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Studies on Peptides. LV.[1,2] Total Synthesis of Porcine Motilin, a Gastric Motor Activity Stimulating Polypeptide

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Synthesis of the docosapeptide corresponding to the newly revised sequence of porcine motilin, a gastric motor activity stimulating polypeptide, was accomplished. Synthetic peptide exhibited characteristic contractile activity patterns of the stomach and the duodenum in dogs, which were quite similar to those of the burst activity of these organs.

Following to the preceding paper,[1] we wish to describe the synthesis of the docosapeptide corresponding to the newly revised sequence of porcine motilin (I).[4] Synthetic route to motilin is illustrated in Fig. 1. As mentioned previously, four peptide fragments, V (sequence 1—5), IV (sequence 6—8), III (sequence 10—13) and II (sequence 14—22), were selected as those necessary to construct the entire amino acid sequence of motilin. To unit these fragments, the azide procedure modified by Honzl and Rudinger[5] was employed exclusively. Only one amino acid residue, Z(OMe)-Glu(OBzl)-OH at position 9, was introduced by the *p*-nitrophenyl ester procedure.[6] Thus the risk of racemization during the condensation reaction is avoided. With this care, the synthesis of the protected tetradecapeptide, Z(OMe)-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH, was prepared[1] and this served here as the starting material for the total synthesis of motilin. Two peptide fragments, IV and V, were newly synthesized.

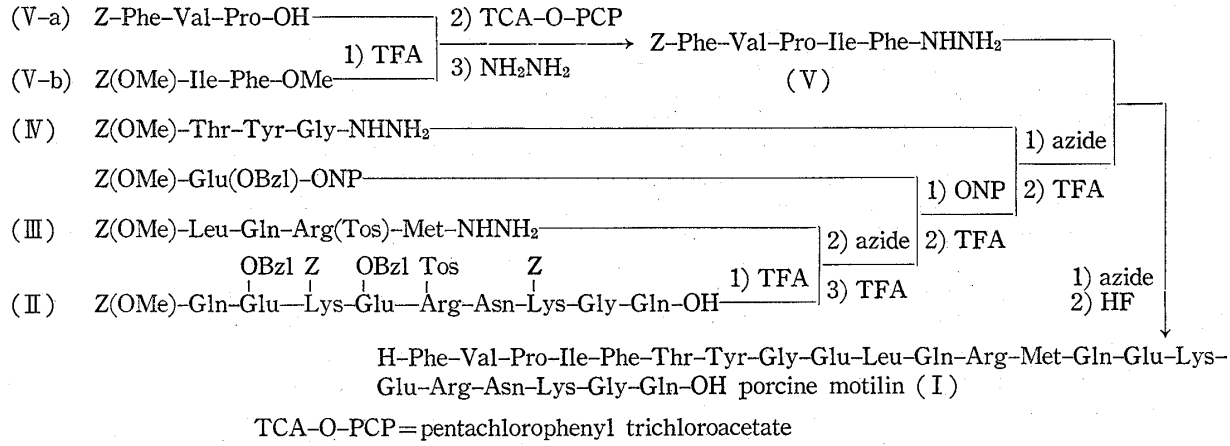

Fig. 1. Synthetic Route to Motilin

1) Part LIV: Y. Kai, H. Kawatani, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2339 (1975).
2) Amino acid, peptides and their derivatives mentioned in this communication 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, OBzl=benzyl ester, Tos=*p*-toluenesulfonyl, OPCP=pentachlorophenyl ester.
3) Location: *a) Sakyo-ku, Kyoto; b) 3-39, Showa-cho, Maehashi, Gunma.*
4) H. Schubert and J.C. Brown, *Can. J. Biochem.*, **52**, 7 (1974).
5) J. Honzl and J. Rudinger, *Collection Czech. Chem. Commun.*, **26**, 2333 (1961).
6) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

