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# Studies on Peptides. LXXVII.[1,2] Synthesis of the Protected Heptadecapeptide corresponding to Positions 13—29 of Avian Glucagon (Duck)

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As a starting material for the total synthesis of duck glucagon, the C-terminal protected heptadecapeptide, Boc–Tyr–Leu–Asp(OBzl)–Thr–Arg(MBS)–Arg(MBS)–Ala–Gln–Asp(OBzl)–Phe–Val–Gln–Trp–Leu–Met(O)–Ser–Thr–OH (position 13—29), was synthesized by successive azide condensation of 4 peptide fragments; (position 13—14), (15—19), (20—23) and (24—29).

**Keywords**——duck glucagon; avian pancreatic hormone; sodium perborate oxidation of Met to Met(O); protected duck glucagon (13—29); $N^{\alpha}$-deprotection of Trp peptides; trichloroethyloxycarbonylhydrazine

The entire amino acid sequence of duck glucagon was determined by Sundby *et al.*[4] in 1972 and species variation from mammalian glucagon was pointed out at two positions, 16 and 28. Duck glucagon possesses Thr and Ser at these positions respectively, while in mammalians, Ser and Asn are placed, regardless of porcine,[5] bovine[6] and human origins.[7]

Synthesis of porcine glucagon was first accomplished by Wünsch *et al.*[8] in 1968, using protecting groups removable by TFA. In 1975, the Chinese research group[9] reported an alternative synthesis of porcine glucagon, in which the fragment condensation procedure on the polymer support explored by us[10] in 1974 was adopted and the hydrogen fluoride procedure[11] was employed for the final deprotection. More recently Fujino *et al.*[12] synthesized this pancreatic hormone alternatively by applying the MSA procedure[13] for the final deprotection. We have extended this MSA procedure to the synthesis of structurally related duck glucagon.

Since we reported in 1975, that MSA cleaved various protecting groups currently employed in peptide synthesis,[13] usefulness of this acid as a deprotecting reagent at the final step for the synthesis of biologically active peptides has been investigated. First, we confirmed that N-alkylation, such as formation of Lys(Bzl) from Lys(Z), was not observed in the MSA-

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1) Part LXXVI: H. Yajima, T. Sasaki, H. Ogawa, N. Fujii, T. Segawa, and Y. Nakata, *Chem. Pharm. Bull.* (Tokyo), **26**, 1231 (1978).
2) Amino acids, peptides and their derivatives mentioned are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, Bzl=benzyl, MBS=*p*-methoxybenzenesulfonyl, TFA=trifluoroacetic acid, DMF=dimethylformamide, MSA=methanesulphonic acid, ESA=ethanesulphonic acid, EDT=ethanedithiol, TFE=trifluoroethanol.
3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
4) F. Sundby, E.K. Frandsen, J. Thomsen, K. Kristiansen, and K. Brunfeldt, *FEBS Letters*, **26**, 298 (1972).
5) W.W. Bromer, L.G. Sinn, and O.K. Behrens, *J. Am. Chem. Soc.*, **79**, 2807 (1957).
6) W.W. Bromer, M.E. Boucher, and J.E. Kofferberger, Jr., *J. Biol. Chem.*, **246**, 2822 (1971).
7) J. Thomsen, K. Kristiansen, K. Brunfeldt, and F. Sundby, *FEBS Letters*, **21**, 315 (1972).
8) E. Wünsch and G. Wendlberger, *Chem. Ber.*, **101**, 3659 (1968).
9) Synthetic Protein Research Group, China Biochem. Inst., *Acta Biochim. Biophys. Sinica*, **7**, 119 (1975).
10) H. Yajima, Y. Kiso, Y. Okada, and H. Watanabe, *J.C.S. Chem. Comm.*, **1974**, 106; H, Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1087 (1974).
11) S. Sakakibara, Y. Shimonishi, Y. Kishida, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
12) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 539 (1978).
13) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).

anisole system.[14] Next, we noticed that for the synthesis of Met-containing peptides, its sulphur atom has to be protected as the sulphoxide, otherwise to which the methyl group is transformed from anisole.[15] Subsequently we devised an easy procedure for the preparation of Met(O) derivatives by oxidation with sodium perborate or sodium metaperiodate.[16] These accumulated data supported our present synthesis of duck glucagon, which possesses both Lys and Met residues.

We wish to report in the two consecutive papers, detailed accounts of the synthesis of the nonacosapeptide corresponding to the entire amino acid sequence of duck glucagon. Amino acid derivatives bearing protecting groups removable by MSA were thus employed, *i. e.*, Asp(OBzl), Lys(Z) and Arg(MBS). The latter compound is the one newly introduced by Nishimura and Fujino[17] and its protecting group was confirmed to be cleaved more smoothly by MSA than Arg(Tos). The Met residue locates at the position 27 in the C-terminal portion of glucagon and this interferes the catalytic deprotection of the $N^{\alpha}$-protecting groups. Thus the acidolytic removal is only the method of choice for elongation of the peptide chain as seen in the synthesis of mammalian glucagon mentioned above. Situation is the same, when Met(O), instead of Met, was employed.

However, this strategy meets with difficulty, since a certain degree of destruction of the acid sensitive Trp residue takes place in each deprotecting step. In order to minimize its destruction, Wünsch *et al.*[8] removed the *o*-nitrophenylsulfenyl (NPS) group with dilute HBr or HCl in the presence of 2-methylindole or indole. This additive reduces the side reaction of the resulting NPS-halide with Trp and seems to minimize the destruction of Trp as well. In our previous syntheses of cholecystokinin-pancreozymin (CCK-PZ)[18] and gastric inhibitory polypeptide (GIP),[19] we obserbed that for this purpose, the presence of EDT[20] in the TFA-anisole system was more effective than mercaptoethanol, when the Z(OMe) group was deprotected from Trp-peptides. However, repeated precipitation was still required for purification of intermediates in some instances. Recently, Shimonishi *et al.*[21] pointed out that acetyl-Trp-OMe in TFA has a tendency to form a dimer, besides the formation of colored substances. More recently, Wünsch *et al.*[22] reported the formation of $N_{in}$-*tert*-butyl-Trp and other alkylated compounds, when Boc-derivatives were treated with TFA. Though these experiments were performed in the absence of the cation scavenger, such as anisole, behavior of Trp in acids was well characterized.

