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Preparation and Determination of Structure of L-3-Deoxymimosine-containing Peptides

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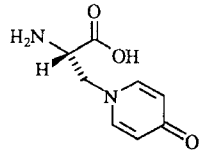
L-3-Deoxymimosine-containing decapeptides were prepared for the development of protein tyrosine kinase (PTK) inhibitors. During the preparation of peptides, several side products were formed. Identification and determination of major peptides generated were reported.

Key words: L-3-Deoxymimosine, Peptide synthesis, Structure determination

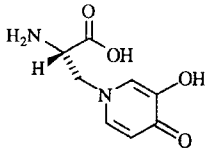
INTRODUCTION

Protein tyrosine kinases (PTK's) play an important role in the regulation of signal transduction pathways (Hunter *et al.*, 1985, Cantly *et al.*, 1991). Specifically, PTK's catalyze phosphorylations of the hydroxyl group on tyrosyl residue in substrate proteins or peptides, which regulate cell proliferation and differentiation. Development of specific inhibitors of PTK's has been of interest not only as a tool to understand the role of tyrosine phosphorylation but also as a connection with PTK's potential use as anti-cancer agents. Results of our prior research (Lee *et al.*, 1994, Kim *et al.*, 1997, Kim *et al.*, 1998) related to the development of PTK inhibitors have been published.

L-3-Deoxymimosine (**1**) is a modified structure of L-mimosine (**2**), a naturally occurring amino acid and toxic principles of Leucaena leucocephala (Lam.) de Wit, which are known to cause fleece-shedding in sheeps (Hegarty *et al.*, 1964). In this research, L-3-deoxymimosine-containing peptides were prepared as potential mechanism-based inhibitors of PTK's to be used in the development of potential anticancer agents. The peptide sequence for incorporation of L-3-deoxymimosine was derived from angiotensin I (**3**) which was reported as being a good substrate for various PTK's (Wong *et al.*, 1984, Zioncheck et al., 1986). The rationale, synthesis and preparation of L-3-deoxymimosine-containing peptide have been previously reported (Lee *et al*, 1994). In these studies, the structures of the major side products resulting during separation of Boc-L-3-deoxymimosine (**4**), a key intermediate for the peptide synthesis, and during the incorporation of Boc-L-3-deoxymimosine into the required sequence were identified and determined. In the present study, the preparation, identification and determination of structures of L-3-deoxymimosine-containing angiotensin I and its major side products are reported.



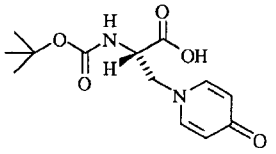
L-3-Deoxymimosine (**1**)



L-Mimosine (**2**)

H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH

Angiotensin I (**3**)



Boc-L-3-deoxymimosine (**4**)

MATERIALS AND METHODS

All reactions were performed with nitrogen or argon, unless stated otherwise, or unless an aqueous reaction medium was employed. Analytical thin-layer chromatography was performed on Merck Kieselgel 60 F254 plates,

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0.25 mm thick or Aldrich Pre-coated TLC sheets, silica gel on polyester, 0.25 mm thickness. Column chromatography was performed on the silica gel, 60-200 or 230-400 mesh. 1H NMR spectra were recorded on a Brucker ARX-300 or a Varian VXR-500 spectrometer. Fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS25 or Kratos MS50 spectrometer, using a glycerol/thioglycerol or dithiothreitol/dithioerythritol (DTT/DTE) matrix. All high resolution mass spectra were recorded on a Kratos MS50 spectrometer. Amino acid analysis was performed with a Beckman 7300 amino acid analyzer. Solid phase peptide synthesis was performed on a Applied Biosystems 430A peptide synthesizer. HPLC tasks were performed on a Rainin HPLC system using the Dynamax program.

Methylene chloride and N,N-dimethylformamide were distilled from calcium hydride. For all analytical and semi-preparative HPLC tasks, double distilled water and HPLC grade acetonitrile and methanol were used.

All the enzymes utilized were purchased from the Sigma Chemical Company. Type I crystalline Carboxypeptidase A was obtained from bovine pancreas and aqueous suspension with toluene added. The activity was 50 units per mg protein. Carboxypeptidase Y was obtained from bakers yeast and lyophilized powder containing approx. 20% protein in balance citrate buffer, pH 5. The activity was 100 units per mg protein. Type III-CP Leucine aminopeptidase, cytosol, was obtained from porcine kidney and chromatographically purified suspension in 2.9 M $(NH_4)_2SO_4$, 0.1 M tris, 5 mM $MgCl_2$ solution, pH 8.0. The activity was 150-300 units per mg protein.

Synthesis

Boc-L-3-Deoxymimosine-containing heptapeptide-Pam resin 8

The heptapeptide-Pam resin **8** was prepared by solid phase synthesis utilizing active ester methodology (Stewart *et al.*, 1984). Boc-L-3-Deoxymimosine (**4**, 0.43 g, 1.53 mmol)

The solid phase synthesis was performed according to the following schedule;

1. DCM wash (3 times)	5 mL × 3	1.5 min × 3	5 min
2. TFA/DCM/m-cresol	5 mL		1.5 min
3. TFA/DCM/m-cresol	5 mL		30 min
4. DCM wash (3 times)	5 mL × 3	1.5 min × 3	5 min
5. DMF wash (3 times)	5 mL × 3	1.5 min × 3	5 min
6. 10% DIEA/DMF	5 mL		45 sec
7. 10% DIEA/DMF	5 mL		1 min
8. 10% DIEA/DMF	5 mL		1.5 min
9. DMF wash (3 times)	5 mL × 3	1.5 min × 3	5 min
10. active ester 5 (0.43 g, 1.53 mmol) in DMF	7 mL		16 h
11. DMF wash (3 times)	5 mL × 3	1.5 min × 3	5 min
12. MeOH wash (3 times)	5 mL × 3	1.5 min × 3	5 min
13. DCM wash (3 times)	5 mL × 3	1.5 min × 3	5 min.

and 1-hydroxybenzotriazole (HOBT) (0.234 g, 1.53 mmol) were dissolved in dry DMF (5.0 mL), and the mixture was chilled to 0°C. 1,3-Dicyclohexylcarbodiimide (DCC) (0.317 g, 1.53 mmol) was added to this mixture, and the mixture was stirred for 40 min at room temperature. After filtering the precipitated dicyclohexyl urea (DCU), the solution containing active ester 5 was immediately used to synthesize the peptides. Boc-protected hexapeptide-Pam resin 6 (0.5 mmol) obtained from the peptide synthesis facility was treated with 10 mL of 33% trifluoroacetic acid (TFA) in dichloromethane (DCM) containing 5% m-cresol for 30 min at room temperature. After washing with dichloromethane (15 mL) and DMF (15 mL), the resin was treated with 10% *N,N*-diisopropylethylamine (DIEA) in DMF (15 mL) for 3 min at room temperature. Resin 7 was washed with DMF (15 mL) and DCM (15 mL) and treated with a solution of active ester **5** in DMF (7 mL) for 16 h at room temperature. The resin was washed with DMF (15 mL), methanol (15 mL) and DCM (15 mL), and dried at high vacuum. The ninhydrin test indicated that 99.4% of the coupling had completed;.

