

THE SYNTHESIS OF A TETRADECAPEPTIDE RENIN SUBSTRATE

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The blood of human beings with hypertensive cardiovascular disease (1), as well as of animals with experimental renal hypertension (2, 3) was found to contain the pressor substance angiotensin¹ (hypertensin) (4). This material was later found to exist in two forms (5). The first of these, the decapeptide angiotensin I, is the product of the action of the renal enzyme renin upon its plasma substrate. Angiotensin I has been purified (6) and its structure determined (7, 8). The second form, the octapeptide angiotensin II, is produced from angiotensin I by a plasma enzyme activated by chloride ions (5). Angiotensin II, which is believed to be the effector substance of the renin-angiotensin system (9, 10), has been purified (11) and its structure (12, 13) confirmed by synthesis (14, 15).

In an effort to discover the structure of the active portion of the renin substrate, tryptic digestion of a plasma protein fraction was found to yield an active polypeptide renin substrate (16). The polypeptide was prepared on a large scale, purified, and its structure was determined. This paper describes the synthesis of this compound.

EXPERIMENTAL

An outline of the steps involved in the synthesis is presented in Fig. 1. The compound numbers and abbreviations correspond to those in the text.

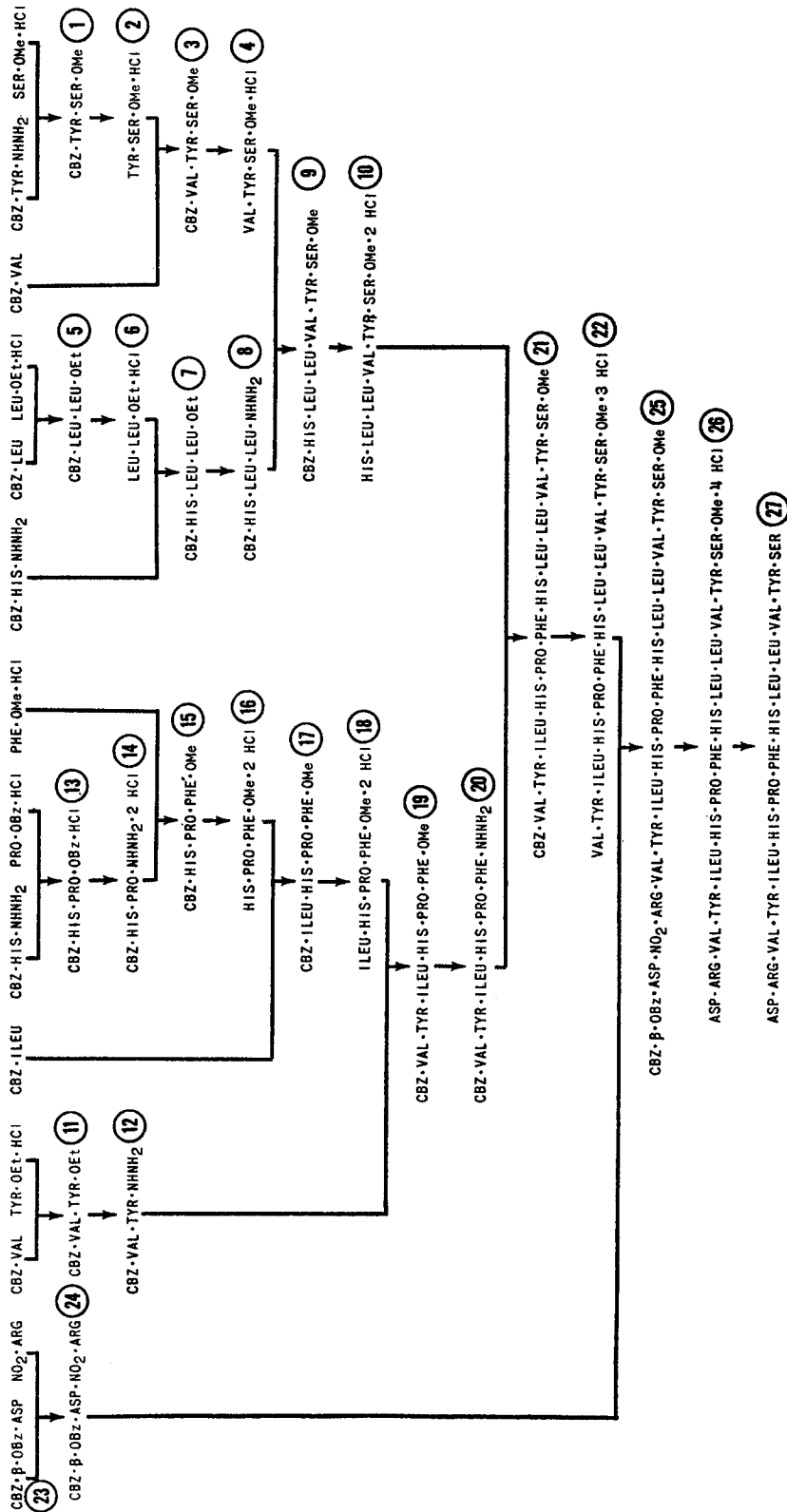
All amino acids used were of the *l* form.

All phases of the preparation of azides from hydrazides were carried out in the cold. The exact amount of NaNO₂ required was determined by use of a solution of 0.5 per cent starch containing 1 per cent KI as an external indicator.

Products were chromatographed on Whatman No. 1 paper as a rapid, but not final, means of testing purity. The solvent butanol-acetic acid-water (4, 1, 5) was used. Chromatograms were developed by heating the papers after dipping in a 0.3 per cent solution of ninhydrin in acetone. Compounds containing histidine were also located by use of Durrum's stain (17).

Prior to chromatography, carbobenzyloxyl compounds (50 to 100 μ M) were reduced in

¹ In conformity with the new nomenclature suggested by Drs. Eduardo Braun-Menendez and Irvine H. Page, the authors will use the hybrid word angiotensin (angiotenin-hypertensin).



KEY
 CBZ = carbobenzyloxy
 OBz = benzyl ester
 OEt = ethyl ester
 OMe = methyl ester
 NO₂ = nitro
 NHNH₂ = hydrazide

Fig. 1 Scheme for the Synthesis of A Tetradecapeptide Renin Substrate

alcoholic solution for 2 hours using 50 pounds of hydrogen pressure in the presence of 200 mg. of palladium and a small excess of acetic acid. Saponification of esters was occasionally performed by treatment with 0.5 N NaOH for 15 minutes followed by neutralization with acetic acid. Hydrolysates (10 μ M) were prepared of all compounds by heating at 105°C. with 1 ml. of 6 N HCl for 22 hours in a sealed tube. After removal of acid by evaporation, the samples were chromatographed using butanol-acetic acid-water, or analyzed by the FDNB² method of Levy (18). In the latter case, His and Arg were sometimes omitted.

Assays of biological activity were performed by direct pressor assay in the rat (19) after incubation of renin substrate preparations with a large excess of renin (16). The results are expressed in Goldblatt units (20).

Ultimate analyses were performed by the Huffman Microanalytical Laboratories of Wheatridge, Colorado.

Optical rotations were determined using two decimeter tubes in a Zeiss polarimeter capable of being read to 0.01°.

Melting points were determined on a Fisher-Johns block and are uncorrected.

Countercurrent distributions were conducted using a 200 tube, 10 ml. phase, automatic Craig-Post apparatus.

