[Chem. Pharm. Bull. 26(2) 539—548 (1978)] UDC 547.466.1.04.09 : 577.154.07

Synthesis of the Nonacosapeptide corresponding to Mammalian Glucagon[1)]

Masahiko Fujino, Mitsuhiro Wakimasu, Susumu Shinagawa, Chieko Kitada,[2a)] and Haruaki Yajima[2b)]

Chemical Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd.[2a)] and Faculty of Pharmaceutical Sciences, Kyoto University[2b)]

(Received July 1, 1977)

The nonacosapeptide corresponding to the entire amino acid sequence of mammalian glucagon was synthesized *via* the corresponding sulfoxide, [Met(O)^{27}]-glucagon. For synthesis of the protected nonacosapeptide six subunits were prepared to serve for building blocks. The condensations of subunits were achieved by the HONB-DCC method. Finally, all the protecting groups of the protected peptide were removed by the treatment with methanesulfonic acid-anisole to give [Met(O)^{27}]-glucagon. The sulfoxide was then treated with 3% aqueous thioglycolic acid to give mammalian glucagon. The product was successfully crystallized from dilute aqueous sodium chloride solution. The N to O acyl migration of serine and threonine residues with methanesulfonic acid treatment was studied in detail.

Keywords——glucagon; HONB-DCC method; [Met(O)^{27}]-glucagon; methanesulfonic acid; nonacosapeptide

In 1968, Wünsch, *et al.*[3)] reported the synthesis of mammalian glucagon using the HOSu–DCC method[4)]; the synthetic peptide was obtained in crystalline form. This was the first example of application of the HOSu–DCC method to prepare a complicated polypeptide. Recently, Chinese peptide chemists[5)] announced the synthesis of this hormone by fragment condensation on solid support using the HOBt–DCC method.[6)] These investigations have clearly shown that the DCC-additive method is useful for preparing complex polypeptides. For debloking, Wünsch, *et al.*[3)] used TFA to remove the protecting groups at final step of the synthesis, whereas the Chinese group[5)] used anhydrous hydrogen fluoride (HF).[7)] One of the present authors (H. Y.) has introduced methanesulfonic acid (MSA)-anisole as a deblocking agent for peptide synthesis,[8)] but this reagent causes a serious side reaction on the methionine residue producing S-methyl methionine.[9)] However, as shown in previous papers[9,10)], this side reaction can be prevented by using methionine sulfoxide instead of

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, BOC=*tert*-butoxycarbonyl, MBS=*p*-methoxybenzene sulfonyl, OBzl=benzyl ester, OBu^t=*tert*-butyl ester, HOSu=N-hydroxysuccinimide, HOBt=1-hydroxybenzotriazole, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, DCC=N,N′-dicyclohexylcarbodiimide, DCU=N,N′-dicyclohexylurea, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=dimethylformamide, THF=tetrahydrofuran, NMP=N-methyl-2-pyrrolidone.
2) Location: *a) Yodogawa-ku, Osaka, 532, Japan; b) Sakyo-ku, Kyoto, 606, Japan.*
3) E. Wünsch and G. Wendlberger, *Chem. Ber.*, **101**, 3659 (1968); E. Wünsch, E. Jaeger, and R. Scharf, *ibid.*, **101**, 3664 (1968).
4) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Naturforsch.*, **21b**, 426 (1966).
5) Chinese group, *Acta Biochim. Biophys. Sin.*, **7**, 120 (1975).
6) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
7) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
8) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
9) H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *Chem. Commun.*, **1976**, 922.
10) M. Fujino, S. Shinagawa, M. Wakimasu, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 101 (1978).

methionine; the sulfoxide in the peptide molecule can be easily reduced by treatment with thiol reagents.[11] In this manner, we have synthesized successfully a docosapeptide corresponding to the amino acid sequence of porcine motilin.[10] The present paper deals with an alternative synthesis of the nonacosapeptide corresponding to the amino acid sequence of mammalian glucagon by the MSA procedure.

Our synthetic scheme of glucagon is outlined in Fig. 1. The α-amino function of most of the intermediates was protected by the TFA-labile BOC group, and amino acid derivatives bearing protecting groups, *e.g.*, Z, OBzl and MBS, which could be removed by treatment with MSA-anisole were used. Among these protecting groups, the MBS group was recently introduced in our laboratory[12] as being useful to protect the guanidino function of arginine.

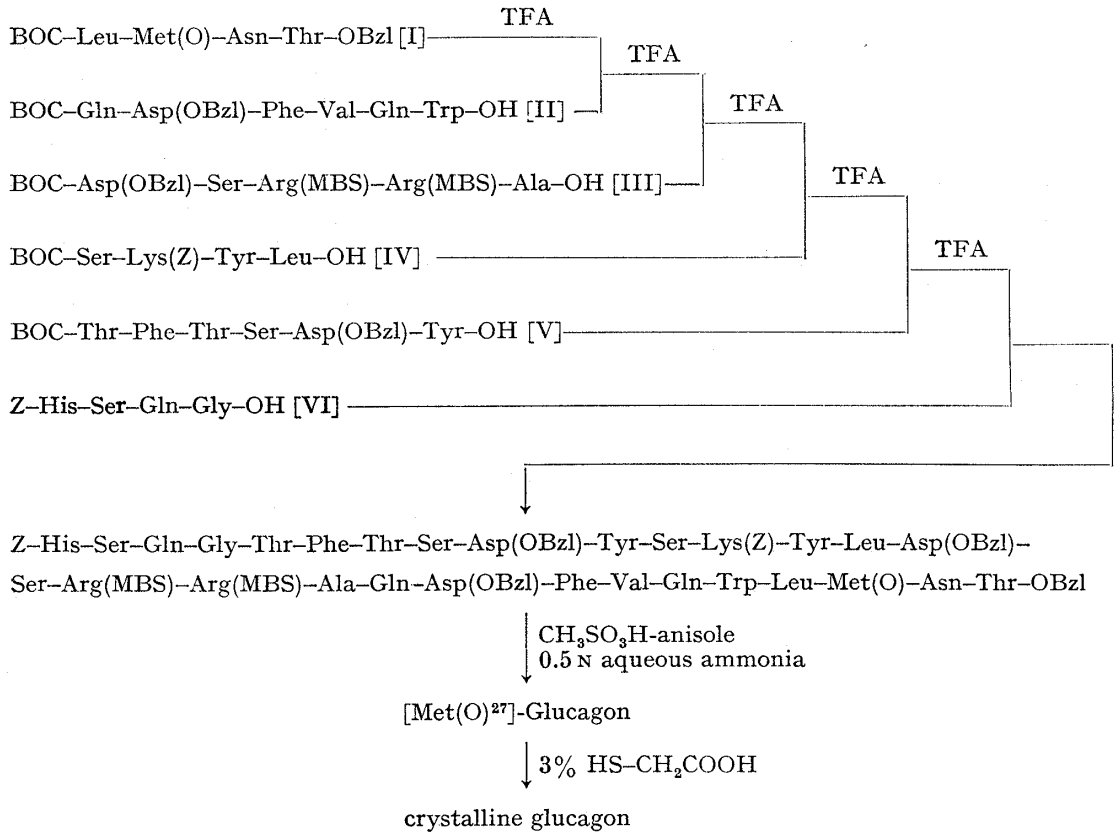

Fig. 1. Synthetic Route to Mammalian Glucagon

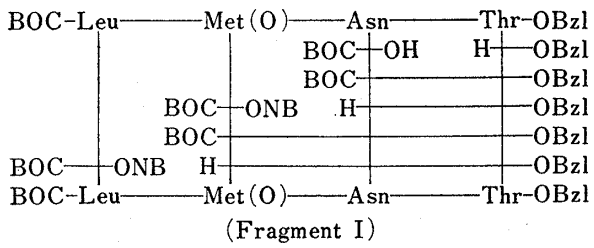

Fig. 2. Preparation of Protected Tetrapeptide (26—29)

