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Synthesis of Des-Pro^2-Bradykinin and Its Behavior in Chromatography[1)]

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Des-Pro^2-Bradykinin was synthesized by the classical solution method as a reference compound for a possible contaminant in synthetic bradykinins, and its behavior in various chromatographic analyses was examined. It showed almost the same *Rf* values as bradykinin under several different conditions in cellulose thin layer chromatography and in paper electrophoresis, but was clearly separable from bradykinin by reversed phase high performance liquid chromatography under specific conditions. Since des-Pro^2-bradykinin was found to have a potent bradykinin-potentiating activity, contamination by this material should be carefully avoided in bradykinin synthesis.

Keywords——des-Pro^2-bradykinin; bradykinin; synthesis; HPLC of bradykinin; ileum contraction; hypotension

Bradykinin is a hypotensive peptide with nine amino acid residues[2)] and has been synthesized by many workers.[3)] However, careful measurement of the biological potency of different products does not always give the same value.[4)] We considered that this discrepancy might be due to the presence of by-products which are difficult to remove from the desired product. Such by-products might contaminate synthetic bradykinin and modify its biological properties. Recently, Arakawa isolated a peptide from a tryptic digest of human sera, and showed that it had hypotensive activity in the rat; the peptide had an amino acid composition corresponding to that of des-proline-bradykinin, but the amino acid sequence has not been determined yet.[5)] Independently, we synthesized des-Pro^2-bradykinin as a possible contaminant of synthetic bradykinins and examined its behavior in chromatographic analyses. Its biological properties have also been studied in our laboratory and will be reported elsewhere.[6)]

Des-Pro^2-bradykinin was synthesized by a classical solution procedure as shown in Fig. 1. The fully protected octapeptide was deprotected by the HF/anisole procedure,[7)] and then purified by ion-exchange chromatography on CM-cellulose followed by column chromato-