1. *Cbz-Tyr-Ser-OMe*.—24.75 gm. (75 mm) of *cbz-tyr-NHNH₂* was dissolved in 1200 ml. of water containing 150 ml. of concentrated HCl. The solution was cooled to 5°C. and 37.5 ml. of 2 M NaNO₂ was added. The azide was extracted into one 300 and two 150 ml. portions of ethyl acetate. The extract was washed twice with 0.5 volume portions of water, once with 1.0 per cent NaHCO₃ and finally twice with 0.5 volumes of water. The solution was dried with Na₂SO₄ and CaSO₄.

10 gm. (95 mm) of serine was suspended in 225 ml. of methanol and treated for 30 minutes with HCl gas. The solution was evaporated to dryness. The residue was again dissolved in methanol, gassed with HCl, and evaporated to dryness. The material was then dissolved in methanol, and dried with CaSO₄. A sufficient amount of sodium methylate was then added to bring the pH to 8.0 (as measured externally on 5-fold dilution with water). The mixture was evaporated to a small volume and some NaCl removed by filtration. The filtrate was evaporated to dryness. The residue was extracted with ethyl acetate. The remaining sodium chloride was removed from the solution by filtration. A chromatogram revealed one ninhydrin spot, *R_f* 0.57 (ser *R_f* 0.34). The cold solutions of the azide and the ester were combined in a volume of 600 ml., kept in the refrigerator for 24 hours and then at room temperature for 12 hours.

The reaction mixture was extracted twice with water, once with 1 N HCl, twice with 0.1 N HCl, and twice with water. The ethyl acetate solution was rapidly stirred with an equal volume of water while the pH was adjusted to 6.0 with 0.1 N NaOH. After removal of the aqueous layer, the process was repeated three times. The extract was finally washed with water and dried with Na₂SO₄. The solution was evaporated to 150 ml., at which point crystallization of the product occurred. A small amount of additional material was obtained from the mother liquor upon addition of petroleum ether. Yield 12.5 gm. (30 mm).

M.p. 153°C. Calculated for C₂₁H₂₄O₇N₂: C, 60.58; H, 5.81; N, 6.73. Found: C, 60.58; H, 5.78; N, 6.92. $[\alpha]_D^{24.5} = -6.25 \pm 0.11$, $c = 4.048$ in dimethylformamide; $[\alpha]_D^{23.0} = -2.49 \pm 0.20$, $c = 4.044$ in methanol. *R_f*, reduced to the dipeptide ester, 0.57; reduced and saponified to the dipeptide, 0.39.

2. *Tyr-Ser-OMe·HCl*.—12.0 gm. (28.8 mm) of *cbz-tyr-ser-OMe* was dissolved in 200 ml. of methanol. 60 ml. of 0.5 N HCl and 2 gm. of palladium were added. The mixture was treated with hydrogen at atmospheric pressure until the consumption of the gas had ceased. The

² FDNB, fluorodinitrobenzene.

palladium was removed by filtration. The solution was evaporated until aqueous. After extraction with ethyl acetate, the water solution was evaporated to dryness and the residue desiccated over P_2O_5 and NaOH. Yield 8.6 gm. (27.0 mm). A sample for analysis was crystallized by slow addition of an alcoholic solution of the ester hydrochloride to cold ether. M.p. 177 to 178°. Calculated for $C_{13}H_{19}O_6N_2Cl$: C, 48.96; H, 6.01; N, 8.79; α -NH₂, 4.40; Cl, 11.12. Found: C, 48.91; H, 6.13; N, 8.76; α -NH₂, 4.36; Cl, 11.10. $[\alpha]_D^{28.5} = +10.93 \pm 0.29$, $c = 1.528$ in methanol. R_f 0.57; reduced and saponified to the dipeptide, 0.39.

3. *Cbz-Val-Tyr-Ser-OMe*.—6.44 gm. (25.6 mm) of cbz-val was dissolved in 160 ml. dimethylformamide. 7.53 ml. (51.2 mm) of triethylamine was added and the solution cooled to $-5^\circ C$. 3.70 ml. (28.3 mm) of isobutylchloroformate was added and the mixture stirred for 10 minutes. At this time, 8.16 gm. (25.6 mm) of tyr-ser-OMe·HCl dissolved in 160 ml. of dimethylformamide was added. The mixture was heated rapidly to $70^\circ C$., cooled, and then poured into 6 liters of water. The pH was adjusted to 2.5. The flocculent precipitate was gathered by filtration. The precipitate was dissolved in 2 liters of ethyl acetate. A small amount of insoluble material was removed by filtration. The ethyl acetate solution was stirred with an equal volume of water while the pH was adjusted to 7.0 with dilute NaOH. This process was repeated five times. The extract was finally washed with water and dried with Na_2SO_4 . The product crystallized upon evaporation of the solution. Yield 5.8 gm. (11.3 mm).

M.p. 206° . Calculated for $C_{26}H_{38}O_8N_3$: C, 60.59; H, 6.46; N, 8.16; OCH₃, 6.01. Found: C, 60.27; H, 6.48; N, 7.71; OCH₃, 6.50. $[\alpha]_D^{25} = -13.5 \pm 0.7$, $c = 3.314$ in dimethylformamide. R_f , reduced to the tripeptide ester, 0.61; reduced and saponified to the tripeptide, 0.49.

4. *Val-Tyr-Ser-OMe·HCl*.—5.78 gm. (11.2 mm) cbz-val-tyr-ser-OMe was dissolved in 500 ml. of methanol, 15 ml. of 1 N HCl, 100 ml. of water, and 1 gm. of palladium were added. The mixture was treated with hydrogen at atmospheric pressure for 5 hours. At this time, the consumption of hydrogen had stopped. The palladium was removed by filtration. The solution was evaporated until aqueous and extracted with ethyl acetate. The aqueous portion was further evaporated to dryness. The residue was desiccated over P_2O_5 and NaOH. Yield 4.28 gm. (10.24 mm). M.p. 142 to 145° .

A sample for analysis was prepared by the slow addition of a 10 per cent methanolic solution of the compound to a large excess of cold ethyl acetate. The resulting precipitate was washed with ether and dried.

M.p. 145 to 146° . Calculated for $C_{18}H_{28}O_6N_3Cl$: C, 51.74; H, 6.75; N, 10.05. Found: C, 51.57; H, 6.95; N, 10.24; $[\alpha]_D^{22.5} = +23.40 \pm 0.50$, $c = 1.123$ in methanol. Molar ratios by FDNB analysis: val, 0.90; tyr, 1.17; ser, 0.93.

5. *Cbz-Leu-Leu-OEt*.—26.5 gm. (100 mm) of cbz-leu was dissolved in 100 ml. of tetrahydrofuran. 14.75 ml. (100 mm) of triethylamine was added and the solution was cooled to $-5^\circ C$. 13.1 ml. (100 mm) of isobutylchloroformate were added and the mixture stirred for 10 minutes. At the end of this time, a cold suspension of 19.57 gm. (100 mm) of leu-OEt·HCl in 100 ml. of tetrahydrofuran containing 14.75 ml. of triethylamine was added. The mixture was stirred for 10 minutes at $0^\circ C$., heated quickly to $65^\circ C$., and cooled to room temperature. The reaction mixture was poured into 1500-ml. of 5 per cent $NaHCO_3$. The aqueous suspension was extracted one time with an equal volume of ether and discarded. The ether layer was washed with water, 0.1 N HCl, water and finally evaporated to dryness. The product was desiccated *in vacuo* over P_2O_5 . M.p. 73 to 75° . Yield 24.7 gm. (60.8 mm).

