

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION, CLEVELAND 6, OHIO]

Synthesis of Isoleucyl<sup>5</sup>-alanyl<sup>7</sup>-angiotensin II<sup>1</sup>BY JUNG HWN SEU,<sup>2</sup> ROBERT R. SMEBY AND F. MERLIN BUMPUS

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The octapeptide isoleucyl<sup>5</sup>-alanyl<sup>7</sup>-angiotensin II has been synthesized in order to study further the significance of structure at the C-terminal end of angiotensin II for biological activity. This replacement of proline, in position 7 of angiotensin II, by alanine, greatly reduces the pressor activity of the peptide. This marked loss of activity, by merely removing two methylene groups of the proline ring, may be caused by changing the conformation at the C-terminus of the peptide.

Isoleucyl<sup>5</sup>-angiotensin II, the octapeptide L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, exhibits marked specificity of structure at the C-terminus for pressor and myotropic activities.<sup>3</sup> Removal of L-phenylalanine<sup>4</sup> or only the aromatic ring of this amino acid<sup>3,5</sup> destroys the biological activities of the peptide. Conversion of the C-terminal carboxyl group to an amide group causes some reduction in biological activities.<sup>6</sup> The phenolic ring of tyrosine<sup>7,8</sup> and the imidazole ring of histidine<sup>9</sup> also have been reported to be essential for the biological activities of the peptide.

Addition of urea to an aqueous solution of angiotensin II greatly reduces its myotropic action and also causes a marked reduction in the degree of order shown by the peptide as studied by optical rotatory dispersion.<sup>10</sup> From this it follows that the conformation of the peptide is an important factor for the myotropic activity.

A conformation recently suggested for angiotensin II,<sup>10</sup> based on the assumption it will form an  $\alpha$ -helix to the greatest extent possible, will explain all physical and biological data presently known for this peptide. In this conformation the groups essential for biological activity are all arranged in close proximity and on the same side of the molecule. Since rupture of the aliphatic ring of proline would change the structure at the C-terminus of the peptide and would alter the relative positions of these essential groups, we have replaced proline in angiotensin II with alanine by the preparation of isoleucyl<sup>5</sup>-alanyl<sup>7</sup>-angiotensin II.

Experimental<sup>11</sup>

**Carbobenzoxyl-L-alanyl-L-phenylalanine Methyl Ester (I).**—To a solution of 22.15 g. (0.1 mole) of carbobenzoxyl-L-alanine in 150 ml. of tetrahydrofuran was added 14 ml.

(0.1 mole) of triethylamine and the solution was cooled to  $-12^\circ$ ; then 9.8 ml. (0.1 mole) of ethyl chloroformate was added and the solution was stirred for 15 minutes at  $-12^\circ$ . A pre-cooled solution of 21.57 g. (0.1 mole) of L-phenylalanine methyl ester hydrochloride in 80 ml. of dimethylformamide containing 14 ml. of triethylamine was then added to the above solution. The mixture was stirred for 1.5 hr. at  $0^\circ$  and then overnight at  $4^\circ$ . The mixture was filtered and the filtrate concentrated to an oil under reduced pressure. The oily material was dissolved in 250 ml. of ethyl acetate, washed once with 30 ml. of water, 3 times with 20 ml. of 1 *N* hydrochloric acid, once with 30 ml. of water, 3 times with 30 ml. of saturated sodium carbonate and 2 times with 30 ml. of water, all at  $4^\circ$ . The ethyl acetate layer was shaken for 20 minutes with anhydrous sodium sulfate and evaporated to an oil after removal of the sodium sulfate by filtration. The oily product was dissolved in ethyl acetate then ether and pet. ether (b.p.  $30-60^\circ$ ) were added to give a final ratio of solvents of 2:1:1; yielding 32.0 g. of crystalline product which melted at  $98-102^\circ$ . This product was recrystallized from the same solvent mixture; yield 30.5 g. (79.9%); m.p.  $99-102^\circ$ ;  $[\alpha]^{25}_D -14.90^\circ$  (*c* 1, in methanol).

*Anal.* Calcd. for  $C_{21}H_{24}N_2O_5$ : C, 65.61; H, 6.29; N, 7.29. Found: C, 65.43; H, 6.29; N, 7.48.

**L-Alanyl-L-phenylalanine Methyl Ester Hydrobromide (II).**—Carbobenzoxyl-L-alanyl-L-phenylalanine methyl ester (9.54 g.) was dissolved in 12 ml. of anhydrous acetic acid, and 60 ml. of 4 *N* hydrogen bromide in anhydrous acetic acid was added. After 45 minutes at room temperature the mixture was evaporated to an oil *in vacuo*. The oily residue was dissolved in methanol, and ethyl acetate and pet. ether (b.p.  $30-60^\circ$ ) added to give a final ratio of solvents of 2:1:1. The crystalline product obtained above was recrystallized from the same solvent mixture; yield, 7.55 g. (91%) m.p.  $149-152^\circ$ . For analysis, a sample was recrystallized from the same solvents, dried *in vacuo* at  $78^\circ$  for 5 hr.; m.p.  $150-152^\circ$ ;  $R_{FAW}$  0.73;  $[\alpha]^{25}_D +14.7^\circ$  (*c* 1, in methanol).