Prior to starting the synthesis of duck glucagon, we have examined more practical and satisfactory conditions for the removal of acid labile protecting groups from Trp. Taking Boc-Trp-OH and Z(OMe)-Trp-OH as model compounds, deprotected products were examined by thin layer chromatography. Among conditions tested, the amounts of side products, at least two, were less in 4 N ESA[23] in TFE-anisole system rather than the TFA-anisole system and decreased immensely by addition of EDT and further by addition of skatole in both systems to almost negligible extent. Effect of skatole is not the established one. However,

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14) N. Fujii, S. Funakoshi, T. Sasaki, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **25**, 3096 (1977).

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).

16) N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 650 (1978).

17) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **24**, 1568 (1976).

18) H. Yajima, Y. Kiso, Y. Mori, K. Koyama, T. Tobe, M. Setoyama, H. Adachi, T. Kanno, and A. Saito, *Chem. Pharm. Bull.* (Tokyo), **24**, 1110 (1976); *idem, ibid.*, **24**, 2794 (1976).

19) H. Yajima, H. Ogawa, M. Kubota, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, H. Adachi, H. Imura, and T. Taminato, *J. Am. Chem. Soc.*, **97**, 5593 (1975).

20) J.J. Sharp, A.B. Robinson, and M.D. Kemen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).

21) Y. Omori, Y. Matsuda, S. Aimoto, Y. Shimonishi, and M. Yamamoto, *Chem. Letters*, **1976**, 805.

22) E. Wünsch, E. Jager, L. Kisfaludy, and M. Low, *Angew. Chem.*, **89**, 330 (1977).

23) H. Yajima, H. Ogawa, N. Fujii, and S. Funakoshi, *Chem. Pharm. Bull.* (Tokyo), **25**, 740 (1977).

it was used by the reason that when proteins are hydrolyzed with 4 N MSA,[24] recovery of Trp is known to be improved by addition of indole derivatives, such as 2-aminoethylindole. In addition, this may have a property to share, with anisole and EDT, to trap the alkyl cation formed. We wish to examine the chemical nature of this deprotecting reaction in a more detail in future.

In a practical standpoint, we decided to use in this synthesis the Boc group for the $N^{\alpha}$-protection of necessary peptide fragments in expecting more soluble intermediates than the corresponding Z(OMe)-peptides. As the deprotecting reagent, the 4N ESA in TFE-anisole (containing 0.1% EDT)-skatole system was employed in the first few steps, but in the latter stages, the TFA-anisole (containing 0.1% EDT)-skatole system had to be employed, depending upon the solubility of intermediates, which decreased progressively by the chain elongation, despite of the use of the Boc group. Concentration of EDT was reduced from 2% to 0.1%, since higher concentration reduced partially the Met(O) residue to Met during this deprotecting period.

After performing these preliminary experiments, synthesis of duck glucagon by the MSA procedure was undertaken as illustrated in Fig. 1. As the building blocks, 7 peptide fragments were selected to construct the entire amino acid sequence of this avian glucagon by the Rudinger's azide procedure[25] exclusively. In this paper, synthesis of the C-terminal heptadecapeptide was described.

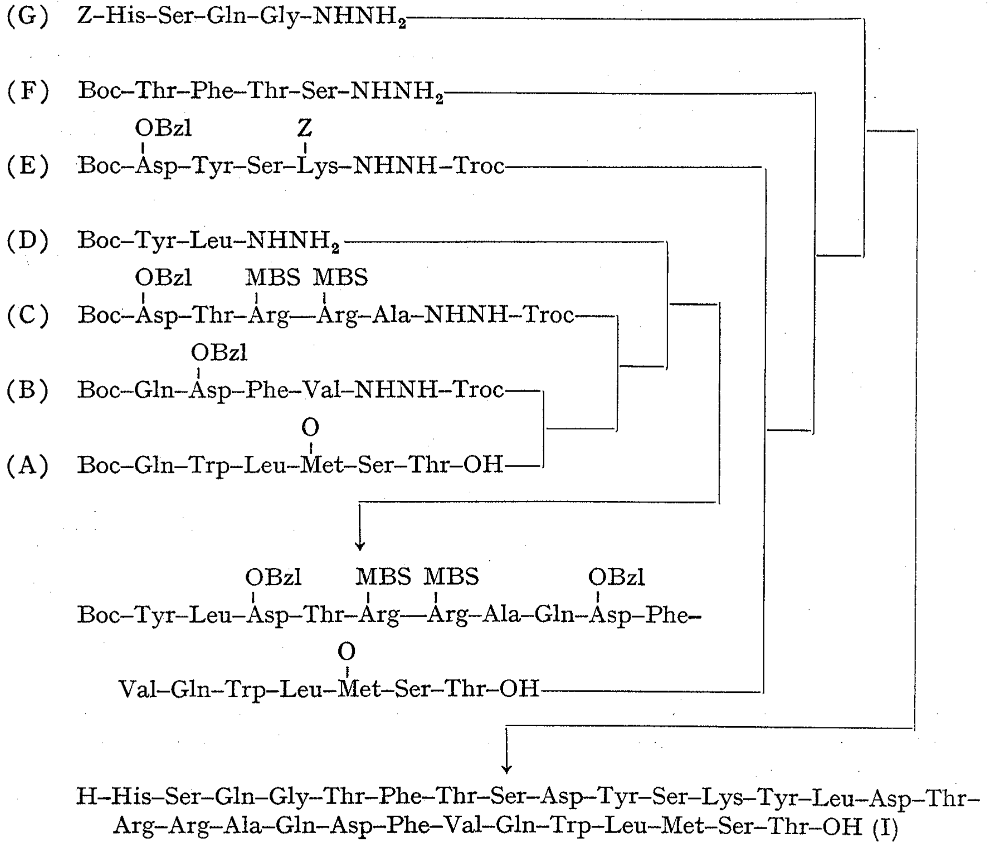

Fig. 1. Synthetic Route to Duck Glucagon

First, the C-terminal hexapeptide, Boc-Gln-Trp-Leu-Met(O)-Ser-Thr-OH (A), *i. e.*, Boc-(duck glucagon 24—29)-OH, was synthesized according to the scheme illustrated in Fig. 2.

