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Studies on Peptides. LXXVIII.[1,2] Synthesis of the Nonacosapeptide corresponding to the Entire Amino Acid Sequence of Avian Glucagon (Duck)

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The nonacosapeptide corresponding to the entire amino acid sequence of duck glucagon was synthesized using methanesulphonic acid as a deprotecting reagent at the final step of the synthesis. Synthetic duck glucagon was obtained in a crystalline form and exhibited the biological activity equivalent to that of porcine glucagon. However, the immunological property of synthetic duck glucagon was found to differ from the mammalian origin.

Keywords——avian pancreatic hormone; duck glucagon; [27-Met(O)]-duck glucagon; methanesulphonic acid as a deprotecting reagent; deprotection of Boc by 4 N ethanesulphonic acid in trifluoroethanol; cation scavenger: anisole containing 0.1% ethanedithiol and skatole; conversion of the N→O shift; lipolytic activity; immunological property

Following to the preceding paper,[1] we wish to describe the synthesis of the nonacosapeptide corresponding to the entire amino acid sequence of duck glucagon.[4] Synthetic scheme we employed for this synthesis is illustrated in Fig. 1 and the synthetic peptide, duck glucagon, was obtained in a crystalline form.

As mentioned previously, 7 peptide fragments, A (position 24—29), B (20—23), C (15—19), D (13—14), E (9—12), F (5—8) and G (1—4), were selected as those necessary to construct the entire amino acid sequence of duck glucagon by the Rudinger's azide procedure[5] exclusively.

This scheme is different from those employed for the syntheses of structurally related mammalian glucagon by other authors.[6–8] In these examples, the DCC condensation of suitable peptide fragments was performed in the presence of a racemization suppressor, such as N-hydroxysuccinimide[6] or N-hydroxy-5-norbornene-2,3-dicarboximide.[9] The chinese group[8] employed N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline[10,11] as the condensation reagent of peptide fragments on the polymer support.

1) Part LXXVII: H. Ogawa and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 1540 (1978).
2) Amino acids, peptides, and their derivatives are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl Z(OMe)=*p*-methoxybenzyloxycarbonyl, Boc=*t*-butoxycarbonyl, Bzl=benzyl, MBS=*p*-methoxybenzenesulfonyl, NP=*p*-nitrophenyl, Troc=*β,β,β*-trichloroethyloxycarbonyl, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, DMF=dimethylformamide, NMP=N-methylpyrrolidone, EDT=ethanedithiol, TFE=trifluoroethanol, ESA=ethanesulphonic acid, MSA=methanesulphonic acid.
3) Location: *a*), *b*) *Sakyo-ku, Kyoto, 606, Japan.*
4) F. Sundby, E.K. Frandsen, J. Thomsen, K. Kristiansen, and K. Brunfeldt, *FEBS Letters*, **26**, 298 (1972).
5) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, **26**, 2333 (1961).
6) E. Wünsch and G. Wendlberger, *Chem. Ber.*, **101**, 3659 (1968).
7) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 539 (1978).
8) Synthetic Protein Research Group, China Biochem. Inst., *Acta Biochim. Biophys. Sinica*, **7**, 119 (1975).
9) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974).
10) B. Bellou and G. Malek, *J. Am. Chem. Soc.*, **90**, 1651 (1968).
11) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **19**, 1905 (1971).

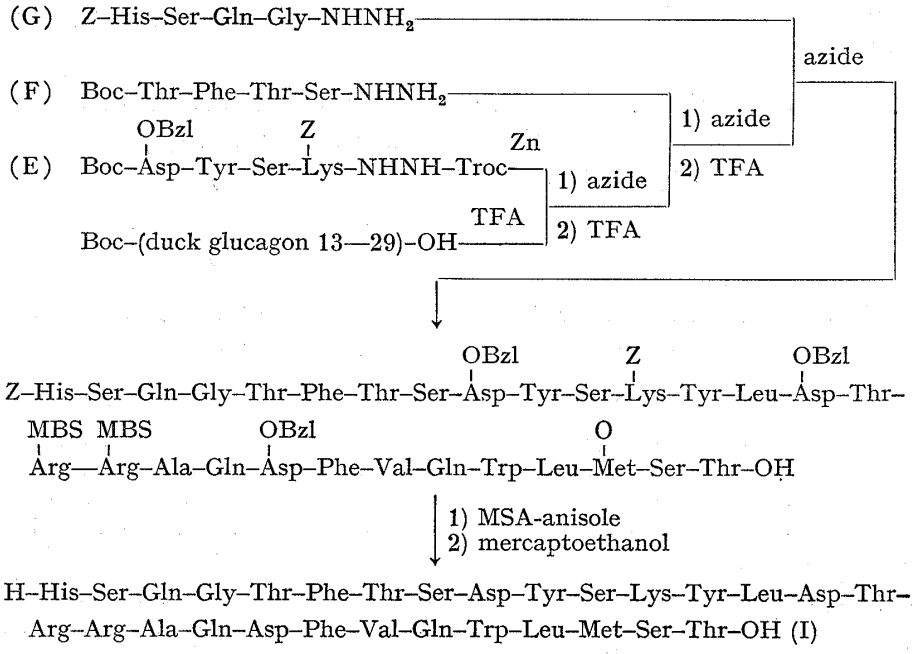

Fig. 1. Synthetic Route to Duck Glucagon

Our present synthesis was intiated using amino acid derivatives bearing protecting groups removable by MSA,[12] *i. e.*, Asp(OBzl), Lys(Z) and Arg(MBS).[13] In addition, Met(O) prepared by the sodium perborate oxidation[14] was adopted to prevent the alkylation at its sulphur atom in the MSA-anisole system[15] for the final deprotection. The Boc group served as the N^{α}-protection of peptides containing Trp, from which this group can be removed without producing any considerable side products and brown color by 4 N ESA—TFE or TFA in the presence of skatole and anisole containing 0.1% EDT.[1]