*Anal.* Calcd. for  $C_{18}H_{18}N_2O_3 \cdot HBr$ : C, 47.14; H, 5.78; N, 8.46; Br, 24.13. Found: C, 47.15; H, 5.82; N, 8.61; Br, 24.07.

**Carbobenzoxyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester (III).**—A solution of 11.9 g. (37 mmoles) of carbobenzoxyl-L-histidine hydrazide in 85 ml. of 2 *N* hydrochloric acid was cooled to  $-10^\circ$  and 20 ml. of a cold 2 *M* sodium nitrite solution was added. After 5 minutes, 100 ml. of 50 per cent. potassium carbonate was added and the aqueous solution extracted 4 times with 600 ml. of cold ethyl acetate. The combined ethyl acetate extracts were washed twice with 50 ml. of saturated sodium chloride solution, dried over anhydrous sodium sulfate for 5 minutes and filtered through cotton into a cold ( $0^\circ$ ) solution of 12.3 g. (37 mmoles) of L-alanyl-L-phenylalanine methyl ester hydrobromide and 9 ml. (37 mmoles) of tributylamine in 50 ml. of dimethylformamide. The reaction mixture was stirred for 1 hr. at  $0^\circ$  and then overnight (16 hr.) at room temperature. After concentrating the mixture *in vacuo*, the oily residue was dissolved in methanol. Ethyl acetate and ether were added to give a final ratio of solvents of 1:1:1. The crystalline product obtained above was recrystallized twice from the same solvent mixture; yield, 17.2 g. (90%), m.p.  $180-$

*RpDw.* The conditions used for paper electrophoresis were: solvent, 95 ml. of acetic acid and 36 ml. of formic acid diluted to 2 liters with distilled water; pH 2.1; voltage, 14.8 volts per cm. of paper. Electrophoretic mobilities are reported as the ratio: distance the peptide moved/distance glutamic acid moved and abbreviated as *B<sub>G</sub>*.

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(11) All melting points were taken on a Kofler hot-stage and are corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Paper chromatograms were developed using butanol:acetic acid: water (4:1:5) abbreviated as *R<sub>BAW</sub>*; acetonitrile:water (3:1) abbreviated as *R<sub>TAW</sub>*; dimethylformamide:water (15:1) abbreviated as

182°;  $R_{\text{fAW}}$  0.90. For analysis it was recrystallized from the same solvents and dried at 110° for 5 hr. *in vacuo*; m.p. 182–183°;  $[\alpha]^{25}_{\text{D}} - 19.05^\circ$  ( $c$  1.1, in methanol).

*Anal.* Calcd. for  $\text{C}_{27}\text{H}_{31}\text{N}_5\text{O}_6$ : C, 62.17; H, 5.99; N, 13.43. Found: C, 62.20; H, 6.20; N, 13.55.

**L-Histidyl-L-alanyl-L-phenylalanine Methyl Ester Dihydrobromide (IV).**—Carbobenzoxy-L-histidyl-L-alanyl-L-phenylalanine methyl ester (6.64 g.) was dissolved in 20 ml. of glacial acetic acid and 40 ml. of 4 *N* hydrogen bromide in glacial acetic acid was added. After 45 minutes at room temperature the peptide was precipitated by the addition of 350 ml. of absolute ether. The precipitate was collected by filtration and washed with a large volume of absolute ether. It was dissolved in absolute methanol and precipitated by the addition of ethyl acetate. This precipitation was conducted two more times and the final product dried over sodium hydroxide and phosphorus pentoxide *in vacuo*; yield, 6.67 g. (96.3%); m.p. 138–145°;  $R_{\text{fAW}}$  0.64. This product was used directly to prepare the tetrapeptide below without analysis.

