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Synthesis of the Nonacosapeptide corresponding to the Proposed Amino Acid Sequence of Turkey Glucagon[1)]

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The nonacosapeptide corresponding to the proposed amino acid sequence of turkey glucagon was synthesized *via* the corresponding sulfoxide, [Met(O)^{27}]-turkey glucagon. For the synthesis of the protected nonacosapeptide six peptide fragments were prepared and the condensations of the fragments were achieved by the HONB–DCC method. Finally, all the protecting groups were removed by the treatment with anhydrous hydrogen fluoride-anisole to give [Met(O)^{27}]-turkey glucagon. The sulfoxide was then treated with aqueous thioglycolic acid to give turkey glucagon, which was successfully crystallized from dilute aqueous sodium chloride solution. The biological activity, lipolysis on rat adipocytes, of the synthetic turkey glucagon was as potent as that of mammalian glucagon.

Keywords——turkey glucagon; chicken glucagon; nonacosapeptide; fragment condensation; hydrogen fluoride

After the elucidation of the complete amino acid sequence of porcine glucagon by Bromer *et al.*,[3)] a few structural variations of glucagon, especially in avian species, have been reported. The amino acid sequence of the hormone from turkey[4)] and chicken[5)] differs from porcine glucagon in having serine instead of asparagine at position 28 while that of duck glucagon[6)] has threonine instead of serine at position 16 and serine instead of asparagine at position 28. Fig. 1 shows the structures of glucagons isolated from various species.

$$\overset{1}{\text{H–His}}\text{–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–}\overset{10}{\text{Tyr}}\text{–Ser–Lys–Tyr–Leu–Asp–}\overset{16}{\text{X}}\text{–Arg–}$$

$$\text{Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–}\overset{28}{\text{Y}}\text{–}\overset{29}{\text{Thr}}\text{–OH}$$

	X	Y
turkey and chicken:	Ser	Ser
duck:	Thr	Ser
mammal:	Ser	Asn

Fig. 1. The Structures of Glucagon

Mammalian glucagon has been synthesized by Wünsch *et al.*,[7)] a Chinese group[8)] and us[9)], but syntheses of avian glucagons have not been reported to date. This paper describes the

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*-methoxybenzenesulfonyl, OBzl=benzyl ester, OBu^t=*tert*-butyl ester, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, DCC=N,N′-dicyclohexylcarbodiimide, DCU=N,N′-dicyclohexylurea, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=N,N-dimethylformamide, THF=tetrahydrofuran NMP=N-methyl-2-pyrrolidone.

2) Location: *Yodogawa-ku, Osaka 532, Japan*; *a*) To whom correspondence should be addressed.

3) W.W. Bromer, L.G. Sinn, and O.K. Behrens, *J. Am. Chem. Soc.*, **79**, 2807 (1957).

4) J. Markussen, E.K. Frandsen, L.G. Heding, and F. Sundby, *Horm. Metab. Res.*, **4**, 360 (1972).

5) H.G. Pollock and J.R. Kimmel, *J. Biol. Chem.*, **250**, 9377 (1975).

6) F. Sundby, E.K. Frandsen, J. Thomsen, K. Kristiansen, and K. Brunfeldt, *FEBS Letters*, **26**, 289 (1972).

7) E. Wünsch, E. Jaeger, and R. Scharf, *Chem. Ber.*, **101**, 3664 (1968).

8) Chinese group, *Acta Biochim. Biophys. Sin.*, **7**, 120 (1975).

9) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 539 (1978).

synthesis of the nonacosapeptide corresponding to the proposed amino acid sequence of turkey glucagon. The nonacosapeptide was synthesized in a manner similar to that described for our synthesis of porcine glucagon. The hormone was prepared *via* the corresponding sulfoxide as outlined in Fig. 2.

