Synthesis of Isoleucine[5]-Tyrosine[8]-Angiotensin II and Isoleucine[5]-Hydroxyproline[7]-Angiotensin II*

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ABSTRACT: Isoleucine[5]-tyrosine[8]-angiotensin II and isoleucine[5]-hydroxyproline[7]-angiotensin II were synthesized by azide condensation of three dipeptides to give the protected C-terminal hexapeptides. The ocatapeptides were obtained by successive mixed anhydride condensations of the final two amino acids. These peptides were homogenous by paper chromatography, and gave correct amino acid composition and C, H, and N analysis.

Isoleucine[5]-tyrosine[8]-angiotensin II possesses 83% and isoleucine[5]-hydroxyproline[7]-angiotensin II 7% of the pressor activity of isoleucine[5]-angiotensin II.

Several analogs of isoleucine[5]- or valine[5]-angiotensin II [asp-arg-val-tyr(ileu or val)his-pro-phe-OH][1] have been synthesized and biological activities determined to study the correlation of amino acid structure with biological activity (Page and Bumpus, 1961; Schwyzer and Turrian, 1960). This work has revealed that the structure of the amino acids in positions 4 and 6–8 can drastically affect biological activity. In contrast, the biological activity appears much less dependent on the structure of amino acids in positions 1–3 and 5. To examine the influence of structure of amino acids 7 and 8 of this peptide on biological activity in more detail, isoleucine[5]-hydroxyproline[7]- and isoleucine[5]-tyrosine[8]-angiotensin II has been prepared. Each of these analogs has a hydroxyl group added to the structure of the naturally occurring peptide.

Results and Discussion

The synthetic route used in this work was the same for both octapeptides utilizing the proper C-terminal dipeptide as starting material. Thus, carbobenzoxy-isoleucylhistidine hydrazide was coupled by the azide procedure with either hydroxyprolylphenylalanine *p*-nitrobenzyl ester or prolyltyrosine methyl ester to yield the tetrapeptides. The hexapeptides were obtained by coupling carbobenzoxyvalyltyrosine hydrazide by the azide procedure to esters of the tetrapeptides. Carbobenzoxynitroarginine was coupled to the hexapeptide esters using the mixed anhydride procedure to yield the protected heptapeptides. Similarly, the mixed anhydride procedure was used to couple carbobenzoxy-β-benzyl-aspartic acid to the heptapeptide esters.

Isoleucine[5]-hydroxyproline[7]-angiotensin II was obtained from the protected octapeptide by catalytic reduction followed by chromatography on carboxymethyl cellulose using an acetic acid gradient elution technique. The final product was homogeneous by paper chromatography and gave the correct amino acid ratios on acid hydrolysis and the correct C, H, and N analyses. Crude leucine aminopeptidase from kidney, which probably contained prolidase and prolinase (Schwarz and Bumpus, 1959), released only the first five amino acids from the N-terminal end of isoleucine[5]-hydroxyproline[7]-angiotensin II even after prolonged incubation (96 hr). Isoleucine[5]-angiotensin II and isoleucine[5]-tyrosine[8]-angiotensin II were readily degraded to the eight component amino acids by this enzyme preparation. Hog kidney prolidase hydrolyzes glycylproline faster than glycylhydroxyproline or glycylmethoxyproline (Adams *et al.*, 1954). The low affinity between the enzyme and the latter two substrates may be due to steric factors rather than a difference in peptide bond stability. Similarly, the complete resistance of the histidyl-hydroxyproline bond toward hydrolysis is likely due to steric factors and may be related to an interaction between the histidine imidazole and the hydroxyl group of hydroxyproline residue. Racemization of histidine in the histidyl-hydroxyproline bond probably did not occur since the same synthetic procedures were used to prepare isoleucine[5]-tyrosine[8]-angiotensin II and the histidyl-proline bond of this peptide is split by our enzyme preparation. Carboxypeptidase readily splits phenylalanine from the C-terminal end of isoleucine[5]-hydroxyproline[7]-angiotensin II.

