BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 39 882—888 (1966)

The Syntheses of Peptides Related to the *N*-Terminal Structure of Corticotropin. VII. The Synthesis of Peptide Derivatives Corresponding to Positions 11 to 18 in the Corticotropin Molecule

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(Received August 27, 1965)

The derivatives of lysyl-prolyl-valyl-glycyl-arginyl-arginine, lysyl-prolyl-valyl-glycyl-lysyl-arginyl-arginine and lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine, which correspond to the amino acid sequences in positions 11—14 plus 17—18, 11—14 plus 16—18, and 11—14 plus 11—18 of the corticotropin molecule respectively, have been obtained as intermediates for the synthesis of adrenocorticotropically active peptides.

As a step toward our synthesis of the adrenocorticotropically active peptides involving a hexadecapeptide $\alpha^{1-14,17-18}$-Gly^1-ACTH,[1] a heptadecapeptide $\alpha^{1-14,16-18}$-Gly^1-ACTH-18-amide,[2] and octadecapeptides α^{1-18}-ACTH and α^{1-18}-ACTH-18-amide,[3] we prepared the peptide intermediates

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[1] H. Otsuka, K. Inouye, M. Kanayama and F. Shinozaki, This Bulletin, **38**, 679 (1965).

[2] H. Otsuka, K. Inouye, M. Kanayama and F. Shinozaki, ibid., **38**, 1563 (1965).

[3] H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, *J. Biochem.*, **58**, 512 (1965).

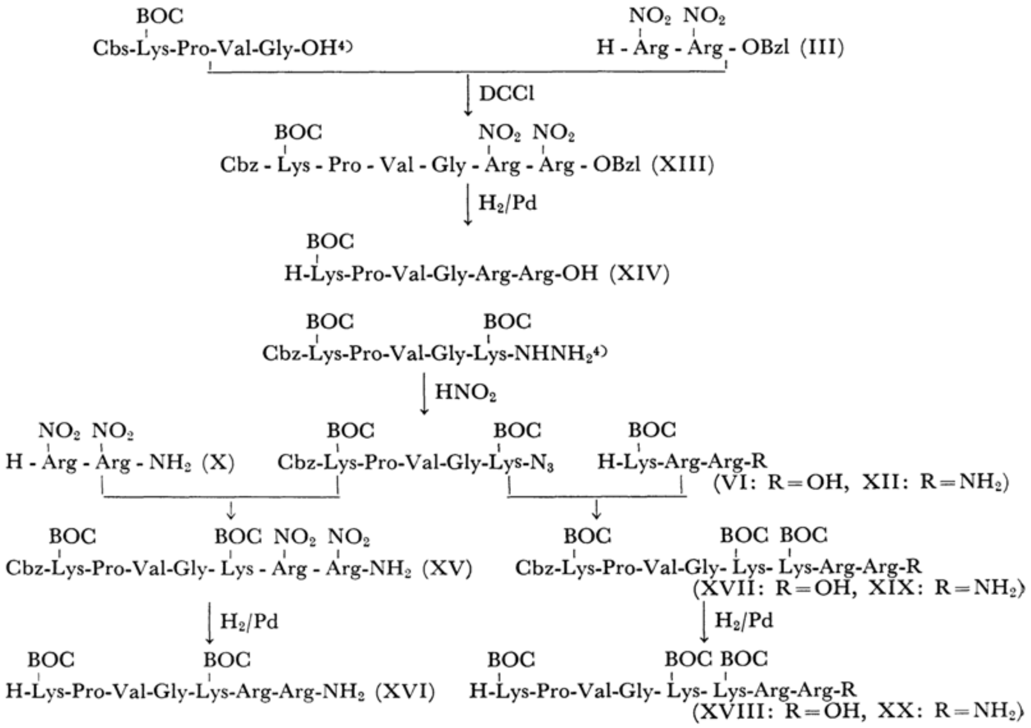


Fig. 1. Outline of the synthetic procedure for the hexapeptide, heptapeptide and octapeptide derivatives. Cbz, carbobenzoxy; BOC, *t*-butyloxycarbonyl; Bzl, Benzyl; DCCl, *N*, *N'*-dicyclohexylcarbodiimide.

corresponding to the natural and the partially-altered sequences of positions 11 to 18 in the corticotropin (ACTH) structure; namely, N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-arginyl-arginine (XIV), N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide (XVI), and N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine (XVIII) and its amide (XX).[*1]

An outline of the synthetic procedure is shown in Fig. 1. The heptapeptide (XV) and the octapeptides (XVII and XIX) were built by the idea of the coupling of Lys-Pro-Val-Gly-Lys with Arg-Arg and with Lys-Arg-Arg respectively, while the hexapeptide (XIII) was prepared from Lys-Pro-Val-Gly and Arg-Arg. The synthesis of the sequences of Lys-Pro-Val-Gly and Lys-Pro-Val-Gly-Lys has already been reported.4) Therefore, we wish to describe here the synthesis of arginyl-arginine and lysyl-arginyl-arginine fragments. We wish also to describe in detail the procedures for preparing the longer peptides from them.