A sample for analysis was prepared by dissolving the product in 70 per cent ethanol, adding water to turbidity and chilling in a dry-ice-alcohol bath with vigorous stirring. After diluting with an equal volume of water, the solid was filtered, washed with water, and dried over P_2O_5 . M.p. 85° . Calculated for $C_{22}H_{34}O_5N_2$: C, 65.01; H, 8.43; N, 6.89; OC₂H₅, 11.07. Found: C, 65.21; H, 8.36; N, 7.21; OC₂H₅, 10.45. $[\alpha]_D^{28.5} = -36.34 \pm 0.27$, $c = 4.144$ in ethanol. R_f , reduced to the dipeptide ester, 0.88; reduced and saponified to the dipeptide, 0.83.

6. *Leu-Leu-OEt·HCl*.—24.3 gm. (59.8 mm) of *cbz-leu-leu-OEt* was dissolved in 175 ml. of methanol. 60 ml. of *N* HCl and 1 gm. palladium were added and the mixture treated with hydrogen at atmospheric pressure. At the end of 5 hours, the consumption of hydrogen had stopped. The palladium was removed by filtration and methanol by evaporation. The aqueous solution was extracted with an equal volume of ether. The ether phase was discarded and the aqueous layer evaporated to dryness and desiccated over P_2O_5 and NaOH. Yield 17.0 gm. (55.0 mm). M.p. 155 to 156°. Calculated for $C_{14}H_{23}O_3N_2Cl$: C, 54.42; H, 9.46; N, 9.06; α NH_2 , 4.53; Cl, 11.48. Found: C, 54.52; H, 9.51; N, 9.15; α NH_2 , 4.40; Cl, 11.79. $[\alpha]_D^{25} = -10.85 \pm 0.34$, $c = 1.908$ in methanol. R_f , 0.87; saponified to the dipeptide, 0.84.

7. *Cbz-His-Leu-Leu-OEt*.—13.45 gm. (44.3 mm) of *cbz-his-NHNH_2* was dissolved in 140 ml. of 1 *N* HCl. The solution was cooled to 2°C. and overlaid with an equal volume of ethyl acetate. 11 ml. of 4 *M* $NaNO_2$ was added and, after 3 minutes, 56 ml. of 50 per cent K_2CO_3 . The ethyl acetate solution of the azide was separated and the aqueous layer extracted with two 25 ml. portions of ethyl acetate. The aqueous phase was discarded and the ethyl acetate portions combined, washed with water, and dried with $CaSO_4$.

14.4 gm. (46.6 mm) of *leu-leu-OEt·HCl* was dissolved in 100 ml. of water. The solution was adjusted to pH 10 with 50 per cent K_2CO_3 and extracted three times with an equal volume of ether. The ether extracts were combined, washed with water, and evaporated to dryness. The oily residue was dissolved in ethyl acetate and dried with $CaSO_4$.

The cold azide and ester solutions were mixed and allowed to stand for 18 hours in the refrigerator.

The reaction mixture was diluted to 750 ml. with ethyl acetate and extracted seven times with 250 ml. portions of 0.01 *N* HCl. The ethyl acetate solution was then washed three times with 250 ml. of 1 per cent $NaHCO_3$. After washing with water, the solution was evaporated to dryness. The residue was dissolved in 200 ml. of ethyl acetate and the product precipitated by dropwise addition to 800 ml. of petroleum ether. Yield 9.6 gm. (17.7 mm). M.p. 170 to 173°. Calculated for $C_{28}H_{41}O_6N_5$: C, 61.86; H, 7.60; N, 12.88. Found: C, 61.92, H, 7.61; N, 12.89. $[\alpha]_D^{25.5} = -45.6 \pm 1.2$, $c = 4.372$ in methanol.

8. *Cbz-His-Leu-Leu-NHNH_2*.—8.6 gm. (15.8 mm) of *cbz-his-leu-leu-OEt* was dissolved in 60 ml. of hot methanol. 2.5 ml. of hydrazine hydrate was added and the solution boiled for 3 minutes, and then allowed to stand at room temperature for 24 hours. The solution was poured into 1 liter of water and the oily precipitate extracted into 750 ml. of ethyl acetate. The extract was washed with 100 ml. of water and dried with Na_2SO_4 . Upon evaporation, the product crystallized yielding 5.17 gm. (9.76 mm). M.p. 167 to 168°. The material was recrystallized from hot ethyl acetate. M.p. 169 to 170°. Yield 2.91 gm. (5.5 mm). A second crop yield 1.8 gm. (3.4 mm). M.p. 169 to 170°. Calculated for $C_{26}H_{39}O_5N_7$: C, 58.95; H, 7.43; N, 18.53. Found: C, 58.41; H, 7.59; N, 18.38. $[\alpha]_D^{20} = -42.54 \pm 0.57$, $c = 2.052$ in methanol. R_f , reduced to the tripeptide hydrazide, 0.74; Durrum's positive.

9. *Cbz-His-Leu-Leu-Val-Tyr-Ser-OMe*.—2.13 gm. (4.05 mm) of *cbz-his-leu-leu-NHNH_2* was dissolved in 50 ml. of water with the aid of 4 ml. of 5 *N* HCl. The solution was cooled to 5°C. and 4.0 ml. of 1 *M* $NaNO_2$ was added. The pH of the mixture was adjusted to 9.5 using 50 per cent K_2CO_3 and the azide extracted into one 100 and two 50 ml. portions of ethyl acetate. The extract was washed twice with 100 ml. portions of water and dried with Na_2SO_4 and $CaSO_4$.

2.19 gm. (5.24 mm) of *val-tyr-ser-OMe·HCl* was dissolved in 100 ml. of anhydrous methanol. 0.77 ml. of triethylamine was added and the solution evaporated to a small volume. 100 ml. of chloroform was then added and the mixture evaporated again to a small volume. The process was repeated until the methanol solvent had been replaced with chloroform. The precipitated ester was removed by filtration, washed with chloroform, and desiccated over P_2O_5 . M.p. 183 to 184°.

The ester was dissolved in 50 ml. of dimethylformamide and added to the solution of the

azide in ethyl acetate. The ethyl acetate was removed from the reaction mixture by evaporation at less than 5°C., and the remaining dimethylformamide solution placed in the refrigerator for 3 days.

The solution was diluted with 1 liter of water. 300 gm. of NaCl was added and the pH adjusted to 6.5. The precipitate was collected by filtration and washed thoroughly with water. The material was dissolved in methanol and dried with Na₂SO₄. Upon evaporation to 200 ml., the product crystallized. Yield 1.64 gm. (1.86 mm). M.p. 231 to 232°. Calculated for C₄₄H₆₂O₁₁N₈: C, 60.11; H, 7.11; N, 12.74; OCH₃, 3.53. Found: C, 59.13; H, 7.06; N, 12.65; OCH₃, 3.31. $[\alpha]_D^{25} = -29.54 \pm 0.22$, $c = 1.01$ in dimethylformamide.