With these considerations and cares, the protected heptadecapeptide corresponding to the C-terminal portion of duck glucagon, Boc-Tyr-Leu-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-

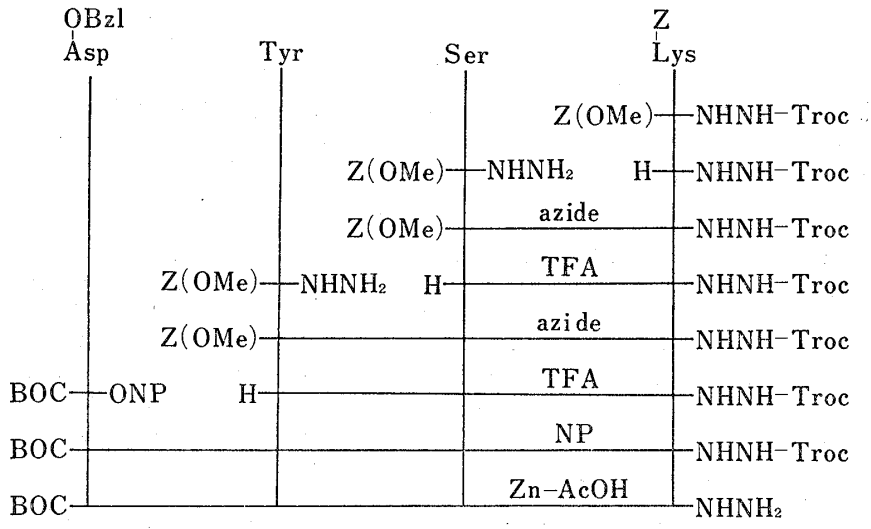

Fig. 2. Synthetic Scheme of the Protected Tetrapeptide Hydrazide Boc-(Duck Glucagon 9—12)-$NHNH_2$ (E)

12) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
13) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **24**, 1568 (1976).
14) N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 650 (1978).
15) H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *J.C.S. Chem. Comm.*, **1976**, 922; *idem*, *Chem. Pharm. Bull.* (Tokyo), **25**, 2929 (1977).

Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, was synthesized by the successive assembling of 4 peptide fragments, A, B, C, and D, as reported[1] and this served here as the starting material for the total synthesis of this avian pancreatic hormone. Thus, 3 peptide fragments, E, F, and G were newly synthesized. The N-terminal portion of duck glucagon covered with these three fragments is identical with that of mammalian glucagon. However, the fragments selected here are different from those employed by other investigators.[6-8]

Synthetic scheme of the fragment E, Boc-Asp(OBzl)-Tyr-Ser-Lys(Z)-$NHNH_2$, is illustrated in Fig. 2. In order to prepare the peptide hydrazide containing Asp(OBzl), this tetrapeptide hydrazide was synthesized starting with Troc-$NHNH_2$.[16] First, Z(OMe)-Lys(Z)-OH was condensed with Troc-$NHNH_2$ by DCC[17] and the Z(OMe) group was removed from the resulting Z(OMe)-Lys(Z)-NHNH-Troc by the usual TFA treatment.[18] Next, the azide procedure was adopted to introduce Tyr and Ser residues successively and the *p*-nitrophenyl ester procedure[19] to Asp(OBzl). Especially, in the latter step, the use of excess triethylamine was avoided to suppress the possible o-acylation at the Tyr residue.[20] The Troc group was removed from Boc-Asp(OBzl)-Tyr-Ser-Lys(Z)-NHNH-Troc by treatment with zinc[21] in a mixture of acetic acid and DMF and the last trace of the contaminated zinc was removed by ethylenediamine tetraacetate (EDTA).

The fragment F, Boc-Thr-Phe-Thr-Ser-$NHNH_2$, was synthesized by the azide condensation of two dipeptide units as shown in Fig. 3. Two hydrazide derivatives of Thr were condensed with H-Phe-OMe and H-Ser-OMe by the azide procedure respectively. The resulting Boc-Thr-Phe-OMe was converted to the corresponding hydrazide, Boc-Thr-Phe-$NHNH_2$, which was then condensed with the TFA treated sample of Z(OMe)-Thr-Ser-OMe obtained by the above coupling reaction. The resulting tetrapeptide ester, Boc-Thr-Phe-Thr-Ser-OMe, was similarly converted to the corresponding hydrazide (F) in the usual manner. For the preparation of peptides containing the hydroxyl group, the azide method seems to be still a preferable tool to prepare peptides avoiding the risk of the O-acylation.[22]

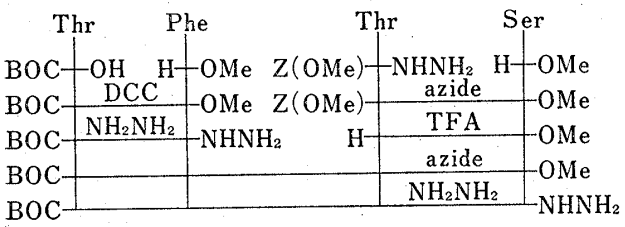

Fig. 3. Synthetic Scheme of the Protected Tetrapeptide Hydrazide Boc-(Duck Glucagon 5—8)-$NHNH_2$ (F)

