Studies on Peptides. LXX.[1,2] Synthesis of Two Octadecapeptides corresponding to the Entire Amino Acid Sequence of Camel β-Melanocyte-stimulating Hormones

Fusako Tamura, Hiroshi Ogawa, Nobutaka Fujii, Haruaki Yajima,[3a]
Kenji Miyata, Masuhisa Nakamura, and Akira Tanaka[3b]

*Faculty of Pharmaceutical Sciences, Kyoto University[3a] and
Shionogi Research Laboratory, Shionogi Co., Ltd.[3b]*

In a conventional manner, two octadecapeptides corresponding to the entire amino acid sequences of camel β_1 and β_2-MSHs were synthesized. Synthetic peptides exhibited the *in vitro* MSH activities of 5.1×10^9 and 1.1×10^9 MSH unit/g, respectively.

In 1975, Li, *et al.*[4] elucidated the complete amino acid sequences of two species of β-melanocyte-stimulating hormones (β_1 and β_2-MSH) from camel pituitary glands and they synthesized these melanotropic principles by the solid phase procedures.[5] These structures are quite similar to that of porcine β-MSH,[6] except that Glu residue at position 2 of porcine β-MSH is replaced by Gly in both camel β-MSHs (I and II) and in addition, Gln, instead of Glu, is placed at position 8 in camel β_1-MSH (I). We have synthesized two camel β-MSHs in a conventional manner using available peptide fragments for our recent synthesis of porcine β-MSH.[7] Both camel β-MSHs contain the Asp-Gly bond at the N-terminus. It is known that establishment of this particular peptide bond, without accompanying the contaminant formation of the cyclic succinimide derivatives, is extremely difficult to perform in peptide synthesis,[8] since this side reaction is catalysed by both acid and base. Therefore purity of the desired peptide depends upon conditions employed to introduce the Asp-Gly residue to the peptide chain and to remove all protecting groups at the final step of the syn-

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

2) Amino acids, peptides and their derivatives mentioned in this communication 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, Tos=p-toluenesulfonyl, Troc=β,β,β-trichloroethyloxycarbonyl, OBzl=benzyl ester, ONP=p-nitrophenyl ester, DCC=dicyclohexylcarbodiimide, HOBT=N-hydroxybenzotriazole, TFA=trifluoroacetic acid, THF=tetrahydrofuran, DMF=dimethylformamide.

3) Location: a) *606, Sakyo-ku, Kyoto*; b) *553, Fukushima, Osaka*.

4) C.H. Li, W.O. Danho, D. Chung, and A.J. Rao, *Biochem.*, **14**, 947 (1975).

5) C.H. Li, D. Yamashiro, and S. Lemaire, *Biochem.*, **14**, 953 (1975); S. Lemaire, D. Yamashiro, and C.H. Li, *J. Med. Chem.*, **19**, 373 (1976).

6) J.I. Harris and P. Ross, *Nature*, **179**, 90 (1956), *idem, Biochem. J.*, **71**, 434 (1959); I.I. Geshwind, C.H. Li, and L. Bernafi, *J. Am. Chem. Soc.*, **78**, 4494 (1956); I.I. Geshwind and C.H. Li, *ibid.*, **79**, 615 (1957).

7) H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).

8) A.B. Battersby and J.C. Robinson, *J. Chem. Soc.*, **1955**, 239; E. Sondheimer and R.W. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954); S.A. Bernhard, A. Berger, J.H. Carter, E. Katchalski, E. Sela, and Y. Shalitin, *ibid.*, **84**, 2421 (1962); G. Folsch, *Acta Chem. Scand.*, **20**, 459 (1966); E.E. Haley and B.J. Corcoran, *Biochem.*, **6**, 2668 (1967); K. Weber and W. Konigsberg, *J. Biol. Chem.*, **242**, 3563 (1967); M. Bodanszky and S. Natarajan, *J. Org. Chem.*, **40**, 2495 (1975) and see other references thereon.

thesis by acid, in our case by hydrogen fluoride.[9,10] Ondetti, et al.[11] synthesized porcine secretin after introduction of the N-terminal tetrapeptide containing Asp-Gly by the azide procedure[12] and mentioned that by other means, such as carbonyldiimidazole[13] or the mixed anhydride,[14] complicated mixtures were formed. Referring to these results, we decided to use the N-terminal tripeptide hydrazide as a building block for the synthesis of both camel β-MSHs.

Synthetic scheme of the N-terminal tripeptide hydrazide, Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH$_2$, is illustrated in Fig. 1. Since the usual hydrazine treatment of Asp(OBzl)-peptide esters is not possible, Troc-hydrazine,[15] a substituted hydrazine introduced by us in 1971, served to prepare this hydrazide. Practical application of this substituted hydrazine was previously demonstrated in two synthetic examples: dogfish MSH[16] and gastric inhibiting
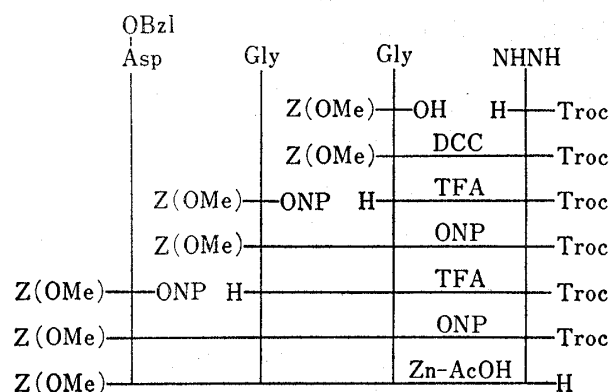