Preparation of L-3-deoxymimosine-containing angiotensin I (9)

The crude peptide **9** (50 mg) obtained from the resin was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 m, 4.6 × 250 mm) and FABMS. A gradient elution of 25% to 55% solvent B in solvent A in 20 min at a flow rate of 1 mL/min was used, where solvent A was 0.25 N triethylamine phosphate buffer (pH 2.25) solution and solvent B was 40% solvent A in acetonitrile. There were three major peaks having retention times of 8.350 min (peptide **12**), 9.545 min (peptide **10** and **11**) and 10.940 min (peptide 9), which were identified through FABMS, protein sequencing analysis, enzymatic reactions and chemical reactions. The peptides **9** and a mixture of peptides **10** and **11** were isolated by semi-preparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 m, 10 × 250 mm). A gradient elution of 20% to 33% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 4.5 mL/min was used. Fractions corresponding to each peak (UV detector at 230 nm) of retention time 9.885 min (peptide 10 and **11**) and 11.050 min (peptide **9**) were collected. The combined fractions were lyophilized to afford peptides **9** (10.1 mg, 20.2%) and a mixture of peptides 10 and 11 (13.4 mg, 26.8%) as a white solid: Low resolution FABMS m/e, peptide **9** 1297 (MH^+), peptide **10** and **11** 1297 (MH^+), 1198 (MH^+); High resolution FABMS peptide **9** m/e ($C_{61}H_{88}O_{14}N_{18}$) calculated 1297.68 (MH^+), found 1297.66 (MH^+); Amino acid analysis, peptide **9** (expected/

found) Asp (1.0/0.9), Pro (1.0/1.1), Val (1.0/ 1.0), Ile (1.0/1.0), Leu (1.0/1.1), Phe (1.0/1.0), His (2.0/ 2.0), Arg (1.0/1.1).

N-(tert-Butoxycarbonyl)-D-, and -L-3-deoxymimosyl-L-isoleucyl methyl ester (14 and 15)

In argon atmosphere, Boc-L-3-deoxymimosine (**4**, 71.0 mg, 0.25 mmol) and 1-hydroxybenzotriazole (HOBT) (36.5 mg, 0.27 mmol) were dissolved in dry DMF (2.0 mL), and the mixture was chilled to 0°C. 1,3-Dicyclohexylcarbodiimide (DCC) (55.5 mg, 0.27 mmol) was added to this mixture, and the mixture was stirred for 40 min at room temperature. After filtration of the precipitated dicyclohexyl urea (DCU), the solution containing active ester **5** was added to a solution of L-isoleucyl methyl ester hydrogen chloride (45.4 mg, 0.25 mmol) in the presence of N-methylmorpholine (25.3 mg, 27.5 mL, 0.25 mmol) in DMF (0.5 mL). The mixture was stirred for 16 h at room temperature. After the precipitate was filtered off, the solvent was removed at high vacuum. The residue was purified by column chromatography (silica gel, 230-400 mesh), eluting with ethyl acetate/methanol (2:1), to afford a diastereomeric mixture of dipeptide (L,L-form and D,L-form) as a gummy oil (21.4 mg, 20.9%). HPLC analysis on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient of 40% to 80% solvent B in solvent A in 15 min at a flow rate of 1 mL/min (UV detector at 254 nm) where solvent A was water and solvent B was 66% aqueous acetonitrile. These mixtures indicated a mixture of 56.6% of L,L-form dipeptide **14** and 43.4% of D,L-form dipeptide **15**. Separation of the mixture (16 mg) was performed on a semi-preparative column with a gradient elution of 40% to 80% solvent B in solvent A in 15 min at a flow rate of 4.5 mL/min where the solvent system was the same as above. Fractions corresponding to each peak of retention times, 9.680 min and 10.515 min, were collected and lyophilized to afford dipeptides **14** (8 mg) and **15** (5 mg) as white solids: TLC (silica gel, EtOAc /MeOH=2:1) peptide **14** and **15**, Rf 0.49; ^{1}H NMR (500 MHz, $CDCl_3$) peptide **14** δ 0.842 (m, 6 H, CH_3), 1.183 (m, 1 H, CH), 1.297 (s, 9 H, CH_3), 1.399 (m, 1 H, CH_2), 1.805 (m, 1 H, CH_2), 3.623 (s, 3 H, OCH_3), 3.778 (d of d, 1 H, CH_2, J_H=10.62 Hz, J_H=13.83 Hz), 4.072 (d of d, 1 H, CH_2, J_H=4.03 Hz, J_H=13.92 Hz), 4.235 (t, 1 H, CH, J_H=7.42 Hz), 4.438 (t of d, 1 H, CH, J_H=4.03 Hz, J_H=9.98 Hz), 6.048 (d, 2 H, CH=, J_H=7.51 Hz), 7.130 (d, 1 H, NH, J_H=9.15 Hz), 7.588 (d, 2 H, CH=, J_H= 7.51 Hz), 8.311 (d, 1 H, NH, J_H=7.97 Hz), peptide **15** δ 0.823 (t, 6 H, CH_3), 1.148 (m, 1 H, CH), 1.291 (s, 9 H, CH_3), 1.336 (m, 1 H, CH_2), 1.774 (m, 1 H, CH_2), 3.651 (s, 3 H, OCH_3), 3.763 (d of d, 1 H, CH_2, J_H= 10.71 Hz, J_H=13.64 Hz), 4.053 (d of d, 1 H, CH_2, J_H= 4.30 Hz, J_H=13.73 Hz), 4.279 (d of d, 1 H, CH, J_H= 6.32 Hz, J_H=8.43 Hz), 4.512 (t of d, 1 H, CH, J_H= 4.21 Hz, J_H=10.16 Hz), 6.044 (d, 2 H, CH=, J_H=7.60 Hz), 7.082 (d, 1 H, NH, J_H=9.43 Hz), 7.597 (d, 2 H, CH=, J_H=7.69 Hz), 8.482 (d, 1 H, NH, J_H=8.61 Hz); FABMS, peptide **14** and **15** *m/e* 410 (MH^+).

L-3-deoxymimosyl-L-isoleucyl methyl ester trifluoroacetate (16)

Thirty-three percent TFA containing 5% *m*-cresol in dichloromethane (0.5 mL) was added to an analytical amount of *N*-(*tert*-Butoxycarbonyl)-L-3-deoxymimosyl-L-isoleucyl methyl ester (**14**) (ca. 0.5 mg), and the mixture was stirred for 30 min at room temperature. Diethyl ether (2 mL) was added to induce precipitation of the product as a white solid. The precipitate was filtered and washed with diethyl ether (3 × 2 mL), and dissolved in water (0.1 mL). Product **16** was analyzed and separated by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm). A gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min was used. The fractions corresponding to the peak of the retention time 9.490 min were collected and lyophilized to afford peptide **16** (<0.5 mg): FABMS *m/e* 310 (MH^+).

