Syntheses of Peptides Related to the *N*-Terminal Structure of Corticotropin. V. Synthesis of the Peptide Fragments, His-Phe-Arg-Try, His-Phe-Arg-Try-Gly and Glu-His-Phe-Arg-Try-Gly

By Ken INOUYE

(Received November 11, 1964)

It has been shown in our previous communications that the tetrapeptide sequence His-Phe-Arg-Try, which occupies the positions 6 to 9 in the molecules of corticotropin and the α-type of melanocyte-stimulating hormone (α-MSH), provides an essential core for eliciting the MSH and the lipolytic activities with these hormones.[1] In connection with the synthesis of the tetrapeptide L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan (I), we have next achieved the synthesis of a pentapeptide L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine (II) and a hexapeptide L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine (III), which corresponds to the amino acid sequences of positions 6—10, and 5—10, respectively, of the corticotropin and α-MSH molecules, together with a new synthesis of the tetrapeptide I, in order to try the comparative tests of the biological properties among these three peptides. An intermediate product to the hexapeptide III, namely γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine (XVII), is a key fragment for the synthesis of adrenocorticotropically active peptides.[2,3] The present synthesis has improved the procedure to obtain this key material.

The tetrapeptide and the pentapeptide were built up stepwise from their carboxyl terminal residues, where *t*-butyloxycarbonyl-nitro-L-arginine (IV) was utilized to incorporate the arginine residue and the *N*, *N'*-dicyclohexylcarbodiimide (DCCI) method[4] was employed except for the last coupling step. In the last step to introduce the histidine, a crystalline active ester N^{α}, N^{Im}-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester (V), gave excellent results. The protected hexapeptide was synthesized by coupling of the phenylalanyl tetrapeptide ester (X) with carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidine azide which was derived from a new crystalline compound carbobenzoxy-γ-*t*-butyl-L-glutamyl-N^{Im}-carbobenzoxy-L-histidine methyl ester (XIII).

Figure 1 outlines the synthetic steps for the

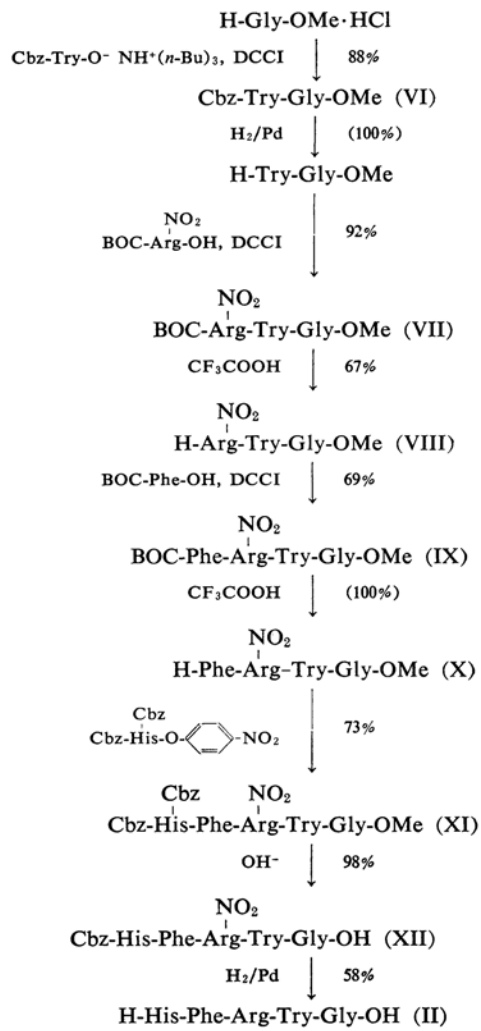

Fig. 1. Scheme for the synthesis of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine. Cbz, carbobenzoxy; BOC, *t*-butyloxycarbonyl; Me, methyl; Bu, butyl; DCCI, *N*, *N'*-dicyclohexylcarbodiimide

1) H. Otsuka and K. Inouye, This Bulletin, **37**, 289 (1964); **37**, 1465 (1964).
2) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **46**, 1550 (1963).
3) R. Geiger, K. Sturm and W. Siedel, *Chem. Ber.*, **97**, 1207 (1964).
4) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

pentapeptide II. Carbobenzoxy-L-tryptophylglycine methyl ester (VI) was obtained in an excellent yield (88%) by coupling of the hydrochloride of glycine methyl ester with the soluble tri-*n*-butylamine salt of carbobenzoxy-L-tryptophan, which was formed in a methylene chloride solution. *t*-Butyloxycarbonyl-nitro-L-arginine (IV), which crystallized from an ethyl acetate solution, was found to combine a half mole of the solvent molecule. Compound IV was linked with the dipeptide ester, which was prepared from the hydrogenolysis product of compound VI, to obtain *t*-butyloxycarbonyl-nitro-L-arginyl-L-tryptophyl-glycine methyl ester (VII). Compound VII failed to crystallize, but it could easily be purified by reprecipitation from ethyl acetate. The trifluoroacetic acid treatment[5] of VII afforded the tripeptide trifluoroacetate (VIII). A small amount of a colored by-product, which gave an anomalous color reaction with the Ehrlich reagent and which ran a little faster than the desired product VIII on paper in the system of 1-butanol - acetic acid - water (4 : 1 : 2), was removed by simple extraction of the aqueous solution with ethyl acetate. The purified tripeptide ester was then condensed with *t*-butyloxycarbonyl-L-phenylalanine to obtain the crystalline protected tetrapeptide *t*-butyloxycarbonyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine methyl ester (IX). The diacyl-L-histidine *p*-nitrophenyl ester V was prepared in the form of fine needles by two different methods. One of these was the ordinary DCCI method, by which N^{α}, N^{Im}-dicarbobenzoxy-L-histidine[6] was condensed with *p*-nitrophenol in an excellent yield. Another procedure involved transesterification between the diacyl-L-histidine and *p*-nitrophenyl trifluoroacetate, which was very recently utilized by Sakakibara and Inukai for the preparation of carboxylic acid *p*-nirophenyl esters.[7] The latter method also gave the desired compound in a moderate yield, especially with high purity. This active ester was allowed to react in an acetonitrile solution with the phenylalanyl tetrapeptide ester (X), which was derived from the protected peptide IX by the trifluoroacetic acid treatment, affording in a yield of 72.3% the pentapeptide N^{α},N^{Im}-dicarbobenzoxy-L-histidy-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine methyl ester (XI), which crystallized as the monohydrate upon treating with hot 90% methanol. Saponification of the methyl ester XI yielded carbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine (XII), accompanied by splitting of the N^{Im}-carbobenzoxy group.

The procedure for synthesis of the hexapeptide III is shown in Fig. 2. Carbobenzoxy-γ-*t*-buty-L-glutamyl-N^{Im}-carbobenzoxy-L-histidine methyl ester (XIII) was synthesized by the coupling of carbobenzoxy-γ-*t*-butyl-L-glutamic acid[8] with N^{Im}-carbobenzoxy-L-histidine methyl ester, the preparation of which has already been described.[6] The yield amounted to 90 per cent after recrystallization. Hydrazinolysis in the next step brought about an immediate cleavage at the N^{Im}-carbobenzoxy bonding to result in the formation of benzyl carbazate which was isolated subsequent to the desired carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidine hydrazide (XIV). Compound XIV