The Synthesis of the Hexapeptide Derivative (XIV).—Attempts to synthesize the nitroarginine benzyl ester were made by three different methods as follows:

(1) $\text{H-Arg(NO}_2\text{)-OH} + \text{C}_6\text{H}_5\text{CH}_2\text{OH}$

$\xrightarrow{\text{CH}_3\text{C}_6\text{H}_4\text{SO}_3\text{H}} \text{H-Arg(NO}_2\text{)-OBzl}\cdot\text{CH}_3\text{C}_6\text{H}_4\text{SO}_3\text{H}$

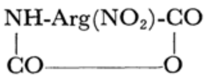
(2) $\text{BOC-Arg(NO}_2\text{)-OH} + \text{C}_6\text{H}_5\text{CH}_2\text{Cl}$

$\xrightarrow{\text{triethylamine}} \text{BOC-Arg(NO}_2\text{)-OBzl}$

$\xrightarrow{\text{CF}_3\text{COOH}} \text{H-Arg(NO}_2\text{)-OBzl}\cdot\text{CF}_3\text{COOH}$

(3) $\text{Cbz-Arg(NO}_2\text{)-OH}$

$\xrightarrow[\text{in dioxane}]{\text{PCl}_5}$



$$\begin{array}{l} \text{NH-Arg(NO}_2\text{)-CO} \\ \vert \qquad\qquad\qquad \vert \\ \text{CO}\text{———————O} \end{array}$$

$\xrightarrow{\text{HCl/C}_6\text{H}_5\text{CH}_2\text{OH}} \text{H-Arg(NO}_2\text{)-OBzl}\cdot\text{HCl}$

In each case, no salt of the ester was crystallized. Of these methods, the first one was found to be the most convenient and to give a moderate yield. The *t*-butyloxycarbonyl-nitroarginine benzyl ester, which was obtained as an intermediate in the second method, was homogeneous in thin-layer chromatography (R_f=0.61, ethyl acetate-methanol (95 : 5)), but it also resisted all attempts at crystallization. In the third method, it seemed that the nitroguanidino function did not decompose upon the treatment with phosphorus pentachloride.

The nitroarginine benzyl ester *p*-toluenesulfonate (I) was, after having been converted into the free

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[*1] All amino acid residues are of the L-configuration, with the exception of glycine.

4) H. Otsuka, K. Inouye and Y. Jono, This Bulletin, **37**, 1471 (1964).

base, combined with *t*-butyloxycarbonyl-nitroarginine to give the *t*-butyloxycarbonyl-nitroarginyl-nitroarginine benzyl ester (II), which failed to crystallize. Compound II was, however, found by thin-layer chromatography to be homogeneous. The *t*-butyloxycarbonyl group was removed with the trifluoroacetic acid treatment; the nitroarginyl-nitroarginine benzyl ester trifluoroacetate (III) was thus obtained. This was then coupled with N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine[4] by the *N*, *N'*-dicyclohexylcarbodiimide (DCCl) procedure, thus affording a hexapeptide derivative, the N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-nitroarginyl-nitroarginine benzyl ester (XIII). Compound XIII crystallized with a 90 per cent yield. The catalytic hydrogenolysis of XIII gave a partially protected hexapeptide N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-arginyl-arginine diacetate (XIV), which was homogeneous in paper chromatography.

The Synthesis of the Heptapeptide Amide Derivative (XVI).—Carbobenzoxy-nitroarginine was treated with ethylchloroformate and tri-*n*-butylamine in a tetrahydrofuran solution in order to convert it into a mixed anhydride, which was then allowed to react with a small excess of aqueous ammonia to produce carbobenzoxy-nitroarginine amide (VII) in an 81 per cent yield. Compound VII crystallized readily from aqueous ethanol and had a melting point of 220—221°C. Van Orden and Smith gave a m. p. of 219—220°C for the same compound.[5] The treatment of VII with hydrogen bromide in acetic acid gave the crystalline monohydrobromide of nitroarginine amide (VIII) in an 85 per cent yield. Carbobenzoxy-nitroarginine and VIII were coupled by the mixed anhydride procedure to produce carbobenzoxy-nitroarginyl-nitroarginine amide (IX), which crystallized from dimethylformamide, combining two molecules of the solvent. The carbobenzoxy group was removed from compound IX by hydrogen bromide in acetic acid. The resultant dipeptide amide nitroarginyl-nitroarginine amide monohydrobromide (X) failed to crystallize, but it was found by paper chromatography to be homogeneous. Compound X was then added to an aqueous tetrahydrofuran solution of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysine azide, which was derived from the corresponding hydrazide;[4] they were then allowed to react in the presence of sufficient triethylamine to produce a hyptapeptide derivative N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-nitroarginyl-nitroarginine amide (XV). A pure sample of compound XV, which was easily obtained by reprecipitation from *n*-butanol in a moderate yield (66%), was found by thin-layer chromatography to be homogeneous. The catalytic hydrogenolysis of XV yielded the partially protected heptapeptide amide N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide triacetate (XVI), which had no absorption in the 250—390 $m\mu$ region and which gave a single ninhydrin and Sakaguchi reactive spot on paper.

In our previous communication, N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysine methyl ester was synthesized from the carboxyl terminal by a step-by-step procedure.[4] Now this protected pentapeptide has also been obtained in an excellent yield (86%) by the DCCl-mediated condensation from N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine[4] and the N^{ε}-*t*-butyloxycarbonyl-lysine methyl ester.[5] The product was found to be identical with that obtained previously.