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[24] R.J. Simpson, M.R. Neuberger, and T.Y. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).
[25] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, **26**, 2333 (1961).

Z(OMe)-Met-OH and H-Ser-OMe was condensed by the DCC procedure[26] and the resulting dipeptide ester, Z(OMe)-Met-Ser-OMe, was converted to the corresponding hydrazide, Z(OMe)-Met-Ser-$NHNH_2$, in the usual manner. This hydrazide was then condensed with the triethylammonium salt of Thr *via* the azide to give Z(OMe)-Met-Ser-Thr-OH. The product is partially soluble in water and therefore the salt out with sodium chloride was required for isolation. The Z(OMe) group was removed from this tripeptide by the usual TFA treatment in the presence of anisole and the deprotected peptide, after neutralization with triethylamine, was coupled with Z(OMe)-Leu-OH by the *p*-nitrophenyl ester procedure[27] to give Z(OMe)-Leu-Met-Ser-Thr-OH. The Met residue was oxidized at this step to the corresponding sulphoxide by sodium perborate, since the oxidation of the former tripeptide gave a very much water soluble compound and isolation of the desired product from the inorganic salt was difficult. The Met(O) residue seems to be a mixture of equal amount of two diastereoisomers, as examined preliminarily in oxidation of Z(OMe)-Met-OH.[16] Next, Boc-Trp-OH, instead of Z(OMe)-Trp-OH, was introduced to the deprotected tetrapeptide by the *p*-nitrophenyl ester procedure. The product was purified by extraction with *n*-butanol, since it was not soluble in ethyl acetate. Removal of the Boc group from the protected pentapeptide, Boc-Trp-Leu-Met-(O)-Ser-Thr-OH, was performed under special cares by the reason stated above. The Boc group was removed smoothly by treatment with 4 N ESA–TFE in the presence of skatole (1.5 equiv.) and anisole containing 0.1% EDT in an ice-bath for 30 minutes without producing any detectable side products and any brown color. The deblocked peptide precipitated by ether as a fine powder, was then submitted to the next coupling reaction with Boc-Gln-ONP. The homogeneous compound was obtained after purification by extraction with *n*-butanol followed by repeated precipitation from DMF with ethyl acetate. The content of Trp in the product (A) was determined by hydrolysis with 4 N MSA mentioned above. Under this condition, Met(O) was converted partially to Met. This was confirmed by hydrolysis of the standard sample of Met(O) under identical conditions.

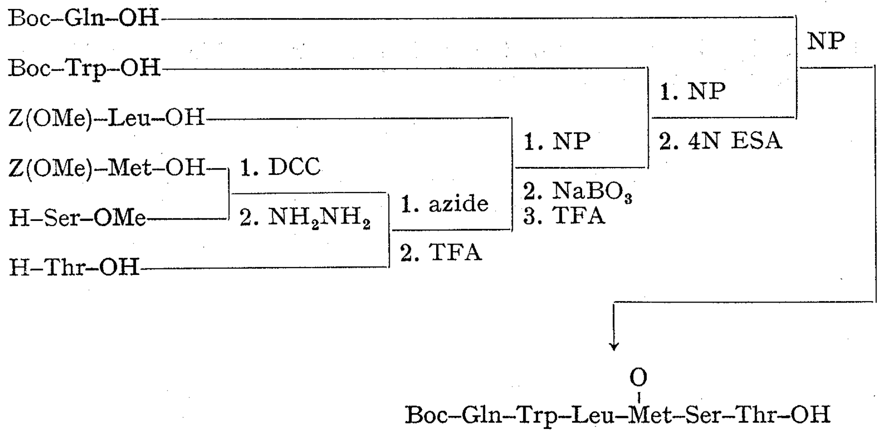

Fig. 2. Synthetic Scheme of the Protected Hexapeptide Boc-(Duck Glucagon 24—29)-OH (A)

The next fragment (B), Boc-Gln-Asp(OBzl)-Phe-Val-$NHNH_2$, was prepared starting with the avialable fragment, Z(OMe)-Asp(OBzl)-Phe-Val-NHNH-Troc, used in our previous synthesis of GIP.[19] This, after treatment with TFA, was condensed with Boc-Gln-ONP to give Boc-Gln-Asp(OBzl)-Phe-Val-NHNH-Troc, from which the Troc group was removed by treatment with Zn[28] in acetic acid and DMF. The last trace of metal contamination

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26) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

27) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

28) R.B. Woodward, K. Heusler, J. Gosteli, W. Oppolzer, R. Ramage, S. Ranganathan, and H. Vorbruggen, *J. Am. Chem. Soc.*, 83, 1991 (1961).

was removed by treatment with ethylenediamine tetraacetate (EDTA). Thus, the Asp(OBzl) residue was incorporated into the hydrazide fragment as shown in Fig. 3.