10. *His-Leu-Leu-Val-Tyr-Ser-OMe*·2 HCl.—1.60 gm. (1.82 mm) of cbz-his-leu-leu-val-tyr-ser-OMe was suspended in 125 ml. of methanol. 1.2 ml. of 1.6 N methanolic HCl, 2.5 ml. 1 N HCl, and 1 gm. of palladium were added. The mixture was treated with hydrogen under 50 pounds pressure for 6 hours. The palladium was removed by filtration and the solution evaporated to dryness. The product was desiccated over P₂O₅ and NaOH. Yield 1.46 gm. (1.69 mm). M.p. 247 to 249°. Calculated for C₃₆H₅₈O₉N₈Cl₂: C, 52.86; H, 7.15; N, 13.70; OCH₃, 3.79. Found: C, 53.44; H, 7.17; N, 13.42; OCH₃, 3.50. $[\alpha]_D^{31.5} = -28.1 \pm 0.4$, $c = 2.000$ in dimethylformamide. R_f 0.71, Durrum's positive.

11. *Cbz-Val-Tyr-OEt*.—25.11 gm. (100 mm) of cbz-val was dissolved in 100 ml. of tetrahydrofuran. 14.75 ml. (100 mm) of triethylamine was added, and the solution cooled to -5°C. 14.4 ml. (110 mm) of isobutylchloroformate was added and the mixture stirred for 10 minutes.

27.0 gm. (110 mm) of tyr-OEt·HCl was powdered and suspended in 100 ml. of cold tetrahydrofuran. 16.2 ml. of triethylamine (110 mm) was added and the suspension added to the mixed anhydride solution. The mixture was stirred rapidly for 10 minutes while at -5°C., then heated rapidly to reflux, and finally cooled to room temperature.

The reaction mixture was diluted with 1.0 liter of water and placed in the refrigerator. The crystalline mass which formed overnight was gathered, washed with cold 0.1 N NaHCO₃ 0.1 N HCl, and water.

The solid was dissolved in 750 ml. of ethyl acetate. The solution was washed three times with an equal volume of 0.1 N HCl, six times with cold 0.1 N NaHCO₃, and finally twice with water. After drying with Na₂SO₄, the solution was evaporated to a small volume. The crystals which formed during the evaporation were filtered off, washed with ether, and dried. Yield 28.34 gm. (64.1 mm). M.p. 155 to 157°. Calculated for C₂₄H₃₀O₆N₂: C, 65.16; H, 6.81; N, 6.34. Found: C, 65.17; H, 6.89; N, 6.41. $[\alpha]_D^{20} = +55.8 \pm 3.9$, $c = 1.008$ in chloroform. R_f , reduced to the dipeptide ester, 0.79; reduced and saponified to the dipeptide, 0.68.

12. *Cbz-Val-Tyr-NHNH₂*.—17.8 gm. (40.2 mm) of cbz-val-tyr-OEt was dissolved in 90 ml. of warm anhydrous methanol. 10 ml. of hydrazine hydrate was added and the mixture allowed to stand at room temperature for 48 hours. The crystalline mass was collected by filtration, washed with 1.0 liter of water, 20 ml. of cold ethanol, ether, and dried. Yield 17.2 gm. (40.1 mm). M.p. 248 to 250°.³ Calculated for C₂₂H₂₈O₅N₄: C, 61.67; H, 6.59; N, 13.08. Found: C, 61.80; H, 6.70; N, 13.02. $[\alpha]_D^{27.5} = -12.95 \pm 0.10$, $c = 4.246$ in dimethylformamide. R_f , reduced to the dipeptide hydrazide, 0.68.

13. *Cbz-His-Pro-OBz*.—30.3 gm. (100 mm) of cbz-his-NHNH₂ was dissolved in 300 ml. of 1 N HCl. The solution was cooled to 5°C. and 26 ml. of 4 M NaNO₂ was added with vigorous stirring. After 2 minutes, 400 ml. of cold ethyl acetate was added and the mixture titrated to pH 9.5, using 120 ml. of 50 per cent K₂CO₃. The ethyl acetate solution of the azide was separated, washed twice with 200 ml. of cold water, and dried with Na₂SO₄.

26.6 gm. (110 mm) of pro-OBz·HCl was dissolved in 120 ml. of cold chloroform. 16.2 ml. (110 mm) of triethylamine was added, followed by 800 ml. of cold ethyl ether. The precipitated triethylamine hydrochloride was removed by filtration, and, after washing with 200 ml.

³ Reference 15 gives m.p. 239 to 241° and $[\alpha]_D^{25} = -13.7$, $c = 3.6$ in dimethylformamide.

of ethyl ether, was discarded. The combined ether solutions of the ester were evaporated to dryness. The resulting oil was dissolved in 100 ml. of cold ether. The solution was dried with CaSO_4 . A chromatogram revealed one yellow ninhydrin spot, R_f 0.77.

The cold azide and ester solutions were mixed and placed in the refrigerator. After 48 hours, the mixture was extracted ten times with 0.2 M sodium phosphate buffer pH 6.0. The solution was washed with water, dried with Na_2SO_4 , and evaporated to dryness. The resulting oil was desiccated over P_2O_5 . Yield 28.5 gm. or 59.9 mM.

A sample for analysis was prepared by dissolving in chloroform, adding ether until a faint turbidity persisted, and gassing with dry HCl. The precipitate was filtered off, washed with ether, and desiccated. M.p. 84 to 85°. Calculated for $\text{C}_{26}\text{H}_{29}\text{O}_5\text{N}_4\text{Cl}$: C, 60.90; H, 5.69; N, 10.93; Cl, 6.91. Found: C, 60.81; H, 5.54; N, 10.81; Cl, 6.90. $[\alpha]_D^{25} = -76.21 \pm 0.28$, $c = 1.768$ in water. R_f reduced⁴ to the dipeptide, 0.20, Durrum's positive.

14. *Cbz-His-Pro-NHNH₂·2 HCl*.—28.5 gm. (59.9 mM) *cbz-his-pro-OBz* was dissolved in 90 ml. of anhydrous methanol. 10 ml. of hydrazine hydrate was added and the mixture allowed to stand at room temperature for 1 week. At the end of this time, the reaction mixture was diluted with 5 volumes of saturated NaCl solution, and extracted four times with ethyl acetate. The ethyl acetate solution of the hydrazide was washed with one-fifth volume of saturated NaCl solution, treated with sodium sulfate, and evaporated to dryness. The residue was dissolved in 50 ml. of methanol. 41.5 ml. (66.4 mM) of 1.6 N methanolic HCl was added, and the solution was evaporated to dryness. The residue was dissolved in 200 ml. of cold methanol and the hydrazide precipitated as an oily mass by the addition of 2500 ml. of cold ethyl acetate. The precipitate was dissolved in 100 ml. of cold methanol and reprecipitated by slow addition to 800 ml. of cold ethyl ether. The white precipitate was gathered by filtration, washed with ether, and desiccated over P_2O_5 . Yield 17.3 gm. (36.6 mM). M.p. 170°. Calculated for $\text{C}_{19}\text{H}_{26}\text{O}_4\text{N}_6\text{Cl}_2$: C, 48.22; H, 5.54; N, 17.77. Found: C, 48.44; H, 5.84; N, 17.21. $[\alpha]_D^{28} = -46.74 \pm 0.15$, $c = 3.132$ in methanol.