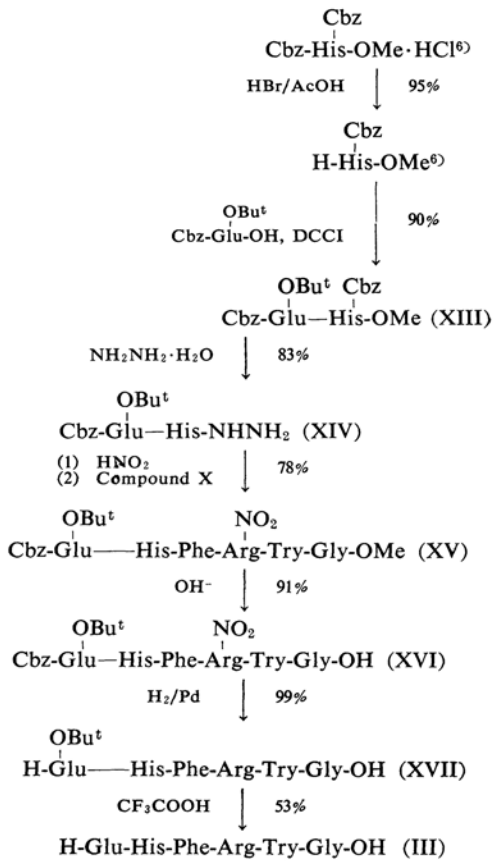

Fig. 2. Scheme for the synthesis of L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine.
Cbz, carbobenzoxy; Me, methyl; Bu^t, *t*-butyl; DCCI, *N*, *N'*-dicyclohexylcarbodiimide

5) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1453 (1960).
6) K. Inouye and H. Otsuka, *J. Org. Chem.*, **27**, 4236 (1962); **26**, 2613 (1961).
7) S. Sakakibara and N. Inukai, This Bulletin, **37**, 1231 (1964).
8) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961); H. Kappeler and R. Schwyzer, ibid., **44**, 1136 (1961).

was obtained in an 83 per cent yield as the monohydrate which melted first at 85—95°C, resolidified at 105—115°C and melted again at 144—146°C. The hydrazide XIV and the phenylalanyl tetrapeptide ester trifluoroacetate X (1.1 : 1.2 molar ratio) were converted into the corresponding azide and the free base, respectively, and these were allowed to react in an acetonitrile-ethyl acetate (2 : 5 by volume) mixture to obtain the protected hexapeptide carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine methyl ester (XV) in an excellent yield (80%). The methyl ester of compound XV was preferentially saponified to give carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine (XVI), which was purified by reprecipitation from dimethylformamide-methanol to remove the unreacted diester XV. The monoacid monoester XVI gave a single round spot (R_f=0.68) on silica gel in the system of dimethylformamide-ethyl acetate (1 : 1 by volume), whereas the diester XV had an R_f value of 0.92 in the same solvent system.

Figure 3 summarizes the present synthesis of the tetrapeptide I, an outline of which has

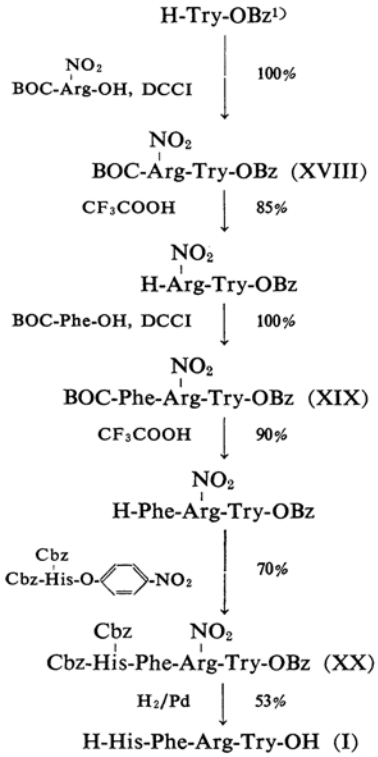

Fig. 3. Scheme for the synthesis of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan.
Cbz, carbobenzoxy; BOC, *t*-butyloxycarbonyl, Bz, benzyl; DCCI, *N*, *N'*-dicyclohexylcarbodiimide.

already been cited in the previous paper dealing with the first synthesis of I.[1] The synthetic steps for each intermediate product are substantially identical with those employed in the course of the synthesis of compound XI. The carbodiimide coupling reactions afforded the protected dipeptide *t*-butyloxycarbonyl-nitro-L-arginyl-L-tryptophan benzyl ester (XVIII) and the tripeptide *t*-butyloxycarbonyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophan benzyl ester (XIX) in quantitative yields; the occurrence of unfavorable side reactions seemed to be slight since they were not detectable by thin-layer chromatography on silica gel. Both of these products, however, resisted all attempts at crystallization. The protected tripeptide XIX was found to be contaminated with a small amount of an impurity which gave an anomalous yellow color reaction with the Ehrlich reagent and which had chromatographic behaviors very similar to the desired product. It appeared most likely that the impurity was derived from a side product which had formed through the trifluoroacetic acid treatment prior to the coupling reaction. Compound XIX was then used in the subsequent reaction without further purification. The reaction of N^{α} N^{Im}-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester (V) with the tripeptide ester, which was derived form XIX in the usual way, was carried out in an acetonitrile solution to obtain the protected tetrapeptide N^{α}, N^{Im}-dicarbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophan benzyl ester (XX), the greater part of which separated in a pure form from the reaction medium. In contrast to the tosylated tetrapeptide derivative N^{α}, N^{Im}-dicarbobenzoxy-L-histidyl-L-phenylalanyl-N^{G}-*tosyl*-L-arginyl-L-tryptophan benzyl ester,[1] which was freely soluble even in ethyl acetate, compound XX had very low solubilities in most of the ordinary solvents.

The carbobenzoxy, nitro, and benzyl ester groups were removed by subsequent catalytic hydrogenolysis to liberate the free tetrapeptide (I) and the free pentapeptide (II) from compounds XX and XII, respectively, and the partially protected hexapeptide (XVII) was obtained from compound XVI in the same way. The free hexapeptide (III) was obtained from XVII by the treatment with trifluoroacetic acid.

Each of these fully deblocked peptide preparations was purified on a carboxymethyl cellulose (CMC) column by means of step-by-step elution with ammonium acetate buffers, where it was found especially in case of the tetrapeptide that a clear-cut separation of the pure material by the CMC column chromatography could be easily performed when the crude sample had previously been purified by partitioning on a

Sephadex column with a solvent system such as 1-butanol - acetic acid - water (4 : 1 : 2 by volume). The results typical of the chromatographic experiments with the tetrapeptide are presented in Figs. 4 and 5. The purified peptides I, II and III gave in each case a single ninhydrin, Pauly, Ehrlich, and Sakaguchi reactive spot on paper chromatography in the different solvent systems and on paper electrophoresis at different pH values. These peptide preparations were also found to be completely digestible by leucine aminopeptidase to give the amino acids in molar ratios consistent with the theoretical values. The biological investigations of these synthetic peptides will be presented in a separate communication in the near future.

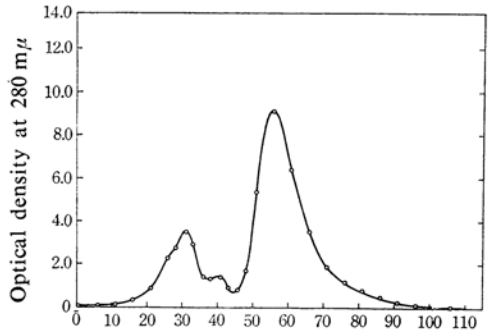

Fig. 4. Partition chromatography of crude L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan on a Sephadex G-25 column.
Material: Synthetic tetrapeptide, 0.350 g.
Column: Sephadex G-25 (coarse), 2.8×105 cm.
Solvent system: 1-Butanol - acetic acid - water (4 : 1 : 2 by vol.)
Flow rate: 0.8 ml./min.

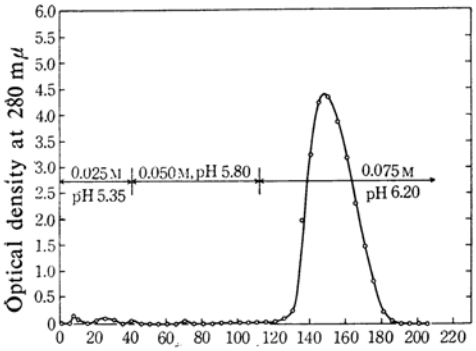

Fig. 5. Rechromatography of purified L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan on a carboxymethyl cellulose column.
Material: Synthetic tetrapeptide, 0.430 g.
Column: CM-cellulose (Serva, 0.44 meq./g.), 1.7×40 cm.
Buffer: Ammonium acetate
Flow rate: 2 ml./min.