Further evidence for a difference between the histidyl-hydroxyproline bond and the histidyl-proline bond was obtained from synthetic studies. When carbobenzoxy-histidylprolylphenylalanine *p*-nitrobenzyl ester was treated with HBr in acetic acid, the expected tripeptide dihydrobromide was readily obtained (Schwarz and Arakawa, 1959). However, similar treatment of carbobenzoxyhistidylhydroxyprolylphenylalanine methyl

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[1] All amino acids are the L isomer.

or *p*-nitrobenzyl ester yielded a mixture of at least four compounds as observed by paper chromatography, and the desired tripeptide dihydrobromide could not be isolated. Under more drastic conditions (Mazur and Schlatter, 1963), histidylprolylphenylalanine was converted to the diketopiperazine of histidylproline with cleavage of phenylalanine.

Isoleucine[5]-tyrosine[8]-angiotensin II was obtained from the blocked octapeptide by catalytic hydrogenation followed by saponification. The peptide was purified by chromatography on carboxymethyl cellulose. The final product was homogeneous on paper chromatography, gave the correct amino acid ratios following acid hydrolysis, and gave correct C, H, and N analysis. This peptide was completely degraded to component amino acids by crude kidney leucine aminopeptidase, but at a slower rate than angiotensin II.

The biological activity of these peptides was determined by pressor assay in the vagotomized, ganglion-blocked rat (Pickens *et al.*, 1965). The tyrosine[8] analog has only slightly less pressor activity than angiotensin II while the hydroxyproline[7] analog has only 7% of the angiotensin activity. Recently asparagine[1]-valine[5]-tyrosine[8]-angiotensin II was reported to have 10% of the pressor activity of the natural peptide (Schröder and Hempel, 1965). Thus, the hydroxyl group in position 7 exerts a marked influence on the biological as well as the chemical properties of the peptide while the hydroxyl group in position 8 has very little effect. A more detailed biological study of these peptides will be presented elsewhere.

Experimental Section[2]

Hydroxyprolylphenylalanine p-Nitrobenzyl Ester Hydrobromide. Hydroxyproline (10.0 g, 0.076 mole) was carbobenzoxylated as previously described (Patchett and Witkop, 1957). The product (19.0 g), however, was an oil and could not be crystallized. It was dissolved in 30 ml of anhydrous tetrahydrofuran with 16.0 ml (0.076 mole) of tri-*n*-butylamine and cooled to −10°. The mixture was stirred with 7.2 ml (0.076 mole) of ethyl chloroformate for 10 min and then phenylalanine *p*-nitrobenzyl ester hydrobromide (26.0 g, 0.0683 mole) dissolved in tetrahydrofuran (130 ml) and dimethylformamide (100 ml) containing 14.8 ml (0.07 mole) of tri-*n*-butylamine was added. This mixture was stirred for 30 min at −5° and 2.5 hr at room temperature. Ethyl acetate and water were then added, shaken, and the two phases separated. The aqueous phase was extracted twice with ethyl acetate. The combined organic phase was washed successively with 1 N hydrochloric acid, water, saturated aqueous solution of sodium bicarbonate, and water. Then it was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo* to yield 38.5 g of a pale yellow gummy substance which did not solidify easily. It was dissolved in 75 ml of anhydrous acetic acid and added to 75 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 50 min at room temperature, anhydrous ether (1 l.) was added and the product precipitated as a gummy solid, which solidified on cooling for 1 hr at 2°. The solid was washed several times with anhydrous ether, dried over sodium hydroxide *in vacuo*, and crystallized from hot ethanol to yield 26.0 g (77% based on phenylalanine *p*-nitrobenzyl ester hydrobromide) of product, mp 202–204°. An analytical sample obtained after three recrystallizations from the same solvent had mp 206–208°; $[\alpha]^{23}D$ −25.3° (*c* 1.3, dimethylformamide) R_{FBAW} 0.8; BAW = 1-butanol–acetic acid–water.

Anal. Calcd for $C_{21}H_{23}N_3O_6 \cdot HBr$: C, 51.02; H, 4.89; N, 8.50; Br, 16.17. Found: C, 51.12; H, 5.18; N, 8.63; Br, 16.06.

Carbobenzoxyisoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester. Carbobenzoxyisoleucylhistidine hydrazide (4.99 g, 0.012 mole) was dissolved in 18 ml of 2 N hydrochloric acid and 15 ml of ethyl acetate, cooled to 0°, and added to 6.5 ml of cold (0°) 2 M sodium nitrite solution. After 5 min, cold 50% potassium carbonate (25 ml) was added, followed by 100 ml of cold ethyl acetate. The two phases were separated, and the aqueous phase was extracted twice with 100-ml portions of ethyl acetate. The combined ethyl acetate extract was washed twice with 100-ml portions of water and dried over anhydrous sodium sulfate. This ethyl acetate solution was added to a cold (0°) solution of hydroxyprolylphenylalanine *p*-nitrobenzyl ester hydrobromide (5.4 g, 0.011 M) in 30 ml of dimethyl formamide containing 2.33 ml (0.011 M) of tri-*n*-butylamine. The mixture was stirred overnight (16 hr) at 2° and for 4 hr at room temperature. It was then washed with 1 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, water, and dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue, after one precipitation from ethyl acetate by ether, yielded 6.1 g of product which gave a slight yellow coloration with ninhydrin. It was dissolved in 250 ml of ethyl acetate and washed again with 1 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water, and dried over anhydrous sodium sulfate, and the solvent was removed. The peptide was then precipitated once from ethyl acetate with ether to yield 5.5 g (62.7%) of pale yellow solid, mp 102–106°, R_{FBAW} 0.9. An analytical sample purified from the same solvent mixture had mp 108–110°, $[\alpha]^{23}D$ −47.8° (*c* 0.9; ethanol).

Anal. Calcd for $C_{41}H_{47}N_7O_{10}$: C, 61.72; H, 5.94; N, 12.29. Found: C, 61.90; H, 6.13; N, 12.49.

Isoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester Dihydrobromide. To carbobenzoxyisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester (3.4 g, 4.25 mmoles) in 15 ml of anhydrous

[2] Melting points were taken on a Leitz melting point apparatus and are not corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Paper chromatograms were developed using 1-butanol–acetic acid–water (4:1:5) and results are abbreviated as $R_{F\ BAW}$.

acetic acid[3] was added 10 ml of 4 N hydrogen bromide in anhydrous acetic acid. The mixture was allowed to stand at room temperature for 50 min. The dihydrobromide was precipitated by the addition of anhydrous ether and purified by precipitation from absolute ethanol with absolute ether; yield, 3.3 g (94.3%), mp 164–166°. A sample was precipitated several times using the same solvent mixture for analysis, mp 169–171°, $[\alpha]^{23}D$ +30.8° (*c* 1.3, dimethylformamide), R_{FBAW} 0.65.

Anal. Calcd for $C_{33}H_{41}N_7O_8$: C, 48.01; H, 5.25; N, 11.88; Br, 19.36. Found: C, 47.75; H, 5.45; N, 11.71; Br, 19.20.

Carbobenzoxyvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester. Carbobenzoxyvalyltyrosine hydrazide (2.14 g, 0.005 mole) dissolved in a mixture of 20 ml of acetic acid, 15 ml of 5 N hydrochloric acid, and 35 ml of ethyl acetate was stirred with 2.5 ml of 2 M sodium nitrite solution at 0°. After 5 min, ethyl acetate (150 ml) and water (100 ml) were added, the two phases separated, and the aqueous phase extracted twice with ethyl acetate. The combined organic phase was washed at 0° once with water, several times with saturated aqueous sodium bicarbonate solution, and twice with water, and was dried over anhydrous sodium sulfate. The cold solution was added to a cold (0°) solution of isoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester dihydrobromide (3.3 g, 0.004 mole) in 20 ml of dimethylformamide containing 1.7 ml (0.008 mole) of tri-*n*-butylamine. The mixture was stirred for 16 hr at 2° and 3 hr at room temperature. Then it was washed successively with 2 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the residue twice precipitated from methanol with ether to yield 3.9 g (92%) of product, mp 160–164°. An analytical sample prepared by precipitation using the same solvent mixture had mp 162–166°, $[\alpha]^{23}D$ −21.0° (*c* 1.19, dimethylformamide), R_{FBAW} 0.9.

Anal. Calcd for $C_{55}H_{65}O_{13}N_9$: C, 62.30; H, 6.19; N, 11.88. Found: C, 61.92; H, 6.07; N, 11.59.

Valyltyrosylisoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester Dihydrobromide. To carbobenzoxyvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester (3.18 g, 3 mmoles) in 20 ml of anhydrous acetic acid was added 11 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature, anhydrous ether was added to precipitate the product, which was purified by reprecipitation twice from absolute ethanol with absolute ether to yield 3.05 g (93.5%) of dihydrobromide, mp 174–179°, $[\alpha]^{23}D$ +12.8° (*c* 1.4, dimethylformamide), R_{FBAW} 0.80.

