and chloroform and treated with 80% acetic acid at 100° for 90 min. It was concentrated under reduced pressure and the residue was dissolved in water (30 ml). The solution was applied to a column of Dowex 50W-X8 (H^+ form, 2.5 × 50 cm), which was eluted with water. The eluate was concentrated to dryness under reduced pressure and the crystalline residue was recrystallized from the appropriate solvent to give nucleoside 5′-phosphate (III). Characterization of the products was achieved by comparison on paper chromatography, melting point, and ultraviolet spectrum with authentic samples.[8] The results are summarized in Table I.

[Chem. Pharm. Bull. 25(8)2123—2126(1977)]

UDC 547.466.1.04.09 : 615.324.011.5.076.9

Synthesis of the Hexadecapeptide corresponding to Positions 1 through 16 of Porcine Motilin, a Gastric Motor Activity Stimulating Polypeptide[1]

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(Received December 16, 1976)

The hexadecapeptide corresponding to positions 1 to 16 of porcine motilin was synthesized in a conventional manner. The synthetic peptide exhibited the activity of 3% of that of the synthetic motilin, when tested using rabbit duodenal muscle *in vitro*.

Keywords——motilin, a gastric motor activity stimulating polypeptide; motilin (1—16); motilin (5—22); contracting activity *in vitro* rabbit duodenal muscle; hydrogen fluoride deprotection

In 1975, we reported the first synthesis of the docosapeptide[3] corresponding to the revised amino acid sequence of a porcine gastric motor activity stimulating polypeptide, termed as motilin.[4] Shortly after our publication, Yamada, *et al.*[5] reported an alternative synthesis of this peptide, in which a new coupling reagent, diethyl phosphorocyanidate, was employed. In addition, two independent research groups, Mihara, *et al.*[6] and Fujino, *et al.*[7] have also synthesized this gut peptide using the liquid ammonia[8] and the methanesulphonic acid procedure[9] respectively.

1) Amino acids, peptides and their derivatives mentioned here 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). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, OBzl=benzyl ester, ONP=*p*-nitrophenyl ester, DMF=dimethylformamide.
2) Location: *a) Sho-machi, Tokushima; b) Kasumi-cho, Hiroshima; c) Sakyo-ku, Kyoto.*
3) H. Yajima, Y. Kai, and H. Kawatani, *J.C.S. Chem. Commun.*, **1975**, 159; Y. Kai, H. Kawatani, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2339 (1975); Y. Kai, H. Kawatani, H. Yajima and Z. Itoh, *ibid.*, **23**, 2346 (1975).
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During the course of our synthetic studies on motilin, we observed that a shorter chain peptide lacking the N-terminal pentapeptide, Phe-Val-Pro-Ile-Phe, retained still the activity of 1/50 of that of the genuine molecule, but further elimination of the tripeptide, Thr-Tyr-Gly, from this hexadecapeptide resulted in the complete loss of the characteristic contracting activity, when tested using rabbit duodenal muscle *in vitro*.[10] It was thus concluded that the N-terminal portion of motilin, especially the pentapeptide, contributes immensely for the docosapeptide to retain the high level of the biological activity. We synthesized next the peptide which covers the N-terminal portion of motilin corresponding to positions 1 to 16 in order to cast a further light on structure-activity correlation of this upper intestinal polypeptide.

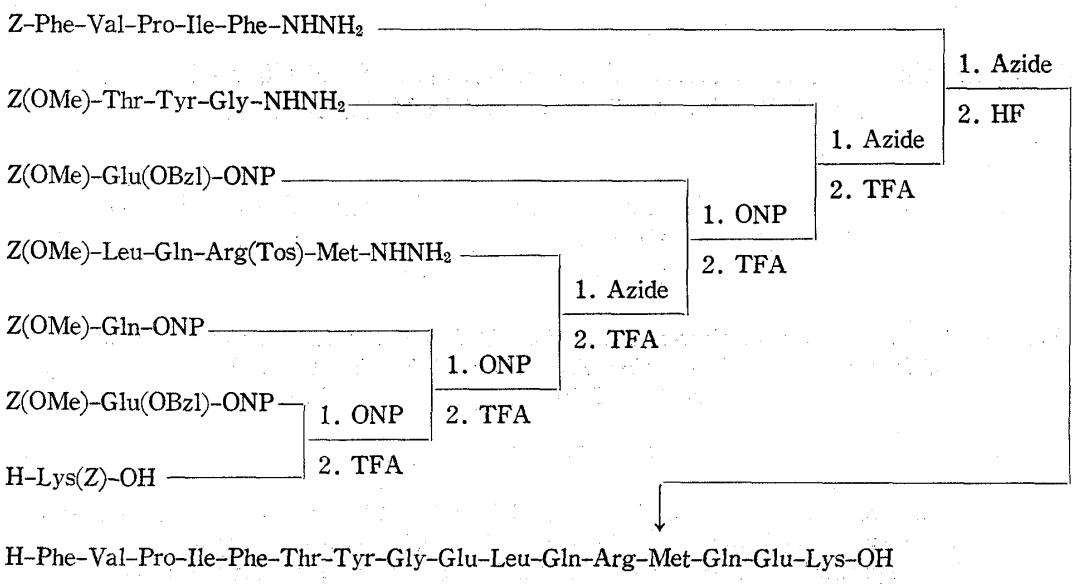

Fig. 1. Synthetic Route to the Hexadecapeptide (motilin 1—16)