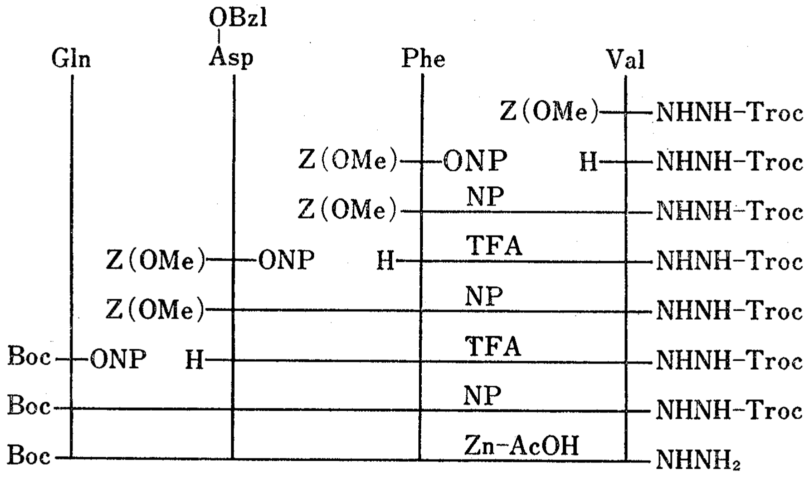

Fig. 3. Synthetic Scheme of the Protected Tetrapeptide Hydrazide Boc-(Duck Glucagon 20—23)-NHNH$_2$ (B)

In order to incorporate the Asp residue at position 15 to the hydrazide fragment, Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-NHNH$_2$ (C) was synthesized starting again with Troc-NHNH$_2$[29] in a stepwise manner as illustrated in Fig. 4. Z(OMe)-Ala-OH was condensed with Troc-NHNH$_2$ by DCC to give Z(OMe)-Ala-NHNH-Troc as a crystalline compound, from which the Z(OMe) group was removed by the usual TFA treatment. To the resulting H-Ala-NHNH-Troc, two residues of Arg(MBS) were introduced successively by the mixed anhydride procedure.[30] By this tool, the homogeneous protected di and tripeptide derivatives were easily obtained. To this tripeptide, Thr and Asp(OBzl) residues were introduced by azide and the *p*-nitrophenyl ester procedures respectively. Removal of the Troc moiety from Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-NHNH-Troc was performed by Zn in essentially the same manner as performed in the preparation of the fragment (B). In this

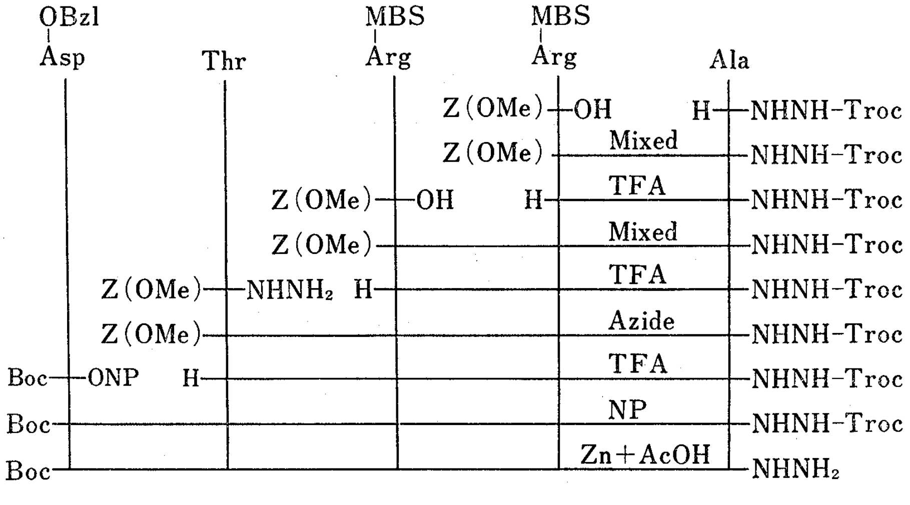

Fig. 4. Synthetic Scheme of the Protected Pentapeptide Hydrazide Boc-(Duck Glucagon 15—19)-NHNH$_2$ (C).

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[29] H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 420 (1971).

[30] Th. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951); R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J.R. Vaughan, *J. Am. Chem. Soc.*, **73**, 3547 (1951).

instance, we noticed that prolonged treatment of this derivative with Zn gave an additional hydrazine test[31] positive substances. The product (C) was purified by repeated precipitation from tetrahydrofuran and ethanol.

The fragment(D), Boc-Tyr-Leu-$NHNH_2$, was prepared by the DCC coupling of Boc-Tyr-OH and H-Leu-OMe followed by treatment of the resulting dipeptide ester with hydrazine hydrate.

The four peptide fragments thus obtained were then assembled according to the scheme illustrated in Fig. 1. The Boc group was removed from the fragment (A) by 4 N ESA in TFE in the presence of skatole and anisole containing 0.1% EDT as stated above and the azide derived from (B) was allowed to react to form the protected decapeptide, Boc-(duck glucagon 20—29)-OH. The product was purified by batchwise washing with 5% citric acid and water followed by precipitation from DMF with ethyl acetate. Such purification procedures, *i. e.*, batchwise washing and precipitation procedures, were extended to the latter steps of the synthesis also.

Next, the deprotecting reagent had to be changed to TFA, because of the less solubility of the protected decapeptide in the TFE system under cooling with ice. Skatole and anisole containing 0.1% EDT was used during this TFA deprotection of the Boc group as mentioned above. We could perform this deprotection under cooling without producing any observable difference from the former conditions. The resulting deprotected decapeptide was allowed to react with the azide derived from the fragment (C) to form the protected pentadecapeptide, Boc-(duck glucagon 15—29)-OH, which was purified by batchwise washing followed by precipitation from DMF with ethanol.