The Synthesis of the Octapeptide Derivatives (XVIII and XX).—The N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysine *N*-hydroxysuccinimide ester (XI), which had been prepared from the diacyl-lysine and *N*-hydroxysuccinimide by the DCCl-mediated reaction,[6] was utilized for the synthesis of the Lys-Arg-Arg sequence. Compound XI was allowed to react with III and with X, each of which had been neutralized with triethylamine, in aqueous tetrahydrofuran to yield, respectively, the benzyl ester (V) and the amide (XI) of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-nitroarginyl-nitroarginine in 70 per cent yields. Compound XI was also obtained by the mixed anhydride procedure, and the product was identical in optical rotation to that obtained by the active ester method. Compounds V and XI were hydrogenolyzed to obtain the partially deblocked tripeptides, N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine diacetate (VI) and N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide triacetate (XII) respectively. Both of these were found by paper chromatography to be homogeneous.

The reaction of the pentapeptide azide with the partially deblocked tripeptide VI or XII proceeded smoothly, affording an octapeptide, N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valylglycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine (XVII) or its amide (XIX). The octapeptides XVII and XIX, which were purified by simple extraction (see "Experimental") were, found to be homogeneous to ninhydrin and Sakaguchi reagent in thin-layer chromatography. Judging from the number of positive charges, compounds XVII and XIX would form the monoacetate and the diacetate, respectively, if they were isolated from the aqueous acetic acid solutions. It was, however, a fact that compound XVII combined with one molecule of acetic acid, whereas the acid was not detected in XIX, when

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[5] H. van Orden and E. L. Smith, *J. Biol. Chem.*, **208**, 751 (1954).

[6] G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964); **85**, 3039 (1963).

they had been dried ordinarily (at 60°C in vacuo). Compounds XVII and XIX were then submitted to catalytic hydrogenolysis, which gave N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine (XVIII) and its amide (XX) respectively. Compound XX was obtained as the triacetate, but XVIII did not contain so much acetic acid as had been expected theoretically. This anomalous phenomenon, which has been observed only with the octapeptides, may suggest that they possess a certain kind of unusual conformation within their molecules. Compound XX was homogeneous in paper chromatography, but XVIII was found to be contaminated by a trace of impurity, which had a little higher R_f value than that of XVIII.

TABLE I. COMPOSITIONS OF BASIC AMINO ACIDS IN ACID HYDROLYSATES OF THE HEXAPEPTIDE (XIV), HEPTAPEPTIDE (XVI) AND OCTAPEPTIDES (XVIII AND XX)

Acid hydrolysate of	Molar ratio of basic amino acids		
	Lys	Arg	NH_2
Compound XIV	1.00 (1)	1.99 (2)	0.17 (0)
Compound XVI	2.00 (2)	1.96 (2)	1.13 (1)
Compound XVIII	3.00 (3)	1.96 (2)	0.33 (0)
Compound XX	3.00 (3)	2.11 (2)	1.23 (1)

Numbers in parentheses are theoretical values.

The compositions of basic amino acids in the acid hydrolysates of the synthesized peptide derivatives (XIV, XVI, XVIII and XX) were determined by an automatic analyzer.7) The results are shown in Table I, where it may be found that the heptapeptide (XVI) and the octapeptide (XX) each contain one amide group per molecule, as was expected.

Experimental

All melting points are uncorrected.

Nitroarginine Benzyl Ester *p*-Toluenesulfonate (I).—A mixture of 10.96 g. (0.05 mol.) of nitroarginine and 14.25 g. (0.075 mol.) of *p*-toluene-sulfonic acid monohydrate in 100 ml. of benzyl alcohol was stirred at 100°C. To this solution 50 ml. portions of carbon tetrachloride were added at about 40-min. intervals and then allowed to distill out. After five hours the reaction mixture was cooled, and 500 ml. of anhydrous ether was introduced. The supernatant was removed by decantation, and the sirupy residue was thoroughly washed with ether and dried in vacuo, thus affording 25.3 g. of the crude ester *p*-toluenesulfonate.

A 5 g. sample of the crude product was dissolved in 20 ml. of *n*-butanol, which had been saturated with water, and the solution was washed three times with water and evaporated at 45°C in vacuo to give an oily residue. The pure material was precipitated by the addition of ether; 4.36 g. (91.7% based on nitroarginine used); a highly hygroscopic powder, $[\alpha]_{D}^{25.5}$ $-1.4\pm1°$ (*c* 1.970, methanol). A single ninhydrin reactive spot was visible on paper, at $R_f=0.83$ (BAPW).[*2]

Found: C, 49.98; H, 5.71; N, 12.77; S, 7.06. Calcd. for $C_{13}H_{19}N_5O_4\cdot C_7H_8O_3S$: C, 49.89; H, 5.65; N, 14.54; S, 6.65%.

***t*-Butyloxycarbonyl-nitroarginyl-nitroarginine Benzyl Ester (II).**—Compound I (4.10 g., 8.5 mmol.) in water-*n*-butanol-ethyl acetate (1 : 1 : 4) was cooled in an ice-bath; to this 5 ml. of ice-cold 50% potassium carbonate was added, and the mixture was shaken vigorously. The aqueous phase was extracted again with *n*-butanol-ethyl acetate (1 : 4). The organic extracts were combined, washed with water, dried over anhydrous sodium sulfate, and concentrated in vacuo. The nitroarginine benzyl ester (free base) was precipitated by the addition of ether; 1.88 g. (71.6%).