The synthetic route to the hexadecapeptide is illustrated in Fig. 1., in which three peptide fragments, Z–Phe–Val–Pro–Ile–Phe–$NHNH_2$, Z(OMe)–Thr–Tyr–Gly–$NHNH_2$ and Z(OMe)–Leu–Gln–Arg(Tos)–Met–$NHNH_2$, were the same building blocks for our previous synthesis of motilin.[3] The C-terminal tripeptide unit, Z(OMe)–Gln–Glu(OBzl)–Lys(Z)–OH, was newly synthesized by the consecutive *p*-nitrophenyl ester procedure[11] starting with H–Lys(Z)–OH. Chain elongation of the tripeptide to the hexadecapeptide was carried out in essentially the same manner as described previously; *i.e.*, deprotection of the Z(OMe) group by TFA,[12] subsequent condensation of the peptide fragments by the Rudinger's azide procedure[13] and introduction of a single amino acid residue, Z(OMe)–Glu(OBzl)–OH at position 9, stepwisely by the *p*-nitrophenyl ester procedure. Column chromatography on silica was performed for purification of the protected octapeptide, Z(OMe)–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–OH, using the solvent system of $CHCl_3$–MeOH–H_2O (8:3:1) and a mixture of the above solvent and DMF was used for purification of the protected undeca and hexadecapeptides, Z(OMe)–Thr–Tyr–Gly–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–OH and Z–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu(OBzl)–Leu–Gln–Arg(Tos)–Met–Gln–Glu(OBzl)–Lys(Z)–OH, in order to maintain their adequate solubilities during the chromatography.

The protected hexadecapeptide thus obtained was then exposed to hydrogen fluoride[14] in an ice-bath for 60 minutes to remove all protecting groups and the deprotected peptide

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was purified by column chromatography on Sephadex G-25 and subsequently on CM-Sephadex. For elution of the desired compound, 3% acetic acid was employed in the former step and gradient elution with ammonium acetate buffer (pH6.9) in the latter. A thin layer chromatographically homogeneous sample was thus obtained and its homogeneity was further assessed by hydrolysis with 6 N HCl and digestion with aminopeptidase (AP-M).[15]

When the synthetic hexadecapeptide, H–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu–Leu–Gln–Arg–Met–Gln–Glu–Lys–OH, was assayed *in vitro* using rabbit duodenal muscle, its contracting potency was approximately 3% of that of the synthetic motilin.[3] From this result, together with our previous observations, it can be concluded that an active site or core of motilin must reside within the sequence corresponding to positions 6 to 16 of the docosapeptide and for maintenance of the high level of the activity, nearly full chain length seems to be required.

Experimental

The melting points are uncorrected. Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck) and *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 *n*-BuOH–pyridine–AcOH–H_2O (30: 6: 20: 24).

Z(OMe)-Glu(OBzl)-Lys(Z)-OH——Z(OMe)–Glu(OBzl)–ONP[16] (10.45 g) in THF (160 ml) was added to a solution of H–Lys(Z)–OH (5.61 g) in H_2O (100 ml) containing Et_3N (5.6 ml) and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was dissolved in H_2O (100 ml), which after washing with ether, was acidified with citric acid. The resulting precipitate was extracted with AcOEt, which was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. Treatment of the residue with ether afforded the solid, which was recrystallized from AcOEt and ether; yield 7.02 g (53%), mp 80—82°, $[\alpha]_D^{24}$ −2.2° (c=0.9, MeOH). Rf_1 0.28. *Anal.* Calcd. for $C_{35}H_{41}N_3O_{10}\cdot 1/2H_2O$: C, 62.49; H, 6.29; N, 6.25. Found: C, 62.41; H, 6.14; N, 6.21.

