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Synthesis of Peptides related to Corticotropin (ACTH). III.[1] Syntheses of α^{1-23NH_2}-ACTH and β-Alanine[1]-α^{1-23NH_2}-ACTH[2]

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The synthesis of a tricosapeptide amide (α-$^{1-23NH_2}$-ACTH) corresponding to the first 23-amino acid-sequence of corticotropin (ACTH) and β-alanine[1]-α-$^{1-23NH_2}$-ACTH is described. The carbobenzoxy group was used for the protection of the ε-amino function of the lysine residues and the nitro group was used for the protection of the guanido function of the arginine residues. Pentachlorophenyl trichloroacetate was successfully used for the synthesis of pentachlorophenyl active ester of the peptide fragments having *c*-terminal glycine and proline. Finally, all the protecting groups of the protected tricosapeptide amides were deblocked by the hydrogen fluoride method.

Since the first announcement of the synthesis of the polypeptide which possesses the full corticotropic activity by Hofmann and Yajima, *et al.*[4] in 1961, various polypeptides having corticotropic activity have been synthesized.[5,6]

Recently, in a preliminary communication we[7] have reported the synthesis of a tricosapeptide amide corresponding to the first 23 amino acid sequence of ACTH by an essentially different route from those reported by other investigators.[5]

In the previous paper,[1] we described the preparation of six intermediary peptide fragments for the synthesis of α^{1-23NH_2}-ACTH, namely, carbobenzoxy-seryl-tyrosyl-serine hydrazide (I-a), *t*-butyloxycarbonyl-methionyl-γ-*t*-butyl-glutamyl-histidine hydrazide (II), *t*-butyloxycarbonyl-phenylalanyl-nitroarginyl-tryptophyl-glycine (III), *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxy-lysyl-prolyl-valyl-glycine (IV), *t*-butyloxycarbonyl-N$^{\varepsilon}$-carbobenzoxy-lysyl-N$^{\varepsilon}$-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-proline (V) and valyl-N$^{\varepsilon}$-carbobenzoxy-lysyl-valyl-tyrosine amide (VI).

In this paper, we wish to describe in detail the synthesis of α^{1-23NH_2}-ACTH and its β-alanine[1]-analogue.[8] The synthesis of the corresponding protected tricosapeptide amides (XXa, XXb) was achieved by the strategy illustrated in Chart 1.

By the use of the pentachlorophenyl trichloroacetate (TCAOPCP) method,[9] the partially protected pentapeptide (V) was converted to the corresponding pentachlorophenyl ester (VII), which was then coupled with the *c*-terminal tetrapeptide amide (VI) to yield the protected nonapeptide amide (VIII).

1) Part II: M. Fujino, O. Nishimura and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **17**, 2135 (1969).
2) The amino acids, peptides and their derivatives (except glycine and β-alanine) mentioned in this paper are of the L-configuration.
3) Location: *Juso, Higashiyodogawa-ku, Osaka, 532, Japan.*
4) K. Hofmann, H. Yajima, N. Yanaihara, T.Y. Liu and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961).
5) See E. Schröder and K. Lübke, "The Peptides," Vol. II, Academic Press, New York, 1966, p. 194.
6) R.A. Boissonnas, St. Guttmann and J. Pless, *Exper.*, **22**, 526 (1966); H. Kappeler, B. Riniker, W. Rittel, P. Desaulles, R. Maier, B. Schär and M. Staehelin, Proc. 8th Europ. Peptide Symposium, Noordwijk, The Netherlands, Sept. 1966, North-Holland Publ. Comp. Amsterdam, 1967, p. 214.
7) M. Fujino, C. Hatanaka and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 2186 (1969).
8) During preparation of this manuscript, a paper on synthesis and biological activity of this β-alanine[1]-analogue appeared (R. Geiger, H. Schröder and W. Siedel, *Ann. Chem.*, **726**, 177 (1969)).
9) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).

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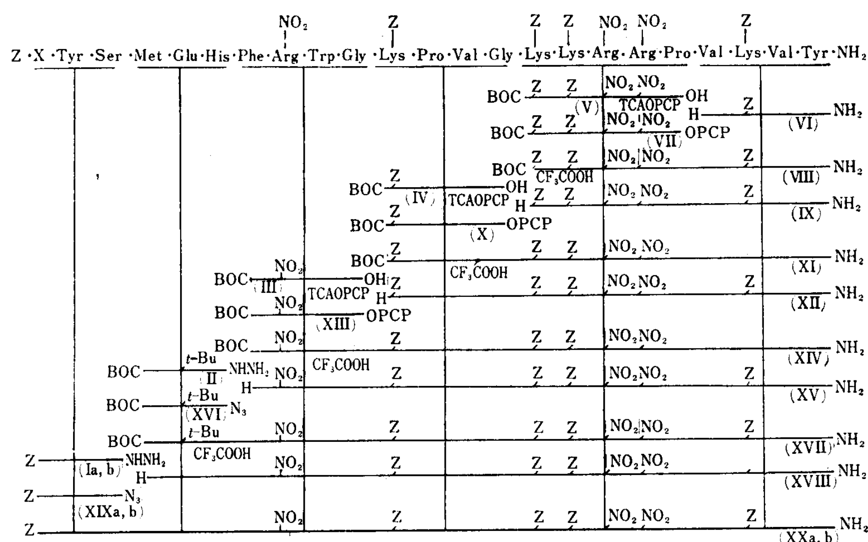