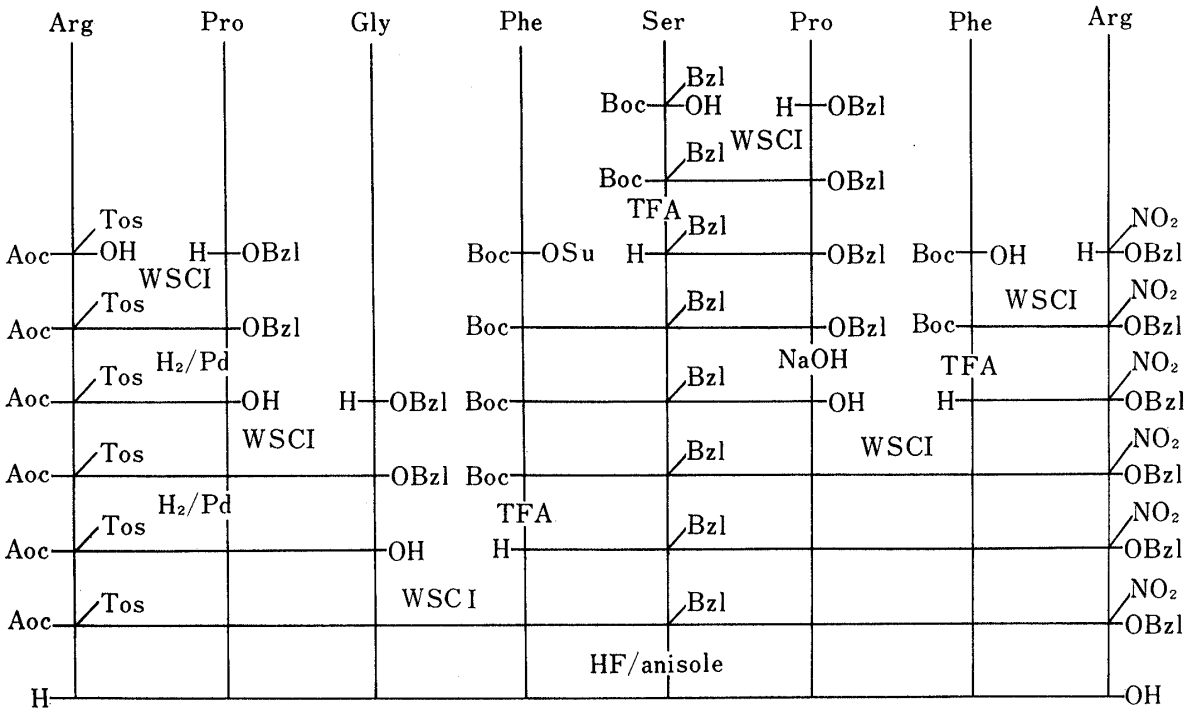

Fig. 1. Synthesis of Des-Pro²- Bradykinin

graphy on Sephadex LH-20. The final product was confirmed to be homogeneous by reversed phase high performance liquid chromatography (HPLC). Thin layer chromatography (TLC) of this material on Merck cellulose plates showed that its *Rf* value was almost the same as that of bradykinin; separation of this material from bradykinin was impossible in ordinary TLC systems such as BuOH: AcOH: water (4: 1: 5 v/v, upper phase) or BuOH: AcOH: pyridine: water (15: 3: 10: 12 v/v). Paper electrophoreses at pH 2, 4.5 and 6.5 also failed to separate this material from bradykinin. Thus, we concluded that if a small amount of this material contaminates synthetic bradykinin, its detection by TLC or by paper electrophoresis would be practically impossible even using highly efficient systems. Effective separation and detection of this material could be achieved only by HPLC, as shown in Fig. 2. Under the conditions used, a small amount of this material, as little as one-hundredth of the amount of bradykinin, was easily detectable from 1: 1: 1 mixture of bradykinin, kallidin, and Met-Lys-bradykinin. Thus, the conditions employed in this study may be useful not only for the micro determination of these compounds in biological mixtures, but also for the detection of des-Pro²-bradykinin in synthetic bradykinins.

As will be reported elsewhere, des-Pro²-bradykinin has no hypotensive activity in

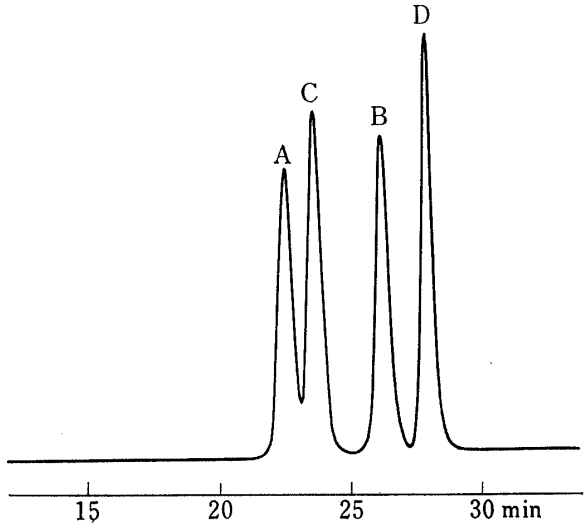

Fig. 2. Separation of Bradykinin Derivatives on HPLC: A, Des-Pro²-Bradykinin; B, Bradykinin; C, Kallidin; D, Met-Lys-Bradykinin

See the text for details of the apparatus and conditions Sample: 2.2 μg each/2 μl. Eluent: 10 mM H_3PO_4–K_2HPO_4 (pH 2.4) buffer containing Na_2SO_4 (50 mM), to which CH_3CN was added as a gradient.

The concentration change of CH_3CN was programed from 13% to 25% within 25 min after sample injection. Flow rate: 1 ml/min. Detection: UV-absorption measurement at 210 nm.

rats at dose levels of up to 1 mg/kg, but shows a rather strong bradykinin-potentiating activity in the guinea pig ileum contraction test and in the rat vasodepressor test.[6] The potentiating activity is almost twice that of natural potentiator B, which is a bradykinin potentiating peptide isolated from the venom of *Agkistrodon halys blomhoffii*.[8] Thus, detection and separation of des-Pro^2-bradykinin from synthetic bradykinin is essential when the synthesis of bradykinin is carried out by attachment of the N-terminal amino acid residues one by one in the final stage, as in the case of the ordinary solid-phase method.

Experimental

Materials and Methods——Boc- or Aoc-amino acids and reagents for peptide synthesis such as HOBt, WSCI and TFA, together with synthetic bradykinin, kallidin and Met-Lys-bradykinin, were obtained from the Peptide Institute, Inc., Osaka. Merck precoated plates, Art 5552, were used for thin layer chromatography. Rf_1 values were measured with a solvent system of *n*-BuOH: AcOH: pyridine: water (15: 3: 10: 12, v/v), and Rf_2 values with *n*-BuOH: AcOH: water (4: 1: 5, v/v, upper phase). HPLC was performed on a Hitachi liquid chromatograph, model 638, which was coupled to a Hitachi multiwavelength UV-monitor, model 635-0900, and a Hitachi chart recorder, model 056. The stainless steel column (4 mm × 150 mm) was slurrypacked with Nucleosil $5C_{18}$ (Machery-Nagel) following the procedure recommended by Hitachi. All runs were performed at ambient temperature. Melting points were measured by the capillary method and are uncorrected. A Daiflon HF-reaction apparatus (Protein Research Foundation) was used for deprotection of the final protected octapeptides.