Enzymatic hydrolysis of peptide 16 with leucine aminopeptidase

A suspension of leucine aminopeptidase (10 mL, 10 units) was added to a solution of analytical amount of peptide **16** (<0.5 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 4 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min. The product was analyzed by calculating the ratio between the reference peak and peptide **16**; peptide **16** was found to be partially hydrolyzed.

D-3-Deoxymimosyl-L-isoleucyl methyl ester trifluoroacetate (17)

To an analytical amount of *N*-(*tert*-butoxycarbonyl)-D-3-deoxymimosyl-L-isoleucyl methyl ester (**15**) (ca. 0.5 mg) was added 33% TFA containing 5% m-cresol in dichloromethane (0.5 mL), and the mixture was stirred for 30 min at room temperature. Diethyl ether (2 mL) was added to induce precipitation of the product as a white solid. The precipitate was filtered and washed with diethyl ether (3 × 2 mL), and dissolved in water (0.1 mL). The product **17** was analyzed and separated by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm). A gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min was used. The fractions corresponding to the peak of the retention time 9.230 min were collected and lyophilized to afford peptide **17** (<0.5 mg): FABMS m/e 310 (MH^+).

Enzymatic hydrolysis of peptide 17 with leucine aminopeptidase

A suspension of leucine aminopeptidase (10 μL, 10 units) was added to a solution of an analytical amount of peptide **17** (< 0.5 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 4 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min. From the analysis of the product by calculation of the ratio between the reference peak and that of peptide **17**, peptide **17** remained unreacted.

Enzymatic hydrolysis of peptide 9 with carboxypeptidase A (peptide 18)

A suspension of carboxypeptidase A (15 μL, 20 units) was added to a solution of an analytical amount of peptide **9** (0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 2 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The HPLC on a Vydac column isolated one major peak of the retention time at 16.280 min. The fractions corresponding to the peak of the retention time 16.280 min were collected and lyophilized to afford peptide **18**: FABMS m/e 890 (MH^+).

Enzymatic hydrolysis of peptide 18 with carboxypeptidase Y (peptide 19)

Carboxypeptidase Y (3 mg, 51 units) was added to a solution of an analytical amount of peptide **18** (<0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 4 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The HPLC on a Vydac column isolated one major peak of the retention time at 17.475 min. The fractions corresponding to the peak of the retention time of 17.475 min were collected and lyophilized to afford peptide **19**: FABMS m/e 666 (MH^+).

Enzymatic hydrolysis of peptide 19 with carboxypeptidase A

A suspension of carboxypeptidase A (25 μL, 33 units) was added to a solution of an analytical amount of peptide **19** (<0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 3 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The analytical HPLC tracing indicated that peptide **19** had been completely hydrolyzed.

Enzymatic hydrolysis of a mixture of peptides 10 and 11 with carboxypeptidase A (peptide 20, 21)

A suspension of carboxypeptidase A (15 μL, 20 units) was added to a solution of an analytical amount of a mixture of peptides **10** and **11** (0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 2 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The HPLC on a Vydac column isolated two major peaks of the retention times at 13.175 and 14.275 min. The fractions corresponding to each peak of the retention times, 13.175 min and 14.275 min, were collected and lyophilized to afford peptides **20** and **21**: FABMS, peptide **20** m/e 801 (MH^+), peptide **21** m/e 890 (MH^+).

Enzymatic hydrolysis of peptide 21 with carboxypeptidase Y (peptide 22)

Carboxypeptidase Y (3 mg, 51 units) was added to a solution of an analytical amount of peptide **21** (<0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 4 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The HPLC on a Vydac column isolated one major peak of the retention time at 11.205 min. The fractions corresponding to the peaks of the retention time of 11.205 min were collected and lyophilized to afford peptide **22**: FABMS m/e 803 (MH^+).

Enzymatic hydrolysis of peptide 22 with carboxypeptidase A

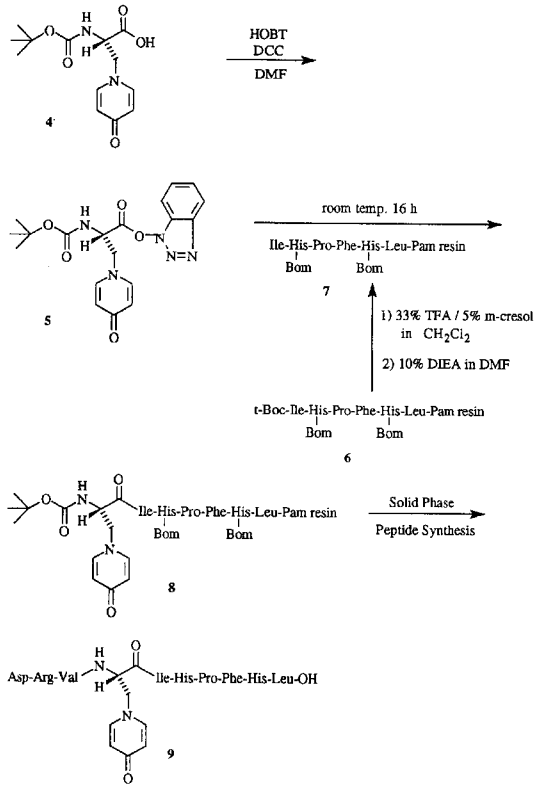
A suspension of carboxypeptidase A (25 μL, 33 units) was added to a solution of an analytical amount of peptide **22** (<0.1 mg) in 0.1 M sodium phosphate buffer solution (0.15 mL, pH 7.2) at room temperature, and the mixture was allowed to stand for 3 h. The reaction mixture was analyzed by HPLC on a Vydac column (218TP54, C-18, 5 μ, 4.6 × 250 mm) with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The analytical HPLC tracing indicated that peptide **22** had not reacted.

RESULTS AND DISCUSSION

Boc-L-3-deoxymimosine (**4**), a key intermediate to be incorporated into the peptide sequence, was synthesized using a method which was previously reported (Lee *et al.*, 1994). The crude product 4 was purified with a silica gel column chromatography using methanol as a eluent.

A Boc-protected hexapeptide-Pam resin 6, prepared in the peptide synthesis facility, was treated with 33% TFA in dichloromethane containing 5% *m*-cresol for 30 min to cleave the Boc group (Scheme 1). It was then treated with 10% DIEA in DMF to generate free amine 7 and was coupled with the active ester of Boc-L-3-deoxymimosine 5 in DMF at room temperature for 16 h to yield Boc-L-3-deoxymimosine-containing heptapeptide 8 (Stewart *et al.*, 1984). The compound **5** was prepared by treatment of Boc-L-3-deoxymimosine (**4**) with 1-hydroxybenzotriazole (HOBT) in the presence of DCC in DMF for 30 min. The coupling was monitored by ninhydrin test which indicated more than 99% completion.