For the synthesis of the N-terminal pentapeptide hydrazide, Z–Phe–Val–Pro–Ile–Phe–$NHNH_2$ (V), two peptide subunits, Z–Phe–Val–Pro–OH (V-a) and Z(OMe)–Ile–Phe–OMe (V-b) were prepared. The latter dipeptide ester (V-b) was obtained by the dicyclohexylcarbodiimide (DCC) condensation of Z(OMe)–Ile–OH and H–Phe–OMe in crystalline form. The former tripeptide (V-a) was prepared in pure form by the modified azide coupling of Z–Phe–Val–$NHNH_2$ with the triethylammonium salt of H–Pro–OH. Prior to this synthesis, we attempted to condense Z–Val–OH with the triethylammonium salt of H–Pro–OH by the N-hydroxysuccinimide ester procedure.[7] However, the product was a mixture of Z–Val–Pro–OH and Z–Val–OH, because of the predominant saponification of the above active ester in alkaline conditions. The DCC condensation of Z(OMe)–Val–OH and H–Pro–OMe gave the product contaminated largely with Z(OMe)–Val-dicyclohexylurea. Addition of N-hydroxybenzotriazole (HOBT)[8] to this coupling reaction did not improve this unfavourable situation. Thus the 2+1 route illustrated in Fig. 2 was judged as the method of choice to prepare the pure sample of Z–Phe–Val–Pro–OH (V-a). Next, by means of pentachlorophenyl trichloro-

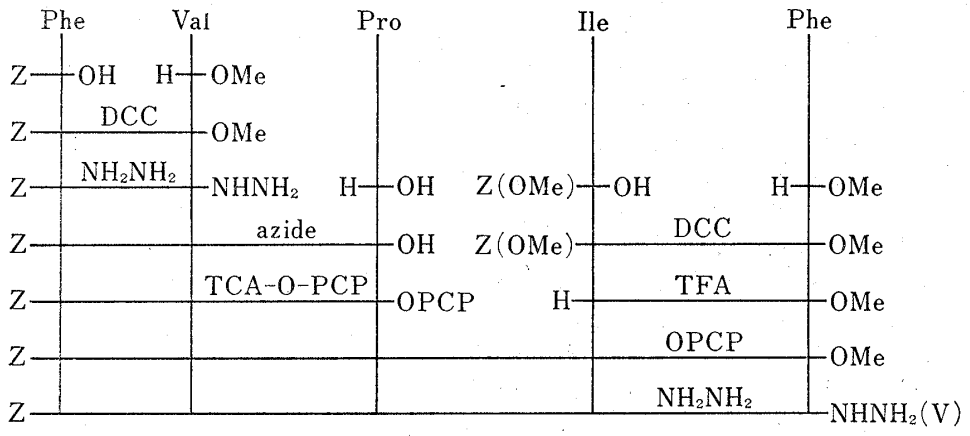

Fig. 2. Synthetic Scheme of the Protected Pentapeptide Hydrazide, Z-(motilin 1—5)-$NHNH_2$

acetate (TCA–O–PCP),[9] V-a was converted to the corresponding pentachlorophenyl ester, which was allowed to react with H–Ile–Phe–OMe derived from V-b by the TFA treatment.[10] The resulting protected pentapeptide ester, Z–Phe–Val–Pro–Ile–Phe–OMe, was converted to V by hydrazine hydrate in the usual manner.

Next, the protected tripeptide hydrazide, Z(OMe)–Thr–Tyr–Gly–$NHNH_2$ (IV) was prepared similarly by the 2+1 coupling method as illustrated in Fig. 3. Z(OMe)–Thr–$NHNH_2$ was condensed with H–Tyr–OMe by the azide procedure and the resulting Z(OMe)–Thr–Tyr–OMe was converted in the usual manner to the corresponding hydrazide, Z(OMe)–Thr–Tyr–$NHNH_2$, which was further condensed with the triethylammonium salt of H–Gly–OH *via* the same azide procedure to give

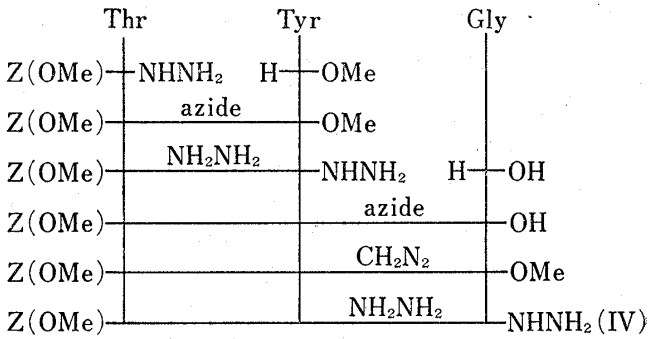

Fig. 3. Synthetic Scheme of the Protected Tripeptide Hydrazide, Z(OMe)-(motilin 6—8)-$NHNH_2$

7) G.W. Anderson, J.E. Zimmerman, and F. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963).
8) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
9) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
10) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

Z(OMe)–Thr–Tyr–Gly–OH. Since this peptide unit we selected possesses the Gly residue as the C-terminus, any tool of the carboxyl activation can be applied without racemization. However the phenolic group of the Tyr residue is not the masked form. Therefore, the azide method, rather than the active ester procedure, was judged as more practical for the next coupling reaction. Treatment of Z(OMe)–Thr–Tyr–Gly–OH by diazomethane followed by hydrazine hydrate afforded IV in satisfactory yield.