Fig. 1. Synthetic Scheme of the Protected Tripeptide Hydrazide

polypeptide.[17] Z(OMe)-Gly-NHNH-Troc was obtained as a crystalline compound by the the DCC procedure.[18] This, after deprotection of the Z(OMe) group[19] with TFA, was condensed with Z(OMe)-Gly-OH by the p-nitrophenyl ester procedure[20] to give Z(OMe)-Gly-Gly-NHNH-Troc, to which Z(OMe)-Asp(OBzl)-OH was introduced by the similar deprotection and the active ester procedure. Especially the latter condensation was accelerated by addition of HOBT.[21] The Troc group was then deprotected from the resulting protected tripeptide, Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH-Troc, with Zn-AcOH[22] and

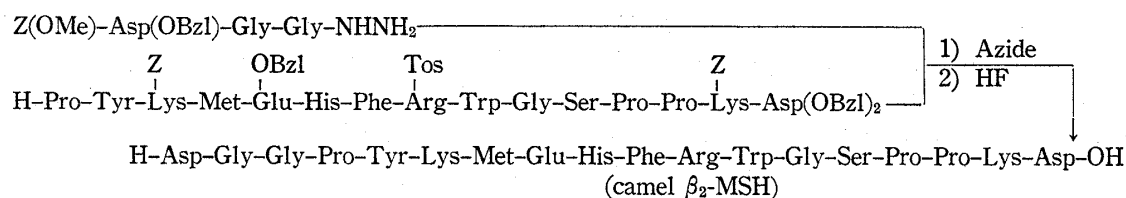Fig. 2. Synthetic Route to Camel β$_2$-MSH (II)

9) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada, and H. Sugihara, Bull. Chem. Soc. Japan, 40, 2164 (1967).

10) T. Baba, H. Sugiyama, and S. Seto, Chem. Pharm. Bull. (Tokyo), 21, 207 (1973).

11) M.A. Ondetti, V.L. Naraganan, M. von Saltza, J.T. Sheehan, E.F. Sabo, and M. Bodanszky, J. Am. Chem. Soc., 90, 4711 (1968); M.A. Ondetti, A. Deer, J.T. Sheehan, J. Pluscec, and O. Kocy, Biochem., 7, 4069 (1968).

12) T. Curtius, Chem. Ber., 35, 3226 (1902).

13) H.A. Staab, Ann. Chem., 609, 75 (1957); G.W. Anderson and R. Paul, J. Am. Chem. Soc., 80, 4423 (1958); ibid., 82, 4596 (1960).

14) Th. Wieland and H. Bernhard, Ann. Chem., 572, 190 (1951).

15) H. Yajima and Y. Kiso, Chem. Pharm. Bull. (Tokyo), 19, 420 (1971).

16) H. Watanabe, M. Kubota, H. Yajima, A. Tanaka, M. Nakamura, and T. Kawabata, Chem. Pharm. Bull. (Tokyo), 22, 1889 (1974).

17) H. Ogawa, M. Kubota, H. Yajima, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, T. Adachi, H. Imura, and T. Taminato, Chem. Pharm. Bull. (Tokyo), 24, 2447 (1976).

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

19) M. Bodanszky, Acta Chem. Hung., 10, 335 (1957).

20) F. Weygand and K. Hunger, Chem. Ber., 95, 1 (1962); H. Yajima, F. Tamura, and Y. Kiso, Chem. Pharm. Bull. (Tokyo), 18, 2574 (1970).

21) W. König and R. Geiger, Chem. Ber., 106, 3626 (1973).

22) R.B. Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganahan, and H. Vorbruggen, J. Am. Chem. Soc., 88, 852 (1966).

contamination of metal was removed by EDTA (ethylenediamine tetraacetic acid disodium salt). Thus Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH$_2$ was obtained in an analytically pure form.

Synthesis of camel β_2-MSH was first carried out as shown in Fig. 2. The modified azide procedure[23] was employed to condense the above stated tripeptide hydrazide with the TFA treated pentadecapeptide ester, H-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)$_2$, a synthetic intermediate of porcine β-MSH.[7] we wish to mention that in this synthesis, we used anisole containing 2% ethanedithiol[24] as a cation scavenger during the Z(OMe) deprotection with TFA in order to minimize destruction of the Trp residue present in the pentadecapeptide, since it was found that this system is much effective than the anisole-mercaptoethanol system[25] employed in the previous synthesis of porcine β-MSH. Thus we could carried out the TFA treatment without producing any brown color. In order to suppress the possible side reaction of the Asp(OBzl)-Gly residue during the azide condensation reaction, the pH of the solution was maintained as possible around 7.5. The product was purified by batchwise washing followed by precipitation from THF with ethyl acetate. Purity of the thin layer chromatographically homogeneous sample of protected octadecapeptide ester, Z(OMe)-Asp(OBzl)-Gly-Gly-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)$_2$ thus obtained, was further assessed by elemental analysis and by hydrolysis with 6 N hydrochloric acid and 3 N p-toluenesulfonic acid.[26] Especially the content of Arg was determined by the former hydrolysis and Trp in the latter.