As shown in Fig. 1, six peptide subunits, BOC-Leu-Met(O)-Asn-Thr-OBzl (I), BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-OH (II), BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-OH (III), BOC-Ser-Lys(Z)-Tyr-Leu-OH (IV), BOC-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH (V) and Z-His-Ser-Gln-Gly-OH (VI) served as building blocks for the construction of the full sequence corresponding to mammalian glucagon. The subunits were prepared by stepwise chain elongation starting from the corresponding carboxy-end amino acid or amino acid ester using the HONB-activated esters of N-protected amino

11) M.L. Dedman, T.H. Farmer, and C.J.O.R. Morris, *Biochem. J.*, **66**, 166 (1957).
12) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **24**, 1568 (1976).

acids.[13] The synthetic routes to these subunits are shown respectively in Fig. 2—7.

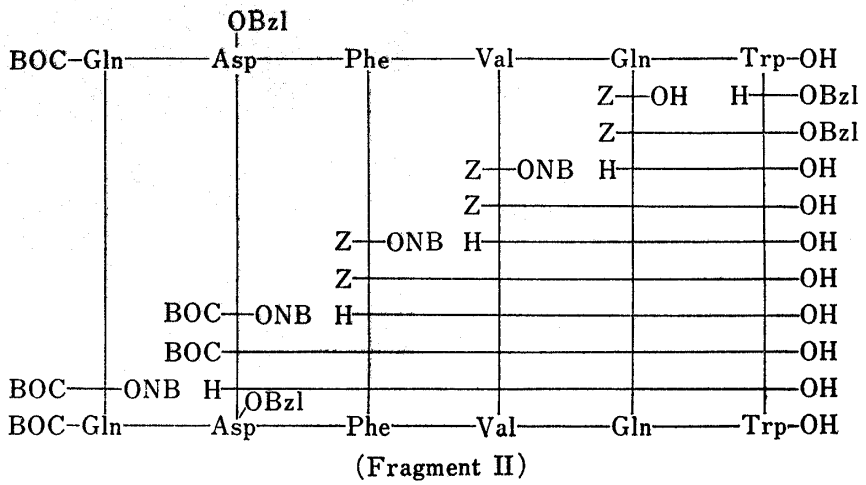

Fig. 3. Preparation of Protected Hexapeptide (20—25)

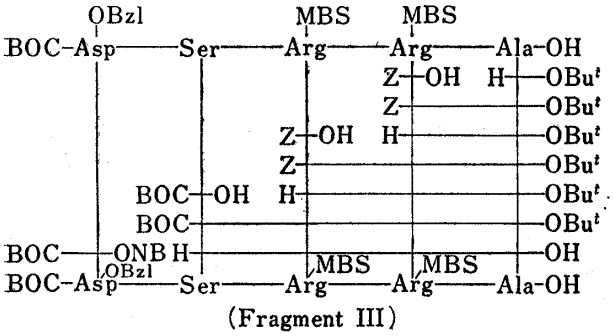

Fig. 4. Preparation Protected of Pentapeptide (15—19)

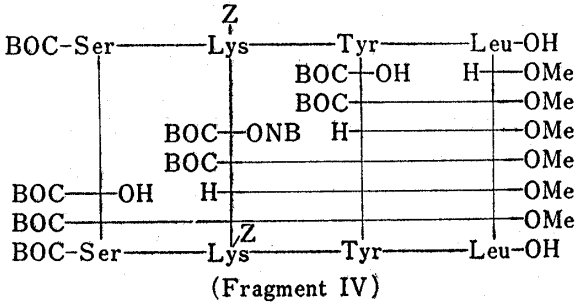

Fig. 5. Preparation of Protected Tetrapeptide (11—14)

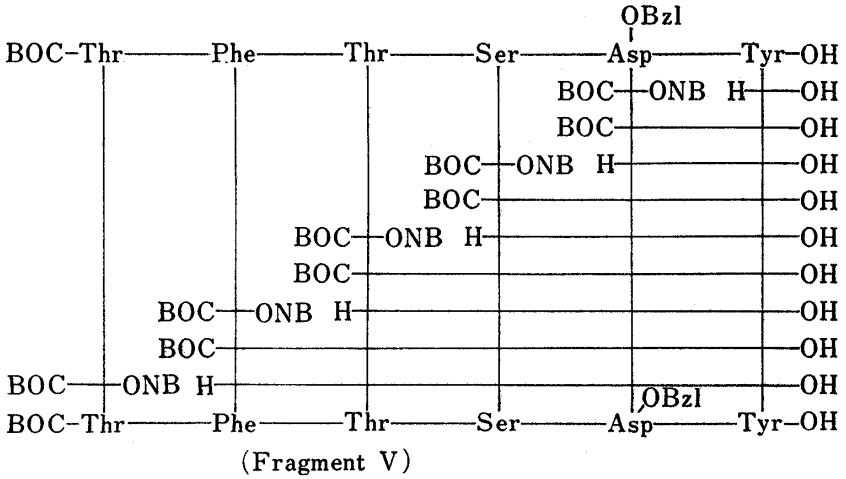

Fig. 6. Preparation of Protected Hexapeptide (5—10)

In synthesizing the entire amino acid sequence of the nonacosapeptide, subunit I was treated with TFA to remove the N^{α}-BOC group and the resulting free base of I was condensed

13) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974).