This free ester (1.88 g.) and 1.815 g. (5.0 mmol.) of *t*-butyloxycarbonyl-nitroarginine9) were dissolved in 10 ml. of dimethylformamide. To this solution there was then added 1.03 g. (5.0 mmol.) of *N*, *N'*-dicyclohexylcarbodiimide (DCCl), dissolved in 15 ml. of methylene chloride, and the reaction mixture was allowed to stand in a refrigerator overnight. After the *N*, *N'*-dicyclohexylurea (DCU) had been removed, the filtrate was evaporated at 40—45°C in vacuo. The resultant sirupy residue was dissolved in *n*-butanol-ethyl acetate (1 : 2), and the solution was washed successively with water, ice-cold N hydrochloric acid, M sodium bicarbonate and water, dried over anhydrous sodium sulfate, and evaporated in vacuo. The desired dipeptide was then precipitated from *n*-butanol as an amorphous solid; 2.42 g. (79.2%), $[\alpha]_{D}^{25}$ $-18.1\pm1°$ (*c* 2.487, methanol). A single spot was visible in thin-layer chromatography, at $R_f=0.40$ (Silica-gel G, methanol-ethyl acetate (15 : 85)).

Found: C, 47.68; H, 6.78; N, 21.42. Calcd. for $C_{24}H_{38}N_{10}O_9$: C, 47.21; H, 6.27; N, 22.94%.

Nitroarginyl-nitroarginine Benzyl Ester-Trifluoroacetate (III).—Compound II (0.916 g., 1.5 mmol.) was dissolved in trifluoroacetic acid, and the solution was allowed to stand at room temperature for 60 min., after which time the dipeptide ester trifluoroacetate was precipitated by the addition of anhydrous ether; 0.892 g. (95.2%).

Found: C, 40.76; H, 5.80; N, 21.57. Calcd. for $C_{19}H_{30}N_{10}O_7\cdot CF_3COOH$: C, 40.39; H, 5.00; N, 22.43%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysine *N*-Hydroxysuccinimide Ester (IV).—To a solution of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysine (prepared from 5.62 g. (0.01 mol.) of the dicyclohexylamine-salt)4) and 1.15 g. (0.01mol.) of *N*-hydroxy-succinimide in acetonitrile 2.06 g. (0.01 mol.) of DCCl was added, and the mixture was kept in a refrigerator overnight. After the urea had been removed by filtration, the filtrate was evaporated in vacuo to give a sirupy residue. An ethyl acetate solution of the residue was washed succesively with ice-cold N hydrochloric acid, water, M sodium bicarbonate and water, and dried over anhydrous sodium sulfate. The removal of the solvent yielded a sirupy residue, which crystallized from ethyl

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

[*2] BAPW = *n*-butanol - acetic acid - pyridine - water (30 : 6 : 20 : 24 by volume).8)

8) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

9) K. Inouye, This Bulletin, **38**, 1148 (1965).

acetate - isopropyl ether; 4.58 g. (95.8%), m. p. 91—94°C. Recrystallization from the same solvent gave 4.00 g. (83.7%); m. p. 94.5—95.5°C, $[\alpha]_D^{27.5}$ −14.8 ±1° (*c* 1.770, dioxane).

Found: C, 57.73; H, 6.73; N, 8.93. Calcd. for $C_{23}H_{31}N_3O_8$: C, 57.85; H, 6.54; N, 8.80%.

N^α-Carbobenzoxy-N^ε-*t*-butyloxycarbonyl-lysyl-nitroarginyl-nitroarginine Benzyl Ester (V).—The dipeptide ester trifluoroacetate (III, 0.843 g., 1.35 mmol.) was dissolved in 10 ml. of 90% tetrahydrofuran, and then 0.38 ml. (2.7 mmol.) of triethylamine was added. After this solution had been cooled in an ice bath, 0.645 g. (1.35 mmol.) of IV was added, and the mixture was kept in a refrigerator overnight. After the solvent had been removed, the residue was redissolved in *n*-butanol - ethyl acetate - water (3 : 2 : 1) and the organic phase was washed successively with ice-cold N hydrochloric acid, water, 5% sodium bicarbonate and saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated in vacuo to give a gelatinous mass. This was reprecipitated from hot acetonitrile to give the desired tripeptide as an amorphous solid; 0.88 g. (74.5%), $[\alpha]_D^{24}$ −20.3 ± 1° (*c* 2.112, methanol).

Found: C, 51.99; H, 6.84; N, 19.00. Calcd. for $C_{38}H_{56}N_{12}O_{12}$: C, 52.28; H, 6.47; N, 19.26%.

N^ε-*t*-Butyloxycarbonyl - lysyl - arginyl - arginine Diacetate (VI).—Compound V (0.740 g.) was hydrogenolyzed in a 90% acetic acid solution in the presence of palladium catalyst at room temperature and at atmospheric pressure for 6.5 hr. After the catalyst had been removed by filtration, the filtrate was evaporated in vacuo to give a sirupy residue, which was lyophilized to afford a colorless powder; 0.578 g., $[\alpha]_D^{24}$ +5.3±1° (*c* 2.132, 50% acetic acid). A single ninhydrin and Sakaguchi reactive spot was visible on paper, at R_f= 0.51 (BAPW).