Thus, two synthetic peptide fragments which cover the N-terminal portion of porcine motilin have been prepared. Next, the protected tetradecapeptide previously prepared, Z(OMe)–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Tos)–Asn–Lys(Z)–Gly–Gln–OH, was treated with trifluoroacetic acid (TFA) in an ice-bath for 60 minutes to remove the Z(OMe) group and the resulting TFA salt was precipitated by addition of dry ether as fine powder. This after neutralization with triethylamine, was submitted to the coupling reaction with the azide derived from 2 equivalent of Z(OMe)–Thr–Tyr–Gly–$NHNH_2$. The Honzl and Rudinger procedure[5] was applied to convert the hydrazide to the corresponding azide as usual and the progress of the reaction was pursued by thin-layer chromatography (TLC). The reaction went smoothly and the product was purified by column chromatography on silica. The rearrangement product of the azide was eluted first from the column by the solvent system of chloroform, methanol and water (8: 3: 1) and the desired compound was then eluted by the mixture of the above solvent and dimethylformamide (DMF) (1: 1). After this elution, the amino component unreacted was emerged as a very minor component. This purification procedure was previously employed for the isolation of the protected tetradecapeptide (II) mentioned above and found here also very much effective than any other purification methods, such as batchwise washing, extraction or recrystallization. Homogeneity of the protected heptadecapeptide thus isolated, Z(OMe)–Thr–Tyr–Gly–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Tos)–Asn–Lys(Z)–Gly–Gln–OH, was assessed by TLC, acid hydrolysis and elemental analysis.

Prior to enter the final coupling reaction, we have examined whether the peptide bond between the Phe residue (position 5) and Thr (position 6) could be established by the azide procedure. It was confirmed that the condensation reaction of Z(OMe)–Ile–Phe–$NHNH_2$ and H–Thr–Tyr–Gly–OH went smoothly to give Z(OMe)–Ile–Phe–Thr–Tyr–Gly–OH in 60% Yield.

Thus, the azide condensation procedure could be extended to the final coupling reaction between the TFA treated sample of the above protected heptadecapeptide and the N-terminal pentapeptide unit, Z–Phe–Val–Pro–Ile–Phe–$NHNH_2$ (V). In this coupling reaction, 2.5 equivalent of the hydrazide were employed and the product was similarly purified by column chromatography on silica. The solvent system of chloroform, methanol and water (8: 3: 1) was effective to elute the rearrangement product of the azide in the front and the desired compound in the next. Thus the protected docosapeptide, Z–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Tos)–Asn–Lys(Z)–Gly–Gln–OH was isolated in thin-layer chromatographically pure form. The sample was hydrolyzed by 3N *p*-toluenesulfonic acid according to Liu and Chang.[11] The hydrolysate contained the constituent amino acids in ratios predicted by theory, indicating that the entire amino acid sequence of motilin was finally constructed by the above coupling reaction. The recovery of Tyr in this hydrolysate was nearly quantitative. As seen in the hydrolysate of the protected heptadecapeptide by 6N hydrochloride, the recovery of Tyr was considerably low, because of the predominant formation of 3-benzyltyrosine as pointed out by Iselin.[12] Thus in cases of hydrolysis of protected peptides containing Tyr, hydrolysis of

11) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
12) B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).

3N *p*-toluenesulfonic acid gives much superior result to that of 6N hydrochloric acid. The empirical formula of the protected docosapeptide was assinged as the pentahydrate.