Boc-Ser(Bzl)-Pro-OBzl (I)——A solution of Boc-Ser(Bzl)-OH[9] (5.9 g) and H-Pro-OBzl·HCl[10] (5.32 g) in CH_2Cl_2 was treated with WSCI (4.0 ml) at −10°C, and the mixture was allowed to react overnight at room temperature. Then, the solvent was removed by evaporation under reduced pressure, and the residue was dissolved in AcOEt. The solution was washed successively with 1 N HCl, 5% $NaHCO_3$ and water. After being dried over $MgSO_4$, the solution was concentrated under reduced pressure to obtain the product as a syrupy material.

Boc-Phe-Ser(Bzl)-Pro-OH (II)——All of the syrupy product I obtained above was treated with TFA (30 ml) at 0°C for 10 min and then at room temperature for 40 min. Excess TFA was removed by evaporation *in vacuo*, the residue was dissolved in 20 ml of DMF, and the solution was carefully neutralized with TEA (3.6 ml). Boc-Phe-OSu[11] (6.5 g) was added to the neutralized solution, and the whole mixture was allowed to react for 2 d at room temperature. Excess AcOEt was added, and the solution was washed successively with 1 N HCl, 5% $NaHCO_3$ and water, dried over $MgSO_4$, and concentrated to a syrup under reduced pressure. The residual syrup was dissolved in methanol, and the solution was treated with 1 N NaOH (20 ml) at room temperature for 3 h, then neutralized to pH 2 with 1 N HCl. The solvent was removed by evaporation, the product was extracted with AcOEt, and the organic layer was washed with water. The washed solution was dried over Na_2SO_4, and concentrated to a residue, which was recrystallized from AcOEt and *n*-hexane; yield 7.2 g (75.0%), mp 168—170°C, $[\alpha]_D^{18}$ −40.4° (c=1, DMF). *Anal.* Calcd for $C_{29}H_{37}N_3O_7 \cdot H_2O$: C, 62.35; H, 6.84; N, 7.53. Found: C, 62.46; H, 7.05; N, 7.54.

Boc-Phe-Ser(Bzl)-Pro-Phe-Arg(NO_2)-OBzl (III)——Boc-Phe-Arg(NO_2)-OBzl[12] (5.56 g) was treated with TFA (30 ml) at 0°C for 10 min and then at room temperature for 40 min. Excess TFA was removed by evaporation, the residue was triturated with 5.5 N HCl in dioxane (2 ml) and ether, and the resulting powder was dried over NaOH pellets *in vacuo*. The dried powder was dissolved in DMF (20 ml) together with II (5.4 g) and HOBt (1.35 g), and the solution was treated with WSCI (1.8 ml) at −10°C. The whole mixture was allowed to react at room temperature for 13 h. Water was added to the mixture to precipitate the product, which was then collected by filtration and recrystallized from AcOEt and *n*-hexane; yield 7.96 g (81.5%), mp 166—170°C, $[\alpha]_D^{25}$ −42.4° (c=1, DMF). *Anal.* Calcd for $C_{51}H_{63}N_9O_{11} \cdot H_2O$: C, 61.37; H, 6.34; N, 12.59. Found: C, 61.49; H, 6.58; N, 12.66.

Aoc-Arg(Tos)-Pro-OBzl (IV)——This material was prepared from Aoc-Arg(Tos)-OH[13] (8.84 g) and H-Pro-OBzl·HCl[10] (5.32 g) using WSCI (4.0 ml) as described for the preparation of I. The product was recrystallized from MeOH and ether; yield 8.98 g (71.4%), mp 166—168°C, $[\alpha]_D^{25}$ −34.6° (c=1, DMF). *Anal.* Calcd for $C_{31}H_{43}N_5O_7S \cdot 1/2H_2O$: C, 58.28; H, 6.94; N, 10.96. Found: C, 58.18; H, 6.90; N, 10.96.