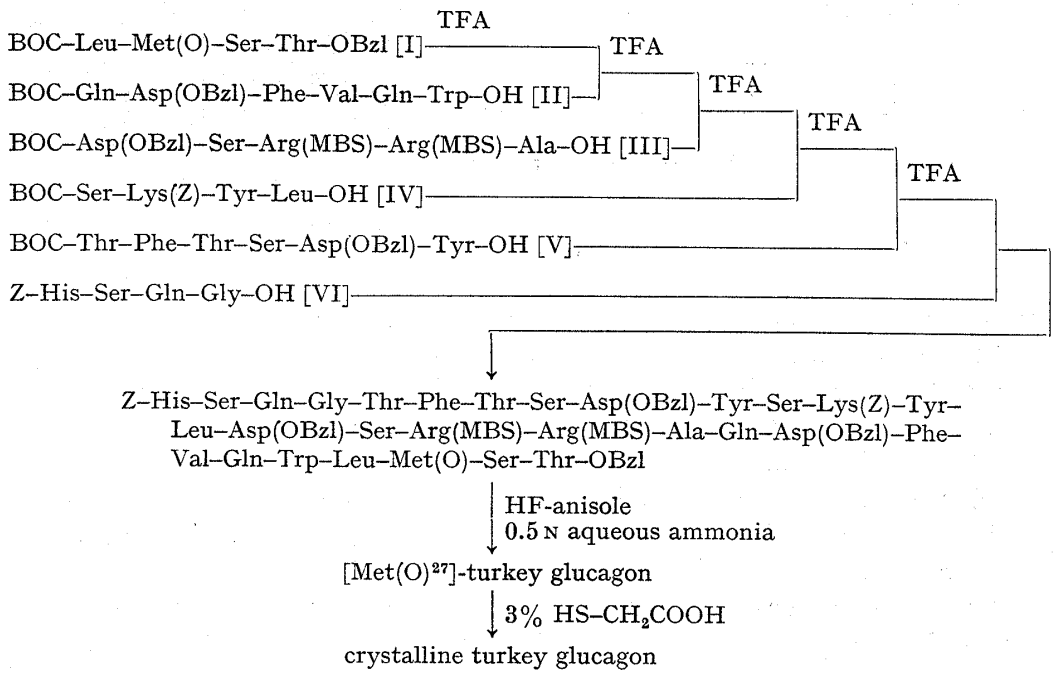

Fig. 2. Synthetic Route to Turkey Glucagon

Six intermediate subunits, BOC-Leu-Met(O)-Ser-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) were chosen to construct the full sequence. Syntheses of subunit II to VI were described in the previous paper.[9] The synthetic route to the C-terminal subunit I is shown in Fig. 3.

The protected C-terminal peptide I was treated with trifluoroacetic acid to remove the BOC group and the resulting product was condensed with subunit II by the HONB–DCC method,[10] giving BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OBzl (VII). The protected decapeptide VII was treated with TFA-anisole under nitrogen gas and the resulting product was coupled with subunit III by the HONB–DCC method to give BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OBzl (VIII). The BOC group of pentadecapeptide VIII was similarly removed and the free base was condensed with subunit IV by the HONB–DCC method to afford BOC-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met-(O)-Ser-Thr-OBzl (IX). The nonadecapeptide IX was again treated with TFA-anisole under nitrogen gas and the resulting product was condensed with subunit V by the HONB–DCC method to give 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)-Ser-Thr-OBzl (X). The BOC group of pentacosapeptide X was removed and the corresponding free base was condensed with the N-terminal subunit VI by the HONB–DCC method to obtain the protected nonacosapeptide corresponding to the entire amino acid sequence of turkey glucagon. The

10) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974); C. Kitada and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **26**, 585 (1978).