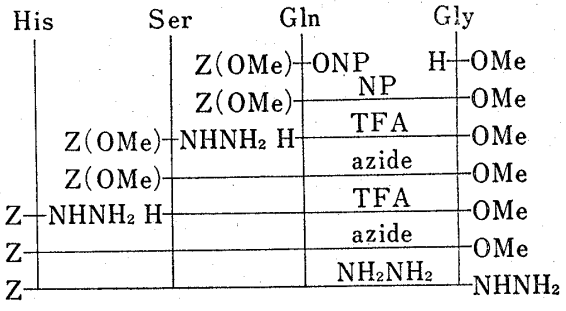

Fig. 4. Synthetic Scheme of the Protected Tetrapeptide Hydrazide Z-(Duck Glucagon 1—4)-$NHNH_2$ (G)

Synthetic scheme of the N-terminal tetrapeptide hydrazide G, Z-His-Ser-Gln-Gly-$NHNH_2$, is illustrated in Fig. 4. The Gly terminal peptides can be condensed by any means without the risk of racemization. However we decided to introduce this unit by the azide procedure

16) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 420 (1971).
17) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **81**, 5688 (1955).
18) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
19) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
20) J. Ramachandran and C.H. Li, *J. Org. Chem.*, **28**, 173 (1963).
21) R.B. Woodward, K. Heusler, J. Gosteli, W. Oppolzer, R. Ramage, S. Ranganathan, and H. Vorbruggen, *J. Am. Chem. Soc.*, **83**, 1991 (1961).
22) M. Bodanszky, M.L. Fink, Y.S. Klausner, S. Natarajan, K. Tatemoto, A.E. Yiotakis, and A. Bodanszky, *J. Org. Chem.*, **42**, 149 (1977).

also, since we started the present synthesis with the free carboxyl group at the C-terminus. This tetrapeptide hydrazide was synthesized stepwisely starting with H-Gly-OMe. The Gln residue was introduced by the *p*-nitrophenyl ester procedure and the subsequent two amino acid residues, Ser and His, by the azide procedure respectively. Conversion of the resulting protected tetrapeptide ester, Z-His-Ser-Gln-Gly-OMe, to the corresponding hydrazide G was performed without particular difficulty.

Three peptide hydrazides thus obtained were then assembled according to the scheme illustrated in Fig. 1. The Boc group was cleaved from the forementioned C-terminal heptadecapeptide, Boc-(duck glucagon 13—29)-OH, by TFA under cooling with ice, instead of 4 N ESA–TFE, as did in the preparation of the protected pentadeca and heptadecapeptides previously.[1] Though TFE is known to be a good solvent for peptides,[23] we could not also bring the above protected heptadecapeptide into a solution of the 4 N ESA–TFE system under cooling with ice. Effect of the additives, skatole and anisole containing 0.1% EDT, was still so evident, that no brown color was produced during this TFA deprotecting step. The deprotected peptide, after neutralization with triethylamine, was submitted to the coupling reaction with the azide derived from E. This coupling reaction had to be performed in a mixture of NMP and DMF, since the deprotected heptadecapeptide was not freely soluble in DMF alone under cooling conditions. The coupling reaction went smoothly and the desired protected heneicosapeptide, Boc-(duck glucagon 9—29)-OH, was obtained in good yield, after purication by batchwise washing with 5% citric acid and water followed by precipitation from hot DMF with ethanol. The homogeneity was assessed by three criteria; *i. e.*, thin-layer chromatography, elemental analysis and hydrolysis with 4 N MSA. As mentioned previously, Met(O) was converted partially to Met under hydrolytic conditions.

The N^{α}-deprotection of Boc-(duck glucagon 9—29)-OH and the subsequent azide condensation of the fragment F were carried out in essentially the same manner as mentioned above. The resulting protected pentacosapeptide was isolated after similar batchwise washing followed by precipitation from hot DMF with methanol. Subsequently the final coupling of the fragment G could also be carried out in a mixture of NMP and DMF in essentially the same manner as mentioned above and the protected nonacosapeptide, Z-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, was obtained in quantitative yield.

As the strategy to construct the nonacosapeptide chain, we assembled relatively small fragments successively by the azide method. This suffers certain disadvantageous feature in a respect of increasing numbers of coupling step, however gave us in turn an advantageous feature in a respect of purification, since small fragments used could be removed after each reaction by washing and precipitation as demonstrated above. Usually efficient tools to purify protected peptides by column chromatography are offenly limited. Such approach seems, in our opinion, still a useful method of choice to construct complex peptides.

For deprotection, the protected nonacosapeptide was then exposed to MSA in the presence of anisole in an ice-bath for 15 minutes and then at room temperature for 45 minutes. The deprotected peptide, precipitated by dry ether, was converted to the corresponding acetate by Amberlite CG-4B and then treated with dilute ammonia for a short period. The latter treatment was performed by the reason that reversible conversion of N→O shift at the Ser and Thr residues, if any, could be achieved, since similar behaviour of Ser and Thr rich peptides in hydrogen fluoride is reported[24] and Fujino *et al.*[7] also mentioned this possibility in the synthesis of mammalian glucagon by the MSA procedure. The treated product was next

23) B. Riniker, B. Kamber, and P. Sieber, *Helv. Chim. Acta*, **58**, 1086 (1975).
24) S. Sakakibara, in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," ed. by B. Weinstein, Marcel Dekker Inc., New York, 1971, Vol. 1, p. 51.