**Carbobenzoxy-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester (V).**—To a cold (–10°) solution of 4.0 g. (15 mmoles) of carbobenzoxy-L-isoleucine and 3.6 ml. (15 mmoles) of tributylamine in 15 ml. of tetrahydrofuran was added 1.45 ml. (15 mmoles) of ethyl chloroformate. After stirring for 15 minutes at –10°, a cold (0°) solution of 5.4 g. (9.5 mmoles) of histidyl-alanyl-phenylalanine methyl ester dihydrobromide, 6 ml. (23 mmoles) of tributylamine and 0.36 ml. of distilled water in 30 ml. of dimethylformamide was added. The mixture was stirred 30 minutes at 0° and overnight (16 hr.) at room temperature. The tetrahydrofuran was removed *in vacuo* at 45° or less, and 150 ml. of ethyl acetate was added. The ethyl acetate solution was washed at 4° with 50 ml. of water, 30 ml. of 1 *N* hydrochloric acid, 30 ml. of water, 30 ml. of saturated sodium carbonate and 30 ml. of water. After drying over anhydrous sodium sulfate, the ethyl acetate was removed *in vacuo*. The oily product was dissolved in ethyl acetate and precipitated by the addition of ether. The product was precipitated again from the same solvents and dried *in vacuo* over phosphorus pentoxide and paraffin; yield of amorphous material, 4.8 g. (78.4%); m.p. 182–191°;  $R_{\text{fAW}}$  0.97;  $R_{\text{fBAW}}$  0.98;  $E_{\text{G}}$  0.87. A small amount of this sample was precipitated two more times as above and the final amorphous product was dried at 64° *in vacuo* for 20 hr. for analysis; m.p. 185–190°;  $[\alpha]^{25}_{\text{D}} - 28.54^\circ$  ( $c$  1.0, in methanol).

*Anal.* Calcd. for  $\text{C}_{35}\text{H}_{42}\text{N}_6\text{O}_7$ : C, 62.44; H, 6.67; N, 13.24. Found: C, 62.19; H, 6.77; N, 13.00.

**L-Isoleucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester Dihydrobromide (VI).**—Carbobenzoxy-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine methyl ester (10.5 g., 16.5 mmoles) was dissolved in 20 ml. of glacial acetic acid for 45 minutes at room temperature. After the addition of 500 ml. of absolute ether, the resulting precipitate was collected by filtration, washed with a large volume of absolute ether and crystallized from absolute methanol with ethyl acetate–absolute ether (1:1). The crystalline product was recrystallized twice from the same solvents; yield, 10.24 g. (93.8%); m.p. 158–162°;  $R_{\text{fBAW}}$  0.51;  $R_{\text{fAW}}$  0.74;  $[\alpha]^{25}_{\text{D}} + 50.91^\circ$  ( $c$  1.0, in pyridine).

*Anal.* Calcd. for  $\text{C}_{25}\text{H}_{33}\text{N}_5\text{O}_5\text{Br}_2$ : C, 45.33; H, 5.78; N, 12.69; Br, 24.13. Found: C, 45.37; H, 5.93; N, 12.74; Br, 24.01.

**Carbobenzoxy-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine-methyl Ester (VII).**—To a cooled (–6°) solution of 9.9 g. (23 mmoles) of carbobenzoxy-L-valyl-L-tyrosine hydrazide in 200 ml. of glacial acetic acid and 200 ml. of 2 *N* hydrochloric acid was added 1.6 g. (23 mmoles) of sodium nitrite in 10 ml. of water. After standing for 5 minutes at –6°, one liter of cold water was added. The precipitated azide was collected by filtration, washed with 500 ml. of 5 per cent. potassium bicarbonate and then with water. It was dissolved in 400 ml. of cold tetrahydrofuran and shaken for 8 minutes with anhydrous sodium sulfate. This solution was filtered into a cooled solution of 9.9 g. (15 mmoles) of tetrapeptide methyl ester dihydrobromide (VI) and 7.2 ml. (30 mmoles) of tributylamine in 30 ml. of tetrahydrofuran. After stirring 3 hr. at 0° and 4 hr. at room temperature, the resulting precipitate was collected by filtration and washed with tetrahydrofuran. The volume of the filtrate was reduced *in vacuo* and

additional precipitate was obtained by addition of tetrahydrofuran. The combined amorphous precipitate showed two spots on paper chromatography:  $R_{\text{fBW}}$  0.74 (ninhydrin and Pauly positive); 0.86 (Pauly positive). After triturating the peptide with 100 ml. of hot methanol–tetrahydrofuran (1:1), the product was dried *in vacuo*; yield 11.0 g. (81.7%) of amorphous product;  $R_{\text{fBW}}$  0.86; m.p. 244–49°;  $[\alpha]^{25}_{\text{D}} - 10.00^\circ$  ( $c$  1.4, in dimethylformamide).

A small sample of this product was prepared for analysis by precipitating twice from dimethylformamide by the addition of methanol and drying at 110° overnight *in vacuo*; m.p. 248–254°;  $E_{\text{G}}$  0;  $[\alpha]^{25}_{\text{D}} - 14.01^\circ$  ( $c$  1.47, in dimethylformamide).

*Anal.* Calcd. for  $\text{C}_{67}\text{H}_{80}\text{O}_{16}\text{N}_8 \cdot \text{H}_2\text{O}$ : C, 61.68; H, 6.82; N, 12.24. Found: C, 61.86; H, 6.83; N, 12.44.

