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

Kenichi AKAI,* a Nobutaka FUKI, a Fuminori TOKUNAGA,b Toshiyuki MIYATA,a 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-fluorenlymethoxycarbonyl (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; peptide amide synthesis; thioanisole-mediated deprotection; trifluoromethanesulfonic acid; trimethylsilyl trifluoromethanesulfonate; 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 (Tachyples 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 Tachyples 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 synthesis 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) resin11) or 2,4-dimethoxybenzhydrylamine (DMBHA) resin12) were 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 DIPCIDI procedure13) with the aid of HOBt.14) The combination of piperidine treatment15) and DIPCIDI plus HOBt procedure served to elongate the peptide chain manually, but in principle, according to the automated program proposed by Sheppard et al.15) The resin became negative to the ninhydrin test16) 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 claimed19) 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-creosol 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](https://example.com/structure.png)

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TMSOTf/TFA and 1 M TFSMA/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 TFSMA-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) 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% CH₂CN in 1 M AcOH. Finally, each crude oxidized peptide was purified by HPLC on an Asahipak ODP-90 preparative column using a gradient of CH₂CN in 0.1% TFA. The yields of these peptides calculated from the protected peptide residues 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 tachypleisin I in the known assay system (Table III). The amount of synthetic tachypleisin I necessary for 50% inhibition (ED₅₀) of 2.5 pg LPS-mediated activation of factor C was nearly the same as that of the natural peptide. In terms of ED₅₀, synthetic tachypleisin I was 2.1 times more active than natural tachypleisin I, but synthetic polyphemusin I was 1.6 times less active.

Thus, the structure of tachypleisin I, tachypleisin II, and polyphemusin I are confirmed by the syntheses described above. In these syntheses, 1 M TFSMA-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...
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 carboxyend.

Experimental

Fmoc-amino acid derivatives were purchased from Kokusai 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. HPLC was conducted with a Waters 2040 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. (IV Table). 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 polyphensin I. The following Fmoc amino acids and derivatives (2.5 eq each) were condensed by the use of DIPCIDI (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). Each 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 0.78 (1), Lys 0.98 (1), Trp N-D, Arg 4.52 (5); and (T-P)-resin: Gly 1.00 (1), Cys N-D, Val 0.88 (1), Ile 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 1 M solution of one of three reagents (TMSBr, TMSOTf, and TMPSA) in TFA (3 ml) in the presence of 1 N HCl. The products, m-cresol and EDT (100 eq) and EDT (300 eq), under the reaction conditions (4°C or 25°C for 4.5—5.5 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 H2O (3 ml). The solution was applied to a column of Sephadex G-10 (2.7 x 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 TMPSA—Thioanisole/TFA Treatment

The protected (T-I)-MBHA resin (500 mg, 70 μmol) was treated with 1 M TMPSA—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 H2O (8 ml) and applied to a column of Sephadex G-10 (3.7 x 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 each fraction determined by a LAPPAP-60 HPLC column (4.6 x 150 mm) with CH2CN (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 (100 ml) with DTT (830 mg, 80 eq) 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 x 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 and thus collected at 280 nm) were combined. The precipitate was adjusted to 7.5 with concentrated NH4OH and the total volume of the solution was brought to 21 with H2O (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 x 150 mm), which was eluted with a gradient of CH2CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min as shown in Fig. 2b.

Finally, the crude air-oxidized peptide was purified by HPLC. A sample (10 mg in 500 μl of H2O) was applied to an Asahipak ODP-90 column (21.5 x 300 mm), which was eluted with a gradient of CH2CN (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 yield was 151 mg (84%). Its gradient elution pattern on an Asahipak ODP-50 HPLC column (4.6 x 150 mm) with CH2CN (10—30%, 30 min) in aqueous 0.1% TFA at the flow rate of 1 ml/min was shown in Fig. 3a.

Preparation of Tachyplesin II and Polyphensin I after 1 M TMPSA—Thioanisole/TFA Treatment

Tachyplesin II and polyphensin 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 TMPSA—thioanisole/TFA (10 ml) in the presence of m-cresol (0.49 ml, 100 eq) and EDT (300 eq) at 25°C for 1.5 h. The product was partially purified by gel-filtration on Sephadex G-10 (3.7 x 50 cm) as stated above; yield 95 mg (94%), for the tachyplesin II derivative and 96 mg (93%) for the polyphensin I derivative. Amino acid ratios of the LAP digest of the peptide are listed in Table II. The gradient elution pattern on an Asahipak ODP-50 HPLC column (4.6 x 150 mm), with CH2CN (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 Dowex H-20+ (200—400 mesh) and Asahipak ODP-90 column (21.5 x 300 mm) using a gradient of CH2CN (20—40%, 40 min) in aqueous 0.1% TFA at the flow rate of 7 ml/min; yield; tachyplesin II 24.5 mg and polyphensin 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 component on a gradient (retention time 17.1 min for tachyplesin II and 17.4 min for polyphensin I), when a Chromosorb 7-OADS-H HPLC column (2.1 x 150 mm) was eluted with a gradient of 80% CH2CN/0.1% TFA at the flow rate of 0.5 ml/min (Fig. 2c).

The predicted sequences of peptides were confirmed by sequencing analyses; the cumulative sequence reviews on tachyplesin II and polyphensin I, 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 (Caled 2264.7), and
2455.2 (M+H)⁺ for polyphemusin 1 (Calcd 2454.9).

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References and Notes
2) Amino acids used here are of the L-configuration. The following abbreviations are used: Boc = tert-butoxycarbonyl, Fmoc = 9-fluorenylmethylloxycarbonyl, Tbu = tert-butyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Acem = acetamidomethyl, EDT = ethanedithiol, DTT = dithiothreitol, TFMSA = trifluoromethanesulfonic acid, TMSBr = trimethylsilyl bromide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TFA = trifluoroacetic acid, DIPCDI = 1,3-diisopropylcarbodiimide, HOBr = N-hydroxybenzotriazol, DMF = dimethylformamide, MBHA = 4-methylbenzhydrylamine, DBMBHA = 2,4'-dimethoxybenzhydrylamine, TEA = triethylamine.