purified by column chromatography on Sephadex G-25 using 0.5% acetic acid as a elunt. A small front peak was discarded, since the material obtained from this fraction was not freely soluble in water. Though, we did not examine this fraction further, it is supposed to be a Trp-modified substance. Similar chromatographic pattern was recorded by Wünsch *et al.*[25] in the purification step of mammalian glucagon. The product obtained from the main peak (Fig. 5-a) was purified further by column chromatography on CM-cellulose using gradient elution with 0.06 M ammonium acetate buffer. The desired product was soon emerged from the column as shown in Fig. 5-b. [27-Met(O)]-duck glucagon thus obtained was incubated

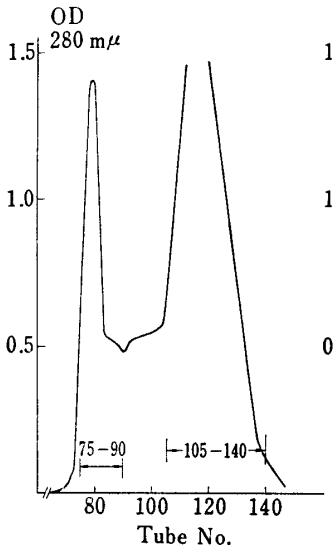

Fig. 5-a. Purification of [27-Met(O)]-duck Glucagon on Sephadex G-25

Sample 226 mg.
Size 3×150 cm.
Fraction 6 ml.
Solvent 0.5% AcOH.

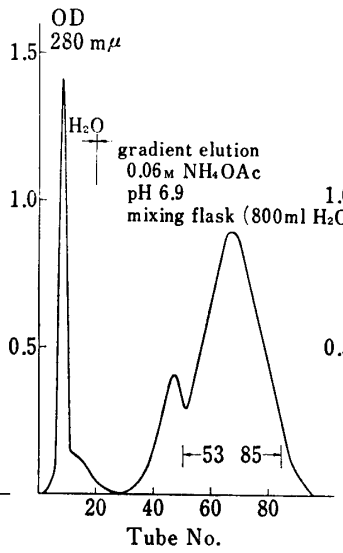

Fig. 5-b. Purification of [27-Met(O)]-duck Glucagon on CM-cellulose

Sample 109 mg.
Size 2.4×6.8 cm.
Fraction 6 ml.

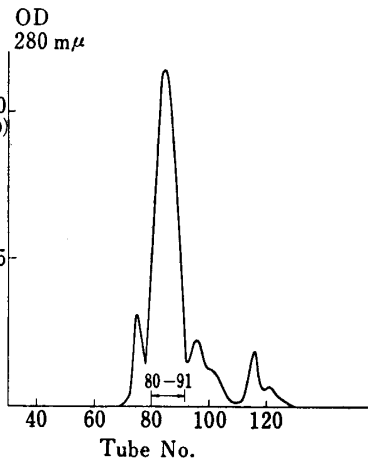

Fig. 5-c. Purification of synthetic Duck Glucagon on Sephadex G-25

Size 2.5×140 cm.
Solvent 0.5% AcOH.
Fraction 6 ml.

with mercaptoethanol to reduce the Met(O) residue to Met according to Iselin.[26] Progress of the reduction was monitored by the Met test on thin-layer chromatography and the reduced material was then purified by column chromatography on Sephadex G-25.

For crystallization, the thin layer chromatographically homogeneous product thus obtained was suspended in 0.02% sodium chloride according to Fujino *et al.*[7] After adjusting at pH 8.5, the solution was stored in a refrigerator for 3 days, while a crystalline material began to precipitate, which was collected by centrifugation and washed with cold 0.02% sodium chloride. (Fig. 6). Homogeneity of synthetic duck glucagon was assessed by disc electrophoresis at pH 2.3, gel electrofocusing and aminopeptidase digestion (AP-M)[27]

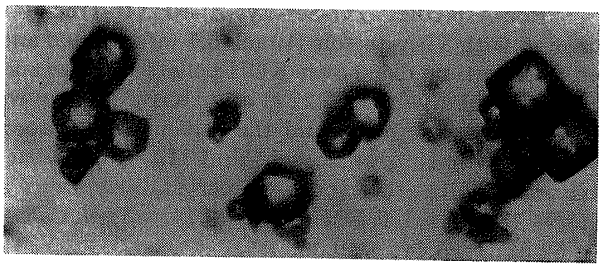

Fig. 6. Crystals of Synthetic Duck Glucagon

25) E. Wünsch, E. Jaeger, and R. Scharf, *Chem. Ber.*, **101**, 3664 (1968).
26) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
27) G. Pfleiderer and G.P. Celliers, *Biochem. Z.*, **339**, 186 (1963).

When the lipolytic activity on adipocytes of Sprague-Dawley rats was examined by Dr. H. Iwatsuka, Takeda Chem. Ind., synthetic duck glucagon was found biologically equivalent to that of porcine glucagon (Sigma G-4250). Sundby *et al.*[4] reported that the immunological properties of duck glucagon was different markedly from those of porcine glucagon, though these two hormones are structurally identical, except for minor alternations at two positions, 16 and 28. When analyzed by radioimmunoassay using anti-porcine glucagon rabbit sera (30K, Lot 25),[28] the cross-reactivity of our synthetic duck glucagon was 46%.

Experimental

General experimental methods employed are essentially the same as described in the Part 62[29] of this series. Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–pyridine–AcOEt–H_2O (1:1:1:1), Rf_3 *n*-BuOH–AcOH–H_2O (4:1:5), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24), Rf_5 *n*-BuOH–pyridine–AcOH–H_2O (30:6:20:24).