**L-Valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester Dihydrochloride (VIII).**—To a solution of 2.0 g. (2.24 mmoles) of carbobenzoxy-hexapeptide (VII) in 40 ml. of glacial acetic acid, 20 ml. of methanol and 2.0 ml. of 6 *N* hydrochloric acid was added 0.4 g. of 10 per cent. palladium on charcoal suspended in a few ml. of water. Hydrogen gas was bubbled through the solution with shaking for 15 hr. at room temperature. Another 10 ml. of methanol and about 0.1 g. of catalyst were added during the hydrogenation. The catalyst was removed by filtration and the filtrate evaporated to an oil under reduced pressure. The oily product showed 2 spots on paper chromatography ( $R_{\text{fBAW}}$  0.44 and 0.54). The residue was precipitated twice from methanol by addition of ether; yield 1.75 g. (93.5%);  $R_{\text{fBAW}}$  0.51. A small sample of this material was precipitated twice from methanol with ether after the addition of one drop of 3 *N* hydrochloric acid and finally dried at 110° for 4.5 hr. *in vacuo* for analysis; m.p. 211–18°;  $[\alpha]^{25}_{\text{D}} - 6.38^\circ$  ( $c$  1.1, in methanol);  $R_{\text{fBAW}}$  0.51;  $R_{\text{fAW}}$  0.54;  $E_{\text{G}}$  1.11.

*Anal.* Calcd. for  $\text{C}_{39}\text{H}_{56}\text{N}_8\text{O}_8 \cdot \frac{1}{2}\text{H}_2\text{O}$ : C, 55.44; H, 6.80; N, 13.26; Cl, 8.39. Found: C, 55.61; H, 6.93; N, 12.82; Cl, 8.55.

**Carbobenzoxy-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester (IX).**—To a cold solution (–5 to –10°) of 1.5 g. (4 mmoles) of carbobenzoxy-nitro-arginine monohydrate in 4 ml. of dimethylformamide, 10 ml. of tetrahydrofuran and 1 ml. of tributylamine (4 mmoles) was added 0.48 ml. (4 mmoles) of ethyl chloroformate. The solution was stirred for 15 minutes at –10° and combined with a cold (0°) solution of 2.05 g. (2.47 mmoles) of hexapeptide methyl ester dihydrochloride (VIII) and 1.2 ml. (4.94 mmoles) of tributylamine in 35 ml. of dimethylformamide. The mixture was stirred 35 minutes at 0° and 2 hr. at room temperature. The addition of 250 ml. of methanol to the reaction mixture yielded a white amorphous precipitate. After 3 hr. at 4°, the product was collected by filtration and washed with 50 ml. of methanol and 100 ml. of ether. The dried, amorphous material (1.9 g.) was precipitated from dimethylformamide by addition of water and then from dimethylformamide with methanol to yield 1.85 g. (68.3%);  $R_{\text{fBAW}}$  0.95;  $E_{\text{G}}$  0; m.p. 236–40°. For analysis, a small sample of this product was dissolved in a large volume of hot methanol and precipitated by cooling. It was dried at 138° overnight *in vacuo* yielding a product melting at 241–244°;  $[\alpha]^{25}_{\text{D}} - 21.89^\circ$  ( $c$  0.8, in formic acid).

*Anal.* Calcd. for  $\text{C}_{63}\text{H}_{77}\text{N}_{13}\text{O}_9 \cdot \text{H}_2\text{O}$ : C, 57.03; H, 6.59; N, 16.31. Found: C, 57.18; H, 6.53; N, 15.93.

**Nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester Dihydrobromide (X).**—To a solution of carbobenzoxyheptapeptide methyl ester (IX) (2.4 g., 2.18 mmoles), in 40 ml. of glacial acetic acid was added 30 ml. of freshly prepared 4.5 *N* hydrogen bromide in glacial acetic acid. After 50 minutes at room temperature 200 ml. of absolute ether was added, and the resulting precipitate was collected by filtration, washed with a large volume of ether in a dry atmosphere and dried over sodium hydroxide *in vacuo*. This product showed 2 spots on paper chromatography;  $R_{\text{fBAW}}$  0.28, 0.51 (both reacted with ninhydrin and Pauly reagent). The slower moving substance was removed by treating a methanolic solution of the peptide with 0.2 g. of Darco G-60 followed by precipitation with ether. Yield of amorphous product was 2.41 g. (98.3%); m.p. 197–203°;  $R_{\text{fBAW}}$  0.51.

A small sample of this product was dissolved in a minimum volume of cold water, the pH adjusted to 9 with

saturated sodium carbonate solution, and the precipitate was collected by centrifugation. This heptapeptide methyl ester free base was washed several times with cold water, precipitated twice from methanol with ether and dried at 100° for 7 hr. *in vacuo*; m.p. 228–232°;  $[\alpha]^{25}_D -10.38^\circ$  (*c* 0.8, in dimethylformamide);  $R_{\text{BAW}}$  0.62;  $E_g$  1.01.

*Anal.* Calcd. for  $C_{48}H_{85}N_{13}O_{11}$ : C, 56.06; H, 6.79; N, 18.89. Found: C, 55.84; H, 7.05; N, 18.89.