Removal of all protecting groups, Z, Bzl and Tos, from the protected docosapeptide was performed by hydrogen fluoride according to Sakakibara, *et al.*[13] In addition to anisole, H–Met–OH was added as scavengers. The deblocked peptide was converted to the corresponding acetate and this was incubated with ethanedithiol to reduce the Met sulfoxide possibly formed during these treatments. Scavengers and ethanedithiol were then removed by Sephadex G-25. The product obtained here was the pure looking substance on TLC This was further purified by CM-Sephadex. Gradient elution was applied to elute the desired compound with 0.2M ammonium acetate buffer and this elution pattern is shown in Fig. 4. A single symmetrical peak was detected by measurement of the ultraviolet (UV) absorbancy at 275 mμ. The fluffy white powder obtained from this peak exhibited a sharp single spot positive to ninhydrin, methionine and Sakaguchi test on TLC. Hydrolysate by 6N hydrochloric acid contained the constituent amino acids in ratios predicted by theory. Despite of the presence of the Pro residue,[14] complete digestion of this peptide with aminopeptidase (AP-M)[15] was possible to identify the presence of Gln and Asn residues in our synthetic motilin. From experimental results cited above it can be concluded that the synthetic docosapeptide thus isolated possesses a high degree of homogeneity and the L-configuration of constituent amino acid residues.

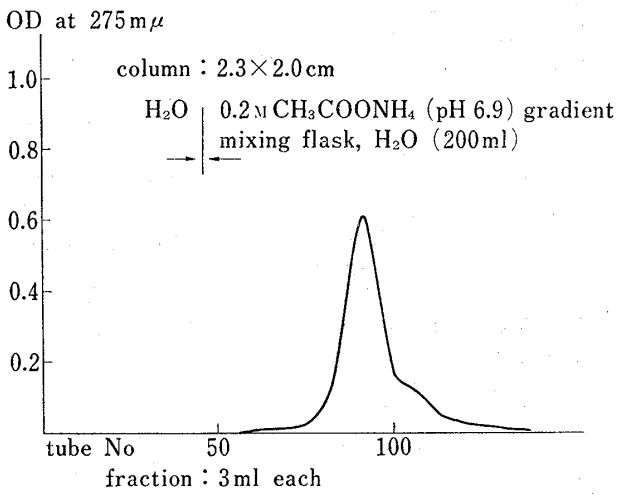

Fig. 4. Elution Pattern of Synthetic Motilin by CM-Sephadex

Biological activity was assayed by one of the authors (Z.I.). It was found that by intravenous infusion to dogs, our synthetic peptide exhibited characteristic contractile activity patterns of the stomach and the duodenum, which were quite similar to those of the burst activity of these organs. Detailed account of these assay results together with observations on the chain length-activity correlationship will be reported in a separate paper. At present, the heptadecapeptide lacking the N-terminal pentapeptide unit was found inactive in the above assay system.

Experimental

Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*–BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf_3 $CHCl_3$–MeOH (95:5), Rf_4 *n*–BuOH–pyridine–AcOH–H_2O (30:6:20:24).

Z-Phe-Val-OMe——The title compound was prepared by the DCC procedure; yield 63%, mp 108—110°, $[\alpha]_D^{24}$ −18.5° (c=1.0, MeOH). Rf_1 0.91. (lit.[16] prepared by the DCC plus N-hydroxybenzotriazole method, mp 111—113°, $[\alpha]_D$ −8.9° in DMF. lit.[17] prepared by the *p*-nitrophenyl ester procedure, mp 113°, $[\alpha]_D$ −16.7° in MeOH). *Anal.* Calcd. for $C_{23}H_{28}O_5N_2$: C, 66.96; H, 6.84; N, 6.79. Found: C, 66.69; H, 6.87; N, 6.55.

13) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
14) E.C. Jorgensen, G.C. Windridge, and W. Patton, *J. Med. Chem.*, **12**, 733 (1969).
15) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963); K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966). Rohm & Haas (Darmstadt), P.R.F. (191226) was employed.
16) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

Z-Phe-Val-NHNH$_2$——In the literature, the title compound was derived from Z-Phe-Val-NHNH-Boc.[17] To a solution of Z-Phe-Val-OMe (5.36 g) in DMF (30 ml), 80% hydrazine hydrate (13 ml) was added. The gelatinous mass formed on standing overnight was collected by filtration and precipitated from MeOH with H_2O; yield 5.13 g (96%), mp 209—214°, $[\alpha]_D^{26}$ −7.7° (c=0.7, DMF). Rf_1 0.56. (lit.[17] mp 214—216°, $[\alpha]_D$ −22.7° in THF). *Anal.* Calcd. for $C_{22}H_{28}O_4N_4$: C, 64.05; H, 6.84; N, 13.58. Found: C, 64.17; H, 6.54; N, 13.61.