Carbobenzoxy - nitroarginine Amide (VII).—Carbobenzoxy-nitroarginine (17.67 g., 0.05 mol.) and 13.1 ml. (0.055 mol.) of tri-*n*-butylamine were dissolved in 130 ml. of anhydrous tetrahydrofuran. To this solution 5.3 ml. (0.055 mol.) of ethyl chloroformate was added drop by drop at an ice-salt bath temperature. After 10 min. aqueous 28% ammonia (9.6 ml.) was introduced, and the mixture was stirred for 2 hr. at 0°C. The precipitates which formed were filtered off, washed with tetrahydrofuran and ether, and dried in vacuo (19.31 g.). These precipitates crystallized from aqueous ethanol to give pure VII; 14.26 g. (80.9%), m. p. 217—219°C. A sample was recrystallized from the same solvent for analyses; there was a recovery of 92.4%, m. p. 220—221°C, $[\alpha]_D^{24}$ +4.9 ± 1° (*c* 1.946, dimethylformamide). The lit.⁵⁾ m. p. 219—220°C, $[\alpha]_D$ not given.

Found: C, 47.40; H, 5.75; N, 24.10. Calcd. for $C_{14}H_{20}N_6O_5$: C, 47.72; H, 5.72; N, 23.85%.

Nitroarginine Amide Hydrobromide (VIII).—To 1.06 g. of VII there was added 8 g. of 40% (w/w) hydrogen bromide in acetic acid; the mixture stood at room temperature for 30 min., after which time anhydrous ether (30 ml.) was introduced. The resultant precipitates were filtered off, washed with anhydrous ether, and dried to give the dihydrobromide as a hygroscopic powder (1.18 g.), which changed to the monohydrobromide upon crystallization from aqueous ethanol; 0.764 g. (85.2%), m. p. 237—239°C. Recrystallization from the same solvent did not alter the melting point; $[\alpha]_D^{26}$ +13.7±0.5° (*c* 2.550, water). A single ninhydrin reactive spot was visible on paper, at R_f=0.38—0.39 (BAPW).

Found: C, 24.09; H, 5.22; N, 28.21; Br, 26.83. Calcd. for $C_6H_{14}N_6O_3 \cdot HBr$: C, 24.09; H, 5.05; N, 28.10; Br, 26.71%.

Carbobenzoxy-nitroarginyl-nitroarginine Amide (IX).—Carbobenzoxy-nitroarginine (7.07 g., 0.02 mol.) was converted into the mixed anhydride in a tetrahydrofuran solution by the same procedure as was employed for the preparation of compound VII. After 20 min. an ice-cold solution of 5.99 g. (0.02 mol.) of VIII and 4.77 ml. (0.02 mol.) of tri-*n*-butylamine in 20 ml. of water and 140 ml. of dioxane was added, and the mixture was stirred for 2 hr. at 0°C. The separated precipitates were filtered off, washed with dioxane and ether, and dried in vacuo to give 13.2 g. (92.1%) of the crude dipeptide amide; m. p. 116—119°C. Recrystallization from hot dimethylformamide gave fine needles which seemed to combine two molecules of dimethylformamide as the crystallization solvent; 9.49 g. (66.1%), m. p. 115—115.5°C, $[\alpha]_D^{25}$ −6.2±1° (*c* 2.165, 50% acetic acid).

Found: C, 43.40; H, 6.75; N, 25.42. Calcd. for $C_{20}H_{31}N_{11}O_8 \cdot 2HCON(CH_3)_2 \cdot H_2O$: C, 43.51; H, 6.60; N, 25.37%.

Nitroarginyl - nitroarginine Amide Hydrobromide (X).—To 5.74 g. (8.0 mmol.) of compound IX there was added 70 g. of 40% (w/w) hydrogen bromide in acetic acid; the mixture stood at room temperature for 40 min., after which time anhydrous ether (150 ml.) was introduced. The resultant precipitates, which were highly hygroscopic, were reprecipitated from aqueous ethanol; 4.21 g. (96.4%), m. p. 147—155°C (decomp.). Reprecipitation from the same solvent gave 3.95 g. (90.5%) of a colorless powder; m. p. 153.5—156°C (decomp.), $[\alpha]_D^{23.5}$ +14.1±1° (*c* 2.026, water). A single ninhydrine reactive spot was visible in paper chromatography, at R_f=0.33 (BAPW).

Found: N, 28.83; Br, 14.24. Calcd. for $C_{12}H_{25}N_{11}O_6 \cdot HBr \cdot C_2H_5OH$: N, 28.25; Br, 14.65%.