15. *Cbz-His-Pro-Phe-OMe*.—17.3 gm. (36.6 mM) of *cbz-his-pro-NHNH₂·2 HCl* was dissolved in 100 ml. of water containing 8.35 ml. of concentrated HCl. The solution was cooled to 3°C. 36 ml. of 1 M NaNO_2 were added and, after overlaying the solution with 200 ml. of cold ethyl acetate, the mixture was adjusted to pH 9.5 with saturated K_2CO_3 . The aqueous portion of the solution was nearly saturated by the addition of 30 gm. of NaCl. The ethyl acetate extract was separated and the aqueous layer re-extracted twice with 200 ml. portions of ethyl acetate. The ethyl acetate extracts containing the azide were combined, washed twice with 125 ml. portions of saturated NaCl, and dried with Na_2SO_4 and CaSO_4 .

10.8 gm. (50.1 mM) of *phe-OMe·HCl* was dissolved in 100 ml. of cold water. After overlaying with 100 ml. of cold ethyl acetate, the solution was adjusted to pH 9.0 with 50 per cent K_2CO_3 . The aqueous layer was saturated with NaCl. The ethyl acetate extract was separated, and the aqueous layer re-extracted with a second 100 ml. portion of ethyl acetate. The two extracts were combined and washed three times with cold water. The extract was dried with Na_2SO_4 and CaSO_4 . A chromatogram revealed one ninhydrin spot, R_f 0.78.

The ester and azide solutions were combined and evaporated at a temperature not greater than 5°C. The concentrated mixture, with a volume of about 200 ml. was placed in the refrigerator.

After 40 hours, the reaction mixture was extracted sixteen times with 500 ml. portions of 0.2 M sodium phosphate buffer pH 6.0. The volume of the ethyl acetate solution was maintained at 250 ml. by frequent additions of fresh solvent. After washing twice with water, the solution was dried with Na_2SO_4 , evaporated to dryness, and desiccated over P_2O_5 . Yield 11.5 gm. (21 mM). M.p. 87 to 89°. Calculated for $\text{C}_{29}\text{H}_{33}\text{O}_6\text{N}_5$: C, 63.62; H, 6.07; N,

⁴ Hydrogenolysis with palladium removes benzyl ester groups in addition to carbobenzyloxy groups.

12.79; OCH₃, 5.66. Found: C, 63.11; H, 6.15; N, 12.45; OCH₃, 5.58. $[\alpha]_D^{26} = -41.59 \pm 0.24$, $c = 2.044$ in methanol. R_f , reduced to the tripeptide ester, 0.45; reduced and saponified to the tripeptide, 0.31; both Durrum's positive. Molar ratios by FDNB analysis: his, 0.941; pro, 1.002; phe, 1.057.

16. *His-Pro-Phe-OMe*·2 HCl.—1.5 gm. (21 mm) of cbz-his-pro-phe-OMe was dissolved in 55 ml. of methanol. 13.1 ml. of 1.6 N methanolic HCl and 10 ml. 2.5 N HCl were added together with 2 gm. of palladium. The mixture was treated with hydrogen at atmospheric pressure for 5 hours. The palladium was removed by filtration using celite. The solution was dried with Na₂SO₄, evaporated to dryness, and desiccated over P₂O₅ and NaOH. Yield 9.64 gm. (19.8 mm). M.p. 145 to 147°. $[\alpha]_D^{26} = -18.94 \pm 0.30$, $c = 1.98$ in methanol.

A sample for analysis was prepared by addition of a 10 per cent methanolic solution of the compound to cold ethyl acetate. The resulting precipitate was washed with ether and dried. M.p. 170 to 172°. Calculated for C₂₁H₂₉O₄N₅Cl₂: C, 51.84; H, 6.00; N, 14.39. Found: C, 52.18; H, 5.74; N, 14.65. R_f , 0.51; Durrum's positive.

17. *Cbz-Ileu-His-Pro-Phe-OMe*.—6.26 gm. of cbz-ileu (23.6 mm) was dissolved in 100 ml. of tetrahydrofuran. After the addition of 3.47 ml. of triethylamine, the solution was chilled to -5°C. 3.09 ml. of isobutylchloroformate was added, and the solution was stirred vigorously for 10 minutes.

7.62 gm. (15.66 mm) of his-pro-phe-OMe·2 HCl was dissolved in 75 ml. of methanol, and the solution cooled to 0°C. 4.63 ml. (31.5 mm) of triethylamine was added, and the mixture evaporated to dryness. The mixture of ester and triethylamine was suspended in 150 ml. of cold tetrahydrofuran and added to the mixed anhydride. Vigorous stirring was continued for 15 minutes at -5°C. The mixture was heated rapidly to 60°C. and then cooled.

The reaction mixture was diluted with an equal volume of water and evaporated to remove tetrahydrofuran. The aqueous solution was extracted with 300, then 150 ml. of ethyl acetate. The two portions of ethyl acetate were combined and washed successively six times with 0.2 M sodium phosphate buffer pH 6.0, three times with cold 0.1 M NaHCO₃, and twice with water. The solution was evaporated to dryness, dissolved in 50 ml. of methanol and treated with 10 ml. of 1.6 N methanolic HCl. The alcohol was removed by evaporation, and the residue dissolved in 300 ml. of water. The solution was extracted three times with ether. The ether was discarded and the aqueous layer clarified by filtration. The solution was chilled to 5°C. and adjusted to pH 8.0 by the slow addition of 1.0 M NaHCO₃. The precipitate was collected by filtration, washed with water, and dissolved in ethyl acetate. The ethyl acetate was dried with Na₂SO₄ and evaporated to a volume of 20 ml. The syrupy solution was then added dropwise to 500 ml. of cold ethyl ether. The resulting precipitate was gathered by filtration, washed with ether, and desiccated over P₂O₅. Yield: 8.98 gm. (13.6 mm). M.p. 100 to 102°.⁵ Calculated for C₃₅H₄₄O₇N₆: C, 63.63; H, 6.71; N, 12.72; OCH₃, 4.69. Found: C, 63.47; H, 6.81; N, 12.68; OCH₃, 4.48. $[\alpha]_D^{21.5} = -58.92 \pm 0.85$, $c = 0.998$ in methanol. R_f , reduced to the tetrapeptide ester, 0.66; Durrum's positive. Molar ratios by FDNB analysis: ileu, 0.90; his, 1.07; pro, 1.00; phe, 1.02.

18. *Ileu-His-Pro-Phe-OMe*·2 HCl.—8.98 gm. (13.6 mm) of cbz-ileu-his-pro-phe-OMe was dissolved in 80 ml. of methanol. 8.25 ml. of 1.6 N methanolic HCl and 17.5 ml. of 1 N HCl were added together with 1.5 gm. of palladium. The mixture was treated with hydrogen at atmospheric pressure for 4.5 hours. The palladium was removed by filtration through celite. The solution was dried with Na₂SO₄, evaporated to dryness, and desiccated over P₂O₅ and NaOH. A chromatogram showed one ninhydrin and Durrum's positive spot R_f 0.65. M.p. 155 to 157°.⁶ Yield 7.96 gm. (13.3 mm).

19. *Cbz-Val-Tyr-Ileu-His-Pro-Phe-OMe*.—7.96 gm. (13.3 mm) of ileu-his-pro-phe-OMe·2

⁵ Reference 14 gives m.p. 105 to 110° and $[\alpha]_D^{22} = -56 \pm 4$, $c = 0.971$ in methanol.

⁶ Reference 14 gives m.p. 130 to 140° for the tetrapeptide dihydrobromide.

HCl was dissolved in 25 ml. of water. The solution was cooled to 5°C. and the pH was adjusted to 9.5 with 50 per cent K_2CO_3 . After saturation with NaCl, the solution was extracted with 250 and then 125 ml. portions of cold chloroform. The extracts were combined and washed twice with 50 ml. portions of cold saturated NaCl solution. The chloroform solution was dried with Na_2SO_4 , evaporated, and the residue desiccated over P_2O_5 .