Chart 1. Synthesis of N-Protected α^{1-23HN_2} ACTH (XX-a, X=Ser) and β-Alanine[1]-α^{1-23NH_2} ACTH (XX-b, X=β-Alanine)

Z=carbobenzoxy, BOC=*t*-butyloxycarbonyl, NO_2=nitro, *t*-Bu=*t*-butylester, OPCP=pentachlorophenyl ester, TCAOPOP=pentachlorophenyltrichloroacetate

After elimination of the *t*-butyloxycarbonyl group from VIII by the acidolysis with cold trifluoroacetic acid,[10] the resulting nonapeptide amide (IX) was coupled with the active ester X, which was prepared from IV and TCAOPCP, to yield the fully protected tridecapeptide amide (XI) in good yield and with a satisfactory purity. XI was treated with cold trifluoroacetic acid[10] to remove the *t*-butyloxycarbonyl group and the resulting XII was coupled with pentachlorophenyl ester (XIII), which was prepared from III by means of the ester–exchange reaction with TCAOPCP, to give the fully protected heptadecapeptide amide (XIV) in good yield.

The esterification of the intermediary acylpeptides with TCAOPCP followed by the coupling afforded more satisfactory results as compared with the coupling of the peptide fragments with dicyclohexylcarbodiimide, because the former procedure was devoid of a trouble such as acylurea formation or contamination of insoluble dicyclohexylurea.

The protected heptadecapeptide amide (XIV) was treated carefully with cold trifluoroacetic acid[10] containing 1.0% thioglycolic acid under nitrogen to remove the *t*-butyloxycarbonyl–group, and the resulting partially protected heptadecapeptide amide (XV) was coupled with the protected tripeptide hydrazide (II) *via* the corresponding azide (XVI) to give the fully protected eicosapeptide amide (XVII).

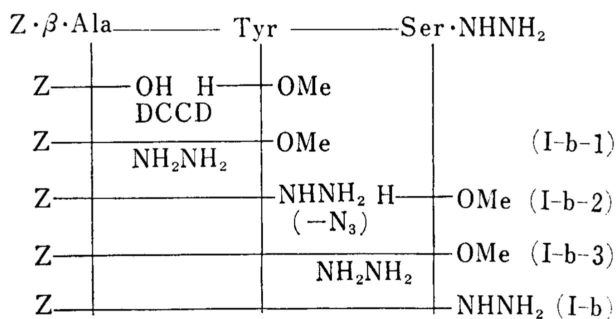

Chart 2. Synthesis of Carbobenzoxy-β-alanyl-tyrosyl-serine Hydrazide (I:-b)

Z: carbobenzoxy-, –OMe: methylester, DCCD: N,N'-dicyclohexylcarbodiimide

XVII thus obtained was then treated with trifluoroacetic acid[10] containing 1.0% thioglycolic acid, and the resulting partially protected eicosapeptide amide (XVIII) was condensed with carbobenzoxy-peptide hydrazide (Ia) *via* the azide (XIX) to give the protected tricosapeptide amide (XXa), which was purified by chromatography on a column of silica gel with ethylacetate–pyridine–acetic acid–water (60:20:6:10 v/v) as the solvent.

10) When the peptide was treated with trifluoroacetic acid at over 20°, some parts of carbobenzoxy–group were cleft.

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On the other hand, to obtain the protected tricosapeptide amide (XXb), carbobenzoxy-β-alanyl-tyrosyl-serine hydrazide (Ib) was prepared according to Chart 2, and coupled with the partially protected eicosapeptide amide (XVIII) by the azide method to give XXb, which was purified by the same manner as described above.

Finally, the removal of the protecting groups from the protected tricosapeptide amides (XXa and XXb) was achieved by means of Sakakibara's hydrogen fluoride method.[11] Thus, XXa and XXb were treated at 0° for one hour with anhydrous hydrogen fluoride which contains an excess amount of either anisole or thioglycolic acid or methionine or tryptophan to avoid undesirable side-reactions.

The resulting peptides, in the form of the hydrofluoride, were exchanged to the corresponding acetates by the treatment with Amberlite IRA-400 (acetate form). Pure samples of α^{1-23NH_2}-ACTH and β-alanine[1]-α^{1-23NH_2}-ACTH were isolated by the chromatography on carboxymethylcellulose using a gradient elution with ammonium acetate. Typical chromatograms are shown in Fig. 1. and 2.

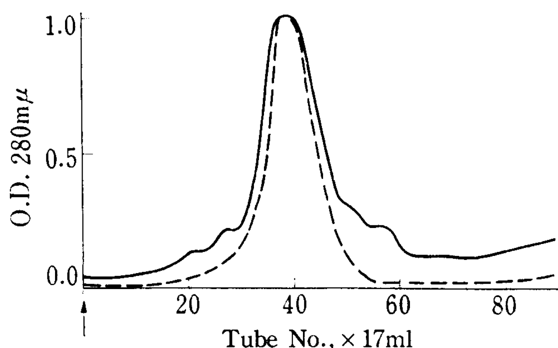

Fig. 1. Purification of Synthetic α^{1-23NH_2}-ACTH-tricosapeptide Amide

Arrow indicates where the gradient elution was started by introducing 0.4M (pH 6.5) ammonium acetate buffer through a 500 ml mixing chamber containing 0.15M (pH 6.5) ammonium buffer.
column: CM-cellulose (2.5 × 18.5 cm)
dotted line: rechromato.