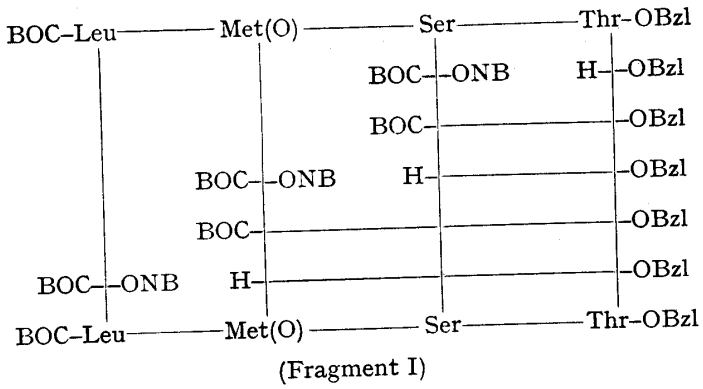

Fig. 3. Preparation of Protected Tetrapeptide (26—29)

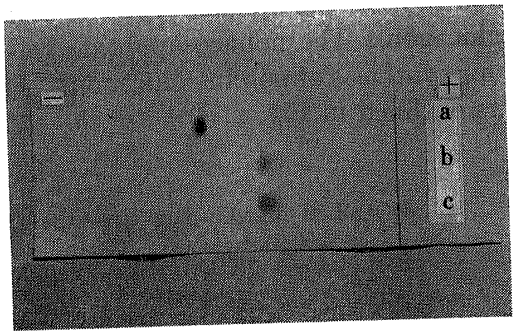

Fig. 4. The Electrophorogram of Glucagon

pH 1.9 HCOOH–AcOH buffer, 600 volt, 60 min.
a: Arg, b: synthetic turkey glucagon, c: natural mammalian glucagon.

protected nonacosapeptide was then treated with anhydrous hydrogen fluoride[11] in the presence of anisole at 0° for 50 min to remove all the protecting groups and the deblocked peptide was converted into the corresponding acetate with Amberlite IRA-410 (acetate form), which was treated with 0.5 N aqueous ammonia at 0° for 30 min to reverse, if any, the undesirable N to O acyl migration of the serine and/or threonine residue. The resulting product was purified by column chromatography on Sephadex LH-20 and carboxymethylcellulose as described in the previous paper,[9] giving purified [Met(O)^{27}]-turkey glucagon. The nonacosapeptide sulfoxide thus obtained was exposed to 3% aqueous thioglycolic acid at 50° for 20 hr and the reduced peptide was passed through a column of Sephadex G-25 to remove the thiol reagent giving chromatographically pure turkey glucagon, which was crystallized from a dilute aqueous sodium chloride solution. The electrophorogram of the crystalline glucagon is shown in Fig. 4. The UV absorption spectrum of the synthetic peptide is also shown in Fig. 5, and a photo of the crystal is presented in Fig. 6. The biological activity, lipolysis on adipocytes of Sprague-Dawley rats,[12] of the synthetic turkey glucagon was as potent as that of mammalian glucagon.[9] These data indicate clearly that the synthetic nonacosapeptide is very pure.

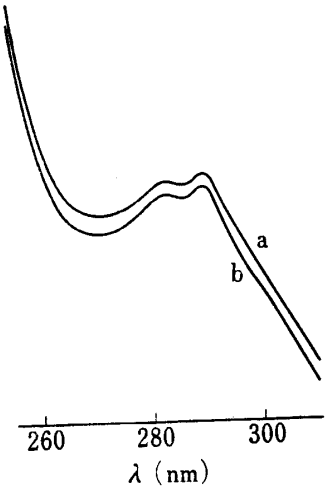

Fig. 5. The UV Absorption Spectrum of Glucagon

a: synthetic turkey glucagon, b: natural mammalian glucagon, solvent: 0.1 N NaOH.

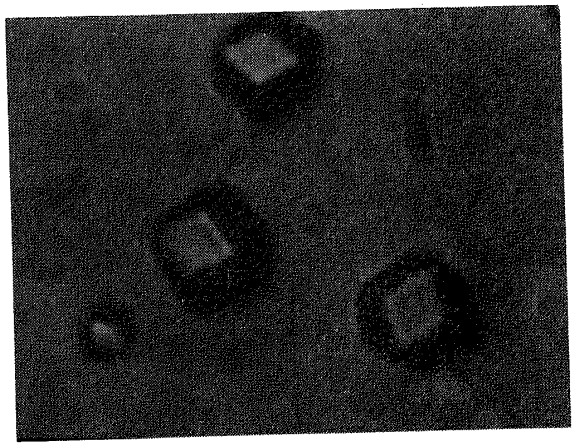

Fig. 6. Crystals of Synthetic Turkey Glucagon

11) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **38**, 1412 (1965).
12) 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.