Elongation of the pentadecapeptide to the heptadecapeptide, Boc-(duck glucagon 13—29)-OH, was carried out in essentially the same manner by deprotection followed by introduction of Boc-Tyr-Leu-$NHNH_2$ (D) *via* the azide. The desired product was isolated as stated above by batchwise washing followed by precipitation from DMF with ethyl acetate and its homogeneity was assessed by thin layer chromatography, hydrolysis with 4 N MSA and elemental analysis. This protected heptadecapeptide served as the starting material for the synthesis of duck glucagon, which we will describe in the second paper.

## Experimental

General experimental methods employed are essentially the same as described in the Part LXII[32] 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–AcOEt–pyridine–$H_2O$ (1: 1: 1: 1), $Rf_3$ $CHCl_3$–MeOH (9: 1), $Rf_4$ *n*-BuOH–AcOH–$H_2O$ (4: 1: 5).

**Z(OMe)-Met-Ser-OMe**——DCC (37.14 g) was added to a mixture of Z(OMe)–Met–OH (47.01 g) and H–Ser–OMe (prepared from 24.58 g of the hydrochloride with 22 ml of $Et_3N$) in DMF–AcOEt (50 ml—500 ml). After stirring at room temperature for 48 hr, the solution was filtered and the filtrate was condensed *in vacuo*. The resulting gel was collected by filtration, washed batchwisely with 10% citric acid and $H_2O$ and then recrystallized from tetrahydrofuran (THF) and AcOEt; yield 46.73 g (75%), mp 167—168°, $[\alpha]_D^{22}$ $-2.6°$ ($c=0.8$, DMF). $Rf_1$ 0.72. *Anal.* Calcd. for $C_{18}H_{26}N_2O_7S$: C, 52.16; H, 6.32; N, 6.76. Found: C, 52.45; H, 6.24; N, 6.82.

**Z(OMe)-Met-Ser-$NHNH_2$**——To a solution of Z(OMe)–Met–Ser–OMe (46.73 g) in THF (500 ml), 80% hydrazine hydrate (30 ml) was added and the solution was kept at room temperature overnight. The gelatinous mass formed was collected by filtration and precipitated from DMF with THF; yield 42.29 g (91%). mp 199—203°, $[\alpha]_D^{22}$ $-2.0°$ ($c=1.0$, DMF). $Rf_1$ 0.55. *Anal.* Calcd. for $C_{17}H_{26}N_4O_6S$: C, 49.26; H, 6.32; N, 13.52. Found: C, 48.98; H, 6.12; N, 13.43.

**Z(OMe)-Met-Ser-Thr-OH**——Under cooling with ice-NaCl, 3.79 N HCl–DMF (30.9 ml) and isoamylnitrite (8.2 ml) were added successively to a solution of Z(OMe)–Met–Ser–$NHNH_2$ (22.95 g) in DMF (200 ml) and the mixture was stirred for 15 min, while the hydrazine test[31] became negative. The solution was neutralized with $Et_3N$ (24.2 ml) and then poured into a solution of H–Thr–OH (7.27 g) in $H_2O$ (50 ml) containing $Et_3N$

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[31] H. Ertel and L. Horner, *J. Chromato.*, 7, 268 (1962).

[32] H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).

(8.5 ml). After stirring at 4° for 48 hr, the solution was condensed *in vacuo* and the residue was treated with 10% citric acid saturated with NaCl and AcOEt. The crystalline mass formed on standing in a refrigerator overnight, was collected by filtration, washed with a small amount of $H_2O$ and then recrystallized from MeOH and AcOEt; yield 11.24 g (40%). mp 148—150°, $[\alpha]_D^{22}$ +6.4° ($c$=0.8, DMF). $Rf_1$ 0.21. *Anal.* Calcd. for $C_{21}H_{31}N_3O_9S$. 1.5 $H_2O$: C, 47.73; H, 6.48; N, 7.95. Found: C, 47.73; H, 6.35; N, 7.91.

**Z(OMe)-Leu-Met-Ser-Thr-OH**——Z(OMe)–Met–Ser–Thr–OH (15.05 g) was treated with TFA (25 ml) in the presence of anisole (16 ml) 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 DMF (150 ml), to which $Et_3N$ (8.4 ml), Z(OMe)–Leu–ONP (14.37 g) and N-hydroxybenzotriazole (HOBT)[33] (4.59 g) were added successively. The mixture was stirred at room temperature for 24 hr and then condensed *in vacuo*. The residue was treated with ether and 10% citric acid and the resulting powder, after washing batchwisely with 10% citric acid and $H_2O$, was recrystallized from MeOH and AcOEt; yield 9.70 g (53%). mp 160—161°, $[\alpha]_D^{22}$ −3.9° ($c$=1.0, DMF). $Rf_1$ 0.37. *Anal.* Calcd. for $C_{27}H_{42}N_4O_{10}S\cdot 1/2H_2O$: C, 51.99; H, 6.95; N, 8.98. Found: C, 52.14; H, 6.89; N, 9.07.

**Z(OMe)-Leu-Met(O)-Ser-Thr-OH**——To an ice-chilled solution of Z(OMe)–Leu–Met–Ser–Thr–OH (12.29 g) in THF (80 ml), $NaBO_3\cdot 4H_2O$ (3.39 g) in $H_2O$ (40 ml) was added and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was treated with hot MeOH and filtered. The filtrate was condensed and the residue, after washing with a small amount of $H_2O$, was recrystallized twice from MeOH and AcOEt; yield 12.70 g (87%). mp 196—198°, $[\alpha]_D^{22}$ +13.2° ($c$=1.0, DMF). $Rf_1$ 0.21. *Anal.* Calcd. for $C_{27}H_{42}N_4O_{11}S\cdot H_2O$: C, 49.99; H, 6.84; N, 8.63. Found: C, 49.99; H, 6.56; N, 8.57.