The protected octadecapeptide ester was next exposed to hydrogen fluoride[9] to remove all protecting groups. The reaction was performed in the presence of anisole, dithiothreitol and skatol[27] in an ice-bath for 60 minutes and the excess hydrogen fluoride was evaporated carefully under cooling with ice *in vacuo* to suppress possible side reactions, which may occur at Glu(OBzl)[28] and Asp(OBzl), since it is known that these anticipated side reactions are both temperature dependent. The deblocked peptide, after conversion to the corresponding acetate with Amberlite CG-4B, was purified by column chromatography on Sephadex G-10 and subsequently on CM-cellulose. To elute the desired compound, 3% acetic acid was used in the former step and gradient elution with 0.1 M ammonium acetate buffer (pH 6.9) in the latter.

Synthetic camel β_2-MSH thus isolated exhibited a single spot on thin layer chromatography in two different solvent systems and migrated as a single component in the disc electrophoresis at pH 2.3. On paper electrophoresis in pyridine acetate buffer at pH 3.6, the synthetic peptide gave a single spot with the Rf value of 0.48 relative to Lys. This value is in closely agreement with that reported by Li, *et al.*[5] at pH 3.7. A hydrolysate with 3 N p-toluenesulfonic acid[26] contained the constituent amino acids in ratios predicted by theory. Aminopeptidase digestion is expected to give us information about a possible content of the succinimide or β-Asp type of analogues in our synthetic peptide. Since the Asp-Gly bond locates at the N-terminal portion of β_2-MSH, recovery of constituent amino acids in a hydrolysate should be decreased depending ,if any, on the amount of peptides containing such unnatural peptide bonds. Beacham, *et al.*[29] mentioned that rate of hydrolysis of the Asp-

23) J. Honzl and J. Ruginder, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).

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

25) G.R. Marshall, *Advan. Exp. Med.*, **2**, 48 (1969); H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **10**, 1333 (1970).

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27) C. Hatanaka, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **23**, 1017 (1975).

28) S. Sano and S. Kawanishi, *J. Am. Chem. Soc.*, **97**, 3480 (1975); R.S. Feinberg and R.B. Merrifield, *ibid.*, **97**, 3485 (1975).

29) J. Beacham, G. Dappis, F.M. Finn, H.T. Story, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Am. Chem. Soc.*, **93**, 5526 (1971).

Gly bond by AP–M[30] is abnormally slow and recovery of 88% was achieved after 40 hr's incubation. When we then examined a 40 hr's hydrolysate, average recovery of amino acids was 87%. The result indicated that the octadecapeptide we isolated is not contaminated with a sizable amount of peptide containing unnatural peptide bonds.

Synthesis of camel β_1-MSH was next carried out according to the scheme illustrated in Fig. 3. Since this hormone possesses the Gln residue at position 8, Z(OMe)-Gln-His-Phe-
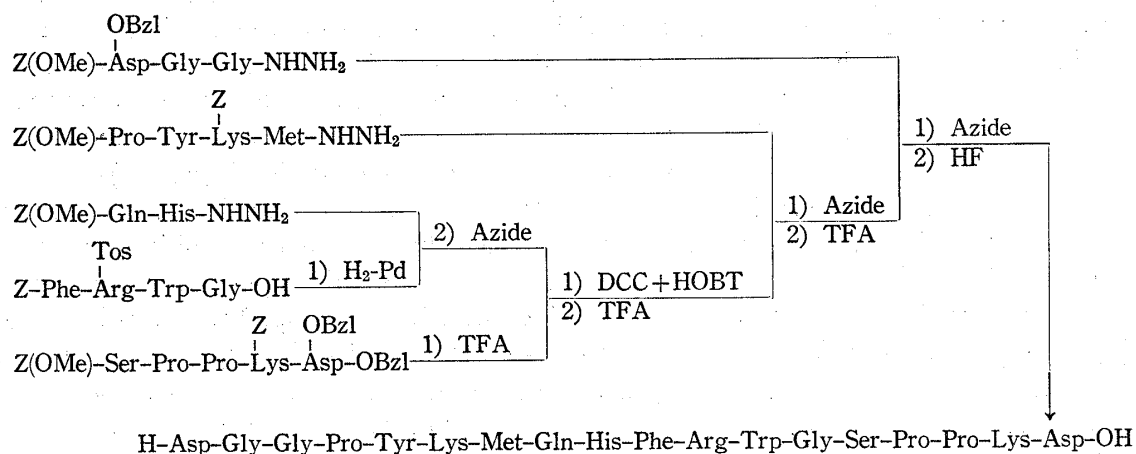

                         OBzl
                          |
       Z(OMe)-Asp-Gly-Gly-NHNH₂ ───────────────────────────────────┐
                                                                    │
                          Z   │ 1) Azide
                          | ├──────────
       Z(OMe)–Pro-Tyr-Lys-Met-NHNH₂────────────────────────┐   │ 2) HF
                                                            │   │
                                                   1) Azide │   │
       Z(OMe)-Gln-His-NHNH₂ ──────────┐   ─────────┤   │
                                      │ 2) Azide 2) TFA   │   │
           Tos   ├──────   │
            | 1) H₂-Pd │   1) DCC+HOBT │   │
       Z-Phe-Arg-Trp-Gly-OH ─────────┘   ───────────┘   │
                                                  2) TFA   │
               Z   OBzl   │
               | | 1) TFA   │
       Z(OMe)-Ser-Pro-Lys-Asp-OBzl─────────   ▼

       H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Gln-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH

             Fig. 3.   Synthetic Route to Camel β_1-MSH (I)

Arg(Tos)-Trp-Gly-OH was first prepared at a building block. This peptide unit was synthesized previously by Kappeler[31] in a form of Z-Gln-His-Phe-Arg-Trp-Gly-OMe in connection with the synthesis of ACTH active peptides. In our present synthesis, Z(OMe)-Gln-His-OMe prepared by the p-nitrophenyl ester procedure, was converted in the usual manner to Z(OMe)-Gln-His-NHNH₂, which was further condensed by the azide procedure with H-Phe-Arg(Tos)-Trp-Gly-OH derived from the Z-derivative.[7] The resulting protected hexapeptide was purified by batchwise washing followed by precipitation from DMF with methanol.