Experimental

All melting points were uncorrected.

***t*-Butyloxycarbonyl-nitro-L-arginine (IV).**—Nitro-L-arginine (32.9 g., 0.15 mol.) and 19.1 g. (0.18 mol.) of sodium carbonate were dissolved in 300 ml. of water at 60—70°C and dioxane (300 ml.) was introduced. To this solution was added - dropwise 25.8g. (0.18 mol.) of *t*-butyl azidoformate[9,10] at a bath temperature of 45°C and the mixture was stirred for 18 hr. at the same temperature. The reaction mixture was then cooled in an ice-bath and neutralized with 90.0 ml. of ice-cold 4 N hydrochloric acid (pH 4). After the fine precipitates of unreacted nitro-L-arginine had been removed by filtration (6.0 g., 0.028 mol.) the filtrate was concentrated to about 200 ml. at 40—45°C in vacuo. The concentrated solution was acidified to below pH 2 with ice-cold 4 N hydrochloric acid in the presence of ethyl acetate (300 ml.) at 0°C. The aqueous phase was extracted three times with 150 ml.-portions of cold ethyl acetate. The ethyl acetate extracts were pooled, dried over anhydrous sodium sulfate, and evaporated at 35°C in vacuo. The resultant sirupy residue crystallized upon treatment with 150 ml. of ethyl acetate. After storage in a refrigerator overnight the crystals were filtered off, washed with cold ethyl aetate and ether, and dried at room temperature in vacuo to give the product, which combined about a half mole of ethyl acetate; yield 30.20 g. (68.2% as calculated for 0.122 mol.), m. p. 98—102°C (resolidified at 165—166°C followed by decomposition at 237—238°C). A methanolic solution of the 0.50 g. sample was evaporated in vacuo and the sirupy residue was crystallized from ethyl acetate and air-dried to give a sample for analyses; m. p. 98—100°C, $[\alpha]_D^{25.5}$ +2.8±1° (*c* 2.296, methanol), $[\alpha]_D^{23.5}$ −3.0±1° (*c* 1.614, water), $[\alpha]_D^{23.5}$ −0.7±0.5° (*c* 1.597, acetic acid).

Found: C, 42.67; H, 7.03; N, 19.69; CH_3CO, 5.21. Calcd. for $C_{11}H_{21}N_5O_6 \cdot 1/2CH_3COOC_2H_5$: C, 42.97; H, 6.93; N, 19.27; CH_3CO, 5.92%.

A sample was treated with trifluoroacetic acid followed by neutralization with ammonia to give, in a 93% yield, nitro-L-arginine which had $[\alpha]_D^{25.5}$ +23.9±0.5° (*c* 4.031, 2 N hydrochloric acid); lit.[11] $[\alpha]_D^{25}$ +24.5° (*c* 4.12, 2 N hydrochloric acid).

***N*$^{\alpha}$, *N*$^{\mathrm{Im}}$-Dicarbobenzoxy - L - histidine *p* - Nitrophenyl Ester (V).**—*By the DCCI Method.*—*N*$^{\alpha}$, *N*$^{\mathrm{Im}}$-Dicarbobenzoxy-L-histidine[6] (12.70 g., 0.03 mol.) and 4.18 g. (0.03 mol.) of *p*-nitrophenol were dissolved in 60 ml. of warm ethyl acetate and to this mixture was added an ethyl acetate solution of 6.20 g. (0.03 mol.) of *N*, *N'*-dicyclohexylcarbodiimide (DCCI) at 0°C. The reaction mixture was then

9) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957); L. A. Carpino, C. A. Giza and B. A. Carpino, ibid., **81**, 955 (1959).
10) K. Inouye, M. Kanayama and H. Otsuka, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **85**, 599 (1964).
11) K. Hofmann, W. D. Reckham and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

allowed to stand in a refrigerator overnight. After the separated *N,N'*-dicyclohexylurea had been removed (6.65 g., 98.8%), the filtrate was evaporated in vacuo to afford an amorphous solid mass. The solid residue was dissolved in 90 ml. of hot anhydrous ethanol and the solution was allowed to cool to separate the crystalline *p*-nitrophenyl ester. After storage in a refrigerator overnight the crystals were filtered off, washed with cold ethanol, and dried in vacuo; yield 13.68 g. (84.0%), m. p. 111—112.5°C. Recrystallization from anhydrous ethanol gave fine needles with m. p. 112—113°C, $[\alpha]_D^{24.5}$ −15.0±0.7° (*c* 3.097, ethyl acetate), $[\alpha]_D^{25}$ −13.8±1° (*c* 2.523, chloroform). Lit. amorphous, m. p. 86—90°C;[12] m. p. 109—110°C.[13]

Found: C, 62.04; H, 4.66; N, 10.02. Calcd. for $C_{28}H_{24}N_4O_8$: C, 61.76; H, 4.44; N, 10.29%.

By the Use of Sakakibara's Reagent.[7]—To a solution of 2.117 g. (5 mmol.) of N^{α}, N^{Im}-dicarbobenzoxy-L-histidine[6] in 5 ml. of pyridine was added 1.176 g. (5 mmol.) of *p*-nitrophenyl trifluoroacetate[7] at 0°C, and the mixture was allowed to stand at room temperature for 30 min. Most of the solvent was removed in vacuo at a bath temperature of 40°C; the oily residue was then treated with 15 ml. of anhydrous ethanol. The Amorphous precipitates which formed crystallized slowly. After storage in a refrigerator overnight the crystals were filtered off, washed with anhydrous ethanol, and dried in vacuo; yield 1.904 g. (70.0%), m. p. 104—106°C. Recrystallization from anhydrous ethanol gave, in a recovery of 85%, a sample with a m. p. of 110—111°C; $[\alpha]_D^{24.5}$ −15.9±1° (*c* 2.018, ethyl acetate), $[\alpha]_D^{24.5}$ −13.3±1° (*c* 1.948, chloroform).

Found: C, 61.72; H, 4.55; N, 10.30. Calcd. for $C_{28}H_{24}N_4O_8$: C, 61.76; H, 4.44; N, 10.29%.

Carbobenzoxy-L-tryptophyl-glycine Methyl Ester (VI).—To a suspension of 4.06 g. (0.012 mol.) of carbobenzoxy-L-tryptophan in 40 ml. of methylene chloride was added 2.86 ml. (0.012 mol.) of tri-*n*-butylamine. The resultant clear solution was kept in an ice-bath and to this were added 1.51 g. (0.012 mol.) of glycine methyl ester hydrochloride and a methylene chloride solution of 2.48 g. (0.012 mol.) of DCCI with stirring. The reaction mixture was then allowed to stand at 0°C overnight.*1 The separated dicyclohexylurea was removed by filtration (2.55 g., 94.8%) and the filtrate was evaporated in vacuo to afford a foamy residue, which was redissolved in ethyl acetate. The solution was washed with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate, and finally with water, and dried over anhydrous sodium sulfate. The crystalline precipitate, which separated during concentration of the ethyl acetate solution, was collected to give a yield of 4.69 g. (95.5%); m. p. 153—157°C. Recrystallization from ethyl acetate-ether gave, in a recovery of 92.5%, crystals with m. p. 156—158°C, $[\alpha]_D^{24}$ −12.1±0.7° (*c* 3.058, glacial acetic acid). Lit. m. p. 158—159°C, $[\alpha]_D^{27}$ −11.0° (*c* 2, glacial acetic acid);[14] m. p. 156—158°C, $[\alpha]_D^{22}$ −11.0±0.5° (*c* 3, glacial acetic acid).[15]

Found: C, 64.47; H, 5.84; N, 10.34. Calcd. for $C_{22}H_{23}N_3O_5$: C, 64.54; H, 5.66; N, 10.26%.