**Boc-Trp-Leu-Met(O)-Ser-Thr-OH**——Z(OMe)–Leu–Met(O)–Ser–Thr–OH (9.07 g) was treated with TFA-anisole (14 ml—7.8 ml) as usual and dry ether was added. The resulting powder isolated as stated above was dissolved in DMF (80 ml), to which $Et_3N$ (4.2 ml), Boc–Trp–ONP (7.35 g) and HOBT (2.21 g) were successively added. The solution was stirred at room temperature overnight and then condensed *in vacuo*. The residue was extracted with *n*-BuOH, which was washed with 10% citric acid, $H_2O$–NaCl and a small amount of $H_2O$, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with AcOEt and precipitated twice from DMF with AcOEt; yield 9.86 g (91%). mp 138—142°, $[\alpha]_D^{22}$ −12.3° ($c$=0.8, DMF). $Rf_1$ 0.08, $Rf_2$ 0.82. Amino acid ratios in 4 N MSA hydrolysate: Trp 1.01, Leu 1.00, Met+Met(O) 0.78, Ser 0.86, Thr 0.93 (average recovery 86%). *Anal.* Calcd. for $C_{34}H_{52}N_6O_{11}S\cdot 2H_2O$: C, 51.76; H, 7.16; N, 10.65. Found: C, 51.45; H, 7.04; N, 11.19.

**Boc-Gln-Trp-Leu-Met(O)-Ser-Thr-OH (A)**——Under $N_2$ gas, Boc–Trp–Leu–Met(O)–Ser–Thr–OH (7.53 g) was treated with 4 N ESA in TFE (30 ml) in the presence of skatole (1.97 g, 1.5 equiv.) and anisole (11 ml) containing 0.1% ethanedithiol in an ice-bath for 30 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 DMF (80 ml), to which $Et_3N$ (2.80 ml), Boc–Gln–ONP (4.22 g) and HOBT (1.53 g) were added. The mixture was stirred at room temperature overnight and the solvent was evaporated. The residue was dissolved in *n*-BuOH, which was washed with 10% citric acid, $H_2O$–NaCl and a small amount of $H_2O$, dried over $Na_2SO_4$ and then precipitated twice from DMF with AcOEt; yield 7.09 g (80%). mp 141—145°, $[\alpha]_D^{22}$ −2.2° ($c$=0.9, DMF). $Rf_1$ 0.09, $Rf_2$ 0.85. Amino acid ratios in 4 N MSA hydrolysate: Glu 1.13, Trp 0.97, Leu 1.00, Met+Met(O) 0.83, Ser 0.84, Thr 0.90 (average recovery 81%). *Anal.* Calcd. for $C_{39}H_{60}N_8O_{13}S\cdot 3.5H_2O$: C, 49.62; H, 7.15; N, 11.87. Found: C, 50.00; H, 6.81; N, 11.46.

**Boc-Gln-Asp(OBzl)-Phe-Val-NHNH-Troc**——Z(OMe)–Asp(OBzl)–Phe–Val–NHNH–Troc (17.29 g) was treated with TFA–anisole (18 ml—12 ml) as usual 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 (140 ml), to which $Et_3N$ (4.4 ml), Boc–Gln–ONP (8.49 g) and HOBT (3.22 g) were added successively. The mixture was stirred at room temperature overnight and the solvent was evaporated. The residue was treated with AcOEt and the resulting powder, after washing batchwisely with 10% citric acid and $H_2O$, was precipitated from DMF with AcOEt; yield 16.36 g (88%). mp 218—222°, $[\alpha]_D^{22}$ −23.5° ($c$=0.9, DMF). $Rf_1$ 0.71. *Anal.* Calcd. for $C_{38}H_{50}Cl_3N_7O_{11}$: C, 51.44; H, 5.68; N, 11.05. Found: C, 51.49; H, 5.80; N, 10.92.

**Boc-Gln-Asp(OBzl)-Phe-Val-NHNH$_2$ (B)**——Zn dust (*ca.* 5 g) was added to a solution of Boc–Gln–Asp(OBzl)–Phe–Val–NHNH–Troc (4.44 g) in DMF–AcOH (20 ml—20 ml) and the mixture was stirred at room temperature for 4 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with a saturated solution of EDTA (150 ml). The gelatinous mass formed on standing in a refrigerator overnight, was collected by filtration, washed with 5% $NaHCO_3$ and $H_2O$ and then precipitated twice from DMF with AcOEt; yield 2.23 g (63%). mp 222—224°, $[\alpha]_D^{25}$ −24.1° ($c$=0.8, DMF), $Rf_1$ 0.65. Amino acid ratios in 4 N MSA hydrolysate: Glu 0.97, Asp 1.01, Phe 1.00, Val 0.90 (average recovery 95%). *Anal.* Calcd. for $C_{35}H_{49}N_7O_9$ C, 59.05; H, 6.94; N, 13.78. Found: C, 59.22; H, 7.12; N, 13.53.

**Z(OMe)-Ala-NHNH-Troc**——DCC (45.39 g) was added to a mixture of Z(OMe)–Ala–OH (50.65 g) and Troc–$NHNH_2$ (41.49 g) in THF (350 ml) and the solution was stirred at room temperature for 20 hr. After

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[33] W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).

filtration, the solution was condensed and the residue was extracted with AcOEt, which was washed with 5% $Na_2CO_3$, 5% citric acid and $H_2O$, dried over $Na_2SO_4$ and then evaporated. The resulting mass was recrystallized from MeOH and AcOEt; yield 69.01 g (78%), mp 142—145°, $[\alpha]_D^{22}$ −33.8° (*c*=0.9, MeOH), *Rf*$_1$ 0.96. *Anal.* Calcd. for $C_{15}H_{18}Cl_3N_3O_6$: C, 40.69; H, 4.10; N, 9.49. Found: C, 40.97; H, 4.19; N, 9.53.