Z-Phe-Val-Pro-OH——To a solution of Z-Phe-Val-NHNH$_2$ (4.12 g) in DMF (40 ml) and 0.75 N HCl-dioxane (26.7 ml), isoamylnitrite (1.6 ml) was added under cooling with ice-NaCl. After stirring for 5 min, when the hydrazine test[18] became negative, the solution was neutralized with Et_3N (2.8 ml) and then combined with a solution of H-Pro-OH (2.30 g) in H_2O (10 ml) containing Et_3N (4.1 ml). The mixture was stirred at 4° for 24 hr and the solvent was condensed *in vacuo*. The residue was dissolved in 5% NH_4OH, which after washing with AcOEt, was acidified with citric acid. The resulting oily precipitate was extracted with AcOEt, which was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 3.00 g (61%), mp 93—95°, $[\alpha]_D^{29}$ −62.0° (c=1.0, MeOH). Rf_1 0.50, Rf_2 0.85. (lit.[19] prepared by diphenyl phosphorazidate, mp 99—102°, $[\alpha]_D^{20}$ −49° in DMF). Amino acid ratios in an acid hydrolysate: Phe 1.02, Val 1.00, Pro 0.96 (average recovery 86%). *Anal.* Calcd. for $C_{27}H_{33}O_6N_3$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.26; H, 6.85; N, 8.19.

Z(OMe)-Ile-Phe-OMe——DCC (35.0 g) was added to a solution of Z(OMe)-Ile-OH (44.25 g) in THF (300 ml). To this solution, H-Phe-OMe (prepared from 32.40 g of the hydrochloride with 20.7 ml of Et_3N) in DMF (300 ml) was combined and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was triturated with ether and petroleum ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized from THF and ether; yield 39.03 g (57%), mp 155—160°, $[\alpha]_D^{27}$ −34.0° (c=1.0, MeOH). Rf_1 0.93. *Anal.* Calcd. for $C_{25}H_{32}O_6N_2$: C, 65.77; H, 7.07; N, 6.14. Found: C, 66.04; H, 7.26; N, 6.44.

Z-Phe-Val-Pro-Ile-Phe-OMe——PCP-O-TCA (0.73 g) was added to a solution of Z-Phe-Val-Pro-OH (0.81 g) in DMF (10 ml) containing Et_3N (0.22 ml) and the solution was stirred in an ice-bath for 60 min. Thin-layer chromatographic examination revealed the appearance of a new spot of Rf_1 0.98 and Rf_1 0.50 of Z-Phe-Val-Pro-OH disappeared. This solution was then combined with a solution of H-Ile-Phe-OMe (prepared from 0.95 g of the Z(OMe) derivative by treatment with 2 ml of TFA in the presence of 0.5 ml of anisole at 0° for 60 min followed by precipitation with dry ether and subsequent neutralization with 0.3 ml of Et_3N) in DMF (10 ml). The mixture, after stirring overnight, was condensed *in vacuo*. Treatment of the residue with ether afforded the solid, which was washed with batchwisely with 5% citric acid and H_2O and then recrystallized from MeOH and ether; yield 0.63 g (51%), mp 164—168°, $[\alpha]_D^{27}$ −75.5° (c=1.0, MeOH). Rf_3 0.55. Amino acid ratios in an acid hydrolysate: Phe 2.16, Val 1.00, Pro 1.08, Ile 1.12 (average recovery 96%). *Anal.* Calcd. for $C_{43}H_{55}O_8N_5$: C, 67.08; H, 7.20; N, 9.10. Found: C, 67.27; H, 7.12; N, 9.00.

Z-Phe-Val-Pro-Ile-Phe-NHNH$_2$——To a solution of Z-Phe-Val-Pro-Ile-Phe-OMe (0.63 g) in MeOH (30 ml), 80% hydrazine hydrate (0.2 ml) was added. The gelatinous mass formed on standing overnight was collected by filtration, washed with EtOH and H_2O and dried over H_2SO_4; yield 0.28 g (45%), mp 255—257°, $[\alpha]_D^{28}$ −50.2° (c=0.7, DMF). Rf_1 0.73. Rf_3 0.12. Amino acid ratios in an acid hydrolysate: Phe 2.02, Val 1.00, Pro 1.20, Ile 0.96 (average recovery 87%). *Anal.* Calcd. for $C_{42}H_{55}O_7N_7$: C, 65.52; H, 7.20; N, 12.74. Found: C, 65.80; H, 7.14; N, 12.50.