***t*-Butyloxycarbonyl-nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester (VII).**—A suspension of 11.25 g. (0.0275 mol.) of compound VI in 180 ml. of methanol containing 3 ml. of acetic acid was hydrogenated over palladium black catalyst for 2.5 hr. After the catalyst had been removed by filtration the filtrate was evaporated in vacuo at 35°C. The resultant foamy residue was dissolved in 50 ml. of water and the aqueous solution was washed with 15 ml. portions of ethyl acetate. A mixture of this aqueous solution and 80 ml. of methylene chloride was shaken with 40 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The aqueous phase was extracted again with methylene chloride. The organic extracts were combined, dried over anhydrous sodium sulfate and concentrated to about 60 ml. at 20°C in vacuo to give a solution of the dipeptide ester. To this was then added a solution of 9.32 g. (0.025 mol.) of compound IV in 25 ml. of dimethylformamide and a methylene chloride solution of 5.68 g. (0.0275 mol.) of DCCI at 0°C. The reaction mixture (total volume ca. 120 ml.) was kept in a refrigerator for 34 hr. The gelatinous precipitate which formed was collected by filtration and washed with cold methylene chloride; the mother liquor and washings were pooled. The precipitate was redissolved in 150 ml. of hot ethyl acetate and the solution was carefully filtered to remove the insoluble urea (5.32 g., 95.0%). The mother liquor was washed successively with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate, and water. The gelatinous precipitate which separated during these washes was collected, washed with water and redissolved in 100 ml. of hot ethyl acetate, and this solution, after being dried quickly over anhydrous sodium sulfate, was combined with the ethyl acetate solution obtained above. The combined solution (total volume 300 ml.) was allowed to stand in a refrigerator overnight and the separated precipitate was filtered off, washed with cold ethyl acetate and with ether, and dried over phosphorus pentoxide at 60°C in vacuo to constant weight; yield 13.21 g. (91.7%), hygroscopic powder, m. p. 111—114°C. Thin-layer chromatography on silica gel in a solvent system of chloroform-methanol (85:15) gave a single round spot (R_f=0.7). A sample was reprecipitated from ethyl acetate, dried as above and stabilized in the air for analyses; m. p. 112—114°C, $[\alpha]_D^{24.5}$ −21.7±0.7° (*c* 3.022, methanol).

Found: C, 51.93; H, 6.68; N, 19.00. Calcd. for $C_{25}H_{36}N_8O_8 \cdot 1/2H_2O$: C, 51.27; H, 6.37; N, 19.14%.

Nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester Trifluoroacetate (VIII).—To a solution of 7.22 g. of the anhydrous compound VII in 7 ml. of glacial acetic acid was added 40 ml. of anhydrous trifluoroacetic acid and the mixture was allowed to

12) F. Sakiyama, This Bulletin, 35, 1943 (1962).
13) J. Meienhofer, *Chimia*, 16, 385 (1962).
*1 At this stage the crystalline dipeptide often separated together with *N,N'*-dicyclohexylurea.
14) K. Hofmann, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, 80, 1486 (1958).
15) R. Geiger, K. Sturm and W. Siedel, *Chem. Ber.*, 96, 1080 (1963).

stand under nitrogen at room temperature for 45 min., after which time 500 ml. of ether was introduced under ice-cooling. The resultant precipitate was collected by filtration, washed thoroughly with ether, and dried to give the crude tripeptide ester trifluoroacetate; yield 6.79 g. (92.0%). This crude preparation was dissolved in 80 ml. of water and the aqueous solution was washed three times with 20 ml. portions of ethyl acetate and then lyophilized, affording a pale yellow fluffy powder (4.93 g.) which gave a single ninhydrin and Ehrlich reactive spot on paper [R_f=0.74 (BAW)]*2; m. p. 122—126°C, $[\alpha]_D^{23.5}$ +16.1±1° (*c* 1.918, methanol).

Found: C, 44.13; H, 5.12; N, 18.78. Calcd. for $C_{20}H_{28}N_8O_6 \cdot CF_3COOH$: C, 44.75; H, 4.95; N, 18.98%.

The ethyl acetate washings were combined and evaporated in vacuo to afford a light brown solid, which gave a minor spot (R_f=0.85) in addition to a major one (R_f=0.74) on paper in the same solvent system as above. The minor component was positive to ninhydrin but it gave an anomalous bluish color reaction with the Ehrlich reagent.

***t*-Butyloxycarbonyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester (IX).**—A solution of 5.02 g. (8.5 mmol.) of compound VIII in 10 ml. of water was mixed with 8 ml. of 1-butanol and 40 ml. of ethyl acetate. The mixture was shaken with 20 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The aqueous phase was extracted again with 1-butanol-ethyl acetate (1:5). The organic extracts were combined, dried over anhydrous sodium sulfate, and evaporated in vacuo. The oily residue was triturated with ether and the resulting white solid was dried over sodium hydroxide pellets in vacuo. The tripeptide ester so obtained was dissolved in acetonitrile together with *t*-butyloxycarbonyl-L-phenylalanine (prepared from 3.795 g. (8.5 mmol.) of the dicyclohexylamine salt)[1] and to this solution was added an acetonitrile solution of 1.75 g. (8.5 mmol.) of DCCI at 0°C. The reaction mixture (total volume ca. 50 ml.) was kept in a refrigerator overnight. The urea which formed was removed (1.73 g., 91.2%) and the solution was evaporated in vacuo. The residue was redissolved in 60 ml. of ethyl acetate and the solution was washed successively with ice-cold N hydrochloric acid, water, M sodium bicarbonate, and water, dried briefly over anhydrous sodium sulfate, and then allowed to stand in a refrigerator overnight. The gelatinous precipitate was filtered off, washed with cold ethyl acetate and ether, and dried in vacuo to afford 5.28 g. (85.9%) of the tetrapeptide, which crystallized readily from hot acetonitrile. Recrystallization from acetonitrile was repeated twice to give colorless needles in a recovery of 80%; m. p. 172—173°C, $[\alpha]_D^{25}$ −20.1±1° (*c* 2.054, methanol).

Found: C, 56.24; H, 6.57; N, 17.35. Calcd. for $C_{34}H_{45}N_9O_9$: C, 56.42; H, 6.27; N, 17.42%.

L-Phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester Trifluoroacetate (X).—To a solution of 1.810 g. of IX in 2 ml. of acetic acid was added 8 ml. of anhydrous trifluoroacetic acid at 0°C and the mixture was kept under nitrogen at room temperature for 45 min., after which time 100 ml. of ether was introduced. The resultant precipitate was filtered off, washed thoroughly with ether, and dried in vacuo to give the tetrapeptide trifluoroacetate as a slightly cream-colored powder; yield 1.765 g. (96.0%). An aqueous solution of a small amount of this crude material was twice washed with ethyl acetate and then lyophilized to give an almost colorless fluffy powder; $[\alpha]_D^{27}$ −1.5 ±1° (*c* 1.384, methanol).

Found: C, 49.27; H, 5.34; N, 16.68. Calcd. for $C_{29}H_{37}N_9O_7 \cdot CF_3COOH \cdot H_2O$: C, 49.79; H, 5.52; N, 16.50%.