N^α - Carbobenzoxy - N^ε - *t* - butyloxycarbonyl - lysyl-nitroarginyl-nitroarginine Amide (XI).—*By the N-Hydroxysuccinimide Ester.*⁶⁾—To a solution of 1.41 g. (2.5 mmol.) of compound X and 1.16 ml. (6.25 mmol.) of tri-*n*-butylamine in a mixture of 3.5 ml. of water and 9 ml. of tetrahydrofuran, 1.19 g. (2.5 mmol.) of the *N*-hydroxysuccinimide ester (IV) was added ; the mixture was then left to stand at room temperature for 4 hr. The solvent was removed by evaporation in vacuo, and the residue was dissolved in a mixture of water, *n*-butanol and ethyl acetate (2 : 1 : 2). The organic phase was washed successively with ice-cold N hydrochloric acid, water, and M sodium bicarbonate, and dried over anhydrous sodium sulfate. After the solvent had been removed by evaporation, a sirupy residue was redissolved in hot *n*-butanol. The protected tripeptide, which separated upon cooling, was collected, washed with cold *n*-butanol and ether, and dried; 1.75 g. (87.4%). Reprecipitation from hot *n*-butanol gave 1.44 g. (71.8%) of a pure material; m. p. 111—114°C, $[\alpha]_D^{25.5}$ −15.4±1° (*c* 2.092, methanol). Thin-layer chromatography gave a single ninhydrin reactive spot when the chromatographed plate had been treated with hydrochloric acid, at R_f=0.45

(Silica-gel G, methanol - ethyl acetate (25 : 75)).

Found: C, 46.83; H, 6.88; N, 22.51. Calcd. for $C_{31}H_{51}N_{13}O_{11}\cdot H_2O$: C, 46.55; H, 6.68; N, 22.77%.

By the Mixed Anhydride Procedure.—To a solution of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysine (prepared from 1.12 g. (2.0 mmol.) of the dicyclohexylamine salt)[4] and 0.48 ml. of tri-*n*-butylamine in 10 ml. of anhydrous tetrahydrofuran, 0.19 ml. (2.0 mmol.) of ethyl chloroformate was added at an ice-salt bath temperature (ca. −10°C). After the mixture had been stirred for 15 min., a solution of nitroarginyl-nitroarginine amide hydrobromide and 0.57 ml. (2.4 mmol.) of tri-*n*-butylamine in 2 ml. of water and 14 ml. of tetrahydrofuran was added. The reaction mixture was stirred for one hour at 0°C and then allowed to stand at room temperature for 3 hr. The solvent was removed by evaporation in vacuo to give a sirupy residue, to which were added 5 ml. of *n*-butanol, 10 ml. of ethyl acetate and 5 ml. of water. The aqueous phase was extracted twice with *n*-butanol - ethyl acetate (1 : 2). The organic extracts were combined, washed successively with ice-cold N hydrochloric acid, M sodium bicarbonate and water, dried quickly over anhydrous sodium sulfate, and evaporated in vacuo at 40—45°C. The residue was reprecipitated from hot *n*-butanol to give the desired tripeptide; 1.05 g. (65.6%), m. p. 110—120°C, $[\alpha]_D^{24.5}$ −15.5±1° (*c* 2.046, methanol), homogeneous in thin-layer chromatography.

Found: C, 46.19; H, 6.74; N, 22.27. Calcd. for $C_{31}H_{51}N_{13}O_{11}\cdot H_2O$: C, 46.55; H, 6.68; N. 22.77%.

N^{ε}-*t*-Butyloxycarbonyl-lysyl-arginyl-arginine Amide Triacetate (XII).—Compound XI (1.00 g.) in 90% acetic acid was hydrogenolyzed in the presence of a palladium catalyst at room temperature and at atmospheric pressure for 5 hr. After the catalyst had been removed by filtration, the filtrate was evaporated in vacuo and the residue was lyophilized to give a colorless powder; 0.913 g., $[\alpha]_D^{23.5}$ +2.7±2° (*c* 1.318, 50% acetic acid). A single ninhydrin and Sakaguchi reactive spot was visible on paper, at $R_f=0.51$ (BAPW).

Found: C, 46.73; H, 8.06; N, 19.99; CH_3CO. 16.24. Calcd. for $C_{23}H_{47}N_{11}O_5\cdot 3CH_3COOH\cdot 2H_2O$: C, 45.01; H, 8.21; N, 19.91; CH_3CO, 16.69%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-nitroarginyl-nitro-arginine Benzyl Ester (XIII).—The dipeptide benzyl ester trifluoroacetate (III) (1.29 g., 2.0 mmol.) in *n*-butanol - methylene chloride (1 : 1) was treated with 50% potassium carbonate at 0°C. The organic phase was, after being dried over anhydrous sodium sulfate, concentrated in vacuo. The free ester of the dipeptide solidified upon the addition of ether. The dipeptide ester thus obtained and 1.11 g. (1.75 mmol.) of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine[4] were dissolved in dimethylformamide, and to this solution there was added a methylene chloride solution of 0.361 g. (1.75 mmol.) of DCCl at 0°C. The reaction mixture was then allowed to stand in a refrigerator for 40 hr. The DCU which formed was filtered off and the filtrate was evaporated in vacuo. The residue was dissolved in 2 ml. of *n*-butanol and 20 ml. of ethyl acetate, and the solution was washed with ice-cold N hydrochloric acid, water, M sodium bicarbonate and with saturated sodium chloride, dried over anhydrous sodium sulfate, and evaporated in vacuo at 40—45°C. The resultant foamy residue crystallized from acetonitrile and ethyl acetate; 1.62 g. (92.8%), m. p. 113—115°C. Recrystallization from the same solvent system gave the pure hexapeptide derivative; 1.49 g. (85.2%), m. p. 114—115°C, $[\alpha]_D^{25}$ −49.4±1° (*c* 1.986, methanol). A single spot was detected in thin-layer chromatography, at $R_f=0.36$ (Silica-gel G, methanol - ethyl acetate (15 : 85)).