The crude L-3-deoxymimosine-containing angiotensin I **9**, which was obtained from the resin, was analyzed by HPLC on a Vydac column with a gradient elution of 25% to 55% solvent B in solvent A in 20 min at a flow rate of 1 mL/min, where solvent A was 0.25 N triethylamine-phosphate buffer (pH 2.25) solution and solvent B was 40% solvent A in acetonitrile. The analytical HPLC tracing indicated the presence of three major peaks having retention times of 8.350 min, 9.545 min and 10.940 min in nearly 2:6:5 ratio (Fig. 1). After collection of fractions corresponding to each peak and lyophilization, FABMS analysis



Scheme 1. Synthesis of L-3-deoxymimosine-containing angiotensin I (**9**)

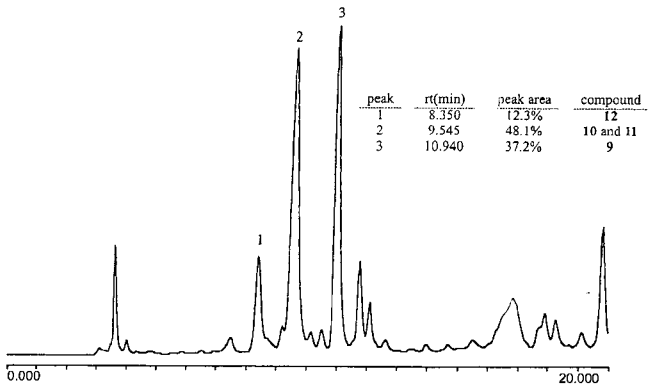


Fig. 1. HPLC analysis of crude peptide **9**. Column; Vydac 218TP54, C-18, 10 µm, 4.6 × 250 mm. A gradient elution of 25% to 55% solvent B in solvent A in 20 min at a flow rate of 1 mL/min was used (detector at 230 nm). For peak 1; peptide **12**, retention time 8.350 min, peak area 12.3%. For peak **2**; peptide **10** and **11**, retention time 9.545 min, peak area 48.1%. For peak **3**; peptide **9**, retention time 10.940 min, peak area 37.2%

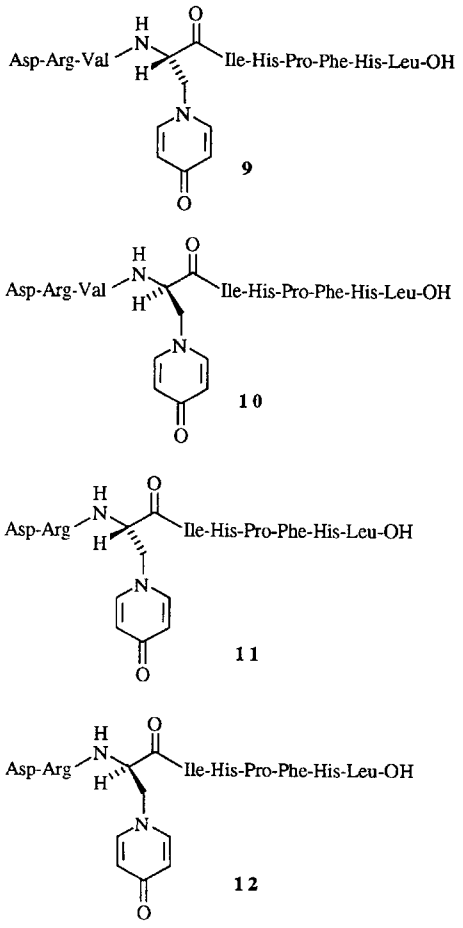
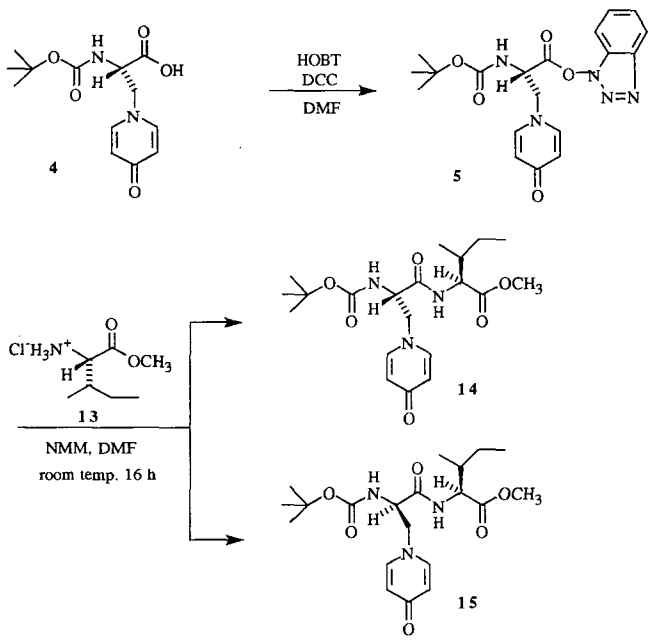


Fig. 2. Structures of crude peptide **9**

and other methods for structural identification were performed. These analyses indicated that the product of the retention time 8.350 min (peak 1, peak area 12.3%) was D-3-deoxymimosine-containing angiotensin I without a valine residue **12** (mw. 1197), which occurred from incomplete coupling during solid phase peptide synthesis (Fig. 1, 2). The products corresponding to the peak of retention time 9.545 min (peak 2, peak area 48.1%) were a mixture of unexpected side products, L-3-deoxymimosine-containing angiotensin I without a valine residue **11** (mw. 1197) and D-3-deoxymimosine-containing angiotensin I **10** (mw. 1296). The product of retention time 10.940 min (peak 3, peak area 37%) was identified as the desired product, L-3-deoxymimosine-containing angiotensin I **9** (mw. 1296). The identification and structural determination of the major products are described in the following section.

The crude peptide **9** was separated by semi-preparative HPLC on a Rainin column (Dynamax-300A, C-18, 12 μ, 10 × 250 mm) with a gradient elution of 20% to 33% aqueous acetonitrile in 0.1% TFA in 13 min at a flow rate of 4.5 mL/min. The fractions corresponding to each peak of the retention time 8.535 min, 9.885 min and 11.050 min were collected. The analytical HPLC tracings of each isolated product indicated the presence of one peak. The product corresponding to the peak of the retention time 8.535 min turned out to be peptide **12** and that of 11.050 min to be the pure, desired peptide **9**. However, the products corresponding to the peak of the retention time of 9.885 min was a mixture of two peptides, **10** and **11**. Considerable efforts were devoted to find a suitable solvent system to remove peptide **11**, but they were fruitless.



Scheme 2. Preparation of L-and D-3-deoxymimosine-containing dipeptide (**14** and **15**)

The yield of isolated peptide **9** was 20.2%; peptide **12** was 8.3% and a mixture of peptides **10** and **11** was 26.8%.

Identification and structural determination of peptides

To identify the major peptides and determine their sturctures, a low and high resolution FABMS analysis, HPLC analysis, protein sequencing analysis, synthesis of D-and L-3-deoxymimosine-containing dipeptides as model peptides, and enzymatic hydrolysis using carboxypeptidase A, carboxypeptidase Y and leucine aminopeptidase were used.