***N*$^{\alpha}$, *N*$^{\mathrm{Im}}$-Dicarbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester (XI).**—A 1.476 g. sample (2.0 mmol.) of the crude trifluoroacetate X obtained just above was dissolved in 4 ml. of 1-butanol and 20 ml. of ethyl acetate, and this solution was shaken vigorously with 5 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The separated organic phase was dried over anhydrous sodium sulfate and evaporated in vacuo at 30°C. The oily residue, which solidified upon addition of ether, was dried in vacuo. The ensuing free ester was dissolved in acetonitrile and this was mixed with an acetonitrile solution of 1.090 g. (2.0 mmol.) of *N*$^{\alpha}$, *N*$^{\mathrm{Im}}$-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester (V). The reaction mixture (total volume 22 ml.) was kept in a refrigerator for 56 hr. The precipitate, which had separated during the storage, was filtered off, washed with cold acetonitrile and ether, and dried in vacuo; wt. 0.800 g., m. p. 145—147C (decomp.) (sintering at 131°C). The filtrate and washings were combined and evaporated in vacuo to give a foamy residue, which was dissolved in ethyl acetate and washed with 12 ml. portions of ice-cold N ammonia (4 times). The precipitate, which separated during these washes, was collected, washed with ethyl acetate and water, and dried over phosphorus pentoxide in vacuo; wt. 0.960 g., m. p. 145—146°C decomp. (sintering at 132°C). Total yield amounted to 1.760 g. (85.9%). A suspension of the product (1.760 g.) in 15 ml. of acetone was stirred for several minutes at the boiling point of the solvent and then refrigerated overnight. The precipitate was treated again with hot acetone to obtain the pure pentapeptide; wt. 1.487 g. (recovery 84.5%), m. p. 157—159°C (decomp.) (sintering at 150°C), $[\alpha]_D^{25.5}$ −22.7±0.5° (*c* 3.179, dimethylformamide).

Found: C, 59.25; H, 5.75; N, 16.20. Calcd. for $C_{51}H_{56}N_{12}O_{12}$: C, 59.52; H, 5.49; N, 16.33%.

An additional treatment with hot 90% methanol caused the compound to crystallize as the monohydrate; recovery 97%, m. p. 158—159°C (decomp.) (sintering at 136°C), $[\alpha]_D^{25.5}$ −21.5±0.5° (*c* 2.429, dimethylformamide).

Found: C, 58.27; H, 6.06; N, 16.00. Calcd. for $C_{51}H_{56}N_{12}O_{12} \cdot H_2O$: C, 58.50; H, 5.58; N, 16.05%.

Carbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine (XII).—To a suspension of 0.515 g. (0.5 mmol.) of compound XI in 6 ml. of 90% dioxane was added 1.5 ml. of N sodium

*2 Abbreviation for the solvent systems employed in paper chromatography is as follows: BAW=1-butanol-acetic acid-water (4:1:2 by vol.). BAPW=1-butanol-acetic acid-pyridine-water (30:6:20:24 by vol.).

hydroxide and the mixture was shaken at room temperature for 30 min., after which time ice-cold water (25 ml.) was introduced and the resultant solution was neutralized (pH 4) with 1.5 ml. of N hydrochloric acid. The gelatinous precipitate was filtered off, washed with water, and then reprecipitated from hot acetic acid - water; yield 0.453 g. (98.0%), m. p. 212—213°C (decomp.). A sample for analyses was reprecipitated again from acetic acid - water, dried over phosphorus pentoxide at 80°C and 2—3 mmHg pressure for several hours, and then equilibrated in the air overnight; m. p. 222—227°C (decomp.), $[\alpha]_D^{25.5}$ $-26.0\pm2°$ (*c* 1.065, dimethylformamide). Lit. m. p. 246—249°C, $[\alpha]_D^{26}$ $-29.2°$ (*c* 1.2, dimethylformamide);[16] m. p. 226—228°C (decomp.), $[\alpha]_D^{22}$ $-26.8\pm0.5°$ (*c* 3, dimethylformamide).[15]

Found: C, 54.79; H, 5.71; N, 18.75; H_2O, 4.60; CH_3CO, 0.00. Calcd. for $C_{42}H_{48}N_{12}O_{10}\cdot2.5H_2O$: C, 54.48; H, 5.77; N, 18.15; H_2O, 4.86; CH_3CO, 0.00%.

Carbobenzoxy-γ-*t*-butyl-L-glutamyl-N^{1m}-carbobenzoxy-L-histidine Methyl Ester (XIII).—To a solution of 4.74 g. (0.01 mol.) of N^{α}, N^{1m}-dicarbobenzoxy-L-histidine methyl ester hydrochloride[6,17] in 10 ml. of glacial acetic acid was added 30 ml. of 33% (w/w) hydrogen bromide in acetic acid and the mixture was kept at room temperature for 15 min. with occasional shaking. At the end of this period anhydrous ether (200 ml.) was introduced and the resultant precipitate was filtered off, washed with methylene chloride and with ether, and dried in vacuo to give the dihydrobromide of N^{1m}-carbobenzoxy-L-histidine methyl ester;[6] wt. 4.40 g. (94.7%). To an aqueous solution of this product in 30 ml. of water were added 60 ml. of methylene chloride and 4 ml. of ice-cold 28% aqueous ammonia, and the mixture was shaken vigorously at 0°C. The aqueous phase was extracted again with cold methylene chloride. The organic extracts were pooled, dried over anhydrous sodium sulfate at 0°C and evaporated in vacuo at a bath temperature of 25°C. The resultant sirupy residue was dissolved in methylene chloride together with carbobenzoxy-γ-*t*-butyl-L-glutamic acid (prepared from 4.15 g. (0.008 mol.) of the dicyclohexylamine salt by treatment with Dowex-50)[8] and to this was added a methylene chloride solution of 1.65 g. (0.008 mol.) of DCCI at 0°C. The reaction mixture was then allowed to stand in a refrigerator overnight. After removal of the separated urea (1.73 g., 96.6%) the solvent was evaporated in vacuo. The sirupy residue was dissolved in ethyl acetate and the solution was washed successively with ice-cold N hydrochloric acid, water, ice-cold 5% sodium bicarbonate, and with water, dried over anhydrous sodium sulfate, and evaporated to afford a sirupy residue, which crystallized from ethyl acetate - petroleum ether; yield 4.57 g. (91.8%), m. p. 100—102°C. Recrystallization from the same solvent gave 4.48 g. (90.0%); m. p. 101—102°C, $[\alpha]_D^{24}$ $-6.1\pm1°$ (*c* 2.475, methanol).

Found: C, 61.87; H, 6.30; N, 9.38. Calcd. for $C_{32}H_{38}N_4O_9$: C, 61.75; H, 6.15; N, 9.00%.

Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidine Hydrazide (XIV).—Compound XIII (12.45 g., 0.02 mol.) was suspended in 60 ml. of anhydrous ethanol and to this was added 6.0 ml. of hydrazine hydrate at 0°C. The reaction mixture was stirred at 0°C until the solid disappeared within several minutes. After the mixture had been allowed to stand in a refrigerator for 68 hr., water (240 ml.) was introduced. The ensuing sirupy precipitate soon crystallized and the crystals were filtered off, washed with cold water and dried; wt. 8.51 g. Concentration of the mother liquor gave an additional 0.735 g. of crystals; total yield amounted to 9.245 g. (91.3%). These crystals were recrystallized from ethanol-water (1 : 4) to give a yield of 8.425 g. (83.2%). The pure sample melted first at 85—95°C, resolidified at 105—115°C, and melted again at 144—146°C, $[\alpha]_D^{23}$ $-41.0\pm1°$ (*c* 2.075, N hydrochloric acid). Lit.[8] m. p. 140—142°C (sintering at 75°C), $[\alpha]_D^{26}$ $-40.5\pm0.5°$ (*c* 1.91, N hydrochloric acid).

Found: C, 54.40; H, 6.98; N, 16.71. Calcd. for $C_{23}H_{32}N_6O_6\cdot H_2O$: C, 54.53; H, 6.77; N, 16.59%.

Refrigeration of the mother liquor, from which the second crop of the compound XIV had been separated, afforded thin needles of *benzyl carbazate*; wt. 1.40 g., m. p. 62—66°C.