The DCC plus HOBT condensation[32] of this hexapeptide with H-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)₂[7] and subsequent azide condensation of Z(OMe)-Pro-Tyr-Lys(Z)-Met-NHNH₂[7] with the undecapeptide ester obtained in the above reaction were carried out in essentially the same manner as described in the synthesis of porcine β-MSH[7] and respective products were purified by column chromatography on silica. Next, the resulting protected pentadecapeptide ester, Z(OMe)-Pro-Tyr-Lys(Z)-Met-Gln-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)₂, was treated with TFA under conditions mentioned above and subsequently condensed with Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH₂ as performed in the synthesis of β_2-MSH with controlling the pH of the solution carefully. The protected octadecapeptide ester, Z (OMe)-Asp(OBzl)-Gly-Gly-Pro-Tyr-Lys(Z)-Met-Gln-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)₂ thus synthesized, was then exposed to hydrogen fluoride and the deblocked peptide was purified in essentially the same manner utilizing experiences obtained above. Homogeneity of the product was assessed by thin–layer chromatography, disc electrophoresis at pH 2.3 and hydrolysis with 3 N p-toluenesulfonic acid as did in the synthetic β_2-MSH. In addition, one mole of Gln in the molecule was confirmed by AP–M digest, in which average recovery of amino acids was 88%. Synthetic β_1-MSH migrated in a little bit faster rate than β_2-MSH on paper electrophoresis at pH 3.6 pyridine acetate buffer as expected from the report of Li, et al.[5]

30) G. Pfleiderer and G.P. Celliers, Biochem. Z., 339, 186 (1963), K. Hofmann, F.M. Finn, M. Limetti, J. Montbeller, and G. Zanetti, J. Am. Chem. Soc., 88, 3633 (1966).
31) H. Kappeler, Helv. Chim. Acta, 44, 476 (1961).
32) W. König and R. Geiger, Chem. Ber., 103, 788 (1970).

MSH activity was determined in vitro using the frog skins (Rana pipiens, six points design) according to Nakamura, et al.[33] When the potency of synthetic a-MSH $(2 \times 10^{10}$ MSH U/g)[34] was taken as a standard, relative potencies of synthetic camel β_1 and β_2-MSH were 0.256 $(5.13 \times 10^9$ MSH U/g) and 0.056 $(1.14 \times 10^9$ MSH U/g) respectively. Thus it can be concluded that β_1-MSH is a much stronger melanotropic principle than β_2-MSH by owing to the single amino acid variation at the position 8 (Gln and Glu). This tendency is well consistent with the report of Li, et al.[4] in the natural hormones.

Experimental

General experimental methods employed here are essentially the same as reported in the Part 62[35] of this series. Rf values refer to the following solvent systems: Rf_1 CHCl$_3$–MeOH–H$_2$O (8: 3: 1), Rf_2 n-BuOH–AcOH–pyridine–H$_2$O (4: 1: 1: 2), Rf_3 n-BuOH–AcOH–pyridine–H$_2$O (30: 6: 20: 24), Rf_4 n-BuOH–AcOH–pyridine–H$_2$O (30: 20: 6: 24).

Z(OMe)-Gly-NHNH-Troc——DCC (4.54 g) was added to a mixture of Z(OMe)-Gly-OH (4.78 g) and Troc–NHNH$_2$ (4.98 g) in AcOEt (100 ml) and the mixture was stirred at room temperature overnight. The solution was filtered and the filtrate was condensed in vacuo. The residue was triturated with ether and petroleum ether and recrystallized from ether; yield 7.52 g (81%), mp 82—83°, Rf_1 0.54. Anal. Calcd. for C$_{14}$H$_{16}$O$_6$N$_3$Cl$_3$: C, 39.22; H, 3.76; N, 9.80. Found: C, 39.20; H, 3.87; N, 9.73.

Z(OMe)-Gly-Gly-NHNH-Troc——Z(OMe)–Gly–NHNH–Troc (7.52 g) was treated with TFA (8.0 ml) in the presence of anisole (5.0 ml) in an ice-bath for 60 min and the excess TFA was removed by evaporation. The residue was washed with petroleum ether, dried over KOH pellets in vacuo for 3 hr and then dissolved in THF (80 ml), to which HOBT (2.16 g), Z(OMe)–Gly–ONP (6.45 g) and Et$_3$N (2.4 ml) were combined. The mixture was stirred at room temperature for 24 hr, the solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ and then evaporated. The residue was triturated with ether and then recrystallized from THF and ether; yield 5.86 g (71%), mp 99—101°, Rf_1 0.57. Anal. Calcd. for C$_{16}$H$_{19}$O$_7$N$_4$Cl$_3$: C, 39.56; H, 3.94; N, 11.54. Found: C, 39.77; H, 4.18; N, 11.73.

Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH-Troc——Z(OMe)–Gly–Gly–NHNH–Troc (4.86 g) was treated with TFA (7 ml) in the presence of anisole (5.4 ml) in an ice-bath for 60 min and the excess TFA was evaporated. The residue was washed with petroleum ether, dried over KOH pellets in vacuo and dissolved in DMF (50 ml), to which Et$_3$N (1.4 ml), HOBT (1.35 g) and Z(OMe)–Asp(OBzl)–ONP (6.10 g) were combined. The mixture was stirred at room temperature for 24 hr and the solvent was evaporated in vacuo. The residue was dissolved in AcOEt, which was washed with 10% citric acid and H$_2$O–NaCl, dried over Na$_2$SO$_4$ and then evaporated. Treatment of the residue with ether afforded a powder, which was recrystallized from MeOH and ether; yield 4.73 g (68%), mp 86—87°, $[\alpha]_D^{20}$ −6.3° (c=0.9, DMF), Rf_1 0.53. Anal. Calcd. for C$_{27}$H$_{30}$O$_{10}$N$_5$Cl$_3$: C, 46.93; H, 4.38; N, 10.14. Found: C, 47.00; H, 4.67; N, 10.02.

Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH$_2$——Z(OMe)–Asp(OBzl)–Gly–Gly–NHNH–Troc (2.76 g) was dissolved in AcOH–DMF (15 ml—5 ml) and Zn powder (2 g) was added and the mixture was stirred at room temperature for 1 hr. After addition of fresh Zn powder (1 g), stirring was continued for an additional 2 hr. The Zn dust was removed by filtration, the filtrate was condensed in vacuo, the residue was treated with a saturated solution of EDTA (20 ml) and the pH of the solution was adjusted with NaHCO$_3$ to 7. The resulting gelatinous mass, after storage in a refrigerator overnight, was collected by filtration, washed batchwisely with H$_2$O, precipitated from DMF with ethanol and recrystallized from hot MeOH; yield 2.06 g (60%), mp 154—158°, $[\alpha]_D^{20}$ −18.9° (c=0.9, DMF), Rf_1 0.57. Anal. Calcd. for C$_{24}$H$_{29}$O$_8$N$_5$: C, 55.91; H, 5.67; N, 13.59. Found: C, 55.77; H, 5.71; N, 13.42.

Z(OMe)-Asp(OBzl)-Gly-Gly-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)$_2$——The protected pentadecapeptide, Z(OMe)–Pro–Tyr–Lys(Z)–Met–Glu(OBzl)–His–Phe–Arg(Tos)–Trp–Gly–Ser–Pro–Pro–Lys(Z)–Asp(OBzl)$_2$[7] (0.27 g) was treated with TFA (0.5 ml) in the presence of anisole (0.3 ml) containing 2% 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 and then dissolved in DMF (2 ml) containing Et$_3$N (0.28 ml). Z(OMe)–Asp(OBzl)–Gly–Gly–NHNH$_2$ (0.10 g) was dissolved in an ice-cold mixture of DMF (2 ml) and 2.98N HCl–DMF (0.15 ml) and isoamylnitrite (0.03 ml) was added. The solution, after stirring for 5 min, was neutralized with Et$_3$N (0.74 ml) and then combined with the above ice-cold solution containing pentadecapeptide ester. The mixture was stirred at 4° for 48 hr, meanwhile the pH of the solution was examined in several times and maintained about at 7.5 by addition of Et$_3$N. The solvent,

33) M. Nakamura, A. Tanaka, M. Hirata, and S. Inouye, Endocrinol Japan, 19, 383 (1972).
34) H. Yajima, K. Kawasaki, Y. Okada, H. Minami, K. Kubo, and I. Yamashita, Chem. Pharm. Bull. (Tokyo), 16, 919 (1968).
35) H. Ogawa, M. Kubota, and H. Yajima, Chem. Pharm. Bull. (Tokyo), 24, 2428 (1976).

after addition of a few drops of AcOH, was evaporated. Treatment of the residue with 3% AcOH afforded a powder, which was washed batchwisely with 3% AcOH and H_2O and then precipitated twice from DMF with ether; yield 0.25 g (80%), mp 170—173°, $[\alpha]_D^{20}$ −34.7° (c=0.3, DMF), Rf_1 0.56. Amino acid ratios in 3N Tos–OH hydrolysate: Asp 2.15, Gly 3.17, Pro 2.71, Tyr 0.87, Lys 1.89, Met 0.80, Glu 1.09, His 1.00, Phe 1.00, Trp 0.94, Ser 0.93 (average recovery 80%). Amino acid ratios in 6N HCl hydrolysate: Asp 2.13, Gly 3.10, Pro 2.67, Tyr 0.75, Lys 1.90, Met 0.89; Glu 1.10, His 1.03, Phe 1.09, Arg 1.00, Ser 0.83 (average recovery 81%). *Anal.* Calcd. for $C_{155}H_{184}O_{36}N_{26}S_2 \cdot 6H_2O$: C, 58.92; H, 6.25; N, 11.53. Found: C, 58.53; H, 5.91; N, 11.84.