Two major products of the crude peptide mixture were initially suspected to be L-and D-3-deoxymimosine-containing angiotensin I **9** and **10**. Dipeptide, Boc-L-3-deoxymimosine-L-isoleucyl methyl ester (**14**) using Boc-L-3-deoxymimosine (**4**) and L-isoleucyl methyl ester hydrogen chloride (**13**) were synthesized to prove this assumption, to study formation of diastereomers, and to observe the formation and amount of diastereomeric dipeptides (Scheme 2).

The dipeptide was prepared under exactly the same conditions employed in the preparation of Boc-L-3-deoxymimosine-containing heptapeptide-Pam resin **8**. L-Isoleucyl methyl ester hydrogen chloride (**13**) was coupled with the active ester of Boc-L-3-deoxymimosine **5** in the presence of NMM in DMF at room temperature for 16 h to yield racemic Boc-L-and Boc-D-3-deoxymimosine-isoleucyl methyl ester (**14** and **15**). Active ester **5** was prepared by treating Boc-L-3-deoxymimosine (**4**) with 1-hydroxybenzotriazole (HOBT) in the presence of DCC in DMF for 30 min. After mixtures **14** and **15** were pre-purified by column chromatography, the ^{1}H NMR and FABMS analysis were performed. The FABMS analysis indicated that each component of the mixture had the same molecular weight of 409. In the ^{1}H NMR spectrum of the mixture, each chemical shift corresponding to the methyl

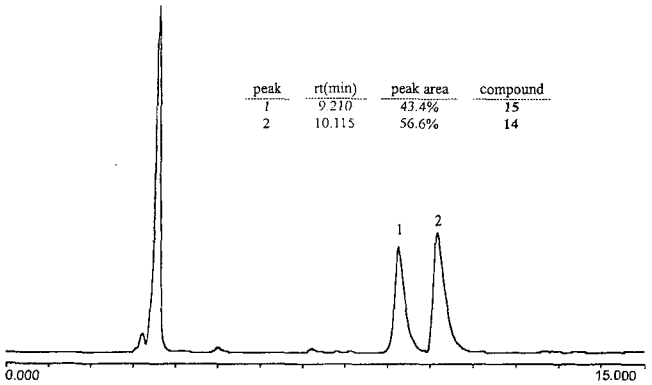
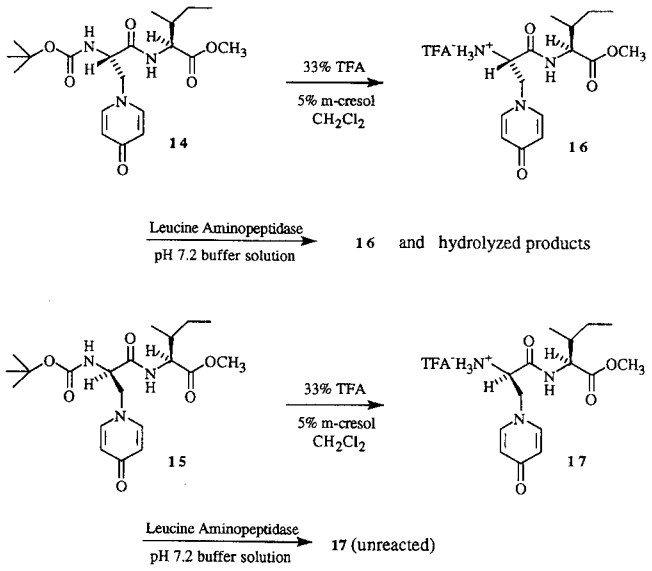


Fig. 3. HPLC analysis of a mixture of dipeptides **14** and **15**. Column; Vydac 218TP54, C-18, 10 μm, 4.6 × 250 × mm. A gradient elution of 40% to 80% solvent B in solvent A in 15 min at a flow rate of 1 mL/min was used (detector at 230 nm). For peak 1; peptide **15**, retention time 9.210 min, peak area 43.4%. For peak 2; peptide **14**, retention time 10.115 min, peak area 56.6%



Scheme 3. Preparation of **16** and **17** and their enzymatic hydrolysis using leucine aminopeptidase

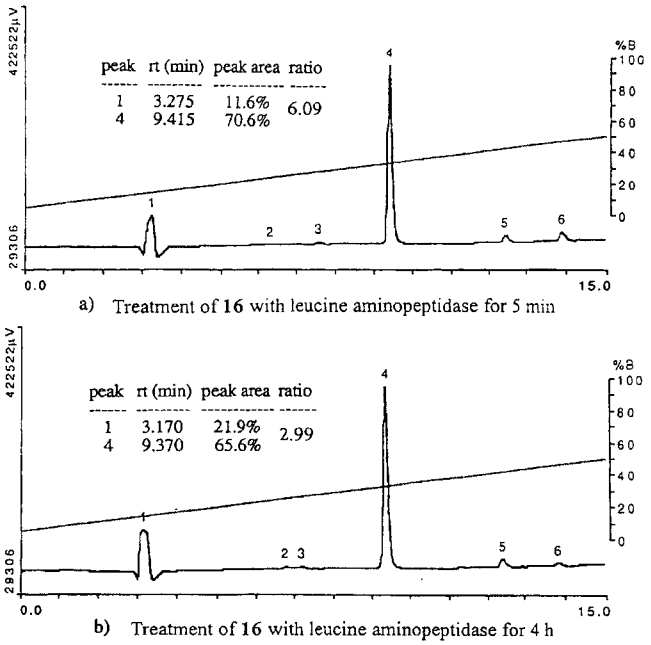


Fig. 4. HPLC analysis of products after treatment of dipeptide 16 with leucine aminopeptidase. a) after 5 min treatment, b) after 4 h treatment. Column; Vydac 218TP54, C-18, 5 μm, 4.6 × 250 mm. A gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min was used (detector at 230 nm)

groups on the methyl ester moieties of D,L and L,L form dipeptides was recorded at 3.649 ppm and 3.621 ppm in nearly 2:3 ratio of integration values, which indicated that the mixture was D,L and L,L form dipeptides. The **14** and **15** was analyzed by HPLC on a Vydac column with a gradient elution of 40% to 80% solvent B in solvent A in 15 min at a flow rate of 1 mL/min, where solvent A was water and solvent B was 66% aqueous acetonitrile. The analytical HPLC tracing (Fig. 3) indicated the presence of two major peaks having retention time of 9.210 min and 10.115 min in nearly 2:3 ratio. Separation of the mixture was performed on a semi-preparative column with a gradient elution of 40% to 80% solvent B in solvent A in 15 min at a flow rate of 4.5 mL/min where the solvent system was the same as above. Fractions corresponding to each peak of the retention times 9.680 min and 10.515 min were collected, and lyophilized. The ^{1}H NMR analysis of each product was performed to compare each diastereomer, which indicated that the two isolated products were diastereomeric isomers presumably D,L and L,L form dipeptides **14** and **15**.