**Z(OMe)-Arg(MBS)-Ala-NHNH-Troc**——Z(OMe)–Ala–NHNH–Troc (10.18 g) was treated with TFA–anisole (10 ml—7.5 ml) as usual and dry *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in THF (30 ml). To this ice-chilled solution, $Et_3N$ and the mixed anhydride (prepared from 11.70 g of Z(OMe)–Arg(MBS)–OH with 3.2 ml of $Et_3N$ and 3.3 ml of isobutylchloroformate) in THF (100 ml) were added. The solution was stirred in an ice-bath for 3 hr, the solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% $NaHCO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with ether and the resulting powder was purified by column chromatography on silica (5.5×10 cm). The desired compound (*Rf*$_1$ 0.81) was eluted with $CHCl_3$–MeOH (9:1) and recrystallized from a mixture of THF and ether; yield 15.25 g (86%), mp 102—106°, $[\alpha]_D^{22}$ −9.6° (*c*=1.0, MeOH). *Rf*$_1$ 0.81. *Anal.* Calcd. for $C_{28}H_{36}Cl_3N_7O_{10}S$: C, 43.73; H, 4.72; N, 12.75. Found: C, 43.75; H, 4.96; N, 12.47.

**Z(OMe)-Arg(MBS)-Arg(MBS)-Ala-NHNH-Troc**——Z(OMe)–Arg(MBS)–Ala–NHNH–Troc (15.25 g) was treated with TFA–anisole (15 ml—6.5 ml) as usual and dry ether was added. The resulting powder isolated as stated above was dissolved in THF (50 ml). To this ice-chilled solution, $Et_3N$ (2.8 ml) and the mixed anhydride (prepared from 9.66 g of Z(OMe)–Arg(MBS)–OH with 2.7 ml of $Et_3N$ and 2.75 ml of isobutylchloroformate) in THF (100 ml) were added. The mixture was stirred in an ice-bath for 3 hr and the solvent was evaporated. The residue was triturated with *n*-BuOH and the resulting powder was washed batchwisely with 10% citric acid, 5% $NaHCO_3$ and $H_2O$. Recrystallization from a mixture of MeOH and *n*-BuOH afforded a powder; yield 14.85 g (68%). mp 133—136°, $[\alpha]_D^{25}$ −9.2° (*c*=1.0, DMF). *Rf*$_1$ 0.74, *Rf*$_3$ 0.25. *Anal.* Calcd. for $C_{41}H_{54}Cl_3N_{11}O_{14}S_2 \cdot C_4H_9OH$:[34] C, 46.21; H, 5.52; N, 13.17. Found: C, 46.04; H, 5.33; N, 13.27.

**Z(OMe)-Thr-Arg(MBS)-Arg(MBS)-Ala-NHNH-Troc**——Z(OMe)–Arg(MBS)–Arg(MBS)–Ala–NHNH–Troc (9.72 g) was treated with TFA–anisole (15 ml—4.8 ml) as usual and dry ether was added. The resulting powder was dissolved in DMF (50 ml) containing $Et_3N$ (1.24 ml). To this ice-chilled solution, the azide (prepared from 3.04 g of Z(OMe)–Thr–$NHNH_2$ with 3.61 ml of 5.96 N HCl–DMF, 1.51 ml of isoamylnitrite and 4.46 ml of $Et_3N$) in DMF (30 ml) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with *n*-propylalcohol. The resulting powder was washed batchwisely as stated above and recrystallized from a mixture of MeOH and *n*-propylalcohol; yield 8.03 g (75%). mp 127—130°, $[\alpha]_D^{25}$ −6.4° (*c*=1.0, DMF), *Rf*$_1$ 0.67. Amino acid ratios in 4 N MSA hydrolysate: Thr 1.11, Arg 2.10, Ala 1.00 (average recovery 97%). *Anal.* Calcd. for $C_{45}H_{61}Cl_3N_{12}O_{16}S_2 \cdot C_3H_7OH$: C, 45.88; H, 5.53; N, 13.38. Found: C, 45.96; H, 5.27; N, 13.70.

**Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-NHNH-Troc**——The above protected tetrapeptide (8.03 g) was treated with TFA–anisole (15 ml-4.5 ml) as usual and dry ether was added. The resulting powder isolated as stated above was dissolved in DMF (50 ml), to which $Et_3N$ (0.94 ml), Boc–Asp(OBzl)–ONP (3.29 g) and HOBT (1.03 g) were added successively. The mixture was stirred at room temperature for 48 hr, the solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with ether and then recrystallized from AcOEt and ether; yield 8.54 g (95%). mp 122—124°, $[\alpha]_D^{26}$ −1.6° (*c*=0.6, DMF), *Rf*$_1$ 0.65. *Anal.* Calcd. for $C_{52}H_{72}Cl_3N_{13}O_{18}S_2$: C, 46.69; H, 5.43; N, 13.61. Found: C, 46.46; H, 5.49; N, 13.32.

**Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-NHNH$_2$ (C)**——The above protected pentapeptide (4.01 g) was dissolved in DMF–AcOH (20 ml-20 ml) and Zn dust (*ca.* 5.0 g) was added and the mixture was stirred at room temperature for 3 hr. The solution was filtered, the filtrate was condensed and the residue was treated with 0.1% EDTA. The resulting gelatinous mass was washed with 5% $NaHCO_3$ and $H_2O$ and recrystallized once from THF and AcOEt and then from THF with EtOH; yield 1.98 g (57%). mp 140—143°, $[\alpha]_D^{25}$ −4.8° (*c*=1.3, DMF). *Rf*$_1$ 0.53. Amino acid ratios in 4 N MSA hydrolysate: Asp 1.18, Thr 1.00, Arg 2.06, Ala 0.92 (average recovery 86%). *Anal.* Calcd. for $C_{49}H_{71}N_{13}O_{16}S_2 \cdot 2H_2O$: C, 49.11; H, 6.31; N, 15.20. Found: C, 49.15; H, 6.24; N, 15.31.