8.48 gm. (19.8 mm) of cbz-val-tyr-NHNH₂ was dissolved in a mixture of 200 ml. of 2.5 N HCl and 200 ml. of glacial acetic acid. The solution was cooled to 3°C. and 20 ml. of 1.0 M $NaNO_2$ was added. After 2.5 minutes, the mixture was diluted with 1 liter of cold water and the azide extracted from the diluted solution into 1 liter of ethyl acetate. The ethyl acetate extract was washed five times with water, once with 1.0 M $NaHCO_3$ and twice with 500 ml. of water. The solution was dried with Na_2SO_4 and $CaSO_4$.

The azide solution with a volume of 500 ml. was added to the ester dissolved in 50 ml. of cold ethyl acetate. Crystallization of the product began within 5 minutes and was complete after standing in the refrigerator for 16 hours.

The crystals were filtered from the reaction mixture, washed with cold ethyl acetate and ether, and dried. Yield 9.07 gm. (9.8 mm). M.p. 184°.⁷ Calculated for $C_{49}H_{62}O_{10}N_8$: C, 63.76; H, 6.77; N, 12.14; OCH_3 , 3.36. Found: C, 63.61; H, 6.81; N, 12.22; OCH_3 , 3.30. $[\alpha]_D^{29.5} = -68.29 \pm 1.03$, $c = 1.006$ in methanol. R_f , reduced to the hexapeptide ester, 0.76; Durrum's positive. Molar ratio by FDNB analysis: val, 1.01; tyr, 1.03; ileu, 1.05; pro, 0.83; phe, 1.07.

20. *Cbz-Val-Tyr-Ileu-His-Pro-Phe-NHNH₂*.—4.61 gm. (5.0 mm) of cbz-val-tyr-ileu-his-pro-phe-OMe was dissolved in 67.5 ml. of warm anhydrous ethanol. After cooling, 7.5 ml. of hydrazine hydrate was added. The solution was allowed to stand at room temperature for 24 hours and in the refrigerator for 36 hours. The resulting crystals were gathered by filtration, washed with cold ethanol, ether and dried. Yield 3.9 gm. (4.22 mm). M.p. 177 to 178°. Calculated for $C_{48}H_{62}O_9N_{10}$: C, 62.44; H, 6.77; N, 15.18. Found: C, 62.51; H, 6.78; N, 15.36. $[\alpha]_D^{28} = -82.18 \pm 0.53$, $c = 0.825$ in methanol. R_f , reduced to the hexapeptide hydrazide, 0.72; Durrum's positive.

21. *Cbz-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe·2HCl*.—1.25 gm. (1.35 mm) of cbz-val-tyr-ileu-his-pro-phe-NHNH₂ was dissolved in a mixture of 5 ml. of 1 N HCl and 5 ml. of glacial acetic acid. The solution was cooled to 5°C. 1.35 ml. of 1 M $NaNO_2$ was added. After 2 minutes, the solution was diluted to 50 ml. with cold water and adjusted to pH 8.5 with 1 M NaOH and $NaHCO_3$. The solution with a volume of 200 ml. was saturated with NaCl and the precipitated azide collected by filtration. The azide was dissolved in 30 ml. of dioxane and diluted with an equal volume of water. The clear solution which was slightly acid was readjusted to pH 8.5 with a few drops of 1 M $NaHCO_3$. The volume was increased to 200 ml. by the addition of water. The solution was saturated with NaCl. The precipitated azide was gathered on a filter, washed with saturated NaCl, water and finally desiccated over P_2O_5 . M.p. 197 to 199°. Yield 1.07 gm. (1.15 mm).

1.04 gm. (1.27 mm) of his-leu-leu-val-tyr-ser-OMe·2 HCl was dissolved in 200 ml. of 0.01 N HCl. The solution was cooled to 5°C. and layered with 200 ml. of secondary butanol. Using vigorous stirring, the pH was adjusted to 9 with 50 per cent K_2CO_3 and the aqueous phase saturated with NaCl. The lower layer was discarded and the butanol extract washed four times with equal volumes of cold, saturated NaCl solution. The extract was dried with Na_2SO_4 and was evaporated to dryness. The residue was desiccated over P_2O_5 . Yield 0.668 gm. (0.896 mm).

The solid azide and ester preparations were dissolved in 30 ml. of dimethylformamide and placed in the refrigerator for 38 hours.

The reaction mixture was evaporated to dryness at low temperature. The residue was dissolved in a mixture of 40 ml. of water, 3 ml. of 1 N HCl, and 40 ml. of secondary butanol.

⁷ Reference 15 gives m.p. 162 to 167° and $[\alpha]_D^{25} = -66.3$, $c = 1.0$ in methanol.

The resulting 2 phase system was adjusted to pH 2.0 and loaded into the countercurrent apparatus. The solvents used were 0.01 N HCl and secondary butanol. The machine was operated for 194 transfers.

The distribution pattern was determined by optical density measurements at 279 μ . One major non-symmetrical band was found ($K = 2.73$). One minor component appeared which was assumed to be peptide ester ($K = 0.21$). All tubes except those containing the major band were emptied and refilled with fresh solvents. The apparatus was then recycled for a total of 1148 transfers. Analysis of the distribution then showed partial separation of a minor component ($K = 3.40$) from a major band ($K = 5.71$). The solutions from the tubes containing the major band were pooled and evaporated to dryness. The residue was dissolved in 80 ml. ethanol. A large excess of cold ether was added and the resulting flocculent precipitate gathered by centrifuging. After washing with ether, the material was dried. Yield 0.406 gm. (0.238 mm). M.p. 192 to 194°. Calculated for $C_{84}H_{118}O_{18}N_{16}Cl_2$: OCH_3 , 1.81. Found: OCH_3 , 1.76, 1.77. $[\alpha]_D^{25} = -73.05 \pm 3.64$, $c = 1.03$ in methanol. R_f , unreduced, 0.91; reduced to the dodecapeptide ester, 0.75; both Durrum's positive. Molar ratios by FDNB analysis: val, 2.26; tyr, 1.77; pro, 1.15; leu + ileu, 3.19; phe, 0.99; ser, 0.75.

22. *Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe*·3 HCl.—0.545 gm. (0.319 mm) of *cbz-val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser-OMe*·2 HCl (some of which were prepared by a second reaction similar to that described) was dissolved in 100 ml. of methanol. 0.7 ml. of 1 N HCl was added together with 800 mg. of palladium. The mixture was treated with hydrogen at 50 pounds pressure for 3 hours. At the end of this time, a chromatogram gave only one Durrum's spot, R_f 0.75 (unreduced compound R_f 0.91). The palladium was removed by filtration. The alcoholic solution was dried with Na_2SO_4 and evaporated to dryness. The residue was desiccated over NaOH and P_2O_5 . Yield 0.499 gm. (0.310 mm). M.p. 232 to 234°. Calculated for $C_{76}H_{111}O_{16}N_{16}Cl_3$: N, 13.90; Cl, 6.61; OCH_3 , 1.92. Found: N, 13.70; Cl, 6.99; OCH_3 , 1.91.