Z(OMe)-Gln-Glu(OBzl)-Lys(Z)-OH——Z(OMe)–Glu(OBzl)–Lys(Z)–OH (6.47 g) was treated with TFA (12 ml) in the presence of anisole (4.5 ml) in an ice-bath for 60 min, when dry ether was added. The resulting powder was collected by filtration, washed with dry ether, dried over KOH pellets *in vacuo* for 5 hr and then dissolved in a mixture of DMF (60 ml) and H_2O (40 ml). To this solution, Et_3N (2.8 ml) and Z(OMe)–Gln–ONP (4.31 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was treated with AcOEt. The resulting solid was washed batchwisely with 5% citric acid and H_2O and then recrystallized from EtOH; yield 5.11 g (64%), mp 178—181°, $[\alpha]_D^{24}$ +21.0° (c=0.4, DMF), Rf_1 0.63. Amino acid ratios in an acid hydrolysate: Glu 2.00, Lys 0.75 (average recovery 93%). *Anal.* Calcd. for $C_{40}H_{49}N_5O_{12}$: C, 60.67; H, 6.24; N, 8.85. Found: C, 60.67; H, 6.50; N, 9.02.

Z(OMe)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-OH——The above protected tripeptide (3.10 g) was treated with TFA (3 ml) in the presence of anisole (1.5 ml) in an ice-bath for 60 min. The fine powder formed by addition of dry ether was collected by filtration and then dissolved in DMF (20 ml). To this ice-chilled solution, Et_3N (1.7 ml) and the azide (prepared from 3.52 g of Z(OMe)–Leu–Gln–Arg(Tos)–Met–$NHNH_2$[3] with 2.0 ml of 5.0 N HCl–DMF, 0.6 ml of isoamylnitrite and 1.4 ml of Et_3N) in DMF (20 ml) were combined and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was washed batchwisely with 5% citric acid and H_2O and precipitated twice from DMF with AcOEt; yield 4.17 g (71%), mp 191—194°, $[\alpha]_D^{24}$ −15.4° (c= 0.5, DMF), Rf_1 0.58. Amino acid ratios in an acid hydrolysate: Leu 1.00, Glu 2.98, Arg 0.99, Met+Met(O) 0.91, Lys 0.93 (average recovery 83%). *Anal.* Calcd. for $C_{69}H_{95}N_{13}O_{19}S_2\cdot 4H_2O$: C, 53.58; H, 6.71; N, 11.78. Found: C, 53.77; H, 6.53; N, 11.73.

Z(OMe)-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-OH——The above protected heptapeptide (4.42 g) was treated with TFA (10 ml) in the presence of anisole (3.0 ml) in an ice-bath for 60 min and the TFA salt isolated as stated above was dissolved in DMF (60 ml) containing Et_3N (1.3 ml). To this solution, Z(OMe)–Glu(OBzl)–ONP (1.73 g) was combined and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was treated with ether and H_2O. The resulting powder 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 (4.0×10 cm), which was eluted with the same solvent system. The eluates which

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16) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1061 (1974).

contained the substance of Rf_1 0.54 were combined and the solvent was evaporated. The residue was treated with H_2O and the resulting powder was precipitated from DMF with AcOEt; yield 3.78 g (75%), mp 175—176°, $[\alpha]_D^{24}$ −26.1° (c=0.5, DMF), Rf_1 0.54. Amino acid ratios in an acid hydrolysate: Glu 4.15, Leu 1.00, Arg 0.92, Met+Met(O) 0.95, Lys 0.94 (average recovery 86%). *Anal.* Calcd. for $C_{81}H_{108}N_{14}O_{22}S_2 \cdot 3H_2O$: C, 55.65; H, 6.57; N, 11.22. Found: C, 55.37; H, 6.47; N, 11.02.

Z(OMe)-Thr-Tyr-Gly-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-OH——Z(OMe)-Thr-Tyr-Gly-$NHNH_2$[3] (1.30 g) was dissolved in DMF (8 ml), to which 5.0 N HCl–DMF (1.0 ml) and isoamylnitrite (0.34 ml) were added under cooling with ice-NaCl. After stirring for 5 min followed by neutralization with Et_3N (0.7 ml), the solution was combined with an ice-chilled solution of H-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-OH (prepared from 3.50 g of the corresponding Z(OMe)-derivative by treatment with 10 ml of TFA in the presence of 3 ml of anisole at 0° for 60 min as stated above, followed by neutralization with 0.7 ml of Et_3N) in DMF (30 ml). The mixture was stirred at 4° for 48 hr and the solvent was evaporated *in vacuo*. The residue, after washing with AcOEt and H_2O, was purified by column chromatography on silica (4.0×9.0 cm) using the solvent system of $CHCl_3$–MeOH–H_2O (8: 3: 1) and DMF (1: 1 v/v). The eluates which contained the substance of Rf_1 0.12 were combined and the solvent was evaporated. The residue was treated with H_2O and the resulting powder was precipitated from DMF with AcOEt; yield 3.76 g (89%), mp 183—187°, $[\alpha]_D^{24}$ −11.8° (c=0.5, DMF), Rf_1 0.12. Amino acid ratios in an acid hydrolysate: Thr 0.75, Tyr 0.55, Gly 1.00, Glu 4.09, Leu 0.99, Arg 1.28; Met 0.87, Lys 1.23 (average recovery 83%).[17] *Anal. Calcd.* for $C_{96}H_{127}N_{17}O_{27}S_2 \cdot 6H_2O$: C, 54.28; H, 6.60; N, 11.21. Found: C, 54.48; H, 6.83; N, 11.55.