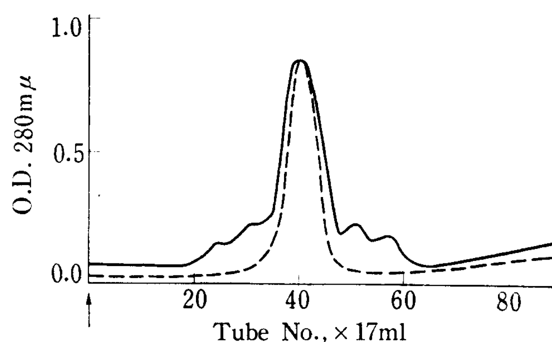

Fig. 2. Purification of Synthetic β-Alanine[1]-α^{1-23NH_2}-ACTH-tricosapeptide Amide

Conditions are the same as in Fig. 1.
column: CM-cellulose (2.5 × 18.5 cm)
dotted line: rechromato.

α^{1-23NH_2}-ACTH and β-alanine[1]-α^{1-23NH_2}-ACTH thus obtained were found to be homogeneous by thin-layer chromatography on silica gel and electrophoresis on paper. The amino

Table I. Amino Acid Composition of The Synthetic α^{1-23NH_2}-ACTH and β-Alanine[1]-α^{1-23NH_2}-ACTH

		Lys	His	Arg	Ser	Glu	Gly	Pro	Val	Met	Tyr	Phe	Trp	β-Ala	Recov.[c] (%)
α^{1-23NH_2}-ACTH	Theor.:	4	1	3	2	1	2	2	3	1	2	1	1	—	
	Found (AH)[a]	4.07	1.03	3.00	2.10	1.00	2.04	2.00	2.80	0.86	2.06	0.99	—	—	98.0
	Found (APM)[b]:	3.00	0.94	2.05	2.04	1.00	1.81	—	3.18	0.95	2.03	0.90	1.00	—	94.6
β-Alanine[1]-analog	Theor.:	4	1	3	1	1	2	2	3	1	2	1	1	1	
	Found (AH)[a]	4.18	1.00	3.17	1.12	1.00	2.02	2.00	2.90	0.81	2.05	1.00	—	0.94	100

a) acid hydrolysate, 110°, 24 hr in 5.7N HCl
b) aminopeptidase-M hydrolysate, E/s = 1.000 mμ/l μmole, 37°, 20 hr
c) Calculated as tricosapeptide octaacetate decahydrate.

11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967); S. Sakakibara, Y. Kishida, R. Nishizawa and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **41**, 438 (1968); S. Sakakibara, N. Nakamizo, Y. Kishida and S. Yoshimura, *Bull. Chem. Soc. Japan*, **41**, 1477 (1968).

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acid composition was determined by a Hitachi model KLA-3B autoanalyzer. The results are shown in Table I, which are in good agreement with the theoretical compositions of the peptides.

For the evaluation of the stereochemical homogeneity, the synthetic α^{1-23NH_2}-ACTH was digested by aminopeptidase-M (Röhm and Hass).[12]

As shown in Table I, all the peptide bonds were completely hydrolyzed by the enzyme with the exception of the lysyl-proline and arginyl-proline bonds.[13] It is interesting to note that the aforementioned two dipeptides having *c*-terminal proline were not digested by the aminopeptidase-M, because Hofmann, *et al.*[14] have reported that their synthetic pyrazolyl-3-alanine^6-α^{1-20NH_2}-ACTH was digested to the corresponding amino acids completely by the same enzyme.

A study on the *in vivo* steroidogenic activity carried out by R. Nakayama, *et al.*[15] in these laboratories demonstrated that our synthetic α^{1-23NH_2}-ACTH possessed the activity of *ca.* 80 units/mg and the β-alanine^1-analog possessed the activity of *ca.* 90 units/mg when assayed at 15 min after administration, but when assays were done at 30—40 min after administration, β-alanine^1-α^{1-23NH_2}-ACTH showed a very high activity (3 times or more active than the 3rd U.S.P. standard preparation). It seems pertinent to assume, therefore, that the presence of an α-amino group at the N-terminal of ACTH molecule is not essential for the biological action of this hormone, and that the introduction of β-alanine at the N-terminal portion would probably have more prevented the molecule from enzymatic degradations in the animal tissues.

Experimental

All melting points are uncorrected. Thin-layer chromatography (TLC) was carried out on Merck's silica gel G with solvent systems of $CHCl_3$–MeOH–AcOH (9:1:0.5, *Rf*^{1}), AcOEt–pyridine–AcOH–H_2O (60:20:6:11, *Rf*^{2}), *n*-BuOH–AcOH–H_2O (4:1:1, *Rf*^{3}) and *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, *Rf*^{4}).