23. *Cbz-β-Benzyl-Asp*.—This compound was prepared from 22.8 gm., 51 mm of *cbz-dibenzyl-asp* by the procedure of Berger and Katchalski (21) with the following modifications. After unreacted dibenzyl ester had been extracted with ether, the aqueous phase was brought to pH 5.8. A four step countercurrent distribution was carried out with equal phases of ethyl acetate and 0.2 M phosphate buffer, pH 5.8. Three of the aqueous fractions showed no turbidity on acidification to pH 3 and were discarded. An oil separated in the fourth fraction; this was extracted into ether and the solution dried over Na_2SO_4 . Evaporation yielded 8.6 gm. of the β -benzyl ester, m.p. 98–100°. Recrystallization from benzene gave the final product, 8.38 g., 23.46 mm, m.p. 110°.⁸ Calculated for $C_{19}H_{19}O_6N$: C, 63.88; H, 5.36; N, 3.92; neutral equivalent, 357.2. Found: C, 63.80; H, 5.36; N, 4.06; neutral equivalent, 357.8. $[\alpha]_D^{25} = +11.18 \pm 0.15$, $c = 10.01$ in glacial acetic acid. R_f , reduced with HBr in glacial acetic acid (22) to β -benzyl aspartic acid, 0.61; in phenol water (100:29), 0.81.

24. *Cbz-β-Benzyl-Asp-Nitro-Arg*.—3.57 gm. (10 mm) of *cbz-β-benzyl-asp* was dissolved in 100 ml. of dimethylformamide. The solution was cooled to -9° , and 1.48 ml. (10.1 mm) of triethylamine were added, followed by 1.31 ml. (10 mm) of isobutylchloroformate. The mixture was then stirred for 10 minutes.

2.63 gm. (12 mm) of nitroarginine was treated with 11 ml. of 1 N NaOH. After thorough stirring, the pH of the mixture dropped to 11.1. The excess nitroarginine was removed by filtration. The filtrate was added to the mixed anhydride and the mixture allowed to rise to room temperature.

After 2.5 hours, the reaction mixture was poured into 1.5 liters of cold water. The pH was adjusted to 3.4. The turbid aqueous solution was extracted four times with 750 ml. of ethyl acetate. The ethyl acetate extracts were combined, washed three times with 300 ml. of water,

⁸ Reference 21 gives m.p. 108°; $[\alpha]_D^{25} = +12.1$, $c = 10$ in glacial acetic acid.

and evaporated to dryness. The residue was dissolved in ethyl acetate and subjected to a 10 tube countercurrent distribution in the system ethyl acetate (90 ml.) — 0.2 M phosphate buffer pH 5.8 (360 ml.). Analyses of the distribution by means of the optical density at 270 μ m (nitroguanidine) revealed one main component in tubes 3 to 8. Impurities present in tubes 0 to 2 and in tube 9 were discarded. The solvents containing the main component were pooled, the pH adjusted to 3.5, and the phases equilibrated. The aqueous phase was discarded and the ethyl acetate layer washed with water, dried with Na_2SO_4 , and evaporated to dryness. Yield 2.67 gm. (4.78 mm). M.p. 83 to 85°. Calculated for $\text{C}_{25}\text{H}_{30}\text{O}_9\text{N}_6$: C, 53.75; H, 5.41; N, 15.05; neutralization equivalent 558.4. Found: C, 53.70; H, 5.40; N, 15.16; neutralization equivalent, 550. $[\alpha]_D^{25} = -3.0 \pm 0.10$, $c = 5.24$ in pyridine. Ultraviolet absorption: E_{max} , 16,450; λ_{max} , 270 μ m, in methanol. R_f , reduced to the dipeptide,¹⁰ 0.11; in phenol-water (100:29), 0.42. R_f , reduced with HBr in glacial acetic acid to β -benzyl-aspartyl-nitroarginine, then saponified to aspartyl-nitroarginine, 0.13.

25. *Cbz- β -Benzyl-Asp-Nitro-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe*. — 279 mg. (0.5 mm) of *cbz- β -benzyl-asp-nitro-arg* was dissolved in 3 ml. of dimethylformamide. 0.735 ml. of 0.68 N (0.5 mm) triethylamine in dimethylformamide was added and the solution was cooled to -12°C . 0.654 ml. of 0.765 N (0.5 mm) isobutylchloroformate in dimethylformamide was added, and the mixture was stirred at -12°C . for 10 minutes.

540 mg. (0.335 mm) of *val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser-OMe-3 HCl* (from two preparations of the ester hydrochloride) was dissolved in 20 ml. of methanol. The solution was cooled to 5°C . and 2.35 ml. of 0.68 N (1.6 mm) triethylamine in methanol was added. The mixture was evaporated to dryness, and desiccated over P_2O_5 . The residue was dissolved in cold dimethylformamide and added to the mixed anhydride. The reaction mixture, with a volume of 10 ml., was stirred at -12°C . for 5 minutes and then allowed to warm to room temperature.

After 20 hours at room temperature, the reaction mixture was diluted with 90 ml. of water. 100 ml. of *n*-butanol was added and the pH adjusted to 2.0. The material was subjected to a six tube countercurrent distribution using as solvents 0.01 N HCl and *n*-butanol. Ten additional transfers were performed using the method of single withdrawal. This procedure accomplished the separation of a minor tyrosine containing component (20 μ m) having a low *K* value which was assumed to be peptide ester. The major component with a very high *K* value was recovered from the distribution system in one 100 ml. butanol phase. This solution was washed with water and evaporated to dryness. The residue was dissolved in 30 ml. of methanol. Water (20 ml.) was added to a slight turbidity. The solution was cooled to 5°C . and the pH was adjusted to 8.0 with 1 M NaHCO_3 . 50 ml. of water was then added. The resulting precipitate was gathered by filtration and washed thoroughly with water. The precipitate was dissolved in 75 ml. of alcohol with the aid of heat. The solution was chilled and 300 ml. of cold ether were added. The flocculent precipitate was collected by centrifugation, washed with ether, and dried. Yield 235 mg. (0.115 mm). M.p. 214 to 215°. Calculated for $\text{C}_{101}\text{H}_{136}\text{O}_{24}\text{N}_{22}$: OCH_3 , 1.52. Found: OCH_3 , 1.73. $[\alpha]_D^{27.5} = -55.93 \pm 1.59$, $c = 0.506$ in 0.1 N methanolic HCl. R_f , reduced to the tetradecapeptide methyl ester, 0.48; Durrum's positive. Molar ratios by FDNB analysis: asp, 0.88; val, 2.06; tyr, 1.72; leu + ileu, 3.32; phe 1.00; pro, 1.05; ser, 0.95.

26. *Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OMe-4 HCl*. — 223 mg. (0.109 mm) of *cbz- β -benzyl-asp-nitro-arg-val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser-OMe* was dissolved in 50 ml. of methanol containing 2 ml. of 0.5 N HCl. 0.5 gm. of palladium was

⁹ Reference 15 gives m.p. 78 to 85° and $[\alpha]_D^{25} = -7.4$, $c = 5.0$ in pyridine for *cbz- β -methyl-asp-nitro-arg*.