Z-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Gln-Glu(OBzl)-Lys(Z)-OH——The above protected undecapeptide (0.34 g) was treated with TFA (1.5 ml) in the presence of anisole (0.5 ml) in an ice-bath for 60 min. Dry ether was added and the resulting TFA salt was dissolved in DMF (10 ml). To this ice-chilled solution, the azide (prepared from 0.15 g of Z-Phe-Val-Pro-Ile-Phe-$NHNH_2$[3] with 0.08 ml of 5.0 N HCl–DMF, 0.03 ml of isoamylnitrite and 0.06 ml of Et_3N) in DMF (10 ml) and Et_3N (0.03 ml) were combined and the mixture was stirred at 4° for 40 hr. After evaporation of the solvent, the residue was treated with 5% citric acid and AcOEt and the resulting powder was purified by column chromatography on silica (4.0×10 cm) using the solvent system of $CHCl_3$–MeOH–H_2O (8: 3: 1) and DMF (1: 1 v/v). The eluates containing the substance of Rf_1 0.19 were combined and the solvent was evaporated. The residue was treated with H_2O and the resulting powder was precipitated from DMF with AcOEt; yield 0.13 g (30%), mp 238—242°, $[\alpha]_D^{20}$ −28.0° (c=0.4, DMF), Rf_1 0.19. Amino acid ratios in an acid hydrolysate: Phe 2.03, Val 0.96, Pro 0.81, Ile 1.02, Thr 0.76, Tyr 0.42, Gly 1.00, Glu 4.30, Leu 1.02, Arg 0.87, Met 0.82, Lys 0.91 (average recovery 87%). *Anal.* Calcd. for $C_{129}H_{170}N_{22}O_{31}S_2 \cdot 2H_2O$: C, 59.01; H, 6.68; N, 11.74. Found: C, 59.17, H, 6.91, N, 11.32.

H-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-OH——The above protected hexadecapeptide (500 mg) was treated with HF (approximately 10 ml) in the presence of anisole (1.0 ml) and Met (150 mg) in an ice-bath for 60 min. The excess HF was evaporated *in vacuo* at 0° and dry ether was added. The resulting powder was collected by filtration, washed with ether and then dissolved in H_2O (20 ml), which was treated with Amberlite CG-50 (acetate form, approximately 3 g). The resin was removed by filtration and the filtrate was lyophilized; yield 318 mg. This deblocked peptide (316 mg) was dissolved in 3% AcOH (8 ml) and the solution was applied to a column of Sephadex G-25 (2.4×144 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and absorbancy at 275 mμ was determined. Fractions corresponding to the front main peak (tube No. 75—96) were combined and the solvent was removed by lyophilization; yield 200 mg. The powder obtained here was then dissoved in a small amount of H_2O and the solution was applied to a column of CM-Sephadex (3.5×2.5 cm), which was eluted with 0.1 M ammonium acetate buffer (pH 6.9, 500 ml) through a mixing flask containing H_2O (250 ml). Individual fractions (5 ml each) were collected and absorbancy at 275 mμ was determined. A single peak present in the gradient eluates (tube No. 115—152) was detected and these eluates were combined. The solvent and most of ammonium acetate were removed by lyophilization and the last trace of the salt was finally removed by column chromatography on Sephadex G-25 under conditions stated above to give a fluffy white powder; yield 117 mg (31%), $[\alpha]_D^{24}$ −49.3° (c=0.2, 3% AcOH). Rf_2 0.77, Rf_3 0.89. Amino Acid ratios in an acid hydrolysate and aminopeptidase digest (AP-M, numbers were given in parentheses): Phe 2.07 (2.03), Val 1.03 (1.01), Pro 0.77 (0.99), Ile 1.00 (1.00), Thr 0.79 (0.95), Tyr 0.83 (0.90), Gly 1.07 (0.90), Glu 4.39 (2.35), Leu 1.15 (1.13), Gln (1.77), Arg 1.03 (0.97), Met 0.84 (0.81), Lys 0.95 (0.90). Average recovery 88% (83%). *Anal.* Calcd. for $C_{92}H_{140}N_{22}O_{24}S \cdot 2CH_3COOH \cdot 10H_2O$: C, 50.78; H, 7.46; N, 13.57. Found: C, 50.21; H, 7.24; N, 13.83.

Acknowledgement We wish to express our deep condolence to Dr. Hiroki Kawatani, who had engaged in this synthesis.

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