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 on a Hitachi KLA-3B amino acid analyzer. Acid hydrolyses were carried out according to the method of Matsubara and Sasaki.[13] 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) and silica gel plate was used unless otherwise mentioned. Solvent systems used are: $CHCl_3$–MeOH–AcOH (9:1:0.5, Rf^1), $CHCl_3$–MeOH–H_2O (7:3:0.5, Rf^2), *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, Rf^3), AcOEt–*n*-BuOH–AcOH–H_2O (1:1:1:1, Rf^4).

BOC-Ser-Thr-OBzl (Ia)——BOC–Thr–OBzl (60.0 g) was dissolved in TFA (150 ml) and the solution was allowed to stand at room temperature for 10 min and evaporated. The residue was dried over NaOH pellets *in vacuo* and then dissolved in THF (300 ml) together with TEA (25 ml). To the solution was added BOC–Ser–ONB prepared from BOC–Ser–OH (40.0 g), HONB (39.4 g) and DCC (45.4 g). The mixture was stirred at room temperature for 15 hr and then evaporated to dryness. The residue was dissolved in AcOEt (500 ml), washed with 4% aqueous $NaHCO_3$ and water, and dried over anhydr. Na_2SO_4, and the solvent was evaporated. The residue was triturated with ether to give crystals, which were purified by recrystallization from AcOEt–ether: 44.0 g (57.2%), mp 110—111°, $[\alpha]_D^{25}$ −8.4° (c=1.1 in DMF), Rf^1 0.63. *Anal.* Calcd. for $C_{19}H_{28}N_2O_7$: C, 57.56; H, 7.12; N, 7.07. Found: C, 57.83; H, 7.23; N, 7.04.

BOC-Met(O)-Ser-Thr-OBzl (Ib)——Compound Ia (30.0 g) was treated with TFA (200 ml) as described above and the solution was evaporated. The resulting residue was triturated with dry ether to give a powder, which was collected by filtration and dissolved in DMF (200 ml) together with TEA (11.6 ml). To this was added BOC–Met(O)–ONB prepared from BOC–Met(O)–OH (20.0 g) by the usual manner. The mixture was stirred at room temperature for 15 hr and the solvent was evaporated. The residue was dissolved in AcOEt (500 ml) and the solution was washed with saturated aqueous $NaHCO_3$ and NaCl solution and dried over anhydr. Na_2SO_4. Then the solvent was evaporated. The residue was triturated with ether to give a powder, which was purified by crystallization from acetonitrile: 28.0 g (68.3%), mp 103—105°, $[\alpha]_D^{25.5}$ −1.5° (c=1.1 in DMF), Rf^1 0.56. *Anal.* Calcd. for $C_{24}H_{37}N_3O_9S$: C, 53.02; H, 6.86; N, 7.73; S, 5.90. Found: C, 52.71; H, 6.95; N, 7.72; S, 5.70.

BOC-Leu-Met(O)-Ser-Thr-OBzl (I)——Compound Ib (28.0 g) was treated with TFA (150 ml) and the resulting residue was dissolved in DMF (200 ml) together with TEA (7.2 ml). To this was added BOC–Leu–ONB prepared from BOC–Leu–OH (12.0 g) and the solution was stirred at room temperature for 15 hr. After evaporation and the usual work-up, the material was obtained as a powder, which was purified by washing with ether: 17.7 g (50.9%), mp 150—151°, $[\alpha]_D^{25.5}$ −13.2° (c=0.9 in DMF), Rf^1 0.60. *Anal.* Calcd. for $C_{30}H_{48}N_4O_{10}S\cdot H_2O$: C, 53.39; H, 7.47; N, 8.30; S, 4.75. Found: C, 53.47; H, 7.25; N, 8.33; S, 4.75.