***t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxy-lysyl-N$^\varepsilon$-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-proline Pentachlorophenyl Ester (VII)**——To a solution of *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxy-lysyl-N$^\varepsilon$-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-proline (V, 2.28 g, 2 mmole) in a mixture of pyridine (4 ml) and DMF (3 ml) was added with stirring at 0° triethylamine (2.8 ml) and pentachlorophenyl trichloroacetate[9] (1.4 g, 2.4 mmole), and the mixture was further stirred for 50 min at room temperature. The product precipitated by the addition of ice-water (80 ml) was collected by filtration and washed with H_2O. After being dried *in vacuo*, the product was purified by reprecipitation from acetone–ether (1:5). Yield, 2.27 g (90%), mp 115° (decomp.), $[\alpha]_D^{23}$ −33.6° (*c*=0.5 in DMF). *Rf*^{1}; 0.68.

***t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxy-lysyl-N$^\varepsilon$-carbobenxozy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N$^\varepsilon$-carbobenzoxy-lysyl-valyl-tyrosine Amide Hydrate (VIII)**——To a solution of valyl-N$^\varepsilon$-carbobenzoxy-lysyl-valyl-tyrosine amide (VI, 1.53 g, 2.4 mmole) in DMF (25 ml) was added the pentachlorophenylester (VII 1.53 g, 2.4 mmole) with stirring at 0°, and the mixture was stirred for 10 hr at room temperature. To the reaction mixture was added ice–water (100 ml) and the precipitate was collected by filtration and washed with H_2O and ether. After dryness *in vacuo*, the resulting powder was purified by reprecipitation from AcOH–MeOH (1:10). Yield 3.21 g (76%), mp 189—191° (decomp.) (179° sinter), $[\alpha]_D^{22}$ −34.6° (*c*= 0.5 in DMF). *Rf*^{1}; 0.12, *Rf*^{2}; 0.80, *Rf*^{3}; 0.95. *Anal.* Calcd. for $C_{83}H_{121}O_{22}N_{21}\cdot H_2O$: C, 55.9; H, 7.0; N, 16.5. Found: C, 55.7; H, 7.0; N, 16.5.

***t*-Butyloxycarbonyl-N$^\varepsilon$-carbobenzoxy-lysyl-prolyl-valyl-glycine Pentachlorophenyl Ester (X)**——To a solution of *t*-butyloxycarbonyl-N$^\varepsilon$-carbobenzoxy-lysyl-prolyl-valyl-glycine (IV, 1.98 g, 3 mmole) in DMF (5 ml) was added triethylamine (0.52 ml) and pentachlorophenyl trichloroacetate[9] (1.24 g, 3 mmole) with stirring at 0°, and the mixture was stirred for 20 min at room temperature. The product was precipitated

12) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966). (*cf.* G. Pfleiderer and P.G. Gelliers, *Biochem. Z.*, **339**, 186 (1963); G. Pfleiderer, P.G. Gelliers, M. Stanulovic, E.D. Wachsmuth, H. Determann and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)).
13) A similar result was found by Dr. S. Sakakibara of Osaka University in case of the hydrolysate of Angiotensin II by the aminopeptidase-M: Private communication. E.C. Jorgensen, G.C. Windridge, W. Patton and T.C. Lee, *J. Med. Chem.*, **12**, 733 (1969).
14) K. Hofmann, H. Bohn and R. Andreatta, *J. Am. Chem. Soc.*, **89**, 7126 (1967).
15) R. Nakayama, M. Shikata and R. Kubota, unpublished.

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by adding ice–water (20 ml), collected by filtration and dried over P_2O_5 *in vacuo*. The dried crystals were triturated with MeOH (20 ml) and then collected by filtration. Yield 2.0 g (74%), mp 188—190° (decomp.), $[\alpha]_D^{23}$ −36.7° (c=1.0 in DMF). Rf^1; 0.89. *Anal.* Calcd. for $C_{37}H_{46}O_9N_5Cl_5$: C, 50.4; H, 5.3; N, 8.0; Cl, 20.1. Found: C, 50.2; H, 5.4; N, 8.0; Cl, 20.3.

***t*-Butyloxycarbonyl-N^ε-carbobenzoxy-lysyl-prolyl-valyl-glycyl-N^ε-carbobenzoxy-lysyl-N^ε-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N^ε-carbobenzoxy-lysyl-valyl-tyrosine Amide Hydrate (XI)**——VIII (1.77 g, 1 mmole) was dissolved in ice–cooled CF_3COOH and the mixture was stirred for 20 min at 10—15°. After being concentrated to a half volume by evaporation *in vacuo* at temperature not exceeding 10° in approx. 7 min, the product was precipitated by the addition of ether (60 ml), collected by filtration and dried over NaOH *in vacuo*, to give a homogeneous powder (IX), which revealed a single spot in TLC with two solvents (Rf^2; 0.45, Rf^3; 0.61). The dried IX was dissolved in DMF (10 ml), and to this was added X (883 mg, 1 mmole) and triethylamine (0.15 ml). The mixture was stirred for 12 hr at room temperature and diluted with 10% aqueous solution of NH_4OAc (60 ml) to form a fine precipitate. The precipitate was collected by filtration and purified by reprecipitation from AcOH–MeOH (5:40). Yield 1.85 g (81%), mp 194—196° (decomp.), $[\alpha]_D^{23}$ −36.0° (c=0.5 in DMF). Rf^2; 0.75, Rf^3; 0.81. *Anal.* Calcd. for $C_{109}H_{158}O_{28}N_{26}\cdot H_2O$: C, 57.0; H, 7.0; N, 15.8. Found: C, 56.7; H, 7.0; N, 15.9.