To confirm the absolute configuration of each dipeptide for structural determination, dipeptides **14** and **15** were treated with 33% TFA containing 5% m-cresol in dichloromethane for 30 min to cleave the Boc-protecting group and to afford **16** and **17**, respectively (Scheme 3). Each product, **16** and **17**, was analyzed by HPLC on a Vydac column with a gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min. The analytical HPLC tracings indicated that each product corresponding to the peak of retention time times, 9.490 min and 9.230 min, was either dipeptide **16** and **17**.

To confirm the absolute configuration of each dipeptide, fractions corresponding to the peak having retention times of 9.490 min and 9.230 min were collected and lyophilized. Each dipeptide was treated with leucine aminopeptidase (Himmelhoch *et al.*, 1970, Hanson *et al.*, 1976), which acts as an exopeptidase specifically to cleave the *N*-terminus of L-form amino acids in proteins or peptides, in pH 7.2 buffer solution for 4 h. The products were analyzed by HPLC on a Vydac column with a gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min. The analytical HPLC tracings indicated poor enzymatic hydrolysis of both dipeptides, since considerable amounts of dipeptides **16** and **17** were detected in the HPLC tracings. An alternative method, which measured the ratio between the reference peak area and unhydrolyzed dipeptide peak area, was used to observe the process of hydrolysis. Fig. 4 and 5 shows the analytical HPLC tracings of the crude product of dipeptides **16** and **17**, formed by treatment with enzyme. In Fig. 4a), which shows the HPLC tracing after 5 min treatment of dipeptide **16** with aminopeptidase, the ratio between peak area of the retention time 3.275 (reference peak, 11.6%) and that of the retention time 9.415 (dipeptide 16, 70.6%) was 70.6/11.6=6.09. In Fig. 4b), which shows the HPLC tracing the 4hr treatment of dipeptide **16** with aminopeptidase, the ratio between peak area of the retention time 3.170 (reference peak, 21.9%) and that of the retention time 9.370 (dipeptide

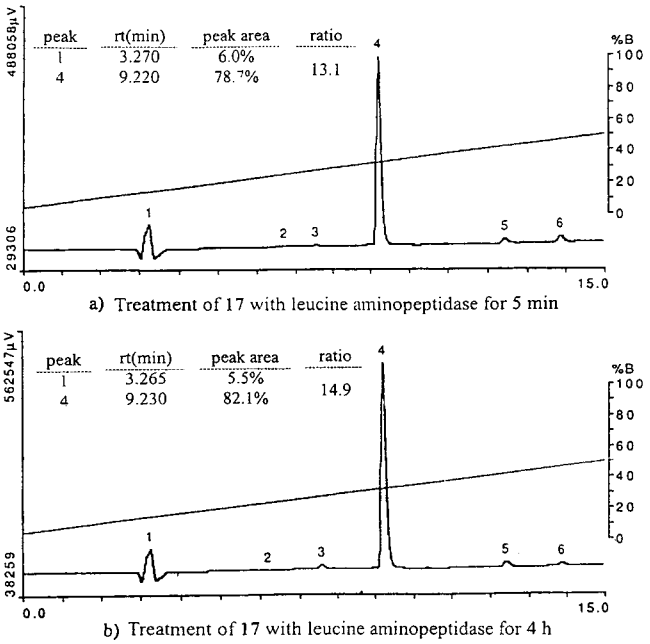
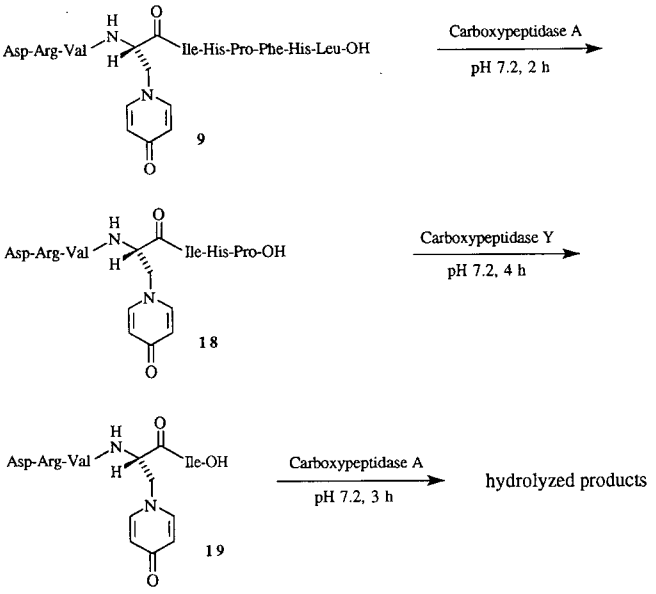


Fig. 5. HPLC analysis of products after treatment of dipeptide 17 with leucine aminopeptidase. a) after 5 min treatment, b) after 4 h treatment. Column; Vydac 218TP54, C-18, 5 μm, 4.6 × 50 mm. A gradient elution of 5% to 50% aqueous acetonitrile in 0.1% TFA in 15 min at a flow rate of 1 mL/min was used (detector at 230 nm)



Scheme 4. Enzymatic hydrolysis of peptide **9**

16, 65.6%) was 65.6/21.9=2.99. This result indicated significant decrease in the amount of dipeptide **16** by enzyme hydrolysis. In Fig. 5a), which shows the HPLC tracing after the 5 min treatment of dipeptide **17** with aminopeptidase, the ratio between the peak area of the retention time 3.270 (reference peak, 6.0%) and that of the retention time 9.220 (dipeptide **17**, 78.7%) was 78.7/6.0=.13.1. In Fig. 5b), which shows the HPLC tracing after a 4h-enzyme treatment of dipeptide **17** with aminopeptidase, the ratio between the peak area of the retention time 3.265 (reference peak, 5.5%) and that of retention time 9.230 (dipeptide **17**, 82.1%) was 82.1/5.5=14.9. This result indicated that dipeptide **17** was stable in an enzymatic hydrolysis. In conclusion, dipeptide 16 was identified as L,L form, and **17**, D,L form. This result indicated that the key intermediate, Boc-L-3-deoxymimosine (**4**) was a mixture of racemate, or racemization which occurred during peptide incorporation.

The protein sequencing analysis was performed on peptide **9** and mixture of peptides **10** and **11**. The retention times and patterns of the peaks of the fourth amino acid residues, which corresponded to the 3-deoxymimosine residues, in peptide **9** were almost the same as in mixture of peptides. Also, these results were consistent with those of the dipeptides **16** and **17**, indicating that the structural difference between peptides **9** and **10** was due to the D- and L-configurations of the 3-deoxymimosine residues.