Z(OMe)-Thr-Tyr-OMe——To a cold solution of Z(OMe)-Thr-NHNH$_2$ (29.73 g) in DMF (250 ml), 3.75 N HCl-DMF (26.7 ml) and isoamylnitrite (13.3 ml) were added and the mixture was stirred at −5° for 5 min, when the hydrazine test became negative. The solution was then neutralized with Et_3N (27.6 ml) and poured into a solution of H-Tyr-OMe (prepared from 23.17 g of the hydrochloride with 27.6 ml of Et_3N) in DMF (200 ml). The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was extracted with AcOEt, which was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from MeOH and ether; yield 31.11 g (68%), mp 133—134°, $[\alpha]_D^{25}$ +0.8° (c=1.0, MeOH). Rf_1 0.63. *Anal.* Calcd. for $C_{23}H_{28}O_6N_2$: C, 59.99; H, 6.13; N, 6.08. Found: C, 60.07; H, 6.02; N, 6.07.

Z(OMe)-Thr-Tyr-NHNH$_2$——In the usual manner, 90% hydrazine hydrate (18.6 ml) was added to a solution of Z(OMe)-Thr-Tyr-OMe (31.11 g) in MeOH (200 ml) and the solid formed on standing overnight was recrystallized from MeOH; yield 27.07 g (86%), mp 215—218°, $[\alpha]_D^{28}$ +4.1° (c=0.6, DMF). Rf_1 0.48, Rf_2 0.85. *Anal.* Calcd. for $C_{22}H_{28}O_7N_4 \cdot H_2O$: C, 56.29; H, 6.31; N, 11.71. Found: C, 56.09; H, 6.30; N, 11.75.

Z(OMe)-Thr-Tyr-Gly-OH——As stated above, isoamylnitrite (3.4 ml) was added to a cold solution (−5°) of Z(OMe)-Thr-Tyr-NHNH$_2$ (11.50 g) in a mixture of DMF (100 ml) and 3.75 N HCl-DMF (13.4 ml). After stirring for 5 min, when the hydrazine test became negative, the solution was neutralized with Et_3N (6.9

17) E. Schnabel, H. Klostermeyer, J. Dahlmans, and H. Zahn, *Ann. Chem.*, **707**, 227 (1967).
18) H.E. Ertel and L. Horner, *J. Chromatog.*, **7**, 268 (1962).
19) S. Yamada, N. Ikota, and T. Shioiri, "Psoc. 12th Symp. Peptide Chem.," ed. by H. Yajima, Protein Research Found., 1974, p. 45.

ml) and then combined with a solution of H-Gly-OH (2.80 g) and Et_3N (8.7 ml) in H_2O. The mixture was stirred at 4° for 48 hr and the solvent was evaporated. The residue was dissolved in 3% NH_4OH, which after washing with AcOEt, was acidified with citric acid. The resulting oil was extracted with AcOEt and the organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and then recrystallized from MeOH and ether; yield 8.31 g (66%), mp 153—157°, $[\alpha]_D^{27}$ −24.3° (c=1.0, MeOH). Rf_1 0.12. Rf_2 0.80. *Anal.* Calcd. for $C_{24}H_{29}O_9N_3 \cdot 1/2H_2O$: C, 56.24; H, 5.90; N, 8.20. Found: C, 56.36; H, 5.93; N, 8.47.

Z(OMe)-Thr-Tyr-Gly-OMe——An etheral solution of diazomethane was added to a solution of Z(OMe)-Thr-Tyr-Gly-OH (3.02 g) in MeOH (30 ml). The yellow colour of the solution was persisted for 30 min and a few drops of AcOH was added. The solvent was evaporated and the residue was triturated with ether; yield 2.75 g (89%), mp 141—142°, $[\alpha]_D^{28}$ −22.5° (c=1.0, MeOH), Rf_1 0.60. *Anal.* Calcd. for $C_{25}H_{31}O_9N_3$: C, 58.02; H, 6.04; N, 8.12. Found: C, 57.87; H, 5.97; N, 8.07.

Z(OMe)-Thr-Tyr-Gly-NHNH$_2$——To a solution of Z(OMe)-Thyr-Tr-Gly-OMe (2.76 g) in MeOH (30 ml), 80% hydrazine hydrate (1.5 ml) was added and the solution was kept on standing overnight. The solid mass formed was recrystallized from MeOH; yield 2.06 g (75%), mp 212—213°, $[\alpha]_D^{25}$ −1.2° (c=0.7, DMF). Rf_1 0.42. Amino acid ratios in an acid hydrolysate: Thr 0.97, Tyr 0.48, Gly 1.00 (average recovery 89%). *Anal.* Calcd. for $C_{24}H_{31}O_8N_5 \cdot 1/2H_2O$: C, 54.74; H, 6.13; N, 13.30. Found: C, 54.81; H, 5.87; N, 13.17.