***t*-Butyloxycarbonyl-phenylalanyl-nitroarginyl-tryptophyl-glycine Pentachlorophenyl Ester (XIII)**——To a solution of *t*-butyloxycarbonyl-phenylalanyl-nitroarginyl-tryptophyl-glycine (III, 1.43 g, 2 mmole) in pyridine (5 ml) was added triethylamine (0.28 ml) and pentachlorophenyl trichloroacetate[9] (1.24 g, 3 mmole) with stirring at 0°. After stirring at room temperature for 40 min, the reaction mixture was diluted with ice–water (20 ml) and stored at 4° for 4 hr. The crystals collected by filtration, washed thoroughly with H_2O, and dried over P_2O_5 *in vacuo*. The dried crystals were then washed with hot MeOH:. Yield 1.80 g (90.5%), mp 169.5—170.5° (dec.), $[\alpha]_D^{22}$ −8.2° (c=1.0 in DMF). *Anal.* Calcd. for $C_{39}H_{42}O_9N_5Cl_5$: C, 48.9; H, 4.4; N, 13.2; Cl, 18.5. Found: C, 48.7; H, 4.5; N, 13.0; Cl, 18.5.

***t*-Butyloxycarbonyl-phenylalanyl-nitroarginyl-tryptophyl-glycyl-N^ε-carbobenzoxy-lysyl-prolyl-valyl-glycyl-N^ε-carbobenzoxy-lysyl-N^ε-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N^ε-carbobenzoxy-lysyl-valyl-tyrosine Amide Tetrahydrate (XIV)**——XI (1.14 g, 0.5 mmole) was dissolved in ice–cooled CF_3COOH (10 ml) and stirred at 10—15° for 20 min under nitrogen. After the solution had been evaporated to a half volume *in vacuo* under cooling (10°, approx. 6 min), the product was precipitated by adding ice–cooled ether (50 ml), collected by filtration, washed with dry ether and then dried over NaOH *in vacuo*.

The dried powder (XV), which was homogeneous in TLC with two solvents (Rf^2: 0.45, Rf^3; 0.61), was dissolved in DMF (5 ml) and to this solution was added XIII (550 mg, 0.55 mmole) and 10% triethylamine-DMF (0.75 ml).

The mixture was stirred at room temperature for 6 hr, and was then diluted with ice-cooled 1N NH_4OH (30 ml) to yield a fine precipitate.

The precipitate was collected by filtration and then purified by reprecipitation from AcOH–MeOH (10:40). Yield 1.21 g (83.3%), mp 186—187° (decomp.), $[\alpha]_D^{21}$ −32.4° (c=0.5 in DMF). Rf^1; 0.0, Rf^2; 0.77, Rf^3; 0.91. *Anal.* Calcd. for $C_{137}H_{191}O_{34}N_{35}\cdot 4H_2O$: C, 55.9; H, 6.8; N, 16.7. Found: C, 55.8; H, 6.8; N, 16.7. Amino acid ratios of acid hydrolysate: $Lys_{3.9}$ $Arg_{3.0}$ $Gly_{2.0}$ $Pro_{2.0}$ $Val_{2.8}$ $Phe_{0.9}$ $Tyr_{0.2}$.[16]

***t*-Butyloxycarbonyl-methionyl-γ-*t*-butyl-glutamyl-histidyl-phenylalanyl-nitroarginyl-tryptophyl-glycyl-N^ε-carbobenzoxy-lysyl-prolyl-valyl-glycyl-N^ε-carbobenzoxy-lysyl-N^ε-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N^ε-carbobenzoxy-lysyl-valyl-tyrosine Amide (XVII)**——a) Amine Component XV: The compound XIV (2.3 g, 0.8 mmole) was dissolved in ice-cooled CF_3COOH (20 ml) containing 1% thioglycolic acid, and the solution was stirred at 10—15° for 25 min under nitrogen. The solution was concentrated to *ca.* 8 ml *in vacuo* under cooling (10°, 12 min). A fine white product was precipitated by adding dry ether (50 ml), collected by filtration and dried over NaOH *in vacuo*.

The dried powder (XV, Rf^2; 0.50, Rf^3; 0.78) was dissolved in DMF (30 ml) together with triethylamine (0.12 ml).

b) Azide XVI: A 1N aqueous solution of sodium nitrite (1.2 ml) was added to a stirred solution, cooled to −10°, of *t*-butyloxycarbonyl-methionyl-γ-*t*-butyl-glutamyl-histidine hydrazide (II, 600 mg, 1 mmole) dissolved in a mixture of 1N HCl (10 ml) and NaCl–saturated H_2O (12 ml). The mixture was stirred at −5° for 20 min, then cooled to −20°, and $NaHCO_3$ (700 mg) was added to the mixture. The resultign azide of II (XVI) was extracted with cold AcOEt (10 ml×2) from the reaction mixture. The AcOEt solution was washed with a cold 4% aqueous solution of $NaHCO_3$ and then with H_2O, and dried over Na_2SO_4.

c) Coupling: The dried azide solution was filtered and the filtrate was added to the cold DMF solution of the amine component XV, and the mixture was stirred for 1 hr at −5° and then for additional 12 hr at 2°.