H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH, Camel β_2-MSH (II)

——The above protected octadecapeptide ester (232 mg) was treated with HF (approximately 5 ml) in the presence of anisole (0.5 ml), skatole (50 mg) and dithiothreitol (40 mg) in an ice-bath for 60 min. The excess HF was evaporated at 0° and AcOEt was added. The resulting powder was collected by filtration and dissolved in H_2O (20 ml), which was treated with Amberlite CG–4B (type II, acetate form, *ca* 3 g) for 30 min and then filtered. The filtrate was lyophilized and the residue (178 mg) was dissolved in a small amount of 3% AcOH. The solution was applied to a column of Sephadex G-10 (3.0 × 140 cm), which was eluted with 3% AcOH. Individual fractions (5 ml) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the front main peak (tube 77—130) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 141 mg (deblocking step 88%). This product (100 mg) was dissolved in H_2O (5 ml) and the solution was applied to a column of CM-cellulose (2.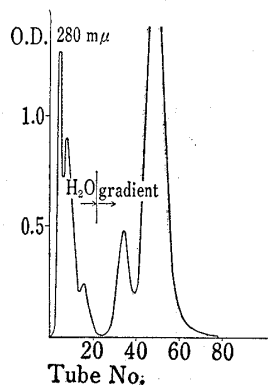2 × 4.0 cm), which was eluted first with H_2O (120 ml) and then by gradient elution with 0.1M AcONH₄ (pH 6.9, 800 ml) through a mixing flask containing 0.01M AcONH₄ (pH 6.9, 200 ml). Individual fractions (8 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the main peak (Fig. 4-a, Tube No 39—58) were combined and the solvent was removed by lyophilization. The residue was dissolved in 3% AcOH (2 ml) and the solution was again applied to a column of Sephadex G-10 (3.0 × 140 cm) for desalting. The product was isolated as stated above and finally lyophilized as a fluffy white powder; yield 38 mg (purification step 38%), $[\alpha]_D^{20}$ −68.1° (c=0.3, H_2O), Rf_3 0.33. Disc electrophoretic mobility on 15% polyacrylamide gel (0.5 × 6.0 cm, 5 mA/tube) at pH 2.3 (0.37M glycine–AcOH buffer) was 3.6 cm after 130 min to the cathod. Paper (Toyo No. 51) electrophoretic mobility: Rf 0.48 × Lys at pH 3.6 pyridine acetate buffer (pyridine–AcOH–H_2O=1: 10: 289, 1100 V, 10 mA, 2 hr). (lit.[5]) Rf 0.48 × Lys at pH 3.7). Amino acid ratios in 3N Tos–OH hydrolysate: Asp 2.00, Gly 3.13, Pro 3.12, Tyr 1.00, Lys 1.87, Met 0.88, Glu 1.00, His 1.08, Phe 1.11, Arg 1.21, Trp 0.72, Ser 0.88 (average recovery 83%). Amino acid ratios in AP–M digest: Asp 2.27, Gly 2.43, Pro 2.38, Tyr 1.00, Lys 1.90, Met 0.97, Glu 1.24, His 0.93, Phe 1.10, Arg 1.17, Trp 0.81, Ser 0.90 (average recovery of amino acids except Asp and Gly 86%). *Anal.* Calcd. for $C_{95}H_{134}O_{27}N_{26}S \cdot 3CH_3COOH$: (dried over P_2O_5 at 100° for 3 hr *in vacuo*): C, 53.10; H, 6.44; N, 15.94. Found: C, 53.74; H, 5.83; N, 15.91.

Fig. 4-a. Purification of Synthetic Camel β_2-MSH on CM-Cellulose

sample 100 mg
column 2.2 × 4 cm
Gradient buffer 0.1M AcONH₄ pH 6.9
fraction: 8 ml
mixing flask 0.01M AcONH₄ (200 ml)

Z(OMe)-Gln-His-OMe——Z(OMe)–Gln–ONP (6.17 g) was added to a solution of H–His–OMe (prepared from 3.15 g of the hydrochloride with 3.6 ml of Et₃N) in DMF (50 ml) and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was treated with AcOEt to form a gelatinous mass, which was collected by filtration and then dissolved in 10% citric acid. The solution, after filtration, was basified with NaHCO₃. The resulting gelatinous mass was washed batchwisely with H_2O and precipitated twice from DMF with AcOEt; yield 4.45 g (74%), mp 176—179°, $[\alpha]_D^{20}$ +4.9° (c=1.0, DMF). Rf_1 0.52. *Anal.* Calcd. for $C_{21}H_{27}O_7N_5$: C, 54.65; H, 5.90; N, 15.18. Found: C, 54.40; H, 5.67; N, 14.98.

Z(OMe)-Gln-His-NHNH₂——To a solution of Z(OMe)–Gln–His–OMe (6.45 g) in DMF–MeOH (50 ml—10 ml), 80% hydrazine hydrate (7 ml) was added and the mixture, after standing overnight, was condensed *in vacuo*. The gelatinous mass formed by treatment with MeOH, was precipitated from DMF with MeOH; yield 4.61 g (70%), mp 191—197°, $[\alpha]_D^{24}$ +11.8° (c=0.7, DMSO), Rf_1 0.25. *Anal.* Calcd. for $C_{20}H_{27}O_6N_7 \cdot 1/2H_2O$: C, 51.05; H, 6.00; N, 20.84. Found: C, 51.05; H, 6.22; N, 20.83.