Found: C, 57.71; H, 6.21; N, 16.95. Calcd. for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86%.

Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine Methyl Ester (XV).—To a solution of 5.57 g. (0.011 mol.) of the hydrazide XIV in ca. 10 ml. of dimethylformamide, which had been previously cooled in an ice-salt bath, were added 45 ml. of ice-cold N hydrochloric acid and 6.05 ml. (0.012 mol.) of ice-cold 2 M sodium nitrite, and the mixture was stirred for 4 min., after which time 45 ml. of ice-cold M sodium carbonate and 30 ml. of ice-cold saturated sodium chloride were added. The ensuing precipitate of the azide was taken up into ice-cold ethyl acetate. The ethyl acetate extracts were pooled, dried over anhydrous sodium sulfate, and mixed with a solution of L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine methyl ester (prepared from 8.87 g. (0.012 mol.) of the tritfluoroacetate X in the same manner as described in the synthesis of XI) in 95ml. of acetonitrile. The reaction mixture (total volume 350 ml.) was then kept at 0—4°C for 48 hr. The gelatinous precipitate, which formed, was filtered off, washed with ethyl acetate and with ether, and dried; yeild 9.52 g. (80.1%), m. p. 183—186°C (sintering at 173°C). The crude product (9.42 g.) was suspended in 110 ml. of acetonitrile and this was stirred for several minutes at the boiling point of the solvent to afford a homogeneous mixture, which was allowed to stand in a refrigerator overnight. The precipitate was filtered off, washed with acetonitrile and with ether, and dried to give the pure hexapeptide derivative; wt. 9.21 g. (recovery 97.8%), R_f=0.92 in thin-layer chromatography on silica gel in the system of dimethylformamide - ethyl acetate (1 : 1 by vol.), m. p. 185—186°C (sintering at 180°C), $[\alpha]_D^{27.5}$ $-33.0\pm1°$ (*c* 1.999, dimethyl-

16) K. Hofmann and S. Lande, *J. Am. Chem. Soc.*, **83**, 2286 (1961).
17) A. Patchornik, A. Berger and E. Katchalski, ibid., **79**, 6416 (1957).

formamide), $[\alpha]_D^{27.5}$ $-33.1\pm4°$ (*c* 0.592, methanol). Lit.[8] m. p. 176—178°C (sintering at 170°C), $[\alpha]_D$ $-30.9\pm0.5°$ (*c* 2.01, methanol).

Found: C, 56.83; H, 6.24; N, 16.83. Calcd. for $C_{52}H_{65}N_{13}O_{13}\cdot H_2O$: C, 56.87; H, 6.15; N, 16.58%.

Carbobenzoxy-γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophyl-glycine (XVI).—Compound XV (3.85 g., 3.5 mmol.) was dissolved in 50 ml. of 75% dioxane and to this was added 4.0 ml. of N sodium hydroxide. The mxiture was shaken at room temperature for 30 min., after which time ice-cold water (300 ml.) was introduced. The solution was then neutralized with 4.15 ml. of N hydrochloric acid to separate the desired product as a gelatinous precipitate. After refrigeration for a few hours the precipitate was filtered off, washed with water and dried to give 3.76 g. (99.2%), m. p. 215—216.5°C (decomp). (sintering at 190°C). The precipitate was reprecipitated from 15 ml. of dimethylformamide and 105 ml. of methanol; yield 3.44 g. (90.8%), m. p. 217—218°C (decomp.). A small sample was reprecipitated from a large volume of 90% methanol resulting in partial crystallization as needles; R_f= 0.68 in thin-layer chromatography on silica gel in the system of dimethylformamide-ethyl acetate (1 : 1 by vol.), m. p. 217°C (decomp.), $[\alpha]_D^{26.5}$ $-26.8\pm1°$ (*c* 2.141, dimethylformamide), lit.[8] m. p. 210°C, $[\alpha]_D$ $-22.2\pm1°$ (*c* 1.056, dimethylformamide).

Found: C, 56.12; H, 6.46; N, 16.46; H_2O, 2.89. Calcd. for $C_{51}H_{63}N_{13}O_{13}\cdot1.5H_2O$: C, 56.03; H, 6.09; N, 16.66; H_2O, 2.47%.

γ-*t*-Butyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine Monoacetate (XVII).—A solution of 1.084 g. (1.0 mmol.) of XVI in 40 ml. of 90% acetic acid was stirred under hydrogen in the presence of palladium-black catalyst for 17 hr. Fresh catalyst was added after 6 hr. of hydrogenolysis. The catalyst was removed from the clear solution which was then evaporated at a bath temperature of 40—45°C, and the resultant oily residue was dissolved in a small volume of methanol. The peptide was precipitated by the addition of ether, lyophilized, and dried over phosphorus pentoxide and sodium hydroxide pellets at room temperature in vacuo; yield 0.977 g. (99.4%). A sample for analyses was dried over phosphorus pentoxide at 60°C and 2—3 mmHg pressure for 8 hr. and then equilibrated in the air overnight. Paper chromatography: R_f=0.60 (BAPW); 0.375 (BAPW) after the trifluoroacetic acid treatment. $[\alpha]_D^{27}$ $-10.3\pm4°$ (*c* 0.624, dimethylformamide); lit.[15] $[\alpha]_D^{20}$ $-13.5\pm1°$ (*c* 1, dimethylformamide).

Found: C, 54.26; H, 7.30; N, 17.13; CH_3CO, 4.16. Calcd. for $C_{43}H_{58}N_{12}O_9\cdot CH_3COOH\cdot2.5H_2O$: C, 54.48; H, 6.81; N, 16.94; CH_3CO, 4.34%.

***t*-Butyloxycarbonyl-nitro-L-arginyl-L-tryptophan Benzyl Ester (XVIII).**—A 4.00 g. sample (0.011 mol.) of *t*-butyloxycarbonyl-nitro-L-arginine (IV) was dissolved in 30 ml. of hot acetonitrile and the solution was cooled in an ice-bath. To this were added 3.24 g. (0.011 mol.) of L-tryptophan benzyl ester[1] and an acetonitrile solution of 2.27 g. (0.011 mol.) of DCCI, and the mixture was kept in a refrigerator overnight. The urea which formed was removed by filtration (2.28 g., 92.7%) and the filtrate was evaporated in vacuo. The resultant foamy residue was dissolved in ethyl acetate and this was washed successively with ice-cold N hydrochloric acid, water, M sodium bicarbonate and water, dried over anhydrous sodium sulfate and evaporated in vacuo. The residue was redissolved in a small volume of acetonitrile and a small amount of urea, which separated upon chilling, was filtered off. Evaporation of the solvent gave a foamy residue, which was dried over phosphorus pentoxide in vacuo to give an amorphous powder; yield 6.77 g. (100%), homogeneous to the Ehrlich reagent in thin-layer chromatography on silica gel in ethyl acetate, $[\alpha]_D^{22}$ $-4.2\pm1°$ (*c* 2.565, methanol).

Found: C, 59.16; H, 6.61; N, 15.61. Calcd. for $C_{29}H_{37}N_7O_7$: C, 58.48; H, 6.26; N, 16.46%.

The above analysis suggests that the product is contaminated with a small amount of urea, but it was utilized in the subsequent reaction without further purifiaction.

***t*-Butyloxycarbonyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophan Benzyl Ester (XIX).**—Compound XVIII (4.47 g., 7.5 mmol.) was dissolved in 4 ml. of acetic acid and to this solution was added 25 g. of anhydrous trifluoroacetic acid. The mixture was allowed to stand at room temperature for 60 min. under dry nitrogen. The dipeptide ester trifluoroacetate was then precipitated by the addition of 200 ml. of ether. The precipitate was filtered off, washed with cold methylene chloride and ether, and dried in vacuo; wt. 3.89 g. (85.2%), $[\alpha]_D^{25}$ $+11.6\pm1.5°$ (*c* 1.409, methanol).