**Boc-Tyr-Leu-NHNH$_2$ (D)**——DCC (4.25 g) was added to a mixture of Boc–Tyr–OH·DCHA (8.28 g) and H–Leu–OMe hydrochloride (3.25 g) in DMF (80 ml) and the solution was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and then evaporated. The oily residue was dissolved in MeOH (50 ml), to which 80% hydrazine hydrate (4.5 ml) was added. The solution, after standing at room temperature overnight, was condensed and the residue was triturated with EtOH and then recrystallized from MeOH and EtOH; yield 3.25 g (44%). mp 212—214°, $[\alpha]_D^{22}$ −3.4° (*c*=0.9, DMF). *Rf*$_1$ 0.65. *Anal.* Calcd. for $C_{20}H_{32}N_4O_5$: C, 58.80; H, 7.90; N, 13.72. Found: C, 59.04; H, 7.97; N, 13.59.

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[34] Solvation property of Arg derivative was recorded in ref. 17.

**Boc-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, Boc-(duck glucagon 20—29)-OH**——Boc-Gln-Trp-Leu-Met(O)-Ser-Thr-OH (4.41 g) was treated with 4 N ESA in TFE (25 ml) in the presence of skatole (0.98 g) and anisole (5.5 ml) containing 0.1% ethanedithiol in an ice-bath for 60 min and dry ether was added. The resulting powder was washed with ether, dried over KOH pellets *in vacuo* for 4 hr and then dissolved in DMF (40 ml) containing $Et_3N$ (1.4 ml). To this ice-chilled solution, the azide (prepared from 4.63 g of Boc-Gln-Asp(OBzl)-Phe-Val-$NHNH_2$ with 2.4 ml of 5.96 N HCl-DMF, 0.96 ml of isoamylnitrite and 2.93 ml of $Et_3N$) in DMF (40 ml) was added and the mixture was stirred at 4° for 48 hr. After addition of a few drops of AcOH, the solvent was evaporated *in vacuo* and the residue was treated with 3% AcOH and AcOEt. The resulting powder was washed batchwisely with 5% citric acid and $H_2O$ and then precipitated twice from DMF with AcOEt; yield 4.89 g (67%). mp 238—241°, $[\alpha]^{26}_{D}$ $-18.7°$ ($c$=0.9, DMF). $Rf_2$ 0.78. Amino acid ratios in 4 N MSA hydrolysate: Glu 1.97, Asp 1.21, Phe 1.03, Val 0.85, Trp 1.05, Leu 1.00, Met+Met(O) 1.03, Ser 0.81, Thr 0.87 (average recovery 96%). *Anal.* Calcd. for $C_{69}H_{97}N_{13}O_{20}S \cdot H_2O$: C, 56.04; H, 6.75; N, 12.32. Found: C, 56.18; H, 6.87; N, 12.46.

**Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-Gln-Asp(OBzl)-Phe-Val-Gln-Trp-Leu-Met(O)-Ser-Thr-OH, Boc-(duck glucagon 15—29)-OH**——Boc-(duck glucagon 20—29)-OH (1.46 g) was treated with TFA (4 ml) in the presence of skatole (0.2 g) and anisole (1.5 ml) containing 0.1% ethanedithiol in an ice-bath for 90 min. Dry ether was added and the resulting powder isolated as stated above dissolved in DMF-NMP (10 ml-2 ml). To this ice-chilled solution, $Et_3N$ (0.28 ml) and the azide (prepared from 1.74 g of Boc-Asp(OBzl)-Thr-Arg(MBS)-Arg(MBS)-Ala-$NHNH_2$ as stated above) in DMF (10 ml) were added and 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 AcOEt and 5% citric acid. The resulting powder was washed batchwisely as stated above and then precipitated twice from DMF with EtOH; yield 1.85 g (74%). mp 236—240°, $[\alpha]^{22}_{D}$ $-22.4°$ ($c$=0.9, DMF). $Rf_2$ 0.76, $Rf_4$ 0.42. Amino acid ratios in 4 N MSA hydrolysate: Asp 2.29, Thr 1.81, Arg 2.16, Ala 1.00, Glu 2.03, Phe 1.04, Val 0.92, Trp 0.92, Leu 0.87, Met+Met(O) 0.87, Ser 0.84 (average recovery 81%). *Anal.* Calcd. for $C_{113}H_{156}N_{24}O_{34}S_3 \cdot H_2O$: C, 54.09; H, 6.35; N, 13.40. Found: C, 53.77; H, 6.32; N, 13.69.

**Boc-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 13—29)-OH**——Boc-(duck glucagon 15—29)-OH (2.49 g) was treated with TFA (5 ml) in the presence of skatole (0.26 g) and anisole (3 ml) containing 0.1% ethanedithiol as stated above and dry ether was added. The resulting powder was then dissolved in NMP-DMF (4 ml-6 ml). To this ice-chilled solution, $Et_3N$ (0.28 ml) and the azide (prepared from 0.82 g of Boc-Tyr-Leu-$NHNH_2$ as stated above) in DMF (5 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent, after addition of a few drops of AcOH, was evaporated. The residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely as stated above and then precipitated twice from DMF with AcOEt; yield 2.32 g (84%). mp 236—241°, $[\alpha]^{22}_{D}$ $-11.9°$ ($c$=1.0, DMF). $Rf_4$ 0.39. Amino acid ratios in 4 N MSA hydrolysate: Tyr 1.08, Leu 1.95, Asp 2.03, Thr 1.79, Arg 1.70, Ala 0.83, Glu 2.00, Phe 1.03, Val 1.00, Trp 0.90, Met+Met(O) 0.79, Ser 0.92 (average recovery 81%). *Anal.* Calcd. for $C_{128}H_{176}N_{26}O_{37}S_3 \cdot 4H_2O$: C, 54.14; H, 6.53; N, 12.83. Found: C, 53.91; H, 6.54; N, 12.93.