Z(OMe)-Gln-His-Phe-Arg(Tos)-Trp-Gly-OH——Z–Phe–Arg(Tos)–Trp–Gly–OH[7]) (6.0 g) dissolved in DMF (60 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 8 hr. The catalyst was removed by filtration, the filtrate was condensed and the residue was treated with ether. The resulting powder (yield 4.39 g, 85%) was dissolved in DMF (40 ml), to which the azide (prepared from 3.36 g of Z(OMe)–Gln–His–NHNH₂ with 11.9 ml of 2.03N HCl–DMF, 1.17 ml of isoamylnitrite and 3.32 ml of Et₃N) in DMF (40 ml) was combined. After stirring at 4° for 48 hr, the solution was condensed *in vacuo* and the residue was treated with 3% AcOH. The resulting powder was washed with 3% AcOH and H_2O and then precipitated from DMF with MeOH; yield 5.49 g (76%), mp 166—171°, $[\alpha]_D^{20}$ −25.1° (c=0.9, DMF), Rf_1 0.48. *Anal.* Calcd. for $C_{55}H_{65}O_{13}N_{13}S \cdot 2H_2O$: C, 55.78; H, 5.87; N, 15.38. Found: C, 55.77; H, 5.97; N, 15.21.

Z(OMe)-Gln-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)——Z(OMe)-Ser-Pro-Pro-Lys-(Z)-Asp(OBzl)$_2$[7) (2.23 g) was treated with TFA (4.5 ml) in the presence of anisole (2.5 ml) in an ice-bath for 60 min and the excess TFA was removed by evaporation *in vacuo*. The residue was washed with petroleum ether, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in AcOEt, which under cooling with ice, was basified with 5% NaHCO$_3$. The organic phase was washed with H$_2$O-NaCl, dried over Na$_2$SO$_4$ and then condensed at 30°. The residue was dissolved in DMF (15 ml), to which HOBT (0.3 g), Z(OMe)-Gln-His-Phe-Arg(Tos)-Trp-Gly-OH (1.89 g) and DCC (0.37 g) were added consecutively. The mixture was stirred at room temperature for 48 hr and then filtered. The filtrate was condensed and the residue was treated with H$_2$O. The resulting powder was dissolved in a small amount of the solvent consisting of CHCl$_3$-MeOH-H$_2$O-DMF (60: 10: 1: 20) and the solution was applied to a column of silica gel (2.8×12 cm), which was eluted with the same solvent. Fractions containing the substance of Rf_1 0.54 were combined and the solvent was evaporated. Treatment of the residue with H$_2$O afforded a fine powder, which was precipitated from DMF with ether; yield 1.41 g (32%), 130—132°, $[\alpha]_D^{20}$ −44.0° (c=1.0, DMF), Rf_1 0.54. Amino acid ratios in 6N HCl hydrolysate: Glu 1.02, His 0.95, Phe 0.99, Arg 1.01, Gly 1.00, Ser 0.89, Pro 2.17, Lys 0.98, Asp 1.00 (average recovery 91%). Amino acid ratios in 3N Tos-OH hydrolysate: Glu 1.00, His 0.85, Phe 0.81, Trp 1.14, Gly 1.00, Ser 0.89, Pro 2.00, Lys 0.93, Asp 1.02 (average recovery 82%). *Anal.* Calcd. for C$_{100}$H$_{119}$O$_{23}$-N$_{19}$S·H$_2$O: C, 59.89; H, 6.08; N, 13.27. Found: C, 59.85; H, 6.10; N, 13.51.

Z(OMe)-Pro-Tyr-Lys(Z)-Met-Gln-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)$_2$——The above protected undecapeptide ester (0.92 g) was treated with TFA (1.5 ml) in the presence of anisole (1.0 ml) containing 2% 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 DMF (3 ml), to which Et$_3$N (0.13 ml) and the azide (prepared from 0.51 g of Z(OMe)-Pro-Tyr-Lys(Z)-Met-NHNH$_2$, 0.44 ml of 2.98N HCl-DMF, 0.09 ml of isoamylnitrite and 0.28 ml of Et$_3$N) in DMF (3 ml) were combined. The mixture was stirred at 4° for 48 hr and the solvent was evaporated. Treatment of the residue with H$_2$O afforded a fine powder, which was washed batchwisely with 3% citric acid and H$_2$O and then precipitated twice from THF with AcOEt; yield 1.02 g (84%), mp 155—157°, $[\alpha]_D^{20}$ −44.4° (c=1.0, DMF), Rf_1 0.54. Amino acid ratios in 6N HCl hydrolysate: Pro 2.85, Tyr 1.00, Lys 1.88, Met 0.96, Glu 1.20, His 1.07, Phe 1.04, Arg 1.09, Gly 1.00, Ser 0.77, Asp 0.87 (average recovery 86%). Amino acid ratios in 3N Tos-OH hydrolysate: Pro 3.03, Tyr 1.11, Lys 1.91, Met 0.86, Glu 1.18, His 1.00, Phe 0.97, Trp 1.09, Gly 1.00, Ser 0.83, Asp 0.94 (average recovery 82%). *Anal.* Calcd. for C$_{133}$H$_{162}$O$_{30}$N$_{24}$S$_2$·4H$_2$O: C, 58.88; H, 6.32; N, 12.39. Found: C, 58.76; H, 6.10; N, 12.58.