Found: C, 52.54; H, 6.49; N, 18.52. Calcd. for $C_{50}H_{75}N_{15}O_{15}\cdot H_2O$: C, 52.48; H, 6.78; N, 18.36%.

N^{ε}-*t*-Butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-arginyl-arginine Diacetate (XIV).—Compound XIII (0.687 g.) was hydrogenolyzed in 90% acetic acid in the usual manner for 8 hr. After the catalyst had been removed, the solvent was removed by evaporation in vacuo, and the residue was then lyophilized; 0.586 g. (100%), $[\alpha]_D^{26.5}$ −50.1±1.5° (*c* 1.565, 50% acetic acid).

Found: N, 19.51; CH_3CO, 9.68. Calcd. for $C_{35}H_{65}N_{13}O_9\cdot 2CH_3COOH$: N, 19.54; CH_3CO, 9.24%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-nitroarginyl-nitroarginine Amide (XV).—A solution of 0.876 g. (1.0 mmol.) of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysine hydrazide[4] in 10 ml. of 90% tetrahydrofuran was chilled in an ice-salt bath, and to this 2.5 ml. of ice-cold N hydrochloric acid and 0.55 ml. of ice-cold 2M sodium nitrite were added. After five minutes the mixture was made neutral by the addition of 0.35 ml. of triethylamine. To this mixture there was then added an ice-cold solution o 0.569 g. (1.0 mmol.) of compound X and 0.168 ml. (1.2 mmol.) of triethylamine in 1.5ml. of water and 7.5 ml. of tetrahydrofuran; the reaction mixture was stirred at 0—4°C for 40 hr. After most of the solvent had been removed by evaporation at 30°C, 10ml. of water, 10 ml. of ethyl acetate and 5 ml. of *n*-butanol were added and the mixture was shaken vigorously. The organic phase which separated was cooled in an ice-bath, washed successively with ice-cold N hydrochloric acid, water, and M sodium bicarbonate, dried quickly over anhydrous sodium sulfate, and evaporated at 40—45°C in vacuo. The residue was dissolved in about 20 ml. of hot *n*-butanol, and the solution was refrigerated overnight. The precipitates which separated were collected, washed with cold *n*-butanol, ethyl acetate and ether, and dried in vacuo to give the pure heptapeptide derivative; 0.856 g. (65.6%), m. p. 133—135°C, $[\alpha]_D^{25.5}$ −40.4±1° (*c* 1.751, methanol). A single spot was detected in thin-layer chromatography, at $R_f=$ 0.42 (Silica-gel G, methanol - ethyl acetate (25 : 75)).

Found: C, 49.76; H, 7.29; N, 19.31. Calcd. for $C_{54}H_{90}N_{18}O_{17}\cdot 2^1/_2H_2O$: C, 49.57; H, 7.32; N,19.27.

N^{ε}-*t*-Butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine Amide Triacetate (XVI).—Compound XV (0.75 g.) was hydrogenolyzed in 90% acetic acid in the presence of palladium black for 8 hr. After the catalyst had been removed, the filtrate was evaporated in vacuo to give an oily residue. In order to remove the remaining acetic acid, this residue was dissolved in *n*-butanol and evaporated again in vacuo. The resultant residue was dissolved in water, and the aqueous solution was concentrated to a small volume in order to remove a trace of *n*-butanol, and then lyophilized to give a colorless fluffy powder; 0.747 g. (100%), $[\alpha]_D^{24}$ −44.8

±1° (c 1.530, 50% acetic acid). A single ninhydrin and Sakaguchi reactive spot was visible on paper, at $R_f=0.69$ (BAPW).

Found: N, 17.79; CH_3CO, 11.10. Calcd. for $C_{46}H_{86}N_{16}O_{11}\cdot 3CH_3COOH\cdot 2H_2O$: N, 17.85; CH_3CO, 10.29%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine Monoacetate (XVII).—To a solution of the protected pentapeptide azide (prepared from 0.876 g. (1.0 mmol.) of N^{α}-carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysine hydrazide4) by the procedure described in the synthesis of compound XV) there was added an ice-cold solution of 0.527 g. (0.76 mmol.) of compound VI, which had been dissolved in 1.5 ml. of water and 6.0 ml. of tetrahydrofuran containing 0.21 ml. (1.52 mmol.) of triethylamine. The reaction mixture was then stirred at 0—4°C for 56 hr. After the solvent had been removed by evaporation, the residue was dissolved in 10 ml. of N acetic acid, and the solution was extracted four times with ethyl acetate. The ethyl acetate extracts were then combined and concentrated to about 20 ml. in vacuo. The resultant concentrate was extracted four times with N acetic acid. The equeous extracts were combined and concentrated to about 10 ml. at 40—45°C in vacuo. The resultant aqueous solution was extracted four times with *n*-butanol which had been saturated with water. The butanol extracts were pooled, dried over anhydrous sodium sulfate, and evaporated at 40—45°C in vacuo. The foamy residue was then lyophilized to give a colorless fluffy powder; 0.587 g. (52.8%), $[\alpha]_D^{25.5}$ $-46.1\pm2°$ (c 1.044, 50% acetic acid). Paper chromatography, $R_f=0.92$ (BAPW).

