on an Asahipak ODP-50 HPLC column (4.6 × 150 mm), with CH_3CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min, are shown in Fig. 2a.

As stated above, each Cys(Acm)-peptide in TFA (10 ml) was treated with AgOTf (40 eq) in the presence of anisole (100 μl), followed by DTT (80 eq). After air-oxidation at pH 7.5 for 5 d (Fig. 2b), each product was isolated by adsorption on Diaion HP-20 and purified by HPLC on an Asahipak ODP-90 column (21.5 × 300 mm) using a gradient of CH_3CN (20—40%, 40 min) in aqueous 0.1% TFA at the flow rate of 7 ml/min; yield; tachyplesin II 24.5 mg and polyphemusin I 22.5 mg (27% and 24%, calculated from the protected peptide resin, respectively). A mixture of each synthetic peptide and the corresponding natural peptide gave a single peak (retention time 17.1 min for tachyplesin II and 17.4 min for polyphemusin I), when a Chemcosorb 7-ODS-H HPLC column (2.1 × 150 mm) was eluted with a gradient of 80% CH_3CN/0.1 M TEA (27—50%, 40 min) in aqueous 0.1 M TEA at the flow rate of 0.5 ml/min (Fig. 2c). The predicted sequences of peptides were confirmed by sequencing analyses; the cumulative previews on both sequencings were less than 3%. Amino acid ratios in a LAP digest of both synthetic peptides are listed in Table II. FAB-MS *m/z*: 2265.3 $(M+H)^+$ for tachyplesin II (Calcd 2264.7), and

2455.2 $(M+H)^+$ for polyphemusin I (Calcd 2454.9).

Acknowledgement The authors are grateful to Drs. Nobuhara Shigematsu and Hirokazu Tanaka, Tsukuba Research Laboratory, Fujisawa Pharmaceutical Co., Ltd., for measurement of FAB-MS spectra and Drs. Makoto Ooki and Shigenori Tanaka, Tokyo Research Institute, Seikagaku Kogyo Co., Ltd., for biological assay of our synthetic peptides.

References and Notes

1) Part CLXVII. K. Akaji, Y. Hayashi, N. Fujii, T. Liu, I. Berkower, and H. Yajima, *Chem. Pharm. Bull.*, **37**, 1612 (1989).
2) Amino acids used here are of the L-configuration. The following abbreviations are used; Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl, tBu = *tert*-butyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Acm = acetamidomethyl, EDT = ethanedithiol, DTT = dithiothreitol, TFMSA = trifluoromethanesulfonic acid, TMSBr = trimethylsilyl bromide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TFA = trifluoroacetic acid, DIPCDI = 1,3-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, DMF = dimethylformamide, MBHA = 4-methylbenzhydrylamine, DMBHA = 2,4′-dimethoxybenzhydrylamine, TEA = triethylamine.
3) T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao, and Y. Shimonishi, *J. Biol. Chem.*, **263**, 16709 (1988).
4) T. Nakamura, T. Morita, and S. Iwanaga, *Eur. J. Biochem.*, **154**, 511 (1986).
5) T. Miyata, T. Yoneya, T. Nakamura, S. Iwanaga, T. Takao, and Y. Shimonishi, *Seikagaku* (in Japanese), **60**, 874 (1988); F. Tokunaga, T. Miyata, T. Yoneya, K. Yoshikawa, T. Nakamura, M. Niwa, S. Iwanaga, T. Takao, and Y. Shimonishi, *J. Biochem.*, **106**, 111 (1989).
6) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125; L. Cameron, M. Medal, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, **1987**, 270.
7) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
8) E. Jaeger, P. Thamm, S. Knof, and E. Wünsch, *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 1629 (1978); Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **53**, 464 (1980); N. Fujii, S. Futaki, K. Yasumura, and H. Yajima, *Chem. Pharm. Bull.*, **32**, 2660 (1984).
9) M. Fujino, O. Nishimura, M. Wakimasu, and C. Kitada, *J. Chem. Soc., Chem. Commun.*, **1980**, 668.
10) D. F. Veber, J. D. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **94**, 5456 (1972).
11) G. R. Matsueda and J. M. Stewart, *Peptide*, **2**, 45 (1981).
12) S. Funakoshi, E. Murayama, L. Guo, N. Fujii, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1988**, 382.
13) A. Tartar and J-C. Gesquiere, *J. Org. Chem.*, **44**, 5000 (1979).
14) W. König and R. Geiger, *Chem. Ber.*, **103**, 788, 2024, 2034 (1970).
15) L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, **92**, 5748 (1970); E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 538.
16) E. Kaiser, R. L. Colescott, C. S. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
17) G. S. Lu, S. Mojsov, and R. B. Merrifield, *Int. J. Peptide Protein Res.*, **29**, 545 (1987).
18) J. P. Tam and R. B. Merrifield, "The Peptides," Vol. 9, ed. by S. Udenfriend and J. Meienhofer, Academic Press, New York, 1987, p. 185.
19) E. Atherton, R. C. Sheppard, and J. D. Wade, *J. Chem. Soc., Chem. Commun.*, **1983**, 1060; *idem, J. Chem. Soc., Perkin Trans. 1*, **1985**, 2065.
20) Y. Kiso, K. Ukawa, and T. Akita, *J. Chem. Soc., Chem. Commun.*, **1980**, 101.
21) N. Fujii, A. Otaka, N. Sugiyama, M. Hatano, and H. Yajima, *Chem. Pharm. Bull.*, **35**, 3880 (1987).
22) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakoshi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 274.
23) H. Yajima and N. Fujii, "The Peptides," Vol. 5, ed. by E. Gross and J. Meienhofer, Academic Press, 1983, New York, p. 65.
24) N. Fujii, A. Otaka, T. Watanabe, A. Okamachi, H. Tamamura, H. Yajima, Y. Inagaki, M. Nomizu, and K. Asano, *J. Chem. Soc., Chem. Commun.*, **1989**, 283.
25) T. Obayashi, H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai, *Clin. Chim. Acta*, **149**, 55 (1985).
26) L. Guo, S. Funakoshi, N. Fujii, and H. Yajima, *Chem. Pharm. Bull.*, **36**, 4989 (1988); S. Funakoshi, H. Tamamura, N. Fujii, K. Yoshizawa, H. Yajima, K. Murayama, A. Funakoshi, M. Ohota, Y. Inagaki, and L. A. Carpino, *J. Chem. Soc., Chem. Commun.*, **1988**, 1589.