Z(OMe)-Lys(Z)-NHNH-Troc——DCC (11.35 g) was added to a mixture of Z(OMe)–Lys(Z)–OH (22.22 g) and Troc–$NHNH_2$ (11.41 g) in AcOEt (150 ml) and the solution was stirred at room temperature overnight. After filtration, the filtrate was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and then recrystallized from ether and *n*-hexane; yield 30.19 g (90%). mp 101—105°, $[\alpha]_D^{22}$ −30.9° (c=1.0, MeOH). Rf_1 0.72. *Anal.* Calcd. for $C_{26}H_{31}N_4O_8Cl_3$: C, 49.26; H, 4.93; N, 8.84. Found: C, 49.53; H, 4.88; N, 8.85.

Z(OMe)-Ser-Lys(Z)-NHNH-Troc——Z(OMe)–Lys(Z)–NHNH–Troc (16.75 g) was treated with TFA–anisole (10 ml–8.7 ml) in an ice-bath for 60 min. The excess TFA was removed by evaporation and the residue was washed with *n*-hexane. An oily residue was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml). To this ice-chilled solution, Et_3N (3.5 ml) and the azide (prepared from 7.37 g of Z(OMe)–Ser–$NHNH_2$ with 14.51 ml of 3.79 N HCl–DMF, 3.75 ml of isoamylnitrite and 11.3 ml of Et_3N) in DMF (60 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and then recrystallized from MeOH and ether; yield 15.86 g (81%). mp 147—148°, $[\alpha]_D^{22}$ −15.2° (c=1.0, MeOH). Rf_1 0.57. *Anal.* Calcd. for $C_{29}H_{36}N_5O_{10}Cl_3$: C, 48.31; H, 5.03; N, 9.71. Found: C, 48.51; H, 5.11; N, 9.66.

Z(OMe)-Tyr-Ser-Lys(Z)-NHNH-Troc——Z(OMe)–Ser–Lys(Z)–NHNH–Troc (15.76 g) was treated with TFA-anisole (10 ml–6.6 ml) in the usual manner and the excess TFA was removed by evaporation. The oily residue was washed with ether, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (40 ml). To this ice-chilled solution, Et_3N (2.8 ml) and the azide (prepared from 7.55 g of Z(OMe)–Tyr–$NHNH_2$ with 13.2 ml of 3.79 N HCl–DMF, 3.2 ml of isoamylnitrite and 10.5 ml of Et_3N) in DMF (80 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with AcOEt and 10% citric acid. The gelatinous mass formed on standing in a refrigerator overnight was collected by filtration, washed batchwisely with 10% citric acid and H_2O and then recrystallized from MeOH and AcOEt; yield 14.13 g (75%). mp 183—186°, $[\alpha]_D^{22}$ −3.7° (c=1.1, DMF). Rf_1 0.67. *Anal.* Calcd. for $C_{38}H_{45}Cl_3N_6O_{12}$: C, 51.62; H, 5.13; N, 9.51. Found: C, 51.44; H, 5.31; N, 9.25.

Boc-Asp(OBzl)-Tyr-Ser-Lys(Z)-NHNH-Troc——Z(OMe)–Tyr–Ser–Lys(Z)–NHNH–Troc (11.54 g) was treated with TFA-anisole (10 ml–6.7 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (100 ml), to which Et_3N (1.7 ml), Boc–Asp(OBzl)–ONP (6.22 g) and HOBT (1.84 g) were added successively. The mixture was stirred at room temperature overnight and the solvent was evaporated. The residue was dissolved in AcOEt, which was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and then recrystallized twice from EtOH and ether; yield 7.45 g (61%), mp 116—118°, $[\alpha]_D^{22}$ −15.1° (c=1.1, DMF). Rf_1 0.74. *Anal.* Calcd. for $C_{45}H_{56}Cl_3N_7O_{14}$: C, 52.71; H, 5.51; N, 9.56. Found: C, 52.51; H, 5.44; N, 9.36.

Boc-Asp(OBzl)-Tyr-Ser-Lys(Z)-$NHNH_2$ (E)——Boc–Asp(OBzl)–Tyr–Ser–Lys(Z)–NHNH–Troc (4.10 g) in a mixture of DMF (20 ml) and AcOH (20 ml) was treated with Zn dust (*ca.* 4 g) at room temperature for 3 hr. The solution was filtered, the filtrate was condensed *in vacuo* at 45° and the residue was treated with 0.1% EDTA. The resulting gelatinous mass was washed batchwisely with 5% Na_2CO_3 and H_2O and then recrystallized from MeOH; yield 2.18 g (64%), mp 172—174°, $[\alpha]_D^{20}$ −11.8° (c=0.8, DMF). Rf_1 0.51. Amino acid ratios in 4 N MSA hydrolysate: Asp 1.00, Tyr 1.11, Ser 0.90, Lys 0.94 (average recovery 80%). *Anal.* Calcd. for $C_{42}H_{55}N_7O_{12}$: C, 59.35; H, 6.52; N, 11.54. Found: C, 59.19; H, 6.75; N, 11.43.

28) E.A. Parada, A.M. Eisentraut, and R.H. Unger, *Amer. J. Med. Sci.*, **257**, 415 (1969).
29) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).