Z(OMe)-Ile-Phe-NHNH$_2$——To a solution of Z(OMe)-Ile-Phe-OMe (3.90 g) in THF–DMF (15 ml+15 ml), 80% hydrazine hydrate (2.4 ml) was added and the solid mass formed on standing overnight was recrystallized from MeOH; yield 3.36 g (86%), mp 194—196°, $[\alpha]_D^{27}$ −20.3° (c=0.7, DMF). Rf_1 0.64. *Anal.* Calcd. for $C_{24}H_{32}O_5N_4$: C, 63.14; H, 7.07; N, 12.27. Found: C, 63.15; H, 6.90; N, 12.10.

Z(OMe)-Ile-Phe-Thr-Tyr-Gly-OH——Z(OMe)-Ile-Phe-NHNH$_2$ (1.15 g) was dissolved in 1 N HCl–DMF (5 ml). To this solution, isoamylnitrite (0.3 ml) was added under cooling with ice-NaCl. After the solution was stirred for 5 min, Et_3N (0.7 ml) was added. This solution was then combined with a solution of H-Thr-Tyr-Gly-OH (prepared from 1.01 g of the Z(OMe) derivative by treatment with 2 ml of TFA in the presence of 0.5 ml of anisole at 0° for 40 min followed by neutralization with 0.7 ml of Et_3N as usual) in DMF (5 ml). The mixture was stirred at 4° for 48 hr and the solvent was evaporated *in vacuo*. The residue was treated with H_2O and the resulting powder was washed batchwisely with 5% citric acid and AcOEt and recrystallized from DMF and AcOEt; yield 1.16 g (60%), mp 195—200°, $[\alpha]_D^{27}$ −15.7° (c=0.6, DMF). Rf_1 0.18, Rf_2 0.80. *Anal.* Calcd. for $C_{39}H_{49}O_{11}N_5 \cdot H_2O$: C, 59.59; H, 6.58; N, 8.96. Found: C, 59.97; H, 6.72; N, 9.03.

Z(OMe)-Thr-Tyr-Gly-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH, Z(OMe)-(motilin 6—22)-OH——Z(OMe)-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH[1] (1.00 g) was treated with TFA (4 ml) in the presence of anisole (1 ml) in an ice-bath for 60 min. Dry ether was added and the resulting fine powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml) containing Et_3N (0.15 ml). This solution was then combined with a solution of Z(OMe)-Thr-Tyr-Gly-azide (prepared in the usual manner from 0.37 g of the corresponding hydrazide with 0.38 ml of 3.78 N HCl–DMF, 0.1 ml of isoamylnitrite and 0.2 ml of Et_3N) in DMF (30 ml). The mixture was stirred at 4° for 48 hr and the solvent was evaporated. The gummy precipitate formed by addition of AcOEt was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–H_2O (8: 3: 1) and the solution was applied to a column of silica gel (3.2×13.5 cm), which was first eluted with the same solvent system and then the desired compound was eluted with the mixture of the above solvent and DMF (1: 1, v/v). The eluates which contained the substance of Rf_1 0.25 were combined and the solvent was evaporated. The residue was treated with H_2O and the resulting powder was again dissolved in a small amount of $CHCl_3$–MeOH–H_2O. The solution was filtered, the filtrate was condensed *in vacuo*. Treatment of the residue with ether gave fine powder; yield 0.70 g (63%), mp 144—148°, $[\alpha]_D^{22}$ +0.8° (c=1.0, DMF). Rf_1 0.25, Rf_2 0.77. Amino acid ratios in an acid hydrolysate: Thr 1.05, Tyr 0.46, Gly 2.09, Glu 6.24, Leu 0.88, Arg 2.08, Met 0.76, Lys 2.08, Asp 1.00 (average recovery 90%). *Anal.* Calcd. for $C_{146}H_{193}O_{41}N_{29}S_3 \cdot 2H_2O$:C, 55.80; H, 6.32; N, 12.93. Found: C, 55.82; H, 6.22; N, 13.01.