Found: N, 15.91. Calcd. for $C_{24}H_{29}N_7O_5\cdot CF_3COOH$: N, 16.09%.

A solution of 3.74 g. (6.14 mmol.) of the trifluoroacetate in 50 ml. of 1-butanol-methylene chloride (1 : 3 by vol.) was cooled in an ice-bath and shaken with 10 ml. of 50% (w/v) potassium carbonate. The organic phase was dried over sodium sulfate and evaporated in vacuo to afford a sirupy residue, which was triturated and washed thoroughly with ether, and then dried in vacuo to give nitro-L-arginyl-L-tryptophan benzyl ester (3.19 g.). This free ester was then dissolved in 20 ml. of acetonitrile containing *t*-butyloxycarbonyl-L-phenylalanine (prepared from 2.90 g. (6.5 mmol.) of the dicyclohexylamine salt)[1] and to this was added an acetonitrile solution of 1.34 g. (6.5 mmol.) of DCCI at 0°C. The reaction mixture was allowed to stand in a refrigerator overnight. The dicyclohexylurea, which formed, was removed by filtration (1.34 g., 92.1%) and the filtrate was evaporated in vacuo to give an amorphous solid residue; the subsequent treatment was carried out in the same manner as for XVIII. Yield 4.78 g. (100%), $[\alpha]_D^{25}$ $-11.8\pm1°$ (*c* 2.272, methanol). Thin-layer chromatography on silica gel in ethyl acetate revealed the presence of a small amount of an impurity which moved ahead of and overlapped the desired tripeptide, and which gave a yellow color reaction with the Ehrlich reagent.

Found: C, 62.06; H, 6.73; N, 14.11. Calcd. for $C_{38}H_{46}N_8O_8$: C, 61.44; H, 6.24; N, 15.09%.

N^{α}, N^{1m}-Dicarbobenzoxy-L-histidyl-L-phenylalanyl-nitro-L-arginyl-L-tryptophan Benzyl Ester

(XX).—To a solution of 1.860 g. (2.5 mmol.) of XIX in 2 ml. of acetic acid was added 8 ml. of anhydrous trifluoroacetic acid and the mixture was allowed to stand at room temperature for 45 min. under nitrogen. To this was then added 80 ml. of ether with ice-cooling; the resultant precipitate was collected, washed thoroughly with ether, and dried in vacuo to give 1.695 g. (89.8%) of the tripeptide ester trifluoroacetate. A suspension of the trifluoroacetate (1.695 g., 2.24 mmol.) in 20 ml. of ethyl acetate was shaken with 5 ml. of ice-cold 50% (w/v) potassium carbonate at 0°C. The organic phase was separated, dried over sodium sulfate and evaporated in vacuo. The resultant foamy residue was dissolved in 10 ml. of acetonitrile and this solution was combined with a solution of 1.220 g. (2.24 mmol.) of N^{α}, N^{Im}-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester (V) in 15 ml. of acetonitrile and the mixture was then kept in a refrigerator for 44 hr. The precipitate which formed was filtered off, washed with cold ethyl acetate and with ether, and dried in vacuo; wt. 1.244 g. Evaporation of the filtrate gave a foamy residue, which was washed in an ethyl acetate solution with 12 ml. portions of ice-cold N ammonia (6 times), ice-cold N hydrochloric acid and with water, dried quickly over anhydrous sodium sulfate, and concentrated to about 15 ml. in vacuo. The precipitate, which separated during storage in a refrigerator, was collected and amounted to 0.699 g.; total yield 1.943 g. (82.7%), m. p. 161—163°C (decomp.). A suspension of the product in 20 ml. of ethyl acetate was stirred for several minutes at the boiling point of the solvent and then kept in a refrigerator overnight. The precipitates (1.686 g., m. p. 164—165°C (decomp.)) were treated again with hot ethyl acetate to afford the pure protected tetrapeptide; wt. 1.650 g. (recovery 85.0%), m. p. 167—168°C (decomp.), $[\alpha]_D^{24.5}$ $-12.3\pm1°$ (*c* 2.080, dimethylformamide).

Found: C, 63.25; H, 5.59; N, 14.78. Calcd. for $C_{55}H_{57}N_{11}O_{11}$: C, 63.03; H, 5.48; N, 14.70%.

L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophan Monoacetate (I).—The protected tetrapeptide XX (0.500 g.) was dissolved in 20 ml. of 90% acetic acid and the solution was stirred under hydrogen in the presence of palladium black for 18 hr. After the catalyst had been removed the solvent was evaporated at a bath temperature of 45°C and the residue was lyophilized twice to give 0.375 g. of fluffy powder. This crude tetrapeptide (0.350 g.) was dissolved in 5 ml. of a mixture 1-butanol - acetic acid - water (4 : 1 : 2 by vol.) and the solution was applied onto a column (2.8×105 cm.) of Sephadex G-25 (coarse, dry wt. 180 g.), which had been equilibrated with the BAW mixture. The column was then eluted with the same solvent system at a flow rate of 0.8 ml. per min.; 10 ml. fractions were collected. The chromatogram is shown in Fig. 4. The fractions in Tubes No. 51—95 were pooled and evaporated at 50°C in vacuo. The residue was then lyophilized twice to afford 0.430 g. (recovery 57% as calculated from the absorbance at 280 mμ) of fluffy powder. This purified peptide was next submitted to ion-exchange chromatography for further purification. The partially purified material (0.43 g.) was dissolved in 10 ml. of 0.025 M ammonium acetate (pH 5.35) and the solution was applied onto a column (1.7×40 cm.) of carboxymethyl cellulose (Serva, 0.44 meq./g.) which had been equilibrated with the same buffer solution. The column was then eluted successively with the following ammonium actate buffers: 0.025 M (pH 5.35), 400 ml.; 0.050 M (pH 5.80), 700 ml. (0.075 M (pH 6.20), 1000 ml. Individual fractions (10 ml. each) were collected at a flow rate of approximately 2 ml. per min. The chromatographic pattern is illustrated in Fig. 5. The fractions in Tubes No. 131—185 corresponding to the major peak were pooled and the bulk of water was removed at 50—55°C using a rotatory evaporator. The rest was then lyophilized three times affording a colorless fluffy powder; yield 0.188 g. (53% as based on XX used). The purified peptide behaved as a single ninhydrin, Pauly, Ehrlich, and Sakaguchi reactive spot in paper chromatography [R_f=0.48 (BAW), 0.49 (BAPW)], and in paper electrophoresis at pH 3.8, 6.6 and 11.1. A sample for analyses was dried over phosphorus pentoxide at 60°C and 2—3 mmHg pressure for 8 hr. and then equilibrated in the air overnight. $\lambda_{max}^{0.1N\ HCl}$=280 mμ (ε=5450), 288 mμ (ε=4600); $\lambda_{max}^{0.1N\ NaOH}$=280 mμ (ε=5500), 288 mμ (ε=4700). $[\alpha]_D^{25}$ $-7.8\pm1.5°$ (*c* 1.484, N hydrochloric acid).*3 Amino acid ratios in acid hydrolyzate: His 1.00, Phe 0.91, Arg 0.95, Try 0.71 (decomposed partially with acid); amino acid ratios in leucine aminopeptidase*4 digest: His 1.00, Phe 0.94, Arg 0.94, Try 0.95.[18]

Found: C, 52.30; H, 6.56; N, 18.46. Calcd. for $C_{32}H_{40}N_{10}O_5\cdot CH_3COOH\cdot 4H_2O$: C, 52.57; H, 6.75; N, 18.03%.