Z(OMe)-Asp(OBzl)-Gly-Gly-Pro-Tyr-Lys(Z)-Met-Gln-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Pro-Lys(Z)-Asp(OBzl)$_2$——The above protected pentadecapeptide ester (0.51 g) was treated with TFA (1.0 ml) in the presence of anisole (0.7 ml) containing 2% 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 DMF (2 ml) containing Et$_3$N (0.05 ml). To this solution, the azide (prepared from 0.20 g of Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH$_2$ with 0.28 ml of 2.98N HCl-DMF, 0.06 ml of isoamylnitrite and 0.11 ml of Et$_3$N) in DMF (2 ml) was combined and the mixture was stirred at 4° for 48 hr, meanwhile the pH of the solution was examined in several times and maintained around at 7.5 with Et$_3$N. A few drops of AcOH was added, the solvent was evaporated and the residue was treated with 3% AcOH. The resulting powder was washed batchwisely with 3% AcOH and H$_2$O and then precipitated twice from THF with AcOEt; yield 0.48 g (84%), mp 157—161°, $[\alpha]_D^{20}$ −36.3° (c=1.1, DMF), Rf_1 0.53. Amino acid ratios in an acid hydrolysate: Asp 2.06, Gly 2.82, Pro 2.63, Tyr 0.96, Lys 1.84, Met 1.00, Glu 0.97, His 1.05, Phe 1.09, Arg 1.10, Ser 0.76 (average recovery 86%). Amino acid ratios in 3N Tos-OH hydrolysate: Asp 2.16, Gly 3.03, Pro 2.82, Tyr 1.09, Lys 1.88, Met 0.78, Glu 1.04, His 1.00, Phe 1.07, Trp 0.83, Ser 0.89 (average recovery 84%). *Anal.* Calcd. for C$_{148}$H$_{179}$O$_{35}$N$_{27}$S$_2$·5H$_2$O: C, 58.27; H, 6.25; N, 12.40. Found: C, 58.11; H, 6.23; N, 12.51.

H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Gln-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH, Camel β_1-MSH (I)——The above protected octadecapeptide ester (296 mg) was treated with HF (approximately 5 ml) in the presence of anisole (0.5 ml) skatole (65 mg) and dithiothreitol (24 mg) in an ice-bath for 60 min. The excess HF was evaporated at 0° and dry ether was added. The resulting powder was dissolved in H$_2$O (20 ml), which was treated with Amberlite CG-4B (type II, acetate form, *ca.* 3 g) for 30 min and then filtered. The filtrate was lyophilized, the residue (234 mg) was dissolved in 3% AcOH (5 ml) and the solution was applied to a column of Sephadex G-10 (3.0×140 cm), which was eluted with 3% AcOH. Individual fractions (3 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the front main peak (tube
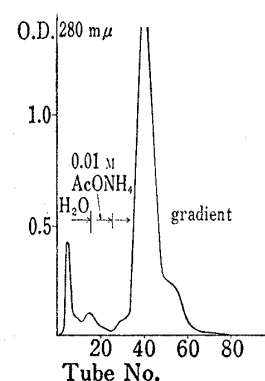

Fig. 4-b. Purification of Synthetic Camel β_1-MSH on CM-Cellulose

sample 100 mg
column 2.2×4 cm
Gradient buffer 0.1M AcONH$_4$ pH 6.9
fraction: 7.5 ml
mixing flask 0.01M AcONH$_4$ (150 ml)

No 71—130) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 205 mg (deblocking step 87%). For the next purification, the product (100 mg) was dissolved in H_2O (5 ml) and the solution was applied to a column of CM-cellulose (2.2×4.0 cm), which was first eluted with H_2O (100 ml) and then with pH 6.9, 0.01M $AcONH_4$ (75 ml). Gradient elution was next established with 0.1M $AcONH_4$ (pH 6.9, 800 ml) through a mixing flask (150 ml) containing 0.01M $AcONH_4$. Individual fractions (7.5 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the main peak (Fig. 4-b, tube No 37—49) were combined and the solvent was removed by lyophilization. The residue was dissolved in a small amount of 3% AcOH and the solution was applied to a column Sephadex G-10 (3.0×140 cm), which was eluted with 3% AcOH for desalting. The product was isolated as stated above and finally lyophilized as a fluffy white powder; yield 31 mg (purification step 31%). $[\alpha]_D^{16}$ −62.9° (c=0.2, H_2O), Rf_3 0.58. Disc electrophoretic mobility on 15% polyacrylamide gel (0.5×6.0 cm, 5 mA/tube) at pH 2.3 (0.37M glycine–AcOH buffer) was 3.5 cm after 130 min to the cathod. Paper electrophoretic mobility at pH 3.6 pyridine acetate buffer: Rf 0.52×Lys (1500 V, 10 mA, 2 hr).. (lit.[5]) natural Rf 0.54×Lys, synthetic 0.52×Lys at pH 3.7). Amino acid ratios in a 3N Tos–OH hydrolysate: Asp 2.21, Gly 3.35, Pro 3.33, Tyr 1.00, Lys 1.95, Met 0.87, Glu 1.14, His 0.80, Phe 0.99, Arg 0.88, Trp 0.86, Ser 1.00 (average recovery 84%). Amino acid ratios in an AP–M digest: Asp 1.82, Gly 2.79, Pro 3.36, Tyr 1.07, Lys 2.30, Met 0.82, Gln 1.06, His 0.92, Phe 1.00, Arg 1.00, Trp 0.92, Ser 1.07 (average recovery of amino acids except Asp and Gly 88%). Anal. Calcd. for $C_{95}H_{135}O_{26}N_{27}S \cdot 3CH_3COOH \cdot 3H_2O$: C, 51.89; H, 6.60; N, 16.18. Found: C, 52.12; H, 6.10; N, 15.92.