Anal. Calcd for $C_{47}H_{61}Br_2O_{11}N_9$: C, 51.88; H, 5.66; N, 11.58; Br, 14.69. Found: C, 51.94; H, 5.56; N, 11.25; Br, 14.70.

Nitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester. Carbobenzoxynitroarginine monohydrate (1.12 g, 3 mmoles) and tri-*n*-butylamine (0.64 ml, 3 mmoles) were dissolved in 5 ml of dimethylformamide, cooled to −10°, and stirred with 0.29 ml (3 mmoles) of ethyl chloroformate for 15 min. A solution of valyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester dihydrobromide (1.63 g, 1.5 mmoles) in 12 ml of dimethylformamide and 0.64 ml (3 mmoles) of tri-*n*-butylamine was cooled to 0° and added to the above solution. The reaction mixture was stirred for 30 min at −10° and 16 hr at room temperature and then poured into a mixture of ethyl acetate (200 ml) and water (100 ml). The two phases were separated and the aqueous phase was extracted twice with 50-ml portions of ethyl acetate. The combined ethyl acetate extract was washed successively with 2 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water (whenever a precipitate appeared during the washing methanol was added to dissolve it). After drying over anhydrous sodium sulfate, the solvent was removed and the residue was precipitated twice from hot methanol by cooling to yield 1.2 g (63.5%) of carbobenzoxynitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester, mp 199–205°, R_{FBAW} 0.9. This was dissolved in 10 ml of anhydrous acetic acid and added to 5 ml of 6 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature, anhydrous ether was added and the precipitated product was collected and purified twice by precipitation from absolute ethanol with anhydrous ether to yield 1.2 g (97.6%) of nitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester dihydrobromide; mp 175–181°, R_{FBAW} 0.75. The dihydrobromide (1.1 g) was dissolved in 15 ml of water at room temperature, cooled in ice, and neutralized with saturated aqueous sodium bicarbonate solution. The resulting precipitate was collected, washed with water, and crystallized from hot ethanol to yield 725 mg (75.1%) of product, mp 180–182°, $[\alpha]^{23}D$ −24.5° (*c* 1.26, dimethylformamide), R_{FBAW} 0.65.

Anal. Calcd for $C_{53}H_{70}N_{14}O_{14}$: C, 56.46; H, 6.27; N, 17.38. Found: C, 56.75; H, 6.34; N, 17.20.

Carbobenzoxy-β-benzylaspartylnitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine p-Nitrobenzyl Ester. Carbobenzoxy-β-benzylaspartic acid (358 mg, 1 mmole) and tri-*n*-butylamine (0.22 ml, 1 mmole) were dissolved in 3 ml of dimethylformamide, cooled to −10°, and then 0.1 ml (1 mmole) of ethyl chloroformate was added. After 15 min of stirring, there was added a cold (−10°) solution of nitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester (542 mg, 0.48 mmole) in 5 ml of dimethylformamide. The mixture was stirred for 30 min at −10° and 16 hr at room temperature. Ethyl acetate (200 ml) was then added and the organic phase was washed with 2 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water (when-

[3] Acetic acid was purified by adding 36 ml of acetic anhydride to 1300 ml of commercial glacial acetic acid and distilling from 28 g of Cr_2O_3. Acetic acid prepared in this way is designated as anhydrous in this paper.

ever a precipitate appeared during the washing, methanol was added to dissolve it). After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* and the residue was purified by precipitation twice from methanol with ether to yield 500 mg (71.1%) of a pale yellow amorphous solid, mp 176–181°, R_{FBAW} 0.9. Following the procedure described above and using 286 mg (0.8 mmole) of carbobenzoxy-β-benzylaspartic acid and 500 mg (0.39 mmole) of nitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester dihydrobromide, 422 mg (74%) of carbobenzoxy-β-benzylaspartylnitroarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester was obtained, mp 176–181°, $[\alpha]^{23}D$ −19.5° (*c* 1.4, dimethylformamide), R_{FBAW} 0.9.

Anal. Calcd for $C_{72}H_{87}N_{15}O_{19}\cdot H_2O$: C, 58.24; H, 6.05; N, 14.14. Found: C, 57.98; H, 6.10; N, 14.12.