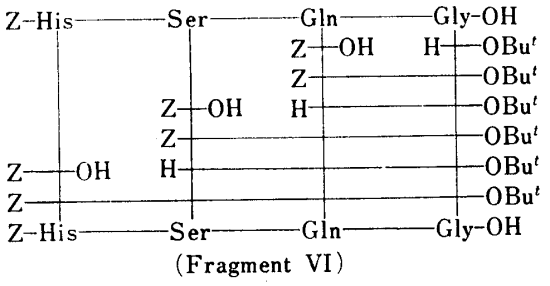

Fig. 7. Preparation of Protected Tetrapeptide (**1**—**4**)

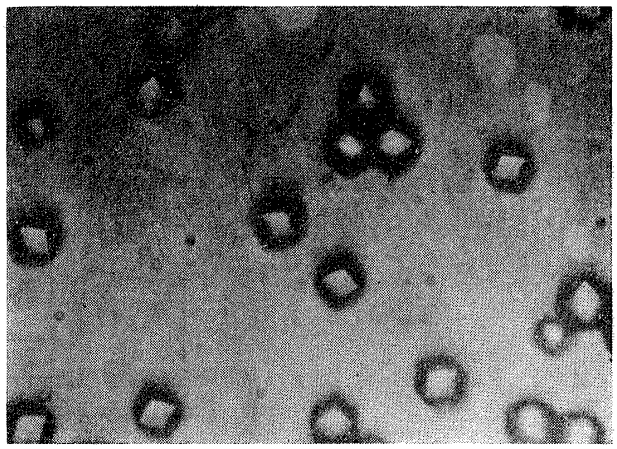

Fig. 8. Crystals of Synthetic Glucagon

with subunit II by the HONB–DCC method to minimize undesirable racemization,[14] giving BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (VII). The BOC group of the decapeptide VII was removed by treatment with TFA in the presence of anisole under nitrogen gas, and the resulting free base of VII was acylated with subunit III by the HONB–DCC method to afford BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (VIII). The BOC group of the pentadecapeptide VIII was removed by treatment with TFA-anisole under nitrogen gas and the resulting N^{α}-free peptide was coupled to subunit IV by the HONB–DCC method giving BOC-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (IX). The nonadecapeptide IX was treated with TFA in the presence of anisole under nitrogen gas to remove the N^{α}-BOC group and the resulting free base was condensed with subunit V to obtain the protected pentacosapeptide, BOC-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln Trp-Leu-Met(O)-Asn-Thr-OBzl (X). The BOC group was again removed and the corresponding free base was condensed with the N-terminal subunit VI by the HONB–DCC method to afford the protected nonacosapeptide (XI) corresponding to the entire amino acid sequence of mammalian glucagon in a crude form. This XI was exposed to MSA in the presence of anisole at room temperature for 60 min to remove all the protecting groups. The deblocked peptide was immediately converted to the corresponding acetate with Amberlite IRA-410 (acetate form) and the acetate was treated with 0.5N aqueous ammonia at 0° for 30 min to reverse the undesirable N→O acyl migration of the serine or threonine residue (see the section below on N→O acyl migration with MSA). The resulting peptide was then purified by column chromatography on Sephadex LH-20 and carboxymethylcellulose (ammonium acetate buffer) to give chromatographically pure [Met(O)^{27}]-glucagon. The product thus obtained was dissolved in 3% aqueous thioglycolic acid and the solution was left standing at 50° for 20 hr to reduce the sulfoxide on the methionine side chain. The reduced peptide was passed through a column of Sephadex G-25 to remove the thiol reagent giving chromatographically pure glucagon as a fine powder, which was successfully crystallized from dilute aqueous sodium chloride solution. Fig. 8 is a photo of the crystal.

The protected [Met(O)^{27}]-glucagon was also deblocked with anhydrous HF in the presence of anisole.[7] The deblocked peptide was purified as described above, then reduced with 3% thioglycolic acid to give glucagon, which was also crystallized from dilute sodium chloride solution.

14) M. Fujino, C. Kitada, and I. Yoshida, "The Proceedings of the 14th Symposium on Peptide Chemistry," ed, by T. Nakajima, Protein Research Foundation, Osaka, Japan, 1977, p. 28.

The physicochemical properties (UV spectra, thin layer chromatography, paper electrophoresis, $[\alpha]_D$, *etc.*) of this crystalline glucagon were identical with those of natural crystalline glucagon (Sigma Chemical, Co.) and the biological activity, lipolysis of adipocytes of Sprague-Dawley rats,[15] was also identical with that of natural glucagon.

N→O Acyl Migration of Serine- and Threonine-containing Peptides with Methanesulfonic Acid

When the deblocked peptide was not treated with 0.5N aqueous ammonia, pure glucagon could not be obtained even after several purifications. This indicates strongly that N→O acyl migration at the position of serine and threonine residues in the peptide might occur due to MSA treatment. To examine the side reaction(s), two model peptides, the protected fragments I and V, were treated with MSA-anisole at room temperature for 60 min, and the resulting products were checked by thin layer chromatography and paper electrophoresis. Both peptides gave a relatively large amount of by-products with much lower *Rf* values on thin-layer chromatograms and more basic properties on electrophorograms than the normal products. These by-products could be reversed to the corresponding normal products by treatment with 0.5 N aqueous ammonia at 0° for 30 min, and therefore seem to be the N to O acyl migration products. The products derived from fragment I were treated with nitrite to eliminate the amino group, and the acid hydrolysate of the resulting product was examined by quantitative amino acid analysis. The recovery of threonine was much lower than that of the other amino acids with the exception of the N-terminal amino acid. This indicates that the main side reaction of the MSA treatment is the N→O acyl shift. Recently, Wakamiya, *et al.*[16] reported that the N→O acyl shift of threonine-containing peptides in conc. H_2SO_4 resulted in a considerable amount of inversion at the β-carbon of threonine residue giving allothreonylpeptides, whereas this type of inversion had not been observed with conc. HCl, HBr or HF. To find out whether such inversion occurs with MSA treatment, some threonine-containing peptides such as BOC-Leu-Met(O)-Asn-Thr-OBzl(I), BOC-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH (Vd) and BOC-Asn-Thr-OBzl (Ia) were treated with MSA. The products were checked with an amino acid analyzer[17] after acid hydrolysis, but only threonine was detected in all cases. Thus, we concluded that inversion at the β-carbon of the threonine residue does not occur with MSA.

Experimental

All melting points were taken in open capillaries and are uncorrected. Rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed with a Hitachi KLA-3B amino acid analyzer. Acid hydrolyses were carried out according to the method of Matsubara and Sasaki.[18] Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35—40°. Catalytic hydrogenations were performed at room temperature with palladium black as catalyst. The purity of the products was tested by thin-layer chromatography (TLC) using Merck precoated silica gel plate 60 F_{254} or/and cellulose plate (Avicel). Solvent systems used are: $CHCl_3$–MeOH–AcOH (9: 1: 0.5, Rf^1), AcOEt–pyridine–AcOH–H_2O (60: 20: 6: 11, Rf^2), $CHCl_3$–MeOH–H_2O (7: 3: 0.5, Rf^3), *n*-BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24, Rf^4), AcOEt–*n*-BuOH–AcOH–H_2O (1: 1: 1: 1, Rf^5). *Rf* values were given using silica gel plate unless otherwise mentioned.