Z(OMe)-Thr-Ser-OMe——The azide (prepared from 5.95 g of Z(OMe)–Thr–$NHNH_2$ with 7.55 ml of 5.96 N HCl–DMF, 2.9 ml of isoamylnitrite and 9.8 ml of Et_3N) in DMF (50 ml) was added to an ice-chilled solution of H–Ser–OMe (prepared from 3.11 g of the hydrochloride with 2.8 ml of Et_3N) in DMF (30 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with 5% citric acid and ether. The resulting crystalline mass was washed batchwisely with 5% citric acid and H_2O–NaCl and then recrystallized twice from MeOH and AcOEt; yield 5.29 g (69%), mp 134—137°, $[\alpha]_D^{22}$ −6.7° (c=0.9, MeOH). Rf_1 0.60. *Anal.* Calcd. for $C_{17}H_{24}N_2O_8$: C, 53.12; H, 6.29; N, 7.29. Found: C, 53.42; H, 6.09; N, 7.31.

Boc-Thr-Phe-$NHNH_2$——To a mixture of the dicyclohexylamine salt of Boc–Thr–OH (5.0 g) and H–Phe–OMe hydrochloride (3.59 g) in DMF (50 ml), DCC (3.94 g) was added and the solution, after stirring at room temperature for 48 hr, was filtered. The filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt. The organic phase was washed with 5% HCl, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and then dissolved in EtOH (60 ml). To this solution, 80% hydrazine hydrate (4.0 ml) was added. The crystalline mass formed on standing at room temperature overnight, was collected by filtration and precipitated from DMF with EtOH; yield 4.0 g (82%), mp 191—195°, $[\alpha]_D^{25}$ −12.3° (c=0.6, DMF), Rf_1 0.64. *Anal.* Calcd. for $C_{18}H_{28}N_4O_5 \cdot 1/4H_2O$: C, 56.16; H, 7.46; N, 14.57. Found: C, 56.13; H, 7.57; N, 14.38.

Boc-Thr-Phe-Thr-Ser-OMe——Z(OMe)–Thr–Ser–OMe (5.77 g) was treated with TFA–anisole (11 ml–8.1 ml) as usual. The excess TFA was removed by evaporation and the residue was treated with ether. The resulting powder, collected by filtration, was dissolved in DMF (45 ml). To this ice-chilled solution, Et_3N (2.1 ml) and the azide (prepared from 3.80 g of Boc–Thr–Phe–$NHNH_2$ with 4.2 ml of 5.96 N HCl–DMF and 1.5 ml of isoamylnitrite and 5.6 ml of Et_3N) in DMF (30 ml) were added. The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was treated with AcOEt/and 5% citric acid. The resulting powder was washed batchwisely with 10% citric acid and H_2O and then recrystallized from MeOH and AcOEt; yield 4.20 g (74%), mp 169—172°, $[\alpha]_D^{26}$ −3.7° (c=0.8, DMF), Rf_1 0.46. *Anal.* Calcd. for $C_{26}H_{40}N_4O_{10} \cdot 1.5H$ O: C, 52.42; H, 7.28; N, 9.41. Found: C, 52.50; H, 7.21; N, 9.46.

Boc-Thr-Phe-Thr-Ser-$NHNH_2$ (F)——The above protected tetrapeptide ester (0.70 g) was dissolved in MeOH (7 ml) and 80% hydrazine hydrate was added. The crystalline mass formed on standing overnight was precipitated from DMF with MeOH; yield 0.29 g (42%), mp 179—184°, $[\alpha]_D^{26}$ −4.4° (c=1.1, DMF), Rf_1 0.51. *Anal.* Calcd. for $C_{25}H_{40}N_6O_9 \cdot 1/4H_2O$: C, 52.39; H, 7.12; N, 14.66. Found: C, 52.39; H, 7.20; N, 14.49.

Z(OMe)-Gln-Gly-OMe——Z(OMe)–Gln–ONP (12.94 g) was added to a solution of H–Gly–OMe (prepared from 4.14 g of the hydrochloride with 6.3 ml of Et_3N) in DMF–H_2O (80 ml–20 ml) and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed batchwisely with 10% citric acid and H_2O and then precipitated from DMF with AcOEt; yield 10.83 g (95%), mp 180—181°, $[\alpha]_D^{22}$ +12.8° (c=0.9, DMF). Rf_1 0.59. *Anal.* Calcd. for $C_{17}H_{23}N_3O_7$: C, 53.53; H, 6.08; N, 11.02. Found: C, 53.71; H, 5.91; N, 11.16.

Z(OMe)-Ser-Gln-Gly-OMe——In the usual manner, Z(OMe)–Gln–Gly–OMe (3.81 g) was treated with TFA–anisole (6 ml–3.3 ml) and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml) containing Et_3N (1.4 ml). To this ice-chilled solution, the azide (prepared from 2.83 g of Z(OMe)–Ser–$NHNH_2$ with 5.8 ml of 3.79 N HCl–DMF, 1.48 ml of isoamylnitrite and 4.88 ml of Et_3N) in DMF (20 ml) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting gel was washed batchwisely as stated above and then precipitated from DMF with AcOEt; yield 3.46 g (74%), mp 156—157°, $[\alpha]_D^{22}$ −1.1° (c=0.9, DMF), Rf_1 0.48. *Anal.* Calcd. for $C_{20}H_{28}N_4O_9 \cdot 1/2H_2O$: C, 50.30; H, 6.12; N, 11.74. Found: C, 50.46; H, 6.98; N, 11.64.