Aspartylarginylvalyltyrosylisoleucylhistidylhydroxyprolylphenylalanine Monoacetate. The protected octapeptide (380 mg) was dissolved in a mixture of 25 ml of glacial acetic acid, 12 ml of methanol, and 6 ml of water and hydrogen was bubbled through the solution for 24 hr in presence of palladium black (100 mg). The catalyst was removed by filtration and the filtrate evaporated to dryness *in vacuo* at 40°. The residue was purified by precipitation from glacial acetic acid with anhydrous ether to yield 285 mg of product which on paper chromatography gave two spots, R_{FBAW} 0.43 and 0.2. The latter spot was positive to ninhydrin but not to Pauly's reagent. This was purified by chromatography on a 2.3 × 70 cm column of carboxymethyl cellulose (55 g). The column was prepared using 0.1 M acetic acid and the sample was applied to the column as a 0.1 M acetic acid solution. The column was developed with 700 ml of 0.1 M acetic acid and then with an acetic acid gradient. To obtain the gradient 500 ml of 0.1 M acetic acid was placed in a flask with good stirring and as it was removed by column flow, an equal volume of 4% acetic acid replaced it. Fractions of 15 ml were collected and from combined fractions numbered 94–141 was obtained by evaporation 205 mg (70.7%) of product, mp 243–248° dec, $[\alpha]^{23}D$ −60° (*c* 0.8, 1 N HOAc), R_{FBAW} 0.425, amino acid ratios in acid hydrolysate, $asp_{1.04}arg_{0.96}val_{1.08}tyr_{1.02}his_{0.97}(OH)pro_{0.96}phe_{1.12}$.

Anal. Calcd for $C_{53}H_{71}N_{13}O_{13}\cdot C_2H_4O_2$: C, 55.64; H, 6.74; N, 16.21. Found: C, 56.14; H, 6.76; N, 16.24.

The peptide possesses about 7% of the pressor activity of isoleucine[5]-angiotensin II. A crude kidney extract which readily hydrolyzed isoleucine[5]-angiotensin II to the component amino acids failed to hydrolyze this peptide completely. Only the five amino acids from the N-terminal end were released readily and even prolonged incubation (48 hr) did not lead to the rupture of the his(OH)–pro bond. On incubation with carboxypeptidase, phenylalanine from the C-terminal end was readily cleaved within 15 min. The incubations were carried out as described previously (Schwarz and Bumpus, 1959).

Carbobenzoxyhistidylhydroxyprolylphenylalanine Methyl Ester. Carbobenzoxyhistidine hydrazide (2.43 g, 8 mmoles) was dissolved in 12 ml of 2 N hydrochloric acid, cooled to 0°, and stirred with 4 ml of 2 M sodium nitrite solution for 5 min. A cold (0°) aqueous solution of 50% potassium carbonate (12 ml) was added and the liberated azide was extracted into ethyl acetate. The extract was washed with water, dried over anhydrous sodium sulfate, and filtered into a cold (0°) solution of hydroxyprolylphenylalanine methyl ester hydrobromide (1.87 g, 5 mmoles) in 18 ml of dimethylformamide and 1.06 ml (5 mmoles) of tri-*n*-butylamine. The mixture was stirred overnight (16 hr) at 2° and for 4 hr at room temperature. Then it was washed with saturated aqueous sodium bicarbonate solution and water. After drying over anhydrous sodium sulfate the solvent was removed *in vacuo* and the oily residue stirred with anhydrous ether to yield 1.86 g (66.2%) of product which was further purified by precipitation from ethyl acetate with anhydrous ether; mp 92–95°, $[\alpha]^{23}D$ −23.3° (*c* 1.2, ethanol), R_{FBAW} 0.85.

Anal. Calcd for $C_{29}H_{33}N_5O_7$: C, 61.08; H, 5.90; N, 12.43; Found: C, 61.25; H, 6.03; N, 12.13.

Treatment of Carbobenzoxyhistidylhydroxyprolylphenylalanine Methyl Ester with HBr–HOAc. To a solution of carbobenzoxyhistidylhydroxyprolylphenylalanine methyl ester (500 mg) in 6 ml of anhydrous acetic acid was added 5 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature the product was precipitated by the addition of anhydrous ether and then purified by precipitation from absolute ethanol with ether; yield, 490 mg. On paper chromatography four spots could be recognized: R_{FBAW} 0.1 (ninhydrin negative and Pauly positive), 0.4, 0.5, 0.55 (ninhydrin and Pauly positive).