¹⁰ The nitroguanidine group is reduced to guanidine by hydrogen in the presence of palladium.

added, and the mixture was treated with hydrogen at 50 pounds pressure for 11 hours. A chromatogram at this point showed a complete disappearance of unreduced compound (R_f 0.88) and the appearance of a new spot (R_f 0.44). However, the optical density as measured at $268\text{ m}\mu$, together with a consideration of the amount of tyrosine present (0.218 mM) indicated that only 50 per cent of the nitro group had been removed. Assays for renin substrate activity showed the presence of 56,000 units. The solution was filtered and 1 gm. of fresh catalyst was added. The mixture was treated with hydrogen for an additional 9 hours. The spectrum at this time showed almost complete removal of the nitro group. The assay value, however, did not increase, remaining at 56,000 units. The palladium was removed by filtration. The filtrate was evaporated to dryness and the residue was dissolved in 30 ml. of 0.01 N HCl and 30 ml. of secondary butanol. This material was loaded into the countercurrent machine. 196 transfers were accomplished using the solvent system 0.01 N HCl and secondary butanol. Analysis of the distribution by means of the optical density at $275\text{ m}\mu$ together with assay of renin substrate activity showed the presence of one biologically active major component ($K = 0.083$). Three minor components were also found ($K = 0.026, 0.225,$ and 11.2) together with other material, also minor in amount, but having an indeterminate K value. All tubes of the apparatus were emptied and refilled excepting those with biological activity. The machine was then operated with recycling until 878 transfers had been accomplished. Analysis then showed a major peak ($K = 0.064$) with slight asymmetry. Throughout the band, the assay values were in reasonable agreement with the optical density. The solutions from the tubes containing this band were pooled and evaporated to dryness. The residue was dissolved in 20 ml. of 75 per cent ethanol. 80 ml. of cold ether was added and the flocculent precipitate which resulted was collected, washed with ether, and dried. Yield 89 mg. (0.046 mM).

Renin substrate assay: 63,600 units, 4600 units per mg. N . M.p. 203 to 205°. Calculated for $C_{36}H_{126}O_{20}N_{21}Cl_4$: N , 15.33; OCH_3 , 1.62. Found: N , 15.36; OCH_3 , 1.59. $[\alpha]_D^{20.5} = -85.20 \pm 1.64$, $c = 0.98$ in 0.01 N HCl. R_f , 0.48; Durrum's positive.

27. *Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser*·2 HCl.—50 mg. (0.026 mM) of *asp-arg-val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser-OMe*·4 HCl was dissolved in 5 ml. of 0.01 N HCl. 5.0 ml. of 0.2 N NaOH was added causing a precipitate to form which immediately dissolved. After 5 minutes, the solution was adjusted to pH 10.5 and was loaded into the countercurrent distribution apparatus. The machine was operated for 237 transfers with recycling using the solvents 0.01 M Na_2CO_3 — 0.01 M $NaHCO_3$ and secondary butanol. Assays of the renin substrate activity showed one symmetrical band. ($K = 0.96$; natural product, $K = 1.03$). Determinations of the tyrosine concentrations ($275\text{ m}\mu$) at various points indicated that an impurity was present in the trailing edge. This portion of the band, containing approximately 25 per cent of the active substance, was discarded. The remaining portion was collected, adjusted to pH 4.0, and evaporated to 35 ml. The pH of the solution was adjusted to pH 7.4. A light flocculent precipitate formed which was collected by centrifugation. The material was washed three times with 5 ml. portions of water, with alcohol-ether, and dried. Yield 15 mg. (0.0082 mM).

Renin substrate assay: 12,600 units, 5760 units per mg. N , natural compound 5900 units per mg. N . Decomposed 219 to 220°; liquified 249 to 252°. Calculated for $C_{85}H_{128}O_{26}N_{21}Cl_2$ (dihydrochloride): N , 16.06; for $C_{85}H_{127}O_{26}N_{21}Cl_4$ (tetrahydrochloride): N , 15.44. Found: N , 15.37. $[\alpha]_D^{28.5} = -58.18 \pm 1.41$, $c = 0.88$ in 0.04 N HCl. R_f 0.50; natural compound, R_f 0.53; mixed synthetic and natural compounds, R_f 0.51. Molar ratios by FDNB analysis: arg, 1.44; asp, 0.88; val, 2.03; tyr, 1.74; leu + ileu, 3.30; his, 2.18; pro, 0.77; phe, 0.87; ser, 0.82.

The supernatant solution and washings from the initial product were combined, acidified, and evaporated to 19 ml. The solution was adjusted to pH 7.4. The precipitate which formed

was collected, washed with water, and desiccated. Yield 10 mg. (0.0055 mm). Decomposes at 217°, liquifies 249 to 252°C. Renin substrate assay: 7500 units, 5140 units per mg. N.

Degradation with Renin.—10.7 mg. (0.0058 mm) of asp-arg-val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser·2 HCl was dissolved in 200 ml. of water. 1.01 ml. of hog renin¹¹ (85 units, 123 units per mg. of dry weight) was added and the solution adjusted to pH 7.4. The mixture was incubated at 37.5°C. for 3 hours. At the end of this time, the solution was acidified and evaporated to a small volume. The material was adjusted to pH 10.5 and loaded in the countercurrent apparatus. The solvent system 0.01 M NaHCO₃–0.01 M Na₂CO₃ and secondary butanol was used. After 196 transfers had been effected, assays for direct pressor activity revealed a single band (K = 0.56; natural angiotensin I, K = 0.55) (11). The solvents containing this band were pooled, adjusted to pH 4.0, and evaporated to 10 ml. The solution was adjusted to pH 1.5 and was saturated with NaCl. The light, flocculent precipitate which appeared was gathered by filtration, washed with saturated sodium chloride, and redissolved in 4 ml. of 0.01 N HCl. Pressor assay: 5200 units. Nitrogen: 0.902 mg. (micro-Kjeldahl). Specific activity: 5760 units per mg. N (natural angiotensin I; 7700 units per mg. N).

R_f 0.34: natural angiotensin I, *R_f* 39; mixed natural and synthetic compounds, one spot, *R_f* 0.34. The solution of the synthetic compound contained some NaCl which may have affected the *R_f* value. Amino acid analysis: arg-asp-val-tyr-ileu + leu-his-pro-phe.

DISCUSSION

The synthetic tetradecapeptide renin substrate possessed a biological activity after reaction with renin essentially equal to substrate isolated from natural sources. Their physical and chemical properties also were essentially the same. Their distribution coefficients were similar (0.96 and 1.03, respectively) when measured in the countercurrent distribution apparatus. The solubility in water of the synthetic material, like that of the natural substrate, was very low, allowing final purification by isoelectric precipitation according to the method used in the isolation of the natural substance. Finally, paper chromatograms of the mixed synthetic and natural compounds yielded a single spot. It would appear probable, therefore, that the synthetic material was identical with the natural product.

Additional evidence in support of the identity of the two compounds was obtained by degradation of the synthetic material with purified renin. The pressor peptide which resulted from this reaction was found to have a distribution coefficient nearly identical with that of natural angiotensin I, (0.55 and 0.56, respectively). The synthetic peptide had a specific activity which was comparable (75 per cent) to that of the natural substance. Chromatograms of the mixed natural and synthetic materials yielded one spot. An analysis of the amino acids in the two compounds was the same. It may be reasonably concluded that the synthetic pressor peptide was, in fact, angiotensin I, produced by enzymatic hydrolysis of the synthetic renin substrate molecule.

¹¹ We are indebted to Drs. Erwin Haas and Harry Goldblatt of Mt. Sinai Hospital, Cleveland, for a supply of purified hog renin.

SUMMARY

A tetradecapeptide renin substrate having a biological activity comparable to the natural product and similar chemical properties has been synthesized by means of the carbobenzyloxyl azide and mixed anhydride methods.

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