Enzymatic hydrolysis of peptide **9** was performed in pH 7.2 buffer solution for 2 h using carboxypeptidase A (Folk et al, 1970) which specifically cleaves the C-terminus of L-form amino acids in proteins or peptides (Scheme 4). The product was analyzed by HPLC on a Vydac column with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The analytical HPLC tracing showed the presence of one major peak having a retention time of 16.280 min (Fig. 6b). FABMS analysis indicated that the major product was peptide **18** of molecular weight of 899. As expected, the enzymatic hydrolysis stopped on the proline residue. After collection of the fractions corresponding to peptide **18** lyophilization, enzymatic hydrolysis was performed in pH 7.2 buffer solution for 4 h using carboxypeptidase Y (Hayashi *et al.*, 1976), which was reported to have the ability to cleave proline residues. The product was analyzed by HPLC as described above. The analytical tracing indicated the presence of one major peak having retention time of 17.475 min (Fig. 6c). FABMS analysis indicated that the major product was peptide **19** having molecular weight of 665. After collection of the fractions corresponding to peptide **19** and lyophilization, enzymatic hydrolysis using carboxypeptidase A was performed in pH 7.2 buffer solution for 3 h. The product was analyzed by HPLC as described above. The analytical HPLC tracing showed that no observable, significant peak was observed (Fig. 6d), indicating that the peptide had been completely hydrolyzed by the enzyme. The compound corresponding to the peak of the retention time 5.095 min was found to be nonpeptidal according to FABMS analysis. These results confirmed that peptide **9** was L-3-deoxymimosine-containing angiotensin I.

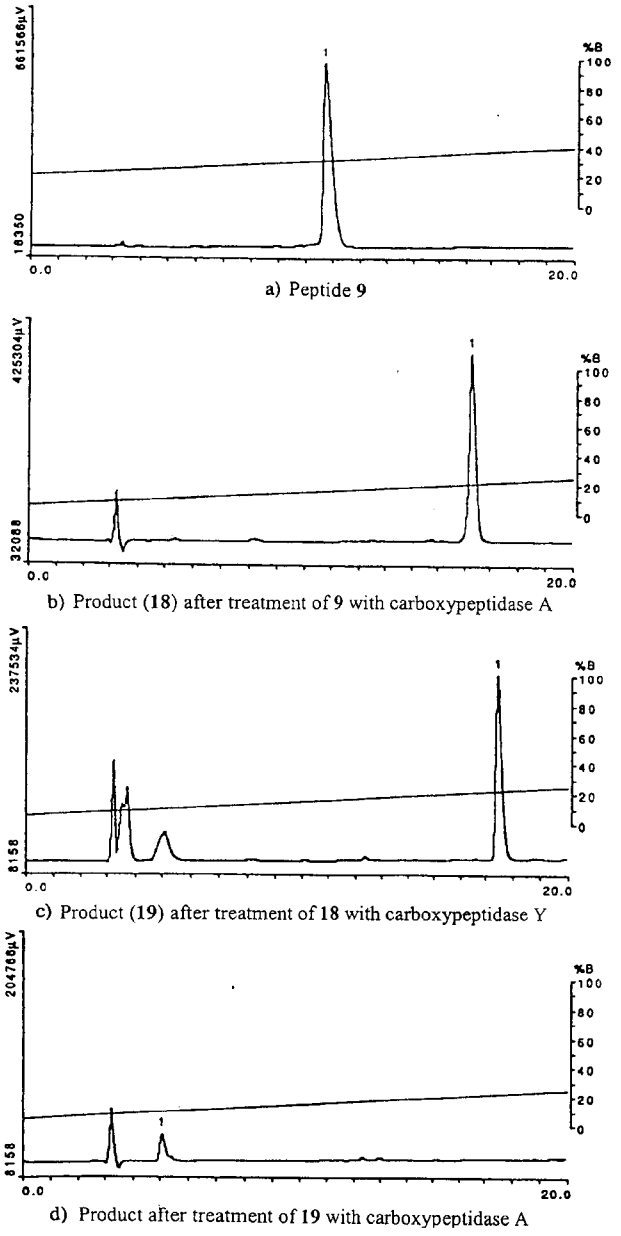
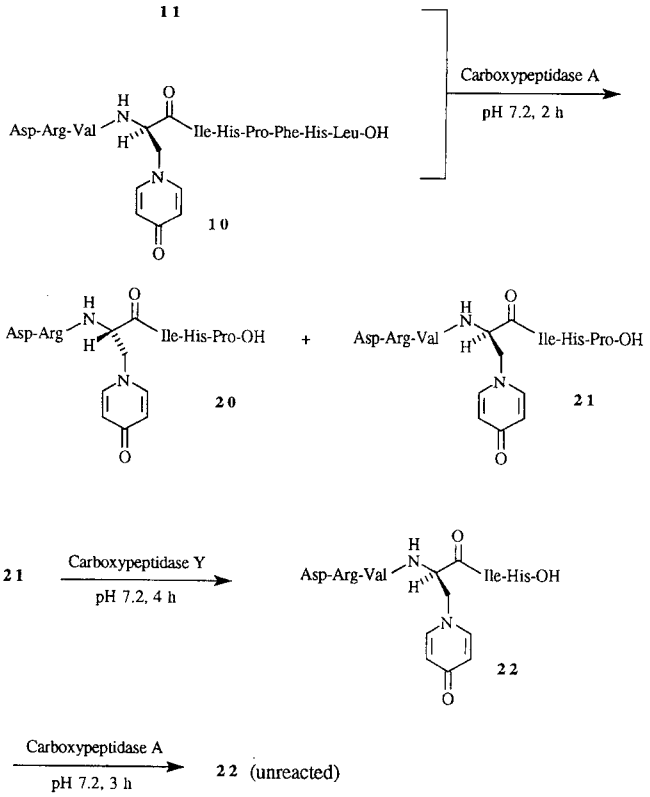


Fig. 6. HPLC analysis of enzymatic hydrolysis of peptide **9**. a) peptide **9** (gradient for HPLC was different from the others), b) product after treatment of peptide **9** with carboxypeptidase A (peptide **18**), c) product after treatment of peptide **18** with carboxypeptidase Y (peptide **19**), d) product after treatment of peptide **19** with carboxypeptidase A

Enzymatic hydrolysis of the mixture of peptides **10** and **11** was performed using carboxypeptidase A in pH 7.2 buffer solution for 2 h (Scheme 5). The products were analyzed by HPLC on a Vydac column with a gradient elution of 5% to 25% aqueous acetonitrile in 0.1% TFA in 20 min at a flow rate of 1 mL/min. The analytical HPLC tracing showed the presence of two major peaks having retention times of 13.175 min and 14.275 min in nearly 3:5 ratio (Fig. 7b). FABMS analysis of each product



Scheme 5. Enzymatic hydrolysis of peptide **10** and **11**

indicated that the product corresponding to the peak of the retention time 13.175 min was a L-form peptide **20** having molecular weight of 800 and that of retention time 14.275 min was peptide **21**. This result proved that the products corresponding to the peak of the retention time of 9.545 min in Fig. 1 were a mixture of peptides **10** and **11** and the ratio of peptide **10**:**9** were 2:3 by calculation. This result also indicated that the peak with the shorter retention time of 8.350 min in Fig. 1 was valine-deleted D-3-deoxymimosine-containing angiotensin I (**12**) and the peak with the retention time of 9.545 min was a mixture of D-3-deoxymimosine-containing angiotensin I (**10**) and valine-deleted L-3-deoxymimosine-containing angiotensin I (**11**). The method for identification will be described later. After separation of peptide **21** by HPLC on a semi-preparative column, enzymatic hydrolysis of **21** was performed in pH 7.2 buffer solution for 4 h using carboxypeptidase Y. The product was analyzed by HPLC as described above. The analytical HPLC tracing indicated the presence of one major peak having the retention time of 11.205 min (Fig. 7c). FABMS analysis indicated that the major product was peptide **22** with a molecular weight of 802, indicating enzymatic hydrolysis only on the proline residue. After collection of the fractions corresponding to peptide **22** and lyophilization, enzymatic hydrolysis using carboxypeptidase A was performed in pH 7.2 buffer solution for 3 h. The product was analyzed by