BOC-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OBzl [VII]——The BOC group of compound I (13.3 g) was removed by treatment with TFA (70 ml) and the TFA salt was then converted to the corresponding free base with TEA (4.5 ml) in DMF, followed by precipitation with ether. The free base was condensed with compound II (20.0 g) in DMF (100 ml) in the presence of HONB (7.2 g) and DCC (6.2 g) at 0° for 48 hr, and then the solvent was evaporated. The residue was triturated with water to give a precipitate, which was collected by filtration and purified by washing with aqueous acetonitrile: 16.7 g (53.1%), mp 227—230° (dec.), $[\alpha]_D^{28.5}$ −9.4° (c=1.1 in NMP), Rf^2 0.66. *Anal.* Calcd. for $C_{76}H_{103}N_{13}O_{20}S$: C, 58.86; H, 6.70; N, 11.74; S, 2.07. Found: C, 58.94; H, 6.78; N, 11.89; S, 1.94.

BOC-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OBzl [VIII]——Compound VII (4.2 g) was treated with TFA (30 ml) in the presence of anisole (1 ml) under nitrogen gas and the TFA salt obtained was converted to the free base with TEA (1.2 ml) in NMP (10 ml), followed by precipitation with ether. The free base was dissolved in DMF (50 ml) together with compound III (3.16 g) and to this were added HONB (0.90 g) and DCC (0.82 g) at −10°. 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 to give a precipitate, which was collected by filtration and washed with aqueous acetonitrile: 3.5 g (50.5%), mp 240—245° (dec.), $[\alpha]_D^{20}$ −4.1° (c=1.0 in NMP), Rf^2 0.64. *Anal.* Calcd. for $C_{119}H_{160}N_{24}O_{34}S_3$: C, 55.68; H, 6.28; N, 13.10; S, 3.75. Found: C, 55.74; H, 6.27; N, 13.52; S, 3.49.

BOC-Ser-Lys(Z)-Tyr-Leu-Asp(OBzl)-Ser-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OBzl [IX]——Compound VIII (3.1 g) was treated with TFA (30 ml) and the TFA salt was converted to the corresponding free base in the same manner as described above. The free base was dissolved in DMF (30 ml) together with compound IV (1.1 g) and to this were added HONB (0.87 g) and DCC (0.50 g) at −10°. 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 to give a precipitate, which was collected by filtration and washed with acetonitrile: 3.0 g (77.2%), mp 226—228° (dec.), $[\alpha]_D^{24}$ +9.2° (c=1.2 in NMP),

13) H. Matsubara, and K. Sasaki, *Biochem. Biophys. Res. Commun.*, 35, 175 (1969).

Rf^2 0.64. *Anal.* Calcd. for $C_{151}H_{203}N_{29}O_{42}S_3 \cdot H_2O$: C, 56.48; H, 6.43; N, 12.65; S, 3.00. Found: C, 56.66; H, 6.40; N, 12.73; S, 3.15.