To the reaction mixture was added the additional azide solution which was prepared from 300 mg of II, and the mixture was stirred for 20 hr at 2°. TLC of an aliquot of the reaction mixture with the solvent

16) Partial alkylation should be occurred within acid hydrolysis. (*cf.* B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962)).

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system 2 and 3 indicated that no more amine component (XV) had remained after 20 hr. The reaction mixture was then diluted with ether (100 ml) to yield a fine precipitate. The precipitate was collected by filtration and was reprecipitated twice from AcOH–MeOH (5:40). Yield 2.25 (95.8%), mp 202—204° (decomp.), $[\alpha]_D^{22}$ $-34.2°$ (c=0.5 in DMF). Rf^2; 0.60, Rf^3; 0.83. *Anal.* Calcd. for $C_{157}H_{222}O_{39}N_{40}S$: C, 56.5; H, 6.7; N, 16.8; S, 1.0. Found: C, 55.9; H, 6.8; N, 16.8; S, 1.2. Amino acid ratios in acid hydrolysate. $Lys_{4.0}His_{1.0}Arg_{2.8}Glu_{1.2}Gly_{2.0}Pro_{1.9}Val_{2.9}Phe_{1.0}Met_{0.6}$[16]$Tyr_{0.2}$.[16]

Carbobenzoxy-seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-nitroargnyl-tryptophyl-glycyl-N^{ε}-carbo-benzoxy-lysyl-prolyl-valyl-glycyl-N^{ε}-carbobenzoxy-lysyl-N^{ε}-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N^{ε}-carbobenzoxy-lysyl-valyl-tyrosine Amide Tetrahydrate (XX-a)——a) Amine Component XVIII: Compound XVII (998 mg, 0.3 mmole) was dissolved in ice-cooled CF_3COOH (10 ml), containing 0.1 ml of thioglycolic acid, and stirred for 15° for 25 min. The solution was concentrated to *ca.* 4 ml (10°, 5 min) *in vacuo*, and a fine white product was precipitated by adding dry ether (50 ml). The precipitate was collected by filtration and dried over NaOH *in vacuo*.

The dried powder (XVIII, Rf^2; 0.0, Rf^3; 0.52) was dissolved in DMF (5 ml). To the solution was added 10% triethylamine in DMF (1.1 ml) and the mixture was then cooled to $-20°$.

b) Azide XIXa: Carbobenzoxy-seryl-tyrosyl-serine hydrazide (Ia, 177 mg, 0.35 mmole) was suspended in DMF (7 ml), and to this suspension was added 6N HCl (0.25 ml) with stirring at $-10°$ to yield a clear solution.

To the solution was added 4N sodium nitrite (0.1 ml) and the mixture was stirred for 30 min at $-10°$. The solution was neutralized by adding triethylamine (0.2 ml) and then dried over Na_2SO_4.

c) Coupling: The dried azide (XIX) solution was filtered and the filtrate was added to the above mentioned DMF solution containing the amine component (XVIII).

The mixture was stirred for 6 hr at $-10°$ and for additional 10 hr at 0°, an azide solution prepared freshly from 90 mg of I was readded to the reaction mixture and the mixture was stirred for additional 24 hr at 0°.

The product was precipitated by adding ice-cooled ether (60 ml) and collected by filtration. The resulting precipitate was dissolved in a mixture of AcOEt, pyridine, AcOH and H_2O (60:20:6:11) and the solution was applied to a column (3×30 cm) of silica gel, which was eluted with the same solvent. Individual fractions (10 ml) were collected and examined by TLC with the solvent systems, 2 and 3. The compound XXa came off mainly in tubes 7—14. The main fraction was pooled and dried *in vacuo*. To the resulting residue was added ice-water to give a fine microcrystalline powder which was collected by filtration and washed with 1% AcOH and then with H_2O; Yield 940 mg (86%), mp 202—203° (decomp., 176° sinter), $[\alpha]_D^{24}$ $-31.8°$ (c=0.5 in DMF). Rf^2; 0.38, Rf^3; 0.73, Rf^4: 0.85. *Anal.* Calcd. for $C_{171}H_{231}O_{45}N_{43}S\cdot 4H_2O$: C, 55.3; H, 6.5; N, 16.2; S, 0.86. Found: C, 55.2; H, 6.5; N, 16.3; S, 0.75.

Carbobenzoxy-β-alanyl-tyrosine Methyl Ester (Ib-1)——Tyrosine methyl ester hydrochloride (27.48 g, 0.12 mole) and triethylamine (16.8 ml) were dissolved in acetonitrile (500 ml), and the mixture was cooled to 0°. To this solution was added carbobenzoxy-β-alanine (22.3 g, 0.1 mole) and N,N'-dicyclohexylcarbodiimide (25 g, 0.12 mole), and the mixture was stirred for 2 hr at 0° and then for additional 12 hr at 5°. The formed dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness.

The residue was dissolved in AcOEt (500 ml) and the solution was washed with 1N HCl, 4% aqueous solution of $NaHCO_3$ and H_2O. After being dried over anhyd. Na_2SO_4, the solvent was evaporated *in vacuo* and the resulting residue was precipitated from AcOEt–pet. ether to yield 33.7 g (94.5%) of Ib-1: mp 78—80°, $[\alpha]_D^{23}$ $+7.3°$ (c=1.0 in MeOH). Rf^1: 0.70. *Anal.* Calcd. for $C_{21}H_{24}O_6N_2$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.99; H, 6.30; N, 7.29.