**Carbobenzoxy- $\beta$ -benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-iso-leucyl-L-histidyl-L-alanyl-L-phenylalanine Methyl Ester (XI).**—To a cold solution ( $-10^\circ$ ) of 1.07 g. (3 mmoles) of carbobenzoxy- $\beta$ -benzyl-L-aspartic acid and 0.72 ml. (3 mmoles) of tributylamine in 7.0 ml. of tetrahydrofuran was added 0.27 ml. (3 mmoles) of ethyl chloroformate. The reaction mixture was stirred for 15 minutes at that temperature and added to a cold (0°) solution of 1.7 g. (1.5 mmoles) of heptapeptide methyl ester dihydrobromide (X) and 0.72 ml. (3 mmoles) of tributylamine in 30 ml. of dimethylformamide. The combined mixture was stirred for 20 minutes in an ice bath and for 3 hr. at room temperature. After addition of 200 ml. of cold water and 15 ml. of cold 1 *N* hydrochloric acid, the precipitate was collected by filtration, washed with a large volume of cold water and with 300 ml. of acetone. After drying at room temperature it was dissolved in 40 ml. of dimethylformamide and precipitated by the addition of 40 ml. of 1 *N* hydrochloric acid and 200 ml. of water. This precipitate was dissolved in 40 ml. of dimethylformamide and precipitated by the addition of 200 ml. of methanol and dried over  $P_2O_5$  *in vacuo*; yield of amorphous product 1.48 g. (71.1%); m.p. 228–230°;  $R_{\text{BAW}}$  0.89;  $E_g$  0.

A small amount of this sample was reprecipitated 3 times from dimethylformamide with methanol and finally washed with water and dried at 110° overnight (16 hr.) *in vacuo* for analysis; m.p. 228–230° (dec);  $[\alpha]^{25}_D -17.65^\circ$  (*c* 0.85, in acetic acid).

*Anal.* Calcd. for  $C_{64}H_{92}N_{14}O_{16} \cdot H_2O$ : C, 58.17; H, 6.41; N, 14.82. Found: C, 58.47; H, 6.39; N, 14.83.

**L-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-iso-leucyl-L-histidyl-L-alanyl-L-phenylalanine Monoacetate (XIII).**—To a solution of 193 mg. of carbobenzoxy- $\beta$ -benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-iso-leucyl-L-histidyl-L-alanyl-L-phenylalanine methyl ester in 15 ml. of glacial acetic acid, and 11 ml. of methanol was added 0.3 g. of 10 per cent. palladium on charcoal suspended in 3 ml. of water. Hydrogen gas was bubbled through this mixture with shaking for 24 hr. Another 10 ml. of water and 5 ml. of methanol were added during the hydrogenation. The reduced product showed one spot on paper chromatography ( $R_{\text{BAW}}$  0.27) and 2 spots on paper electrophoresis. The faster moving spot reacted with ninhydrin, Pauly and Sakaguchi reagents while the other spot reacted only with Pauly reagent. The small slow moving spot on electrophoresis did not disappear on further reduction. After removal of the palladium on charcoal by filtration, the clear solution was evaporated to an oil and triturated with ether. The resulting amorphous solid was collected by filtration, washed with ether and dried; yield, 158 mg. (97%); m.p. 248–255° (decomposition beginning at 220°);  $R_{\text{BAW}}$  0.27;  $E_g$  1.08. This octapeptide methyl ester (100 mg.) (XII) was suspended in 8 ml. of 1 *N* sodium hydroxide solution, and after 40 minutes the material was completely dissolved. After 2.3 hr. at room temperature, the material was precipitated by the addition of about 1 ml. (about 17 mmoles) of glacial acetic acid. The precipitate was collected by filtration, washed with 6 ml. of cold water, 2 ml. of acetone and 15 ml. of ether. This product showed one spot on paper chromatography ( $R_{\text{BAW}}$  0.40) but 2 major spots and a third minor component on paper electrophoresis. The product was dissolved in 10 ml. of 50% acetic acid solution, precipitated by the addition of 20 ml. of acetone, and reprecipitated from a solution of 10 ml. of glacial acetic acid, 3 ml. of water and 30 ml. of acetone. This dried product showed one spot on paper chromatography and paper electrophoresis; yield, 65 mg. (70.6%); m.p. 238–240° (decomposition at 230°). A small sample was dried at 110° for 24 hr. *in vacuo* for analysis; m.p. 248–255° (began decomposition at 223°);  $R_{\text{BAW}}$  0.41;  $E_g$  1.13;  $[\alpha]^{25}_D -20.85^\circ$  (*c* 0.67, in 50% acetic acid).

*Anal.* Calcd. for  $C_{48}H_{85}N_{13}O_{13} \cdot CH_3COOH$ : C, 55.59; H, 6.81; N, 16.85. Found: C, 55.82; H, 6.80; N, 16.75.