Z-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH, Z-(motilin 1—22)-OH——Z(OMe)-(motilin 6—22)-OH (0.40 g) was treated with TFA (2 ml) in the presence of anisole (1 ml) in an ice-bath for 60 min, when 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 (15 ml) containing Et_3N (0.05 ml). To this cooled solution with ice–NaCl, the azide (prepared as usual from 0.25 g of Z(OMe)-Phe-Val-Pro-Ile-Phe-NHNH$_2$ with 3.13 N HCl–DMF, 0.05 ml of isoamylnitrite and 0.1 ml of Et_3N) in DMF (3 ml) was combined and the mixture was stirred at 4° for 48 hr. A few drop of AcOH was added and the solvent was evaporated. The residue, after washing with ether, was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–H_2O (8: 3: 1). This solution was applied to a column of silica gel (3.3×6.4 cm), which was eluted with the same solvent system. The rearrangement product of the azide (Rf_1 0.88) was eluted in the front fractions. The next fractions containing the substance of Rf_1 0.19 were combined, the solvent was evaporated and the residue was treated with H_2O. The resulting powder was dissolved in a small amount of the same solvent system used in the above chromatography. The solution

was filtered and the filtrate was condensed. Treatment of the residue with ether afforded the fine powder; yield 0.28 g (58%), 138—143°, $[\alpha]_D^{27}$ −7.5° (c=0.5, DMF). Rf_1 0.19, Rf_2 0.84. Amino acid ratios in a hydrolysate by p-Tos-OH: Phe 1.89, Val 0.92, Pro 1.15, Ile 0.97, Thr 0.94, Tyr 0.73, Gly 1.97, Glu 5.94, Leu 0.90, Arg(Tos) 1.78, Met 0.89, Lys 1.82, Asp 1.00 (average recovery 84%). *Anal.* Calcd. for $C_{179}H_{236}O_{45}N_{34}S_3 \cdot 5H_2O$: C, 57.02; H, 6.58; N, 12.63. Found: C, 57.07; H, 6.59; N, 12.71.

H-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln-OH (I)——The above protected docosapeptide (197 mg) was treated with HF (approximately 10 ml) in the presence of anisole (1.0 ml) and Met (53 mg) in an ice-bath for 60 min. The excess HF was removed by evaporation under reduced pressure and the residue was dissolved in H_2O (10 ml), which was stirred with Amberlite IR 4B (acetate form, approximately 3 g) for 30 min and then filtered. The pH of the filtrate was adjusted to 6.5 with 3% NH_4OH and a few drops of ethanedithiol was added. The solution was incubated at 40° for 8 hr and then applied to a column of Sephadex G-25 (2.6×34.0 cm), which was eluted with 1% AcOH. Individual fractions collected (3 ml each) were examined by UV absorbancy at 275 mμ. Fractions corresponding to the front main peak (tube No. 29—58) were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 123 mg (85%). This powder (58 mg) was dissolved in H_2O (65 ml) and the solution was applied to a column of CM-Sephadex (2.3×2.0 cm), which was first eluted with H_2O (100 ml) and then with 0.2 M ammonium acetate buffer (pH 6.9) through a mixing flask containing H_2O (200 ml). Individual fractions (3.6 ml each) were collected and the UV absorbancy at 275 mμ was determined. A single peak with a small shoulder was detected in the gradient eluates (tube No. 76—98). These fractions were combined and the solution was applied for desalting to a column of Sephadex G-25 (1.8×128.0 cm), which was eluted with 1% AcOH as stated above. A single symmetrical peak (tube No. 36—62) was detected. Repeated lyophilization of these fractions gave a fluffy white powder; yield 29.5 mg (51% in the purification step). $[\alpha]_D^{27}$ −54.5° (c=0.6, H_2O). Rf_4 0.71. Amino acid ratios in an acid hydrolysate and AP-M digest (number in bracket): Phe 1.78 (1.92), Val 0.84 (0.93), Pro 1.01 (0.89), Ile 0.80 (0.91), Thr 0.89 (Thr+Gln 3.83), Tyr 0.82 (0.90), Gly 2.17 (2.39), Glu 6.12 (3.26), Leu 1.00 (1.00), Arg 2.23 (2.21), Met 0.94 (0.68), Lys 2.04 (2.25), Asn (0.97), Asp 1.00. average recovery 93% (85%). *Anal.* Calcd. for $C_{120}H_{188}O_{35}N_{34}S \cdot 4CH_3COOH \cdot 8H_2O$: C, 49.85; H, 7.19; N, 15.45. Found: C, 49.58; H, 7.26; N, 16.10.

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