BOC-Asn-Thr-OBzl (Ia)——BOC–Thr–OBzl (112 g) was dissolved in TFA (300 ml) and the solution was allowed to stand at room temperature for 20 min. To the solution was added 12 N HCl (30 ml) and evaporated. The residue was dried over NaOH pellets *in vacuo* and then dissolved in THF (1000 ml) together with TEA (50 ml). To the solution were added BOC–Asn–OH (76.7 g), HONB (64.5 g) and DCC (74.3 g) with stirring. The mixture was stirred at 0° for 6 hr and at room temperature for additional 10 hr and then

15) We wish to express our deep appreciation to Dr. H. Iwatsuka and Mr. S. Taketomi, Biological Research Laboratories of this Division, for the biological assay.
16) T. Wakamiya, Y. Tarumi, and T. Shiba, *Chem. Lett.*, **1973**, 233.
17) Column, 0.9×150 cm; buffer, 0.35 N sodium citrate; pH 5.28; column temperature, 55°. The retention times of allothreonine and threonine are 134 min and 140 min, respectively.
18) H. Matsubara and K. Sasaki, *Biochem. Biophys. Res. Commun.*, **35**, 175 (1969).

filtered to remove the formed DCU. The filtrate was evaporated to dryness and the residue was dissolved in AcOEt (1000 ml), washed with 4% aqueous $NaHCO_3$ and 10% aqueous citric acid, dried over anhydr. Na_2SO_4 and then the solvent was evaporated. The residue was triturated with ether to give crystals which were purified by recrystallization from acetonitrile: 105.1 g (75.2%), mp 165—166°, $[\alpha]_D^{23}$ −13.9° (c=0.9 in DMF), Rf^1 0.51. *Anal.* Calcd. for $C_{20}H_{29}N_3O_7$: C, 56.72; H, 6.90; N, 9.92. Found: C, 57.01; H, 6.89; N, 9.94.

BOC-Met(O)-Asn-Thr-OBzl (Ib)——Compound Ia (50.0 g) was treated with TFA (170 ml) as described above and the solution was evaporated. The resulting residue was triturated with dry ether to give a powder whcih was dissolved in THF (400 ml) together with TEA (20 ml) and to this was added BOC-Met(O)-ONB which was prepared from BOC-Met(O)-OH (31.3 g) by the usual manner.[13] The mixture was stirred at room temperature for 15 hr and evaporated. The residue was triturated with a mixture of AcOEt (200 ml) and ether (200 ml) to give a fine powder, which was purified by reprecipitation from acetonitrile: 48.5 g (72.0%), mp 145—147°, $[\alpha]_D^{23}$ −6.5° (c=1.1 in DMF), Rf^1 0.19. *Anal.* Calcd. for $C_{25}H_{38}N_4O_9S$: C, 52.62; H, 6.71; N, 9.82; S, 5.62. Found: C, 52.44; H, 6.73; N, 9.60; S, 5.15.

BOC-Leu-Met(O)-Asn-Thr-OBzl (I)——Compound Ib (15.0 g) was treated with TFA (45 ml) and the free base obtained was dissolved in DMF (50 ml) together with TEA (5.7 ml). To this solution was added BOC–Leu–ONB prepared from BOC–Leu–OH (6.69 g). After the usual work-up the product was purified by reprecipitation from acetonitrile–AcOEt: 15.0 g (83.4%), mp 134—136°, $[\alpha]_D^{24}$ −15.1° (c=1.0 in DMF), Rf^1 0.25. *Anal.* Calcd. for $C_{31}H_{49}N_5O_{10}S$: C, 54.45; H, 7.22; N, 10.24; S, 4.69. Found: C, 54.62; H, 7.60; N, 9.89; S, 3.95.

Z-Gln-Trp-OBzl (IIa)——To a solution of H–Trp–OBzl–p-toluenesulfonate (50 g) and Z–Gln–OH (28.0 g) in THF (500 ml) were added TEA (15.4 ml), HONB (19.7 g) and DCC (22.7 g) at 0°. The mixture was stirred at 0° for 15 hr. After the usual work-up, the material was crystallized from acetonitrile: 46.1 g (82.8%), mp 136—138°, $[\alpha]_D^{23}$ +5.8° (c=1.0 in DMF), Rf^1 0.60. *Anal.* Calcd. for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.80; N, 10.07. Found: C, 66.79; H, 5.71; N, 10.20.

Z-Val-Gln-Trp-OH (IIb)——Compound IIa (50.0 g) was hydrogenolyzed in MeOH (700 ml) for 5 hr. The crystalline product formed was collected by filtration and the crystals were dissolved in DMF (300 ml) together with TEA (13 ml). The mixture was filtered to remove the catalyst and to this was added Z–Val–ONB (37.0 g). The mixture was stirred at room temperature for 10 hr and then acidified with 1 N HCl (100 ml). The solution was diluted with H_2O (500 ml) to give a precipitate which was washed well with MeOH: 43.0 g (84.7%), mp 246—247°, $[\alpha]_D^{23}$ +12.4° (c=0.9 in DMF), Rf^1 0.14. *Anal.* Calcd. for $C_{29}H_{35}N_5O_7$: C, 61.58; H, 6.24; N, 12.38. Found: C, 61.86; H, 6.30; N, 12.36.

Z-Phe-Val-Gln-Trp-OH (IIc)——Compound IIb (5.1 g) was hydrogenolyzed in AcOH (50 ml) for 3 hr. After filtration and evaporation, the resulting residue was suspended in DMF (200 ml). To this solution were added TEA (2 ml) and Z–Phe–ONB prepared from Z–Phe–OH (2.70 g) and the mixture was stirred at room temperature for 7 hr, and then evaporated. The residue was triturated with aqueous AcOH to give a powder which was purified by reprecipitation from MeOH: 5.50 g (84.5%), mp 240°, $[\alpha]_D^{24}$ +4.1° (c=1.0 in DMF), Rf^1 0.15. *Anal.* Calcd. for $C_{38}H_{44}N_6O_8 \cdot 1/2H_2O$: C, 63.23; H, 6.28; N, 11.64. Found: C, 63.11; H, 6.29; N, 11.80.

BOC-Asp(OBzl)-Phe-Val-Gln-Trp-OH (IId)——Compound IIc (8.5 g) was hydrogenolyzed in a mixture of DMF (150 ml) and AcOH (50 ml) for 5 hr. The mixture was then filtered to remove the catalyst and the filtrate was evaporated to dryness. The residue was crystallized from MeOH. The crystals were suspended in DMF (200 ml) and to this were added TEA (3.0 ml) and BOC–Asp(OBzl)–ONB prepared from BOC–Asp(OBzl)–OH (3.90 g). The mixture was stirred at room temperature for 10 hr and then evaporated. The residue was triturated with 5% aqueous AcOH to give a fine powder which was purified by reprecipitation from aqueous DMF: 6.3 g (58.1%), mp 191—192° (dec.), $[\alpha]_D^{24}$ −6.2° (c=1.1 in DMF), Rf^1 0.11. *Anal.* Calcd. for $C_{46}H_{57}N_7O_{11} \cdot 3/2H_2O$: C, 60.64; H, 6.63; N, 10.76. Found: C, 60.30; H, 6.48; N, 11.34.

BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-OH (II)——Compound IId (6.0 g) was treated with TFA (50 ml) under nitrogen gas and the TFA salt of the free base obtained as a fine powder was dissolved in DMF (100 ml) together with TEA (2.0 ml). To the solution was added BOC–Gln–ONB prepared from BOC–Gln–OH (1.76 g) and the mixture was stirred at room temperature for 16 hr, then evaporated. The residue was triturated with 5% aqueous AcOH to give a powder which was purified by reprecipitation from aqueous acetonitrile: 5.50 g (82.6%), mp 210—212° (dec.), $[\alpha]_D^{24}$ −10.1° (c=1.1 in DMF), Rf^1 0.09. *Anal.* Calcd. for $C_{51}H_{65}N_9O_{13}$: C, 60.52; H, 6.47; N, 12.46. Found: C, 60.19; H, 6.37; N, 12.23.

Z-Arg(MBS)-Ala-OBu^t (IIIa)——Z–Arg(MBS)–OH·DCHA (53.0 g) was suspended in AcOEt (500 ml) and the suspension was washed with 10% aqueous citric acid (500 ml × 2) and dried over anhydr. Na_2SO_4. After evaporation, the residue was dissolved in THF (500 ml) together with H–Ala–OBu^t prepared from Z–Ala–OBu^t (31.2 g) by hydrogenolysis. To this solution were added HONB (14.9 g) and DCC (17.1 g) at 0° and the mixture was stirred at 0° for 5 hr and at room temperature for additional 10 hr. After usual work-up the substance was crystallized from MeOH: 44.5 g (66.8%), mp 126—127°, $[\alpha]_D^{25}$ −6.0° (c=1.0 in DMF), Rf^1 0.62. *Anal.* Calcd. for $C_{28}H_{39}N_5O_8S$: C, 55.52; H, 6.49; N, 11.56; S, 5.27. Found: C, 55.71; H, 6.49; N, 11.81; S, 5.29.

Z-Arg(MBS)-Arg(MBS)-Ala-OBu^t (IIIb)——Compound IIIa (43.0 g) was hydrogenolyzed in the usual way and the deblocked peptide was condensed with Z–Arg(MBS)–OH prepared from DCHA salt (49.7 g)

by the HONB/DCC method[13] in DMF (200 ml). After the usual work-up the product was purified by trituration with MeOH: 52.4 g (79.2%), mp 116—118°, $[\alpha]_D^{25}$ −8.8° (c=1.0 in DMF), Rf^1 0.42. *Anal.* Calcd. for $C_{41}H_{57}N_9O_{12}S_2 \cdot 2H_2O$: C, 50.86; H, 6.35; N, 13.02; S, 6.62. Found: C, 51.05; H, 6.08; N, 13.11; S, 6.62.

BOC-Ser-Arg(MBS)-Arg(MBS)-Ala-OBu^t (IIIc)——Compound IIIb (30.0 g) was hydrogenolyzed in DMF–MeOH (80—300 ml). The free base obtained was coupled with BOC–Ser–OH (7.3 g) by the HONB/DCC method in DMF (300 ml). After the usual work-up the substance was chromatographed on silica gel (400 g) and eluted with $CHCl_3$–MeOH–AcOH (9: 0.7: 0.35). The fractions (800—2000 ml) containing the desired product (checked by TLC) were combined and evaporated. The residue was triturated with ether to give a powder: 25.5 g (79.0%), mp 85—88°, $[\alpha]_D^{26}$ −20.9° (c=1.0 in MeOH), Rf^1 0.33. *Anal.* Calcd. for $C_{41}H_{64}N_{10}O_{14}S_2 \cdot H_2O$: C, 49.09; H, 6.63; N, 13.96; S, 6.39. Found: C, 48.96; H, 6.55; N, 13.70; S, 5.84.

BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-OH (III)——Compound IIIc (10.5 g) was treated with TFA (50 ml) for 60 min and the TFA salt of the product was dissolved in DMF (50 ml) together with TEA (4.1 ml). To this was added BOC–Asp(OBzl)–ONB prepared from BOC–Asp(OBzl)–OH (3.4 g) in the usual manner. After 15 hr at room temperature and the usual work-up the material was purified by reprecipitation from acetonitrile–ether: 6.0 g (51.5%), mp 126—130°, $[\alpha]_D^{24}$ +3.5° (c=1.0 in DMF), Rf^1 0.17. *Anal.* Calcd. for $C_{48}H_{67}N_{11}O_{17}S_2 \cdot 2H_2O$: C, 49.26; H, 6.12; N, 13.17; S, 5.48. Found: C, 49.62; H, 5.84; N, 13.00; S, 5.03.

BOC-Tyr-Leu-OMe (IVa)——To a suspension of H–Leu–OMe·HCl (28.5 g) in THF (500 ml) were added TEA (19.8 ml), BOC–Tyr–OH (40.0 g), HONB (28.0 g) and DCC (32.2 g) at 0°. After 5 hr at 0° and 10 hr at 20° and the usual work-up, the material was crystallized from AcOEt–pet. ether: 51.8 g (89.3%), mp 106—108°, $[\alpha]_D^{24}$ −14.7° (c=0.9 in DMF), Rf^1 0.73. *Anal.* Calcd. for $C_{21}H_{32}N_2O_6$: C, 61.74; H, 7.90; N, 6.86. Found: C, 61.86; H, 7.73; N, 6.84.

BOC-Lys(Z)-Tyr-Leu-OMe (IVb)——Compound IVa (28.0 g) was treated with TFA (140 ml) and the powder obtained was dissolved in THF (200 ml) together with TEA (9.6 ml). To this solution was added BOC–Lys(Z)–ONB prepared from BOC–Lys(Z)–OH·DCHA (35.0 g), HONB (12.3 g) and DCC (14.1 g) in THF (300 ml). The mixture was stirred for 10 hr at room temperature and then evaporated. After the usual work-up the material was crystallized from AcOEt–pet. ether: 39.7 g (95.0%), mp 110—111°, $[\alpha]_D^{24}$ −23.1° (c=0.9 in DMF), Rf^1 0.53. *Anal.* Calcd. for $C_{35}H_{50}N_4O_9$: C, 62.67; H, 7.51; N, 8.35. Found: C, 62.39; H, 7.77; N, 8.09.

BOC-Ser-Lys(Z)-Tyr-Leu-OMe (IVc)——Compound IVb (15.0 g) was treated with TFA (50 ml) and the TFA salt was dissolved in DMF (150 ml) together with TEA (3.1 ml). To this were added BOC–Ser–OH (4.6 g), HONB (4.4 g) and DCC (5.1 g) at 0°. After 15 hr the reaction mixture was evaporated and then triturated with H_2O. The powder obtained was further purified by reprecipitation from MeOH–ether: 11.7 g (67.3%), mp 109—111°, $[\alpha]_D^{24}$ −24.2° (c=1.1 in DMF), Rf^1 0.59. *Anal.* Calcd. for $C_{38}H_{55}N_5O_{11} \cdot H_2O$: C, 58.82; H, 7.41; N, 9.03. Found: C, 59.02; H, 7.08; N, 9.15.