Essentially similar results were obtained when carbobenzoxyhistidylhydroxyprolylphenylalanine *p*-nitrobenzyl ester was treated with hydrogen bromide in acetic acid.

Carbobenzoxyprolyltyrosine Methyl Ester. Carbobenzoxyproline (9.98 g, 0.04 mole) and 8.5 ml (0.04 mole) of tri-*n*-butylamine were dissolved in 50 ml of tetrahydrofuran, cooled to −10°, and stirred with 3.8 ml (0.04 mole) of ethyl chloroformate for 15 min. To this solution was added a cold (0°) suspension of 7.81 g (0.04 mole) of tyrosine methyl ester in 100 ml of tetrahydrofuran and the mixture stirred for 15 min at −10° and 2 hr at room temperature. Water and ethyl acetate were then added to obtain two phases. The aqueous phase was separated and extracted twice with ethyl acetate. The combined ethyl acetate extract was washed successively with 1 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo*. The residue was dissolved in a small amount of ethyl acetate and petroleum ether (bp 30–60°) added until a faint haze developed in the solution. On standing a solid separated, yield, 16.85 g (98.7%), mp 72–75°. A sample was crystallized from the same solvent mixture for analysis, mp 74–77°, $[\alpha]^{23}D$ −14.4° (*c* 1.7, dimethylformamide).

Anal. Calcd for $C_{23}H_{26}N_2O_6$: C, 64.77; H, 6.15; N, 6.57. Found: C, 64.88; H, 6.31; N, 6.43.

Prolyltyrosine Methyl Ester Hydrobromide. Carbobenzoxyprolyltyrosine methyl ester (16.63 g, 0.039 mole) was dissolved in 35 ml of anhydrous acetic acid and 32 ml of 4 N hydrogen bromide in anhydrous acetic acid was added. After 28 min at room temperature, anhydrous ether was added to precipitate the product which was crystallized twice from absolute methanol–anhydrous ether to yield 14.0 g (96.1%) of hydrobromide, mp 158–161°, $[\alpha]^{23}D$ −24.3° (*c* 1.5, methanol).

Anal. Calcd for $C_{15}H_{21}BrN_2O_4$: C, 48.26; H, 5.67; N, 7.51; Br, 21.41. Found: C, 48.31; H, 5.71; N, 7.40; Br, 21.20.

Carbobenzoxyisoleucylhistidylprolyltyrosine Methyl Ester. To a cold (2°) solution of carbobenzoxyisoleucylhistidine azide (0.022 M), prepared as described above, was added a cold (2°) solution of prolyltyrosine methyl ester hydrobromide (7.47 g, 0.02 mole) in 45 ml of dimethylformamide and 4.22 ml (0.02 mole) of tri-*n*-butylamine. The mixture was stirred for 16 hr at 2° and 5 hr at room temperature. Ethyl acetate and water were then added and the aqueous phase separated and extracted twice with ethyl acetate. The combined ethyl acetate extract was washed twice with saturated aqueous sodium bicarbonate solution, then with water, dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The residue was purified by precipitation from ethyl acetate with ether to yield 9.2 g (67.9%) of product, mp 194–197°. For analysis a sample was crystallized twice from small amounts of methanol to afford a pale yellow substance, mp 201.5–204.5°, $[\alpha]^{23}D$ −32.6° (*c* 1.3, dimethylformamide), R_{FBAW} 0.9.

Anal. Calcd for $C_{36}H_{44}N_6O_8$: C, 62.11; H, 6.56; N, 12.41. Found: C, 61.96; H, 6.34; N, 12.62.

Isoleucylhistidylprolyltyrosine Methyl Ester Dihydrobromide. To a solution of 8.12 g (0.012 mole) of carbobenzoxyisoleucylhistidylprolyltyrosine methyl ester in 20 ml of anhydrous acetic acid was added 20 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature, the product was precipitated by the addition of anhydrous ether and purified by precipitation from absolute ethanol with anhydrous ether; yield, 7.8 g (92.3%), mp 168–172°, $[\alpha]^{23}D$ +30.6° (*c* 1.0, dimethylformamide); R_{FBAW} 0.65.

Anal. Calcd for $C_{27}H_{40}Br_2N_6O_6$: C, 46.02; H, 5.73; N, 11.92; Br, 22.68. Found: C, 46.10; H, 6.01; N, 11.73; Br, 22.30.