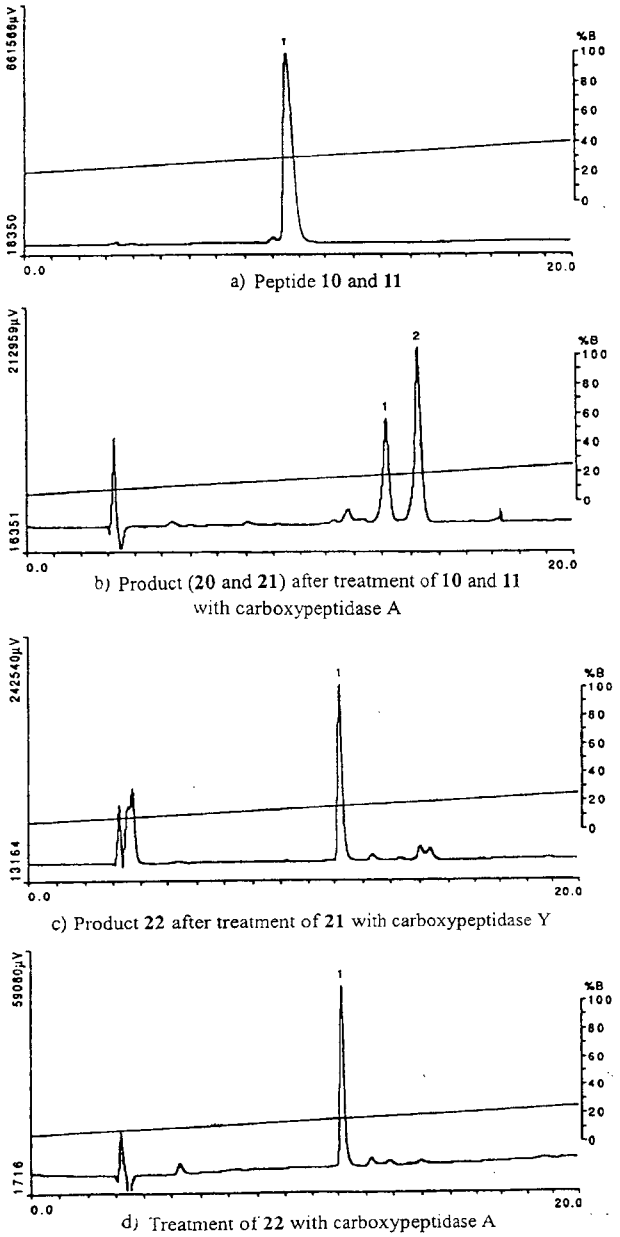


Fig. 7. HPLC analysis of enzymatic hydrolysis of peptide **10**. a) Mixture of peptides, **10** and **11** (gradient for HPLC was different from the others), b) Products after treatment of mixtures, **10** and **11**, with carboxypeptidase A (peptide **21** and **20**), c) Product after treatment of peptide **21** with carboxypeptidase Y (peptide **22**), d) Product after treatment of peptide **22** with carboxypeptidase A

HPLC as described above. The analytical HPLC tracing showed the presence of one major peak corresponding to peptide **22**; hence, no hydrolysis was achieved (Fig. 7d). Enzymatic hydrolysis was expected to stop on the D-3-deoxymimosine residue, directly proving that peptide **10** was D-3-deoxymimosine-containing angiotensin I. But the evidence in support of structure **10** is as follows: 1) FABMS analysis showed that peptides **9** and **10** had the same molecular weight of 1296. 2) Protein sequencing analysis of peptide peptides, **9** and **10**, indicated that the structural difference between the two peptides can be generated from D- and L-form configurations of the 3-deoxymimosine residue. 3) D-L form dipeptide **15** and L-L form dipeptide **14** were formed during preparation under the same conditions employed in the coupling of Boc-L-3-deoxymimosine (**4**) onto hexapeptide-Pam resin 7. 4) The ratio of D-L form **15** to L-L form dipeptide **14** was in nearly 2:3. Likewise, the ratio of peptide **10** to **9** was in nearly 2:3. 5) L-3-deoxymimosine-containing angiotensin I **9** was completely hydrolyzed by enzymatic reactions, but enzymatic hydrolysis of peptide **10** was incomplete. This indicated that peptide **10** was a D-3-deoxymimosine-containing angiotensin I.

The product corresponding to a peak having a shorter retention time (8.350 min) in Fig. 1 was identified as valine-deleted D-3-deoxymimosine-containing angiotensin I (**12**) and that corresponding to a peak having the retention time of 9.545 was determined as a mixture of D-3-deoxymimosine-containing-angiotensin I (**10**) and valine-deleted L-3-deoxymimosine-containing angiotensin I (**11**), revealed by a calculation of the ratio of the peak areas. In Fig. 7b the ratio of peak area of peptide **20** (peak corresponding to shorter retention time) to that of peptide **21** (peak corresponding to longer retention time) was 3:5, indicating that the ratio of peptide **11** (valine-deleted L-3-deoxymimosine-containing angiotensin I) to peptide **10** (D-3-deoxymimosine-containing angiotensin I) was also 3:5 in the peak of the retention time 9.545 min. This corresponded to a mixture of peptides, **10** and **11**, in Fig. 1, and the peak area was 48.1%. The peak area of peptide **11** was calculated to be 18.0%. The peak area of the retention time 8.350 corresponding to peptide **12** was 12.3%. Accordingly the ratio of the amount of peptide **12** to that of peptide **11** was 2:3, indicating that peptide **12** was valine-deleted D-3-deoxy-mimosine-containing angiotensin I and that peptide **11** was valine-deleted L-3-deoxymimosine-containing angiotensin I, since the ratio of D-form to L-form-3-deoxymimosine-containing peptide was consistently 2:3.

In conclusion, the structures of all the major peptides generated during preparation of L-3-deoxymimosine-containing angiotensin I (**9**) were identified and determined. Peptides **11** and **12** whose valine moiety was deleted in their peptide sequence were formed during peptide sequence incorporation because of incomplete coupling. D-3-deoxymimosine-containing peptides **10** and **12** may be generated during purification of Boc-L-3-deoxymimosine (**4**). A previous publication reported that L-3-deoxymimosine-containing angiotensin I (**9**) was the only major product. This method was followed in our study to prepare the peptide, the result should be similar. The only difference was in Boc-L-3-deoxymimosine purification (**4**). The previous report used C-18 reverse-phased column chromatography for purification, but a silica gel column chromatography

using methanol as a eluent was used in our study. Racemization may occur during the purification.

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