After hydrolysis in 6 *N* hydrochloric acid at 111° for 36 hr. under nitrogen, the expected eight amino acids were found on paper chromatography. The pressor activity of this sample was 116 units per mg. The assay was conducted as described previously.<sup>7</sup>

A second preparation of the crude octapeptide, which showed three spots on electrophoresis, was purified in a simpler manner by precipitation from aqueous acetic acid by carefully raising the pH. The octapeptide monoacetate (230 mg.) was dissolved in 55 ml. of 1 *N* acetic acid, and the pH was carefully adjusted to 4.4 with 5 *N* sodium hydroxide. After standing 2 weeks at 4° the resulting precipitate was collected by centrifugation, washed twice with cold water and redissolved in 50 ml. of 1 *N* acetic acid. The pH of this solution was brought to 3.8 and the solution was allowed to remain at 4° for 3 weeks. The resulting precipitate was collected by centrifugation, washed twice with 5 ml. of cold water, dissolved in 20 ml. of 50% acetic acid and taken to dryness by lyophilization. Additional precipitate which separated from the mother liquor on standing 2 additional weeks at 4° was treated as described for the initial precipitate. The final combined product exhibited only one spot on electrophoresis and paper chromatography and could not be separated by these methods from the product analyzed above; yield, 102 mg. (46%); m.p. 248–250°;  $[\alpha]^{25}_D -20.88^\circ$  (*c* 0.66, in 50% acetic acid).

## Discussion

This peptide was prepared following the general plan used recently for the synthesis of isoleucyl<sup>1</sup>-angiotensin II<sup>12</sup> and is outlined in Fig. 1. Beginning with the C-terminal phenylalanine, as the methyl ester, the peptide was built up one amino acid at a time except for the addition of the dipeptide valyl-tyrosine. The mixed anhydride procedure was used for most condensations in which carboxyl-entering group was a single amino acid and the azide procedure was used to add the dipeptide unit. Use of these procedures would avoid racemization.<sup>12–15</sup>

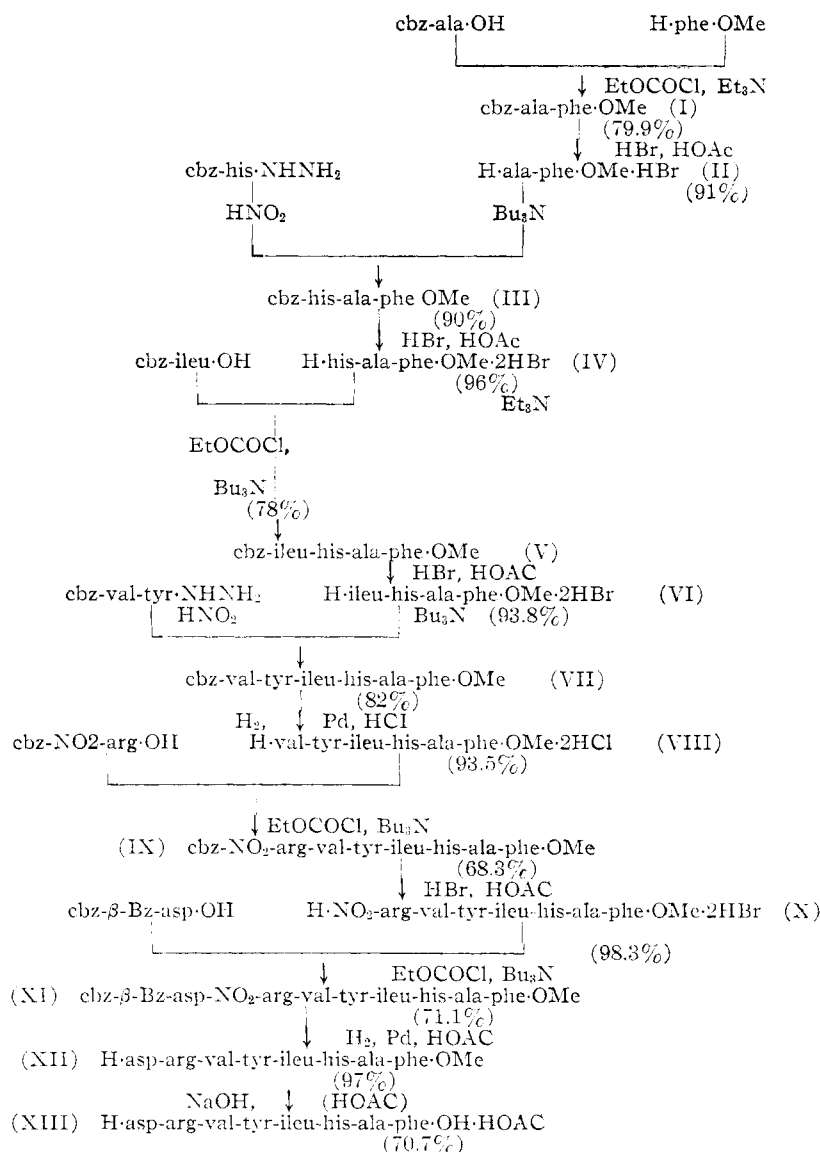
The dipeptide, alanyl-phenylalanine methyl ester hydrobromide (II), was prepared in 73% overall yield from carbobenzoxy-alanine and phenylalanine methyl ester by the mixed anhydride procedure followed by removal of the carbobenzoxy group with hydrogen bromide in acetic acid. This peptide was then coupled to carbobenzoxy-histidine azide to give carbobenzoxy-histidyl-alanyl-phenylalanine methyl ester. The tripeptide dihydrobromide (IV) was prepared by removal of the blocking group with hydrogen bromide in acetic acid in an over-all yield of 86%. Carbobenzoxy-iso-leucyl-histidyl-alanyl phenylalanine methyl ester (V) was prepared from the tripeptide (IV) in 78% yield by the mixed anhydride procedure. Carbobenzoxy-valyl-tyrosine azide was then coupled to the tetrapeptide hydrobromide (VI) to yield, after hydrogenolysis, valyl-tyrosyl-iso-leucyl-histidyl-alanyl-phenylalanine methyl ester dihydrochloride (VIII) in 77% yield. Nitro-arginyl-valyl-tyrosyl-iso-leucyl-histidyl-alanyl-phenylalanine methyl ester dihydrobromide (X) was prepared in 67% yield from carbobenzoxy-nitroarginine and hexapeptide (VIII) by the mixed anhydride procedure followed by removal of the blocking group with hydrogen bromide in acetic acid. Carbobenzoxy- $\beta$ -benzyl aspartic acid was joined to heptapeptide (X) by