Carbobenzoxy-β-alanyl-tyrosine Hydrazide (Ib-2)——To a solution of compound I-b-2 (20.2 g, 50 mmole) in MeOH (100 ml) was added hydrazine hydrate (10 ml) and the reaction mixture was left stand for 12 hr at room temperature. The product was crystallized by adding H_2O (100 ml), collected by filtration and washed with H_2O and then with MeOH; Yield 14.7 g (73%), mp 214—215°, $[\alpha]_D^{24}$ $-6.7°$ (c=1.0 in DMF). *Anal.* Calcd. for $C_{20}H_{24}O_5N_4$: C, 59.99; H, 6.04; N, 13.99. Found: C, 59.84; H, 6.00; N, 13.97.

Carbobenzoxy-β-alanyl-tyrosyl-serine Methyl Ester (Ib-3)——The compound Ib-2 (8.1 g, 20 mmole) was dissolved in a mixture of DMF (80 ml) and 2N HCl (40 ml), and the solution was coled to $-10°$. In this was added slowly an aqueous 5N sodium nitrite (5 ml) and the mixture was stirred for 5 min at $-5°$. The azide solution was diluted with H_2O (200 ml) and the oily azide was extracted with ice-coled AcOEt. The extract was washed with ice-cooled 4% $NaHCO_3$ and then with H_2O and was dried over Na_2SO_4.

The azide solution was added to an ice-cooled AcOEt solution of serine methyl ester which was prepared from 3.12 g (0.02 mole) of the hydrochloride with triethylamine (2.8 ml) in AcOEt (40 ml), and the mixture was stirred for 2 hr at 0° and for 20 hr at 2°. The solvent was removed by evaporation *in vacuo* and the residue was washed by trituration with H_2O.

The resulting product was crystallized from MeOH–AcOEt; yield 6.35 g (64.5%), mp 191—193°, $[\alpha]_D^{24}$ $-1.6°$ (c=1.0 in DMF). Rf^1; 0.46, Rf^2; 0.92. *Anal.* Calcd. for $C_{24}H_{29}O_8N_3$: C, 59.13; H, 6.00; N, 8.62. Found: C, 58.71; ,H 6.06; N, 8.70.

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Carbobenzoxy-β-alanyl-tyrosyl-serine Hydrazide (Ib)——To a solution of the compound Ib-3 (975 mg, 2 mmole) in a mixture of MeOH (10 ml) and DMF (7 ml) was added hydrazine hydrate (1 ml) and the mixture was left to stand for 20 hr at room temperature. The hydrazide which had separated as microcrystals was collected by filtration, washed with MeOH and recrystallized from DMF–H_2O. Yield 750 mg (77%), mp 234° (decomp.), $[\alpha]_D^{24}$ $-4.2°$ ($c=1.0$ in DMF). *Rf*[2]; 0.60, *Rf*[4]; 0.83. *Anal.* Calcd. for $C_{23}H_{29}O_7N_5$: C, 56.66; H, 6.00; N, 14.37. Found: C, 56.46; H, 5.71; N, 14.43.

Carbobenzoxy-β-alanyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-nitroarginyl-tryptophyl-glycyl-N^{ϵ}-carbobenzoxy-lysyl-prolyl-valyl-glycyl-N^{ϵ}-carbobenzoxy-lysyl-N^{ϵ}-carbobenzoxy-lysyl-nitroarginyl-nitroarginyl-prolyl-valyl-N^{ϵ}-carbobenzoxy-lysyl-valyl-tyrosine Amide Tetrahydrate (XX-b)——To a solution of the hydrazide I-b (140 mg, 0.3 mmole) in a mixture of 6N HCl (0.2 ml) and DMF (6 ml) was added 4N sodium nitrite (0.1 ml) at $-10°$ and the mixture was stirred for 30 min at $-10°$. The reaction mixture was neutralized with triethylamine (0.17 ml) and dried over anhyd. Na_2SO_4.

The azide solution was filtered and the filtrate was added to a cold DMF solution of the amine component (XVIII), which was prepared from 665 mg (0.2 mmole) of XVII as described above. The mixture was stirred for 6 hr at $-10°$ and then for additional 10 hr at 0°. The additional azide solution prepared from 600 mg of Ib was added to the reaction mixture.

After the mixture had been left standing 24 hr at 0°, the product was precipitated by adding ice-cooled ether (50 ml) to yield a fine powder.

The precipitate was collected by filtration and washed thoroughly with AcOEt. The fine powder was purified by a column chromatography on silica gel as mentioned above; Yield 640 mg (86%), mp 207—213 (decomp.), (173—182 sinter), $[\alpha]_D^{26.5}$ $-28.6°$ ($c=0.5$ in DMF). *Rf*[2]; 0.60, *Rf*[3]; 0.75; *Rf*[4]; 0.91. *Anal.* Calcd. for $C_{176}H_{237}O_{46}N_{43}S \cdot 4H_2O$: C, 55.6; H, 6.5; N, 16.3; S, 0.87. Found: C, 55.5; H, 6.6; N, 15.6; S, 0.78.