Aoc-Arg(Tos)-Pro-OH (V)——Hydrogen was bubbled through a suspension of 5% palladium-charcoal (5 g) in a solution of IV in methanol (100 ml) at room temperature for 4 h. After the catalyst has been removed by filtration, the solvent was removed by evaporation and the residue was recrystallized from AcOEt and *n*-hexane; yield 5.22 g (96.8%), mp 84—95°C, $[\alpha]_D^{25}$ −27.9° (c=1, DMF). *Anal.* Calcd for $C_{24}H_{37}N_5O_7S \cdot 1/4$ Hexane: C, 54.58; H, 7.27; N, 12.48. Found: C, 54.38; H, 7.47; N, 12.24.

Aoc-Arg(Tos)-Pro-Gly-OH (VI)——A solution of V (2.16 g), H-Gly-OBzl-TosH[14] (1.48 g) and HOBt (0.594 g) in CH_2Cl_2 (40 ml) was treated with WSCI (0.81 ml) at room temperature for 13 h. The solvent was replaced with AcOEt, and the solution was washed successively with 1 N HCl, 5% $NaHCO_3$ and water, then dried over $MgSO_4$. The dried solution was concentrated to a residue, which was dissolved in MeOH

(30 ml). The MeOH solution was subjected to catalytic hydrogenolysis as in the case of V. The product was crystallized from $CHCl_3$ and ether; yield 1.83 g (76.9%), mp 115—121°C, $[\alpha]_D^{25}$ −34.4° (c=1, DMF). *Anal.* Calcd for $C_{26}H_{40}N_6O_8 \cdot 3/2H_2O$: C, 50.25; H, 6.79; N, 13.42. Found: C, 50.07; H, 6.95; N, 13.48.

Aoc-Arg(Tos)-Pro-Gly-Phe-Ser(Bzl)-Pro-Phe-Arg(NO_2)-OBzl (VII)——Compound III (0.98 mg) was treated with TFA (10 ml) as in the case of the synthesis of III. The product was then triturated with 5.5 N HCl in dioxane and ether. The resulting powder was dried over NaOH pellets, and then dissolved in DMF (10 ml) together with HOBt (162 mg) and compound VI (715 mg). The DMF solution was treated with WSCI (0.22 ml) for 13 h at room temperature. The product that precipitated upon addition of water was recrystallized from $CHCl_3$ and ether; yield 1.1 g (75.9%), mp 129—135°C, $[\alpha]_D^{25}$ −33.6° (c=1, DMF). *Anal.* Calcd for $C_{72}H_{93}N_{15}O_{16}S \cdot 2/5H_2O$: C, 57.29; H, 6.41; N, 13.64. Found: C, 57.58; H, 6.58; N, 13.99.

Des-Pro^2-Bradykinin——Compound VII (300 mg) was put in an HF reaction cylinder together with anisole (0.5 ml), then anhydrous HF (10 ml) was introduced into the cylinder at −70°C using a HF reaction apparatus, and the mixture was stirred for one h at 0°C. Excess HF was removed under a vacuum, the residue was extracted with 0.5 M AcOH (15 ml), and the extract was washed with ether. The washed solution was passed through a column of Dowex 1×2 (AcO^- form) and subjected to lyophilization. The resulting product was dissolved in a minimum amount of water, then charged onto a column of CM-cellulose (2.2 cm × 48 cm), which was eluted with a linear gradient of ammonium acetate buffer (pH 4.8) from 0.1 M to 0.6 M. Fractions containing the major peak (fractions 20 to 26) were collected and lyophilized. The product was further purified by gel filtration on a column of Sephadex LH-20 (2.8 cm × 109 cm) using 0.1 M AcOH as the eluent. The product at the major symmetrical peak was lyophilized to obtain a fluffy powder; yield 120 mg (59.4%), Rf_1 0.54, Rf_2 0.15, $[\alpha]_D^{25}$ −58.4° (c=0.5, 1% AcOH). *Anal.* Calcd for $C_{45}H_{66}N_{14}O_{10} \cdot 2AcOH \cdot 5H_2O$: C, 50.15; H, 7.22; N, 16.72. Found: C, 50.31; H, 7.53; N, 17.08. Amino acid ratios in an acid hydrolyzate; Arg 2.00 (2), Ser 0.89 (1), Pro 1.89 (2), Gly 1.00 (1), Phe 2.00 (2).

References and Notes

1) The following abbreviations are used in addition to those recommended by IUPAC-IUB: WSCI=water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=dimethylformamide, HOBt=1-hydroxybenzotriazole, OSu=N-hydroxysuccinimide ester, Aoc=*tert*-amyloxycarbonyl.
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