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Fig. 1.—Outline of the synthesis of isoleucyl<sup>15</sup>-alanyl<sup>17</sup>-angiotensin II.

the mixed anhydride procedure to yield the completely blocked octapeptide (XI).

Hydrogenation of the blocked octapeptide methyl ester (XII) followed by saponification gave crude isoleucyl<sup>15</sup>-alanyl<sup>17</sup>-angiotensin II in 69% yield. This product exhibited a single spot on chromatography in several solvent systems but was resolved into two spots by paper electrophoresis at pH 2.1 indicating the two peptides had very similar solubility properties but differed in isoelectric points and/or charged groups. It was possible to utilize this charge difference for final purification. By dissolving the mixture obtained from saponification in 0.1 *M* acetic acid and carefully raising the pH by the addition of sodium hydroxide, the desired product precipitated first, yielding a homogeneous material.

The hexa-(VIII), hepta- and octapeptide intermediates (IX–XII) are all considerably less soluble in organic solvents than the corresponding peptides prepared in the synthesis of isoleucyl<sup>15</sup>-

angiotensin II. Similarly isoleucyl<sup>15</sup>-alanyl<sup>17</sup>-angiotensin II is considerably less soluble in aqueous systems, such as 0.1 *M* acetic acid, than the natural angiotensin which possibly suggests a greater degree of order for the alanyl<sup>17</sup>-peptide.<sup>16</sup> The imidazole ring of histidine in the completely blocked hexa-, hepta- and octapeptide intermediates (VII, IX, XI) does not protonate readily for these peptides did not migrate on paper electrophoresis strips at pH 2.1, while the completely blocked tetrapeptide (V) did migrate under the same conditions. Even at pH 1.6 these blocked intermediates did not migrate and the blocked octapeptide (XI) precipitated from dilute hydrochloric acid (about 0.1 *N*) as the free base. The reason this imidazole group is so resistant to reaction with the hydrogen ion is not known.

Isoleucyl<sup>15</sup>-alanyl<sup>17</sup>-angiotensin II possesses only about 1% of the pressor activity of isoleucyl<sup>15</sup>-

(16) M. M. Marsh, *J. Am. Chem. Soc.*, **84**, 1896 (1962).

angiotensin II. Rupture of the aliphatic ring of proline causes a marked decrease in the pressor activity of the peptide. Since the removal of this ring would be expected to cause a marked change in

the conformation of the C-terminus of the peptide, this evidence further suggests the importance of conformation in the angiotensin structure for biological activity.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, GREECE]

## On Cysteine and Cystine Peptides. I. New S-Protecting Groups for Cysteine<sup>1,2</sup>

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The problem of the synthesis of unsymmetrical cystine peptides with two or more cystine -S-S- bridges is discussed. For a solution of this problem, the following requirements must be fulfilled: (a) cysteines bearing different S-protecting groups selectively removable must be available and (b) procedures must be worked out for preventing the rearrangement of cystine chains during synthesis until their final incorporation in a multimembered ring system. Concerning the first of the above requirements, S-diphenylmethyl-L-cysteine (I) and S-trityl-L-cysteine (II) are proposed as the most suitable S-protected cysteines for the incorporation of cystine residues in a peptide chain. The S-trityl group can be easily split off with heavy metal salts at room temperature, whereas the removal of the S-diphenylmethyl group is also easily effected by the action of trifluoroacetic acid. The SH- groups thus liberated can be oxidized to the corresponding -S-S- derivatives. Several peptides of cysteine and cystine have been synthesized in this way.