Z-His-Ser-Gln-Gly-OMe——Z(OMe)–Ser–Gln–Gly–OMe (4.78 g) was treated with TFA–anisole (10 ml—5.4 ml) as usual and the excess TFA was removed by evaporation. The residue was treated with dry ether and the resulting powder, isolated as stated above, was dissolved in DMF (20 ml), to which Et_3N (1.4 ml) and the azide (prepared from 3.49 g of Z–His–$NHNH_2$ as stated above) in DMF (30 ml) were added. The mixture was stirred at 4° for 48 hr, the solvent was condensed *in vacuo* and the residue was treated with AcOEt. The resulting gelatinous mass was collected by filtration and recrystallized twice from MeOH and AcOEt; yield 5.40 g (94%), mp 118—122°, $[\alpha]_D^{24}$ −6.7° (c=1.0, DMF), Rf_1 0.24. *Anal.* Calcd. for $C_{25}H_{33}N_7O_9 \cdot 2.5H_2O$: C, 48.38; H, 6.17; N, 15.80. Found: C, 48.32; H, 5.92; N, 15.77.

Z-His-Ser-Gln-Gly-$NHNH_2$ (G)——To a solution of Z–His–Ser–Gln–Gly–OMe (4.30 g) in MeOH (50 ml), 80% hydrazine hydrate (2.3 ml) was added. The crystalline mass formed on standing overnight was collected by filtration and precipitated from DMF with MeOH; yield 3.44 g (80%), mp 208—212°, $[\alpha]_D^{22}$ −9.9° (c=1.3, DMF), Rf_1 0.10. Amino acid ratios in 4 N MSA hydrolysate: His 1.19, Ser 0.91, Glu 1.00, Gly 1.00 (average recovery 99%). *Anal.* Calcd. for $C_{24}H_{33}N_9O_8 \cdot 1/4H_2O$: C, 49.69; H, 5.82; N, 21.73. Found: C, 49.95; H, 6.01; N, 21.17.

Boc-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, Boc-(duck glucagon 9—29)-OH——Boc-(duck glucagon 13—29)–OH (0.72 g) was treated with TFA (2 ml) in the presence of skatole (68 mg) and anisole (1 ml) containing 0.1% ethanedithiol in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by

filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in NMP–DMF (3 ml–2 ml). To this ice-chilled solution, Et_3N (0.07 ml) and the azide (prepared from 0.40 g of Boc–Asp(OBzl)–Tyr–Ser–Lys(Z)–$NHNH_2$ with 0.17 ml of 5.96 N HCl–DMF, 0.07 ml of isoamylnitrite and 0.21 ml of Et_3N) in DMF (5 ml) were added. The mixture was stirred at 4° for 48 hr, the solvent, after addition of a few drops of AcOH, was evaporated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was purified by batchwise washing with 5% citric acid and H_2O followed by precipitation from DMF with EtOH; yield 0.81 g (90%), mp 247—249°, $[\alpha]_D^{26}$ −3.0° (c=0.7, DMF). Rf_2 0.87, Rf_3 0.41. Amino acid ratios in 4 N MSA hydrolysate: Asp 3.29, Tyr 1.79, Ser 2.04, Lys 1.01, Leu 1.74, Thr 1.96, Arg 2.07, Ala 1.00, Glu 2.09, Phe 1.08, Val 1.03, Trp 0.67, Met+Met(O) 0.76 (average recovery 88%). *Anal.* Calcd. for $C_{165}H_{219}N_{31}O_{47}S_3 \cdot 4H_2O$: 55.71; H, 6.43; N, 12.21. Found: C, 55.55; H, 6.68; N, 12.44.

Boc-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, Boc-(duck glucagon 5—29)-OH——Boc–(duck glucagon 9—29)–OH (0.73 g) was treated with TFA (2 ml) in the presence of skatole (55 mg) and anisole (1 ml) containing 0.1% ethanedithiol as stated above and dry ether was added to form a fine powder, which was dissolved in NMP–DMF (2 ml–3 ml). To this ice-chilled solution, Et_3N (0.06 ml) and the azide (prepared from 0.16 g of Boc–Thr–Phe–Thr–Ser–$NHNH_2$ as stated above) in DMF (2 ml) were added. The mixture was stirred at 4° for 48 hr. After addition of a few drops of AcOH, the solvent was evaporated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was purified as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 0.53 g (64%), mp 235—240°, $[\alpha]_D^{26}$ −19.3° (c=0.5, DMF), Rf_2 0.78, Rf_3 0.44. Amino acid ratios in 4 N MSA hydrolysate: Thr 3.59, Phe 1.85, Ser 2.76, Asp 3.26, Tyr 2.10, Lys 0.90, Leu 2.08, Arg 1.74, Ala 1.00, Glu 2.18, Val 1.06, Trp 0.74, Met+Met(O) 0.83 (average recovery 99%). *Anal.* Calcd. for $C_{185}H_{247}N_{35}O_{54}S_3 \cdot 4H_2O$: C, 55.63; H, 6.44; N, 12.28. Found: C, 55.45; H, 6.59; N, 12.28.