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)-Ser-Thr-OBzl [X]——Compound IX (2.8 g) was treated with TFA (20 ml) and the free base obtained in a similar manner as described above was dissolved in DMF (30 ml) together with compound V (1.0 g). To the solution were added HONB (0.65 g) and DCC (0.37 g) at −10° and the mixture was stirred at 0° for 10 hr and at room temperature for additional 48 hr. After filtration and evaporation, the residue was triturated with AcOEt to give a precipitate, which was collected by filtration and purified by washing with acetonitrile: 2.85 g (80.3%), mp 238—240° (dec.), $[\alpha]_D^{24}$ +11.3° (c=1.1 in NMP), Rf^2 0.65. *Anal.* Calcd. for $C_{191}H_{251}N_{35}O_{54}S_3 \cdot 2H_2O$: C, 56.87; H, 6.37; N, 12.16; S, 2.39. Found: C, 56.89; H, 6.16; N, 12.12; 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)-Ser-Thr-OH ([Met(O)^{27}]-Turkey Glucagon)——Compound X (2.5 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 VI (0.37 g). To this were added HONB (0.45 g) and DCC (0.26 g) at 0° and the mixture was stirred at 0° for 10 hr and at room temperature for additional 48 hr. After filtration and evaporation, the residue was triturated with AcOEt to give a precipitate, which was collected by filtration and washed with acetonitrile: 2.7 g (93.5%). The protected nonacosapeptide (500 mg) thus obtained was dissolved in anhydr. HF (4 ml) in the presence of anisole (0.4 ml), and the mixture was stirred at 0° for 50 min. After evaporation, the residue was dissolved in water (30 ml) and the solution was washed with ether. The aqueous solution was then passed through a column (1×10 cm) of Amberlite IRA-410 (acetate form). The passed solution and washings were combined (total volume, 50 ml) and to this was added 3 N aqueous ammonia (10 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 (15 ml) and passed through a column (3.8×200 cm) of Sephadex LH-20 (0.1 N AcOH). The fractions (810—1070 ml) were combined and lyophilized: 40 mg. The powder obtained was again passed through the same column of Sephadex LH-20 (0.1 N AcOH). The fractions containing the desired product were pooled and lyophilized: 20 mg. The powder was dissolved in 0.1 N AcOH (5 ml) and applied to a column (1.6×10 cm) of carboxymethylcellulose, and then eluted with pH 4.6 ammonium acetate buffer (gradient: 0.005 M/0.2 M=250 ml/250 ml). The fractions (185—215 ml) containing the pure product (checked by TLC) were combined and lyophilized: 8.0 mg, $[\alpha]_D^{27}$ −33.5° (c=0.2 in 1% AcOH), Rf^3 (cellulose) 0.56. Amino acid anal: Lys 1.07(1); His 1.01(1); Arg 1.99(2); Trp 0.80(1); Asp 3.44(3); Thr 2.74(3); Ser 3.68(5) Glu 3.01(3); Gly 0.98(1); Ala 0.96(1); Val 1.00(1); Met 0.92(1); Leu 1.88(2); Tyr 1.88(2); Phe 1.91(2) (average recovery, 76%).

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-Ser-Thr-OH (Turkey Glucagon)——[Met(O)^{27}]-Turkey glucagon (8.0 mg) was dissolved in 3% thioglycolic acid (1 ml) and the solution was kept to stand at 50° for 20 hr. The solution was applied to a column (2.5×110 cm) of Sephadex G-25 and eluted with 0.1 N AcOH. The fractions (200—280 ml) containing the desired product were combined and lyophilized: 6.5 mg. The powder obtained was suspended in 0.02% aqueous NaCl solution (0.7 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 HCl, and allowed to stand at room temperature for 24 hr and at 4° for additional 24 hr. The crystals formed were collected by centrifugation and then washed with 0.02% aqueous NaCl solution: 3.0 mg, $[\alpha]_D^{26}$ −34.6° (c=0.2 in 1% AcOH), UV $\lambda_{max}^{0.1N\text{-}NaOH}$ nm ($E_{1\,cm}^{1\%}$) 283.0 (20.57), 289.5 (21.24), Rf^3 (cellulose) 0.61, Rf^4 (cellulose) 0.66. Paper electrophoresis (pH 1.9 HCOOH–AcOH buffer, 600 volt, 60 min) 0.64×Arg. Amino acid ratios in acid hydrolysate: Lys 0.88(1); His 0.80(1); Arg 2.06(2); Trp 0.73(1); Asp 3.20(3); Thr 2.83(3); Ser 3.80(5); Glu 3.39(3); Gly 1.00(1); Ala 1.06(1); Val 1.17(1); Met 1.02(1); Leu 2.07(2); Tyr 2.16(2); Phe 2.07(2) (average recovery, 84%). Amino acid ratios in aminopeptidase M hydrolysate: Lys 1.03(1); His 0.83(1); Arg 2.11(2); Trp 0.78(1); Asp 3.01(3); Thr+Gln 4.10(6); Ser 4.76(5); Gly 1.16(1); Ala 1.00(1); Val 1.06(1); Met 0.76(1); Leu 1.81(2); Tyr 1.95(2); Phe 1.93(2) (average recovery, 79%).

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