Carbobenzoxyvalyltyrosylisoleucylhistidylprolyltyrosine Methyl Ester. To a solution of 4.23 g (0.006 mole) of isoleucylhistidylprolyltyrosine methyl ester dihydrobromide in 30 ml of dimethylformamide and 2.74 ml (0.013 mole) of tri-*n*-butylamine was added an ethyl acetate solution of carbobenzoxyvalyltyrosine azide (0.01 M) prepared as described above at 2°. The mixture was stirred for 16 hr at 2° and 3 hr at room temperature. Ethyl acetate was added and the solution was washed successively with water, 0.5 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water (whenever a precipitate appeared during the washing small amounts of methanol were added to dissolve it). After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* and the residue was purified by precipitation twice from ethanol with ether to yield 4.9 g (87%) of product, mp 164–168°, $[\alpha]^{23}D$ −25.0° (*c* 1.0, dimethylformamide), R_{FBAW} 0.90.

Anal. Calcd for $C_{49}H_{62}N_8O_{11}$: C, 62.66; H, 6.66; N, 11.92. Found: C, 62.41; H, 6.78; N, 12.10.

Valyltyrosylisoleucylhistidylprolyltyrosine Methyl Ester Dihydrobromide. Carbobenzoxyvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester (4.32 g, 0.0046 mole) was dissolved in 20 ml of anhydrous acetic acid and mixed with 15 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature, the product was precipitated by the addition of anhydrous ether and purified twice by precipitation from absolute ethanol with anhydrous ether; yield, 4.2 g (94.4%), mp 181–185°, $[\alpha]^{23}D$ +13.5° (*c* 1.3, dimethylformamide), R_{FBAW} 0.6.

Anal. Calcd for $C_{41}H_{58}Br_2N_8O_9$: C, 50.93; H, 6.05; N, 11.58; Br, 16.53. Found: C, 50.47; H, 6.21; N, 11.51; Br, 16.30.

Carbobenzoxynitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine Methyl Ester. A solution of carbobenzoxynitroarginine monohydrate (2.97 g, 8 mmoles) in 15 ml of dimethylformamide was mixed with 1.7 ml (8 mmoles) of tri-*n*-butylamine, cooled to −10°, and stirred for 15 min with 0.76 ml (8 mmoles) of ethyl chloroformate. A solution of valyltyrosylisoleucylhistidylprolyltyrosine methyl ester dihydrobromide (3.87 g, 4 mmoles) in 30 ml of dimethylformamide and 1.7 ml (8 mmoles) of tri-*n*-butylamine was cooled to 0° and added to the above solution. The mixture was stirred for 15 min at −10° and 16 hr at room temperature. Ethyl acetate was added and the solution was washed successively with 0.5 N hydrochloric acid, water, saturated aqueous sodium bicarbonate solution, and water. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo* and the residue was purified by twice precipitating from methanol with ether; yield, 2.6 g, mp 205–207°, R_{FBAW} 0.9. When the 0.5 N hydrochloric acid wash was neutralized with sodium bicarbonate more of the product separated and it was purified by precipitation from methanol with ether to afford 1.5 g of material; mp 203–205°, R_{FBAW} 0.9; total yield, 4.1 g (89.9%), $[\alpha]^{23}D$ −19.8° (*c* 1.2, dimethylformamide).

Anal. Calcd for $C_{55}H_{73}N_{13}O_{14}\cdot H_2O$: C, 57.02; H, 6.56; N, 15.71. Found: C, 57.23; H, 6.52; N, 16.14.

Nitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine Methyl Ester. A solution of carbobenzoxynitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester (950 mg) in 10 ml of anhydrous acetic acid was mixed with 6 ml of 4 N hydrogen bromide in anhydrous acetic acid. After 40 min at room temperature the product was precipitated by the addition of anhydrous ether and purified twice by precipitation from absolute ethanol with anhydrous ether; yield, 900 mg, mp 195–205°, R_{FBAW} 0.6. This was dissolved in 15 ml of water and the pH was brought to 8 by the addition of sodium bicarbonate. The precipitate was removed by filtration, washed with water, and purified

by precipitation twice from methanol with ether to yield 525 mg (62.5%) of product, mp 174–179°, $[\alpha]^{23}D$ −28.5° (*c* 1.0, dimethylformamide), R_{FBAW} 0.55.