BOC-Ser-Lys(Z)-Tyr-Leu-OH (IV)——Compound IVc (11.0 g) was dissolved in MeOH (200 ml) and to this was added 1 N NaOH (50 ml) at 0°. The mixture was stirred at 10° for 2 hr and then acidified with 1 N HCl (50 ml), and evaporated. The resulting residue was triturated with H_2O to give a powder, which was collected by filtration and washed well with ether: 7.8 g (69.0%), mp 143—145°, $[\alpha]_D^{24}$ −18.4° (c=1.1 in DMF), Rf^1 0.19. *Anal.* Calcd. for $C_{37}H_{53}N_5O_{11} \cdot 2H_2O$: C, 56.98; H, 7.37; N, 8.98. Found: C, 56.60; H, 7.08; N, 8.64.

BOC-Asp(OBzl)-Tyr-OH (Va)——To a solution of H–Tyr–OH (19.9 g) in 2 N NaOH (110 ml) was added a solution of BOC–Asp(OBzl)–ONB (prepared from 32.3 g of BOC–Asp(OBzl)–OH in the usual manner) in THF (300 ml). The mixture was stirred at room temperature for 15 hr and then evaporated. After the usual work-up the material was chromatographed on silica gel (300 mg) and eluted with $CHCl_3$–MeOH–AcOH (20: 1: 0.5). The substance was crystallized from AcOEt–pet. ether: 31.0 g (63.5%), mp 75—77°, $[\alpha]_D^{24}$ −3.4° (c=0.9 in DMF), Rf^1 0.50. *Anal.* Calcd. for $C_{25}H_{30}N_2O_8 \cdot H_2O$: C, 59.51; H, 6.39; N, 5.55. Found: C, 59.66; H, 6.13; N, 5.47.

BOC-Ser-Asp(OBzl)-Tyr-OH (Vb)——Compound Va (14.0 g) was treated with TFA (50 ml) and the TFA salt obtained was coupled with BOC–Ser–OH (5.91 g) in DMF (50 ml) *via* the corresponding HONB–ester in the presence of TEA (7 ml). After the usual work-up the material was crystallized from *n*-BuOH–ether: 8.2 g (48.9%), mp 144—145°, $[\alpha]_D^{24}$ −7.3° (c=0.9 in DMF), Rf^1 0.21. *Anal.* Calcd. for $C_{28}H_{35}N_3O_{10} \cdot 1/2H_2O$: C, 57.72; H, 6.23; N, 7.21. Found: C, 57.47; H, 5.94; N, 7.29.

BOC-Thr-Ser-Asp(OBzl)-Tyr-OH (Vc)——Compound Vb (8.0 g) was treated with TFA (60 ml) and the TFA salt obtained was coupled with BOC–Thr–OH (3.4 g) in DMF (60 ml) *via* the corresponding HONB–ester in the presence of TEA (4.3 ml). After 15 hr and evaporation (DMF), the material was obtained in the usual way and then crystallized from ether: 8.85 g (94.3%), mp 101—102°, $[\alpha]_D^{24}$ −4.7° (c=1.0 in DMF), Rf^1 0.13. *Anal.* Calcd. for $C_{32}H_{42}N_4O_{12} \cdot 1/2H_2O$: C, 56.21; H, 6.34; N, 8.20. Found: C, 56.31; H, 6.28; N, 8.28.

BOC-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH (Vd)——Compound Vc (8.0 g) was treated with TFA (60 ml) and the deblocked material was coupled to BOC–Phe–ONB (5.31 g) in DMF (50 ml). After the usual work-up the material was crystallized from ether: 7.2 g (73.3%), mp 173—174° (dec.), $[\alpha]_D^{24}$ −4.5° (c=1.0 in DMF), Rf^1 0.19. *Anal.* Calcd. for $C_{41}H_{51}N_5O_{13} \cdot H_2O$: C, 58.63; H, 6.36; N, 8.34. Found: C, 58.32; H, 6.22; N, 8.46.

BOC-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH (V)——Compound Vd (6.9 g) was treated with TFA (50 ml)

and the deblocked product was dissolved in DMF (50 ml) together with TEA (2.4 ml). To this solution was added BOC–Thr–ONB prepared from BOC–Thr–OH (2.02 g). After 15 hr and the usual work-up the product was purified by trituration with 5% aqueous AcOH: 7.0 g (88.8%), mp 175—176°, $[\alpha]_D^{25}$ −3.2° (c=1.1 in DMF), Rf^1 0.11. *Anal.* Calcd. for $C_{45}H_{58}N_6O_{15}\cdot H_2O$: C, 57.43; H, 6.43; N, 8.93. Found: C, 57.28; H, 6.31; N, 8.90.

Z-Gln-Gly-OBu^t (VIa)——Z–Gln–OH (28.0 g) was coupled with H–Gly–OBu^t (14.5 g) in THF (300 ml) by the HONB–DCC method. After the usual work-up the material was crystallized from MeOH–AcOEt: 26.0 g (66.0%), mp 170—171°, $[\alpha]_D^{24}$ −6.7° (c=0.9 in DMF), Rf^1 0.57. *Anal.* Calcd. for $C_{19}H_{27}N_3O_6$: C, 58.00; H, 6.92; N, 10.68. Found: C, 57.71; H, 6.71; N, 10.89.

Z-Ser-Gln-Gly-OBu^t (VIb)——Compound VIa (12.0 g) was hydrogenolyzed in MeOH (100 ml) for 5 hr and the free base obtained was coupled with Z–Ser–OH (7.3 g) by the HONB–DCC method in DMF (50 ml). After the usual work-up the material was crystallized from aqueous acetonitrile: 10.0 g (68.2%), mp 156—158°, $[\alpha]_D^{24}$ −0.1° (c=1.0 in DMF), Rf^1 0.25. *Anal.* Calcd. for $C_{22}H_{32}N_4O_8$: C, 54.99; H, 6.71; N, 11.66. Found: C, 55.48; H, 6.90; N, 11.47.

Z-His-Ser-Gln-Gly-OBu^t (VIc)——Compound VIb (4.81 g) was hydrogenolyzed in MeOH (50 ml) for 7 hr and the free base obtained was condensed with Z–His–OH (2.89 g) by the HONB–DCC method in DMF (50 ml). After the usual work-up the material was purified by reprecipitation from MeOH–AcOEt: 5.60 g (90.7%), mp 114—115°, $[\alpha]_D^{25}$ −4.0° (c=1.0 in DMF), Rf^3 0.51. *Anal.* Calcd. for $C_{28}H_{39}N_7O_9$: C, 54.45; H, 6.36; N, 15.88. Found: C, 54.58; H, 6.51; N, 15.65.

Z-His-Ser-Gln-Gly-OH (VI)——Compound VIc (5.5 g) was treated with TFA (40 ml) for 30 min and the powder obtained was dissolved in H_2O and to this was added 15 N aqueous ammonia to give a powder, which was purified by reprecipitation from aqueous MeOH: 3.70 g (69.5%), mp 169—171°, $[\alpha]_D^{25}$ −36.0° (c=1.0 in H_2O), Rf^5 0.70. *Anal.* Calcd. for $C_{24}H_{31}N_7O_9\cdot 2H_2O$: C, 48.23; H, 5.90; N, 16.41. Found: C, 48.00; H, 5.84; N, 16.44.

BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (VII)——The BOC-group of compound I (3.25 g) was removed by treatment with TFA (25 ml). The TFA salt was then converted to the corresponding free base with TEA (2.0 ml) in NMP followed by precipitation with ether. The free base was dissolved in DMF (100 ml) and then condensed with compound II (4.80 g) in the presence of HONB (2.70 g) and DCC (1.55 g) at 0°. After 58 hr and the usual work-up the material was purified by washing with acetonitrile and H_2O: 5.75 g (76.8%), mp 234° (dec.), $[\alpha]_D^{26}$ −15.8° (c=0.4 in AcOH), Rf^3 0.63. *Anal.* Calcd. for $C_{77}H_{104}N_{14}O_{20}S$: C, 58.61; H, 6.64; N, 12.43; S, 2.03. Found: C, 59.13; H, 6.96; N, 12.40; S, 1.70.

BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (VIII)——Compound VII (5.20 g) was treated with TFA (35 ml) in the presence of anisole (1 ml) under nitrogen gas and the resulting TFA salt was converted to the free base by treatment with TEA (2.8 ml) in NMP (20 ml). The free base was obtained as a powder by treatment with dry ether and the powder was dissolved in DMF (150 ml) together with compound III (3.66 g). To this solution were added HONB (2.36 g) and DCC (1.02 g) at −10° and the mixture was stirred at 0° for 10 hr and at room temperature for additional 24 hr. After filtration and evaporation, the residue was triturated with AcOEt (500 ml) to give a powder, which was collected by filtration and washed with aqueous acetonitrile: 5.3 g (61.1%), mp 237° (dec.), $[\alpha]_D^{26}$ −7.1° (c=0.9 in AcOH), Rf^3 0.63. *Anal.* Calcd. for $C_{120}H_{161}N_{25}O_{34}S_3\cdot 2H_2O$: C, 54.82; H, 6.32; N, 13.32; S, 3.57. Found: C, 54.47; H, 6.16; N, 13.07; S, 3.47.

BOC-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (IX)——Compound VIII (3.0 g) was treated with TFA (30 ml) and the TFA salt obtained was converted to the free base by the same manner described above. The free base was dissolved in DMF (40 ml) together with compound IV (0.91 g) and to this were added HONB (0.83 g) and DCC (0.47 g) at −10°. The mixture was stirred at 0° for 10 hr and at room temperature for 24 hr and then filtered to remove the formed DCU. The filtrate was evaporated to dryness. The residue was triturated with H_2O to give a powder, which was collected by filtration and then washed well with aqueous acetonitrile: 2.0 g (53.5%), mp 235° (dec.), $[\alpha]_D^{26}$ +1.7° (c=1.1 in AcOH), Rf^3 0.64. *Anal.* Calcd. for $C_{152}H_{204}N_{30}O_{42}S_3\cdot 3H_2O$: C, 55.76; H, 6.47; N, 12.84; S, 2.94. Found: C, 55.82; H, 6.47; N, 12.77; S, 2.67.

BOC-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (X)——Compound IX (1.90 g) was treated with TFA (20 ml) and the free base obtained in a similar manner as described above was dissolved in DMF (20 ml) together with compound V (0.55 g). To the solution were added HONB (0.42 g) and DCC (0.24 g) at −10° and the mixture was stirred at 0° for 10 hr and at room temperature for 48 hr. After filtration and evaporation, the residue was triturated with AcOEt (50 ml) to give a powder, which was collected by filtration and purified by washing with aqueous acetonitrile: 1.24 g (50.7%), mp 246—249° (dec.), $[\alpha]_D^{26}$ +4.1° (c=1.0 in NMP), Rf^3 0.65. *Anal.* Calcd. for $C_{192}H_{252}N_{36}O_{54}S_3\cdot 6H_2O$: C, 55.80; H, 6.54; N, 12.20; S, 2.33. Found: C, 55.63; H, 6.31; N, 11.83; S, 2.38.

Z-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg-(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OBzl (XI)——Compound X (1.11 g) was treated with TFA (10 ml) and the free base obtained in a similar manner as described above was dissolved in DMF (20 ml) together with compound VI (0.18 g). To this were added HONB (0.22 g) and DCC (0.13 g)

at 0° and the mixture was stirred at 0° for 10 hr and at room temperature for 48 hr. After filtration and evaporation, the residue was triturated with AcOEt to give a powder, which was collected by filtration and purified by washing with aqueous acetonitrile: 0.93 g (73.1%), mp 226—229° (dec.), $[\alpha]_D^{23}$ +12.8° (c=1.0 in NMP), Rf^3 0.62. *Anal.* Calcd. for $C_{211}H_{273}N_{43}O_{60}S_3 \cdot 8H_2O$: C, 54.95; H, 6.32; N, 13.06; S, 2.09. Found: C, 54.62; H, 6.04; N, 13.03; S, 2.02.

H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-OH (Met(O)^{27}-glucagon)——a) Treatment with MSA: Compound XI (460 mg) was dissolved in MSA (5 ml) containing anisole (0.4 ml) at 0° and the mixture was kept to stand at room temperature for 60 min. The mixture was triturated with ether to give an oily residue which was dissolved in H_2O (10 ml). The solution was passed through a column (1×10 cm) of Amberlite IRA-410 (acetate form). The passed solution and washings were combined (total volume, 40 ml), and to this was added 2 N aqueous ammonia (15 ml). The solution was kept to stand at 0° for 30 min, then lyophilized. The crude peptide thus obtained was dissolved in 0.1 N AcOH (5 ml) and passed through a column (4.5×135 cm) of Sephadex LH-20 (0.1 N AcOH). The fractions (540—620 ml) containing the desired product were combined and lyophilized: 75 mg. The powder obtained was dissolved in 0.1 N AcOH (5 ml) and applied to a column (2.4×14 cm) of CM-cellulose, which was eluted with pH 4.6 ammonium acetate buffer (gradient: 0.005 M/0.2 M =500 ml/500 ml). The fractions (330—440 ml) containing the pure product (checked by TLC) were combined and lyophilized: 27 mg, $[\alpha]_D^{24}$ −37.1° (c=0.5 in 1% AcOH), Rf^4 (cellulose) 0.56. Amino acid anal: Lys 1.00 (1); His 1.00 (1); Arg 2.25 (2): Trp 0.88 (1); Asp 4.25 (4); Thr 3.00 (3); Ser 3.63 (4); Glu 3.38 (3); Gly 1.00 (1); Ala 1.00 (1); Val 1.00 (1); Met 1.00 (1); Leu 2.13 (2); Tyr 2.13 (2); Phe 2.13 (z) (average recovery, 79%).

b) Treatment with anhydr. HF: Compound XI (500 mg) was dissolved in anhydr. HF (5 ml) in the presence of anisole (0.5 ml), and the mixture was stirred at 0° for 60 min. After evaporation, the residue was dissolved in H_2O (50 ml) and then washed with ether (40 ml×2). The solution was passed through a column (1×10 cm) of Amberlite IRA-410 (acetate form). The passed solution and washings were combined (total volume, 150 ml) and to this was added 3 N aqueous ammonia (25 ml). The solution was kept to stand at 0° for 30 min and then lyophilized. The crude peptide thus obtained was dissolved in 0.1 N AcOH (5 ml) and passed through a column (4.5×135 cm) of Sephadex LH-20 (0.1 N AcOH). The fractions (560—680 ml) containing the desired product were combined and lyophilized: 91 mg. The powder obtained was further purified with CM-cellulose column (2.4×14 cm) in the same manner as described above, and the fractions (330—420 ml) containing the pure product were pooled and lyophilized: 39 mg, $[\alpha]_D^{24}$ −37.1° (c=0.3 in 1% AcOH), Rf^4 (cellulose) 0.56.