α^{1-23NH_2}-ACTH-tricosapeptide Amide——The protected tricosapeptide XX-a (500 mg, 0.134 mmole), anisole (0.75 ml), thioglycolic acid (0.05 ml), methionine (500 mg) and tryptophan (750 mg) were all placed in a difuron cylinder with CF_3COOH (2 ml) and treated with anhydrous hydrogen fluoride (approximately 20 ml) in an ice-bath for 60 min.

The hydrogen fluoride and the CF_3COOH were removed under reduced pressure. The residue was dried over NaOH *in vacuo* (1 mmHg, 5 hr) and then dissolved in H_2O (25 ml). Some insoluble by-products were removed by filtration with Hyflo supercel, and the filtrate was passed through a column of Amberlite IRA-400 (AcO^-, 75 ml) and the column was washed with H_2O. The total effluent was concentrated to dryness *in vacuo*. The residue was dissolved in 2% aqueous thioglycolic acid (10 ml) and the solution was kept at 50° for 20 hr under nitrogen. The resulting solution was diluted with H_2O (400 ml) and applied to a column of carboxymethylcellulose (2.5 × 18.5 cm) which was eluted in succession with ammonium acetate buffer (pH 6.5): 0.01M (500 ml); 0.1M (500 ml); 0.15M (500 ml). In the chromatography was used an exponential gradient. The gradient was established by adding 0.4M ammonium acetate buffer (pH 6.5) through a mixing flask containing 500 ml of 0.15M ammonium acetate buffer (pH 6.5). Individual fractions of 17 ml each were collected at a flow rate of 1—2 ml per min. Tubes 30—55 from the gradient elution were pooled, the bulk of solvent was removed *in vacuo* and the residue was lyophilized to yield *ca.* 300 mg of colorless fluffy powder. This material was then purified by rechromatography on a column of carboxymethylcellylose by the same manner as described above.

The main fraction (tubes 32—50, 17 ml each) was pooled and lypohilized to constant weight. Yield 235 mg (49.9%), $[\alpha]_D^{22}$ $-76.7°$ ($c=0.5$ in 1% AcOH)[17] (lit.[18] $[\alpha]_D$ $-76.8 \pm 1.5°$ ($c=0.5$ in 1% AcOH)). UV $\lambda_{max}^{0.1N NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.5 (24.99), 290 (25.73). *Rf*[2]; 0.0, *Rf*[3]; 0.0, *Rf*[4]; 0.54. Paper electrophoresis: 2N AcOH (20 v/cm, 1 hr), Mobility (M) = Lys × 0.98; pyridine–AcOH–H_2O (1:10:89 v/v) (13 v/cm, 6 hr), M = Lys × 0.84; pyridine–AcOH–H_2O (10:0.4:90 v/v) (13 v/cm, 3 hr), M = Lys × 0.83. *Anal.* Calcd. for $C_{131}H_{203}O_{30}N_{39}S \cdot 8CH_3COOH \cdot 10H_2O$: C, 50.49; H, 7.35; N, 15.62; S, 0.91; CH_3CO, 9.85. Found: C, 50.31; H, 7.13; N, 15.64; S, 0.86; CH_3CO, 9.76, 10.11.

β-Alanine^1-α-$^{1-23NH_2}$-ACTH-tricosapeptide Amide——The protected tricosapeptide (XX-b) (200 mg) was treated with 20 ml of hydrogen fluoride in the presence of anisole (0.3 ml), thioglycolic acid (0.05 ml), methionine (200 mg), tryptophan (200 mg) and CF_3COOH (1 ml) at 0° for 60 min, and the hydrogen fluoride and the CF_3COOH were removed *in vacuo*. The residue was taken up in H_2O (15 ml) and the solution was passed through a column of Amberlite IRA-400 (AcO, 50 ml). The eluate and washings were evaporated to dryness *in vacuo*. The residue was dissolved in 2% aqueous thioglycolic acid (10 ml) and the solution was kept at 50° for 20 hr under nitrogen. The resulting solution was applied to a column of carboxymethylcellulose (2.5 × 18.5 cm) by the same manner as described above and the main fraction (tubes 33—48, 17 ml each) was lyophilized to yield a colorless fluffy powder (*ca.* 120 mg). This material was then purified by rechromatography on a column of carboxymethylcellulose by the same manner and the main fraction (tubes 35—45, 17 ml each) was lyophilized to a constant weight; Yield 84 mg, $[\alpha]_D^{22}$ $-73.0°$ ($c=0.5$ in 1% AcOH).[19]

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17) As the anhydrous octaacetate.
18) R. Geiger, K. Sturm and W. Siedel, *Ber.*, **97**, 1207 (1964).
19) R. Geiger, H. Schröder and W. Siedel, (ref. 8) give $[\alpha]_D^{20} = -75.0°$ ($c=0.5$ in 1% AcOH).

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UV $\lambda_{max}^{0.1N NaOH}$ mμ ($E_{1cm}^{1\%}$) 284 (25.38), 290 (26.85). Rf^2; 0.0, Rf^3; 0.0, Rf^4; 0.54. Paper electrophoresis: 2N AcOH (20 v/cm, 1 hr), M=Lys×0.98; pyridine–AcOH–H_2O (1:10:89) (13 v/cm, 6 hr), M=Lys×0.84; pyridine–AcOH–H_2O (10:0.4:98) (13 v/cm, 3 hr), M=Lys×0.83.

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