Found: C, 50.66; H, 8.32; N, 15.32; CH_3CO, 2.80. Calcd. for $C_{65}H_{111}N_{17}O_{17}\cdot CH_3COOH\cdot 7H_2O$: C, 50.65; H, 8.18; N, 14.99; CH_3CO, 2.71%.

N^{ε}-*t*-Butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine Acetate (XVIII).—Carbobenzoxy octapeptide (XVII) (0.300 g.) was hydrogenolyzed in 15 ml. of 90% acetic acid over a palladium black catalyst for 4.5 hr. in the usual manner. The catlyst was then filtered off, and the filtrate was evaporated at 40—45°C in vacuo. The residue was treated as was compound XVI in order to remove the remaining acetic acid, and then lyophilized; 0.275 g. (98.9%), $[\alpha]_D^{25.5}$ $-41.6\pm2°$ (c 1.007, 50% acetic acid). Paper chromatography gave a main spot and a faint minor one reactive to both ninhydrin and Sakaguchi reagents, at $R_f=0.86$ (BAPW) and 0.94 (BAPW) respectively.

Found: C, 47.44; H, 8.54; N, 16.71; CH_3CO, 2.42. Calcd. for $C_{57}H_{105}N_{17}O_{15}\cdot {}^3/_4CH_3COOH\cdot 7H_2O$: C, 48.80; H, 8.54; N, 16.54; CH_3CO, 2.24%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine Amide (XIX).—To a solution of the protected pentapeptide azide (prepared from 1.313 g. (1.5 mmol.) of the hydrazide4) by the procedure described in the synthesis of compound XV) there was added a solution of 0.756 g. (1.0 mmol.) of compound XII, which had been dissolved in 2.0 ml. of water and 8.0 ml. of tetrahydrofuran, together with 0.28 ml. (2.0 mmol.) of triethylamine. The reaction mixture was then stirred at 0—4°C for 40 hr., and evaporated at 30°C in vacuo. The residue was purified exactly as was compound XVII. The purified product was then lyophilized to give a colorless fluffy powder; 1.292 g. (84.2%), $[\alpha]_D^{22.5}$ $-43.7\pm1°$ (c 1.745, 50% acetic acid). Thin-layer chromatography gave a Sakaguchi reactive spot, at $R_f=0.76$ (Silica-gel G, dimethylformamide-ethyl acetate-acetic acid (15 : 10 : 2)).

Found: C, 50.85; H, 8.54; N, 16.33; CH_3CO, 0.0%. Calcd. for $C_{65}H_{112}N_{18}O_{16}\cdot 7{}^1/_2H_2O$: C, 50.80; H, 8.33; N, 16.41; CH_3CO, 0.0%.

N^{ε}-*t*-Butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine Amide Triacetate (XX).—A sample of carbobenzoxy-octapeptide amide (XIX) was hydrogenolyzed for 3 hr. in 15 ml. of 90% methanol containing 0.1 ml. of acetic acid in the presence of palladium black. The catalyst was then removed by filtration, and the filtrate was evaporated in vacuo. The lyophilization of the resultant residue gave a colorless fluffy powder; $[\alpha]_D^{24}$ $-41.0\pm2°$ (c 1.210, 50% acetic acid). A single ninhydrin and Sakaguchi reactive spot was visible on paper, at $R_f=0.80$ (BAPW).

Found: C, 50.22; H, 8.22; N, 16.83; CH_3CO, 8.37. Calcd. for $C_{57}H_{106}N_{18}O_{14}\cdot 3CH_3COOH\cdot 2H_2O$: C, 51,00; H, 8.29; N, 16.99; CH_3CO, 8.70%.

N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysine Methyl Ester.—N^{α}-Carbobenzoxy-N^{ε}-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycine (3.17 g., 5.0 mmol.)4) and the N^{ε}-*t*-butyloxycarbonyl-lysine methyl ester (prepared from 1.76 g. (5.5 mmol.) of the acetate)4) were dissolved in methylene chloride, and to this solution a methylene chloride solution of 1.135 g. (5.5 mmol.) of DCCl was added. The mixture was then allowed to stand in a refrigerator overnight. After the DCU which separated had been removed, the filtrate was evaporated in vacuo and the residue was redissolved in ethyl acetate. This solution was then washed successively with ice-cold N hydrochloric acid, 5% sodium bicarbonate and water, and dried over anhydrous sodium sulfate. The gelatinous precipitates separated from the concentrated ethyl acetate solution upon the addition of ether; 4.14 g. (94.6%), m. p. 92—94°C. The amorphous solid was reprecipitated from acetone-ether to give fine crystals with a m. p. of 101—104°C; 3.75 g. (85.5%), $[\alpha]_D^{25}$ $-56.6\pm1°$ (c 2.017, methanol). Lit.4) m. p. 105—107°C, $[\alpha]_D^{25}$ $-55.2\pm1°$ (c 2.126, methanol).

Found: C, 58.72; H, 7.98; N, 11.13. Calcd. for $C_{43}H_{69}N_7O_{12}$: C, 58.95; H, 7.94; N, 11.19%.

Grateful acknowledgement is made to Dr. Mitsuo Ebata and staff for amino acid analyses, to Dr. Kaoru Kuriyama and staff for optical rotation measurements, and to Dr. Satoshi Mizukami and staff for elemental microanalyses.