H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH (Glucagon)——Met(O)^{27}-glucagon (30 mg) was dissolved in 3% aqueous thioglycolic acid (3 ml) and the solution was kept to stand at 50° for 20 hr. The solution was applied to a column (2.4×90 cm) of Sephadex G-25 (0.1 N AcOH). The fractions (190—225 ml) containing the pure glucagon were combined and lyophilized: 20 mg. The powder (15 mg) was suspended in 0.02% aqueous NaCl solution (1.5 ml) and dissolved by the addition of 0.1 N aqueous NaOH (pH 10.5). The solution was then neutralized to pH 8.5 with 0.1 N aqueous HCl, and allowed to stand at room temperature for 24 hr and at 0° for additional 24 hr. The crystals formed were collected by centrifugation and then washed well with 0.02% aqueous NaCl solution: 8.3 mg, $[\alpha]_D^{21}$ −39.9° (c=0.2 in 1% AcOH),[19] UV $\lambda_{max}^{0.1N\ NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.0 (20.85), 289.5 (21.60),[19] Rf^4 (cellulose) 0.60, Rf^5 (cellulose) 0.65,[19] Paper electrophoresis (pH 1.9, HCOOH–AcOH buffer, 600 volt, 60 min) 0.68×Arg.[19] Amino acid ratios in acid hydrolysate: Lys 1.00 (1); His 0.88 (1); Arg 2.13 (2); Trp 0.88 (1); Asp 4.13 (4); Thr 2.88 (3); Ser 3.38 (4); Glu 3.25 (3); Gly 1.00 (1); Ala 1.00 (1); Val 1.00 (1); Met 1.00 (1); Leu 2.13 (2); Tyr 2.13 (2); Phe 2.00 (2) (average recovery, 86%). Amino acid ratios in aminopeptidase M hydrolysate: Lys 1.21 (1); His 0.86 (1); Arg 2.11 (2); Trp 0.99 (1); Asp 3.16 (3); Thr+Gln 5.18 (6); Ser+Asn 4.75 (5); Glu 0.28 (0); Gly 1.00 (1); Ala 1.05 (1); Val 1.13 (1); Met 1.07 (1); Leu 2.11 (2); Tyr 2.08 (2); Phe 2.14 (2) (average recovery, 80%).

The N to O Acyl Migration of Thr or Ser-containing Peptide in MSA——a) Examination of the Products by Thin-layer Chromatography and Paper Electrophoresis: Each sample [100 mg of BOC–Leu–Met(O)–Asn–Thr–OBzl(I), BOC–Asn–Thr–OBzl(Ia), BOC–Thr–Phe–Thr–Ser–Asp(OBzl)–Tyr–OH(V) or BOC–Phe–Thr–Ser–Asp(OBzl)–Tyr–OH(Vd)] was treated with MSA (1 ml) in the presence of anisole (0.1 ml) at room temperature for 60 min. Dry ether was added and the resulting oily precipitate was dissolved in H_2O (5 ml). The solution was passed through a column (1×5 cm) of Amberlite IRA-410 (acetate form). The passed solution and washings were combined and then lyophilized. A part of the powder obtained was examined by TLC (Rf^5) and paper electrophoresis (pH 1.9, HCOOH–AcOH buffer, 600 volt, 60 min). Products were detected by ninhydrin. The results are shown in Table I.

b) Treatment of the Deblocked Peptides with Aqueous Ammonia: Each product (50 mg) obtained above was dissolved in H_2O (10 ml) at 0° and to this was added 2 N aqueous ammonia (3 ml). The solution

19) Natural crystalline glucagon (Sigma Chemical Co.): $[\alpha]_D^{21}$ −39.6° (c=0.2 in 1% AcOH); UV $\lambda_{max}^{0.1N\ NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.0 (20.85), 289.5 (21.50); Rf^4 (cellulose) 0.65; Paper electrophoresis (pH 1.9, HCOOH–AcOH buffer, 600 volt, 60 min) 0.68 x Arg.

was stirred at 0° for 30 min and then lyophilized. A part of the powder obtained was examined by TLC and paper electrophoresis in the same manner as described above. The results are also shown in Table I.

Table I. Chromatographic Behaviour of MSA-treated Glucagon Fragments

Compounds		*Rf* values on TLC[a] of major products	Mobilities (X Arg) on PEP[b] of major products
BOC-Leu-Met(O)-Asn-Thr-OBzl [I]	A)	0.32, 0.19	0.62, 0.55
	B)	0.32	0.55
BOC-Asn-Thr-OBzl [Ia]	A)	0.30, 0.20	0.88, 0.57
	B)	0.30[c]	0.57[c]
BOC-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH [V]	A)	0.49, 0.39	0.73, 0.54
	B)	0.49[c]	0.54[c]
BOC-Phe-Thr-Ser-Asp(OBzl)-Tyr-OH [Vd]	A)	0.59, 0.55	0.78, 0.56
	B)	0.59[c]	0.56[c]

A) Samples were treated with MSA at room temperature for 60 min.
B) Samples (A) were exposed to 0.5 N aqueous ammonia at 0° for 30 min.
a) Thin-layer chromatography; solvent, Rf^5 (AcOEt: *n*-BuOH: AcOH: H_2O=1: 1: 1: 1)
b) Paper electrophoresis; buffer, 0.6 N HCOOH: 2 N AcOH=1: 1 (pH 1.9)
c) The value was identical with that of the corresponding authentic sample.

c) Treatment of the Product obtained from BOC–Leu–Met(O)–Asn–Thr–OBzl (I) with Sodium Nitrite: The deblocked I (3.4 mg) and the ammonia-treated product of the deblocked I (3.2 mg) were dissolved in 50% aqueous AcOH (0.5 ml) together with 1 N HCl (0.5 ml). After addition of 1 N aqueous sodium nitrite (0.4 ml), these solution were stirred at room temperature for 6 hr, and then evaporated. The residues were hydrolyzed with 5.7 N HCl, and threonine contents of both the samples were analyzed by amino acid analyzer: Deblocked I: Thr/Asp=0.45/1.00; Ammonia treated sample: Thr/Asp=0.88/1.00 (No allothreonine was detected).

Acknowledgement We wish to thank Drs. E. Ohmura, K. Morita and M. Nishikawa for their encouragement and useful discussion throughout this work. Thanks are also due to Dr. M. Hori and Mr. I. Yoshida for amino acid analyses.