Z-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp(OBzl)-Tyr-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, Z-(duck glucagon 1—29)-OH——Boc-(duck glucagon 5—29)–OH (0.48 g) was treated with TFA (2 ml) in the presence of skatole (32 mg) and anisole (0.8 ml) containing 0.1% ethanedithiol as stated above and dry ether was added. The resulting powder was then dissolved in NMP (3 ml). To this ice-chilled solution, Et_3N (0.04 ml) and the azide (prepared from 0.17 g of Z–His–Ser–Gln–Gly–$NHNH_2$ as stated above) in DMF–NMP (1 ml–1 ml) were added. The mixture, after stirring at 4° for 48 hr, was neutralized with a few drops of AcOH and the solvent was evaporated. The residue was treated with 5% citric acid and AcOEt and the resulting powder was purified by batchwise washing as stated above followed by precipitation from DMF with EtOH; yield 0.51 g (97%), mp 223—226°, $[\alpha]_D^{24}$ +3.1° (c=1.0, DMF). Rf_2 0.72, Rf_3 0.29. Amino acid ratios in 4 N MSA hydrolysate: His 1.06, Ser 3.67, Glu 3.12, Gly 1.05, Thr 3.43, Phe 1.76, Asp 3.16, Tyr 1.94, Lys 1.01, Leu 1.93, Arg 1.90, Ala 1.00, Val 1.08, Trp 0.79, Met+Met(O) 0.72 (average recovery 98%). *Anal.* Calcd. for $C_{204}H_{268}N_{42}O_{60}S_3 \cdot 5H_2O$: C, 54.99; H, 6.29; N, 13.21. Found: C, 55.16; H, 6.91; N, 13.03.

H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Thr-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Ser-Thr-OH (duck glucagon)——The protected nonacosapeptide, Z-(duck glucagon 1—29)-OH (521 mg), was treated with MSA (4 ml) in the presence of anisole (1 ml) and skatole (80 mg, 5 equiv.) in an ice-bath for 15 min and then at room temperature for 45 min and dry ether was added. The resulting powder was washed with ether and dissolved in H_2O (45 ml), which was treated with Amberlite CG-4B type II (acetate form, approximately 5 g) for 60 min and then filtered. To this filtrate (45 ml) was added 2 N NH_4OH (15 ml) and the solution, after stirring in an ice-bath for 30 min, was lyophilized to give a white powder; yield 472 mg.

The crude deprotected product, [27-Met(O)]-duck glucagon (226 mg) thus obtained, was dissolved in 0.5% AcOH (8 ml). A small amount of the insoluble material was removed by filtration and the filtrate was applied to a column of Sephadex G-25 fine (3×150 cm), which was eluted with the same solvent. Individual fractions (6 ml each) were collected and absorbancy at 280 mμ was determined (Fig. 5-a). The front peak (tube No. 75—90, 35 mg) was discarded and the fractions corresponding to the 2nd peak (tube No. 105—140) were collected and the solvent was removed by lyophilization; yield 207 mg (84%).

Next, the Sephadex purified sample (109 mg) was dissolved in H_2O (4 ml) and the solution was applied to a column of CM-cellulose (2.4×6.8 cm), which was eluted first with H_2O (120 ml) and then by gradient elution with 0.06 M (pH 6.9) ammonium acetate buffer (800 ml) through a mixing flask containing H_2O (800 ml). Individual fractions (6 ml each) were collected and absorbancy at 280 mμ was determined (Fig. 5-b). The solvent of the main peak present in the gradient eluates (tube No. 53—85) was removed by lyophilization. The residue was dissolved in H_2O (3 ml) and the solution was incubated in the presence of mercaptoethanol (0.1 ml) under the N_2 gas at 45° for 48 hr. Meanwhile, the substance of Rf_4 0.53 was disappeared and the Met test positive spot, Rf_4 0.56, was detected on TLC. In order to remove ammonium acetate and mercaptoethanol, the solution was applied to a column of Sephadex G-25 (2.5×140 cm), which was eluted with 0.5% AcOH. The desired fractions (Fig. 5-c, tube No. 80—91) were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 49 mg (45%). Rf_4 0.56, Rf_5 0.96. Amino acid ratios in 4 N MSA hydrolysate: His 0.84, Ser 3.93, Glu 3.06, Gly 1.22, Thr 3.64, Phe 1.91, Asp 3.14, Tyr 1.85, Lys 1.11, Leu 1.73, Arg 2.22, Ala 1.11, Val 1.00, Trp 1.01, Met 0.83 (average recovery 80%).

According to Fujino *et al.*[7] and Wünsch *et al.*,[6] the product (15 mg) was suspended in 0.02% NaCl (1.5 ml) and dissolved by addition of 0.1 N NaOH at pH 10.5. The solution, after adjusting the pH 8.5 with 0.1 N HCl, was allowed to stand at room temperature overnight and then stored in a refrigerator for 3 days. The crystalline precipitate was collected by centrifugation and washed with 0.02% NaCl; yield 6.7 mg. $[\alpha]_D^{24}$ −32.4° (c=0.8, 0.5% AcOH). Amino acid ratios in AP-M digest: His 1.17, Ser 4.04, Gln+Thr 5.88 (calcd. as Thr), Gly 1.31, Phe 1.97, Asp 2.70, Tyr 1.68, Lys 1.18, Leu 1.75, Arg 2.14, Ala 1.14, Val 1.00, Trp 0.99, Met 0.86 (average recovery 82%). Disc electrophoretic mobility on 15% polyacrylamide gel (0.5×6.0 cm, 5 mA/tube for 2 hr) at pH 2.3 (0.37 M glycine-AcOH buffer) was 1.4 cm to the cathod (stained by Coomassie Blue). Gel electrofocussing[30] on acrylamide (0.5×6.0 cm, 1 mA/tube for 1 hr and 100 V. for 1 hr) showed the presence of a single band at 2.9 cm from the origin to the alkaline end of the gel. *Anal.* Calcd. for $C_{159}H_{238}N_{42}O_{55}S \cdot 3CH_3COOH \cdot 16H_2O$: C, 48.49; H, 6.91; N, 14.94. Found: C, 48.19; H, 6.83; N, 15.47.

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