### Introduction

An attempt by Fischer and Gerngross<sup>3</sup> to prepare monoglycyl- and monoleucyl-L-cystine through aminolysis of their respective monohaloacyl-L-cystine precursors resulted in the formation, in each instance, of a dipeptide which was not pure and appreciable amounts of cystine. This unusual aminolysis of an  $\alpha$ -haloacylamino acid can be easily explained<sup>4</sup> by the well-known fact that unsymmetrical open chain derivatives of cystine (Fig. 1) are not stable but rearrange very easily

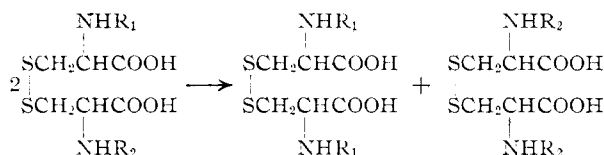


Fig. 1.

to the symmetrical ones.<sup>4,5</sup> The existence of unsymmetrical cystine peptides, as in oxytocin and in vasopressin, may apparently be attributed to the fact that these compounds are of cyclic structure, the only cystine -S-S- bridge being implicated in the ring system. Most of the proteins can be considered, in principle, as unsymmetrical polypeptides of cystine. These proteins, *i.e.*, insulin, whose structure has been elucidated by Sanger, *et al.*,<sup>6</sup> are more or less stable, because in this case more than one cystine -S-S- bridge holds the polypeptide chains together, forcing them to participate in a multimembered ring system.

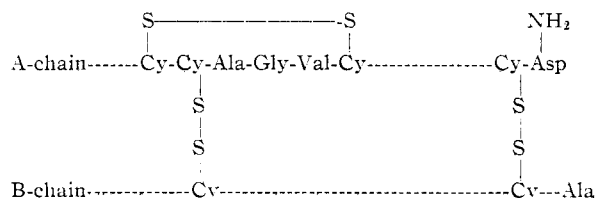


Fig. 2.

Owing to the carbobenzoxy method<sup>7</sup> as well as to various other methods<sup>8</sup> the synthesis of common polypeptide chains (*i.e.*, peptides of different amino acids including cysteine, symmetrical cystine peptides or peptides of the oxytocin type) is no longer a problem, especially since these methods have already been adapted to the peculiarities of some amino acids, as in the case of lysine<sup>9</sup> and arginine<sup>10</sup> and—what was more requisite—of cysteine-cystine.<sup>1,11</sup> However, an inspection of the insulin -S-S- bridge system<sup>6</sup> (Fig. 2) shows that an approach to the synthesis of unsymmetrical cystine peptides with two or more cystine -S-S- bridges would be facilitated if, in addition to the methods mentioned above, the following requirements could be met: (a) the availability at cysteines bearing different S-protecting groups (R,R' Fig. 3)<sup>1a,1b</sup> which could be incorporated into a peptide chain;

(7) M. Bergmann and L. Zervas, German Patent 556,798 (1932); *Ber.*, **65**, 1192 (1932).

(8) (a) A detailed description of the methods for peptide synthesis can be found in J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961; (b) *cf.* also A. Cosmatos, I. Photaki and L. Zervas, *Chem. Ber.*, **94**, 2644 (1961).

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(10) (a) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957). (b) L. Zervas, T. Otani, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959); L. Zervas, M. Winitz and J. P. Greenstein, *ibid.*, **83**, 3300 (1961); M. Bergmann, L. Zervas and H. Rinke, *Z. physiol. Chem.*, **224**, 40 (1934); C. Gros, M. P. de Gaviñhe, A. Costopanagiotis and R. Schwyzler, *Helv. Chim. Acta*, **44**, 2042 (1961).

(11) (a) R. H. Siffert and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935); (b) C. R. Harington and T. H. Mead, *Biochem. J.*, **29**, 1602 (1935).

(1) (a) A summary of a part of this paper was presented at the 3rd European Peptide Symposium, Basle, September, 1960; L. Zervas and I. Photaki, *Chimia*, **14**, 375 (1960). (b) A summary of this paper was presented at the 4th European Peptide Symposium, Moscow, August, 1961; L. Zervas, *Collection Czechoslov. Commun.*, in press.

(2) This investigation was supported by the Royal Hellenic Research Foundation, to which we are greatly indebted.

(3) E. Fischer and O. Gerngross, *Ber.*, **42**, 1485 (1909).

(4) L. Zervas, L. Benoiton, E. Weiss, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 1729 (1959).

(5) F. Sanger, *Nature*, **171**, 1025 (1953); A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955); R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **80**, 1666 (1958).

(6) H. Brown, F. Sanger and R. Kitai, *Biochem. J.*, **60**, 556 (1955).