Anal. Calcd for $C_{47}H_{67}N_{13}O_{12}$: C, 56.10; H, 6.72; N, 18.08. Found: C, 55.67; H, 6.65; N, 17.72.

Aspartylarginylvalyltyrosylisoleucylhistidylprolyltyrosine Monoacetate. Carbobenzoxy-β-benzylaspartic acid (715 ml, 2 mmoles) and 0.43 ml (2 mmoles) of tri-*n*-butylamine were dissolved in 6 ml of dimethylformamide, cooled to −10°, and stirred with 0.19 ml (2 mmoles) of ethyl chloroformate for 15 min. To this solution was added a cold (−10°) solution of nitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester (1.006 g, 1 mmole) in 15 ml of dimethylformamide. The mixture was stirred for 15 min at −10° and 16 hr at room temperature. Ethyl acetate was then added and the solution washed with water and 1 N hydrochloric acid. During the washing, some of the product precipitated and was removed by filtration. This was purified by precipitation from methanol with ether, yield 0.75 g, mp 172–176°, R_{FBAW} 0.9. The filtrate was washed with water, saturated aqueous sodium bicarbonate solution, and water. Then it was dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue was purified by precipitation twice from methanol with ether to yield 300 mg of additional product, mp 172–176°, R_{FBAW} 0.9; total yield of carbobenzoxy-β-benzylaspartylnitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester, 1.05 g (78%).

Following the procedure described above and using 895 mg (2.5 mmoles) of carbobenzoxy-β-benzylaspartic acid in 6 ml of dimethylformamide, 0.53 ml (2.5 mmoles) of tri-*n*-butylamine, 0.24 ml (2.5 mmoles) of ethyl chloroformate, and 1.46 g (1.25 mmoles) of nitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester dihydrobromide in 15 ml in dimethylformamide containing 0.53 ml (2.5 mmoles) of tri-*n*-butylamine, 1.45 g (86.3%) of carbobenzoxy-β-benzylaspartylnitroarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester was obtained, mp 172–176°, R_{FBAW} 0.90.

This protected octapeptide (1.0 g) was dissolved in a mixture of 40 ml of acetic acid, 20 ml of methanol, and 10 ml of water, and hydrogen was bubbled through the solution for 44 hr in presence of palladium black (170 mg). The catalyst was removed by filtration, the filtrate evaporated to dryness *in vacuo*, and the residue purified by precipitation from glacial acetic acid with anhydrous ether to yield 750 mg (93.7%) of aspartylarginylvalyltyrosylisoleucylhistidylprolyltyrosine methyl ester, mp 202–207°; on paper chromatography using BAW system one major spot at R_F 0.45 and two very minor spots at R_F 0.15 and 0.80 were observed. This methyl ester (600 mg) was dissolved in 10 ml of 0.93 N sodium hydroxide. After 75 min, 1 ml of acetic acid was added which caused the solution to become turbid. The precipitate was dissolved by adding more water and then chromatographed over 55 g of carboxymethyl cellulose. The column was packed in 0.01 M acetic acid and the material was applied as a solution in 0.01 M acetic acid. The column was then washed with 3.6 l. of 0.01 M acetic acid to remove sodium acetate present in the product. Then gradient elution was started as described above with 5% acetic acid flowing into 500 ml of 0.01 M acetic acid, and 16-ml fractions were collected. From fractions 63–150, 406 mg (65%) of aspartylarginylvalyltyrosylisoleucylhistidylprolyltyrosine monoacetate was obtained, mp 247–254° dec, R_{FBAW} 0.40, amino acid ratios in acid hydrolysate, $asp_{0.92}arg_{1.02}val_{1.00}tyr_{1.70}ileu_{0.90}his_{0.81}pro_{1.01}$, $[\alpha]^{23}D$ −58.2° (*c* 0.55, 5% acetic acid).

Anal. Calcd for $C_{50}H_{71}N_{13}O_{13}\cdot C_2H_4O_2$: C, 55.64; H, 6.74; N, 16.21. Found: C, 55.47; H, 7.05; N, 16.04.

The peptide was degraded to the component amino acids on incubation with a crude kidney extract within 20 hr and carboxypeptidase cleaved tyrosine from the C-terminal end in 19 min of incubation. The incubations were carried out as described previously (Schwarz and Bumpus, 1959). The peptide possesses 83% of the pressor activity of isoleucine^5-angiotensin II.

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