L-Histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine Monoacetate (II).—A suspension of 0.250 g. of XII in 20 ml. of 90% acetic acid was stirred under hydrogen in the presence of palladium-black catalyst for 14 hr. The catalyst was removed by filtration and the filtrate was evaporated at a bath temperature of 40—45°C in vacuo. The resultant oily residue was lyophilized and dried over phosphorus pentoxide and sodium hydroxide pellets affording 0.220 g. of fluffy powder. This crude pentapeptide was dissolved in 25 ml. of 0.025 M ammonium acetate (pH 5.35) and the solution was aplied to a column (1.2×50 cm.) of carboxymethyl cellulose (Serva, 0.6 meq./g.) which had been equilibrated with the same buffer solution. The column was then eluted successively with the following ammonium acetate buffers: 0.025 M (pH 5.35), 300 ml.: 0.05 M (pH 5.80), 600 ml.; 0.075 M (pH 6.20), 1600 ml. Individual fractions (10 ml. each) were collected at a flow rate of 2 ml. per min. Absorbancy measurements at 280 mμ revealed the presence of one major peak in Tubes No. 201—235 in the chromatogram. These fractions

*3 In our preceding papers the value, $[\alpha]_D^{24.5}$ $-5.4\pm2°$ (*c* 0.947, N hydrochloric acid), was given for this peptide as a result of a single measurement.[1] The present result appeared more reliable than the former, because the value was reproducible in repeated measurements.

*4 LAP Lot No. 5930, Worthington Biochemical Corporation, Freehold, New Jersey, U. S. A.

18) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1191 (1958).

corresponding to the major peak were pooled and the bulk of water was removed at a bath temperature of 50—55°C in vacuo resulting in the separation of a gelatinous precipitate of the peptide. The rest was lyophilized three times to give a colorless fluffy powder; yield 0.125 g. (58% as based on XII used). The purified material behaved as a single nihydrin, Pauly, Ehrlich, and Sakaguchi reactive spot in paper chromatography [R_f=0.38 (BAW), 0.41 (BAPW)], and in paper electrophoresis at pH 3.8, 6.6 and 11.1. A sample for analyses was dried over phosphorus pentoxide at 60°C and 2—3 mmHg. pressure for 8 hr. and equilibrated in the air overnight. $\lambda_{max}^{0.1N\ HCl}$=280 mμ (ε=5500), 288 mμ (ε=4650); $\lambda_{max}^{0.1N\ NaOH}$=280 mμ (ε=5650), 288 mμ (ε=4800). $[\alpha]_D^{26.5}$ −11.9±3° (*c* 0.606, N hydrochloric acid); lit. $[\alpha]_D^{26}$ −11.8° (*c* 0.9, N hydrochloric acid),[16] $[\alpha]_D^{22}$ −11.7±0.5° (*c* 1, N hydrochloric acid).[15] Amino acid ratios in acid hydrolyzate: His 1.00, Phe 0.90, Arg 0.97, Try 0.73 (decomposed patially with acid), Gly 0.98; amino acid ratios in leucine aminopeptidase*4 digest: His 1.00, Phe 0.92. Arg 0.91, Try 0.92, Gly 0.93.[18]

Found: C, 51.71; H, 6.88; N, 18.12; CH_3CO, 6.81; H_2O, 8.62. Calcd. for $C_{34}H_{43}N_{11}O_6 \cdot CH_3COOH \cdot 4H_2O$: C, 51.85; H, 6.65; N, 18.48; CH_3CO, 5.61; H_2O, 8.64%. Calcd. for $C_{34}H_{43}N_{11}O_6 \cdot 1.5CH_3COOH \cdot 4H_2O$: C, 51.44; H, 6.65; N, 17.84; CH_3CO, 7.47; H_2O, 8.34%.

L-Glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine (III).—An 0.250 g. sample of XVII was dissolved in 4 ml. of anhydrous trifluoroacetic acid and the solution was allowed to stand at room temperature for 30 min., after which time, ether was introduced to precipitate the hexapeptide trifluoroacetate. The precipitate was dissolved in a few milliliters of water and the solution was passed through a small Amberlite IR-45 column (acetate form). The column was washed with water and the eluate were pooled and lyophilized, affording the hexapeptide; wt. 0.234 g. This crude peptide was dissolved in 20 ml. of 0.025 M ammonium acetate (pH 5.35) and the solution was added to a column (1.2×50 cm.) of carboxymethyl cellulose (Serva, 0.6 meq./g.) which had been equilibrated with the same buffer solution. The column was then eluted successively with the following ammonium acetate buffers: 0.025 M (pH 5.35), 450 ml.; 0.0375 M (pH 5.35), 1600 ml.; 0.050 M (pH 6.45), 600 ml. Individual fractions (10 ml. each) were collected at a flow rate of approximately 2 ml. per min. Absorbancy measurements at 280 mμ revealed the presence of one major peak in Tubes No. 146—190 in the chromatogram. These fractions corresponding to the major peak were pooled and the bulk of water was removed at a bath temperature of 50—55°C in vacuo. The rest was lyophilized three times affording a colorless fluffy powder; yield 0.199 g. (86% as based on XVII used). This material was twice reprecipitated from water-ethanol to give 0.123 g. (recovery 62%). The purified peptide behaved as a single ninhydrin, Pauly, Ehrlich, and Sakaguchi reactive spot in paper chromatography [R_f=0.30 (BAW), 0.25 (BAPW)], and in paper electrophoresis at pH 3.8, 6.6 and 11.1. A sample for analyses was dried over phosphorus pentoxide at 60°C and 2—3 mmHg pressure for 8 hr. and then equilibrated in the air overnight. $\lambda_{max}^{0.1N\ HCl}$=280.5 mμ (ε=5500), 288 mμ (ε=4700); $\lambda_{max}^{0.1N NaOH}$=280.5 mμ (ε=5600), 288 mμ (ε=4800). $[\alpha]_D^{26.5}$ −18.0±3° (*c* 0.621, N acetic acid); lit. $[\alpha]_D$ −15.1±1° (*c* 0.1042, N acetic acid),[8] $[\alpha]_D^{25}$ −17.3° (*c* 1, acetic acid).[19]

Amino acid ratios in acid hydrolyzate: Glu 1.00, His 1.04, Phe 0.97, Arg 1.07, Try 0.71 (decomposed partially with acid), Gly 1.00; amino acid ratios in leucine aminopeptidase*4 digest: Glu 1.00, His 1.11, Phe 1.03, Arg 1.03, Try 1.02, Gly 0.96.

Found: C, 51.15; H, 6.92; N, 18.13; H_2O, 9.66. Calcd. for $C_{39}H_{50}N_{12}O_9 \cdot 5H_2O$: C, 50.86; H, 6.57; N, 18.25; H_2O, 9.78%.

Summary

1) Synthesis of the peptides L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophan, L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine and L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycine, which corresponded to the amino acid sequences of positions 6—9, 6—10 and 5—10, respectively, of the corticotropin and α-MSH molecules, has been described.

2) The synthetic procedure for γ-*t*-butyl-L-glutamyl-L-histidyl-L-phenylalanlyl-L-arginyl-L-tryptophyl-glycine, a key intermediate to the higher peptides eliciting the adrenocorticotropic activity, has been improved.

3) An active ester N^{α}, N^{Im}-dicarbobenzoxy-L-histidine *p*-nitrophenyl ester has been found to be of value for the synthesis of histidyl peptides.

4) An effective use of partition chromatography on a Sephadex column for purification of peptides has been cited.

The author expresses his deep gratitude to Dr. Hideo Otsuka for unfailing guidance throughout the course of this work. Thanks are also due to Dr. Mitsuo Ebata for amino acid analyses, Dr. Kaoru Kuriyama and staff for optical rotation measurements, Dr. Satoshi Mizukami and staff for elemental microanalyses, and Mr. Makoto Kanayama, Miss Fusako Shinozaki and Miss Yoshiko Jono for valuable technical assistance.

Biochemistry Division
Shionogi Research Laboratory
Shionogi & Co., Ltd.
Fukushima-ku, Osaka

19) C. H. Li, B. Gorup, D. Chung and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).