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Synthesis of Canine Motilin, a Gastric Motor Activity-Stimulating Polypeptide, and Its N^1-Substituted Analogs[1)]

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The docosapeptide corresponding to the entire amino acid sequence of canine motilin, a newly characterized gastric motor activity-stimulating polypeptide, was synthesized by the conventional solution method. All protecting groups employed were removed by 1 M trifluoromethanesulfonic acid–thioanisole–trifluoroacetic acid and the deprotected peptide was purified by gel-filtration on Sephadex G-25, followed by partition chromatography and reverse phase high performance liquid chromatography. When contractile activity on rabbit duodenal muscle was examined, synthetic canine motilin was as active as synthetic porcine motilin. The relative potencies of Lys^1 and Ser^1 derivatives prepared in the same manner as canine motilin were *ca.* 1/1000 and 1/250, respectively.

Keywords——motilin solution synthesis; gastric motor activity-stimulating polypeptide; gastro-intestinal hormone; thioanisole-mediated acidolysis, TFMSA–TFA deprotection; rabbit duodenal muscle contraction

Motilin, a gastric motor activity-stimulating polypeptide, was first isolated from porcine intestine by Brown *et al.*[2)] and its structure (including a minor revision) was finally determined by Brown and Schubert in 1974.[3)] Since then, immuno-reactive motilin-like peptides have been detected in many animal species.[4,5)] In 1983, Poitras *et al.* reported the characterization of canine intestinal motilin.[6)] In their structural studies, small amounts of Lys and Ser, in addition to Phe, were detected in the first Edman sequencing, but the Phe^1-peptide was regarded as the authentic motilin of canine origin. As shown in Fig. 1, within the 22 amino acid residues of canine motilin, five residues are different from those of porcine motilin.

We synthesized canine motilin by a method different from those employed for the previous syntheses of structurally related porcine motilin.[7–11)] For the present synthesis, the thioanisole-mediated TFMSA deprotecting procedure[12)] was employed. In addition, in view of the report by Poitras *et al.* that the N-terminal residue could not be assigned with certainty, we synthesized two derivatives, the Lys^1 and Ser^1-peptides, and examined their activities.

As shown in Fig. 2, the TFA-labile Boc or Z(OMe) group was employed for N^{α}-protection and amino acid derivatives bearing protecting groups removable by TFMSA were employed, *i.e.*, Lys(Z), Glu(OBzl), Ser(Bzl) and Arg(Mts).[13)] To construct the docosapeptide backbone of canine motilin, five fragments, [1] to [5], were selected as building blocks; of

H–Phe–Val–Pro–Ile–Phe–Thr–7–8–Glu–Leu–Gln–12–13–14–
(10)
Glu–Lys–Glu–Arg–Asn–Lys–Gly–Gln–OH
(20)

position	1	7	8	12	13	14
porcine	Phe	Tyr	Gly	Arg	Met	Gln
canine		His	Ser	Lys	Ile	Arg

Fig. 1. Amino Acid Sequences of Porcine and Canine Motilin

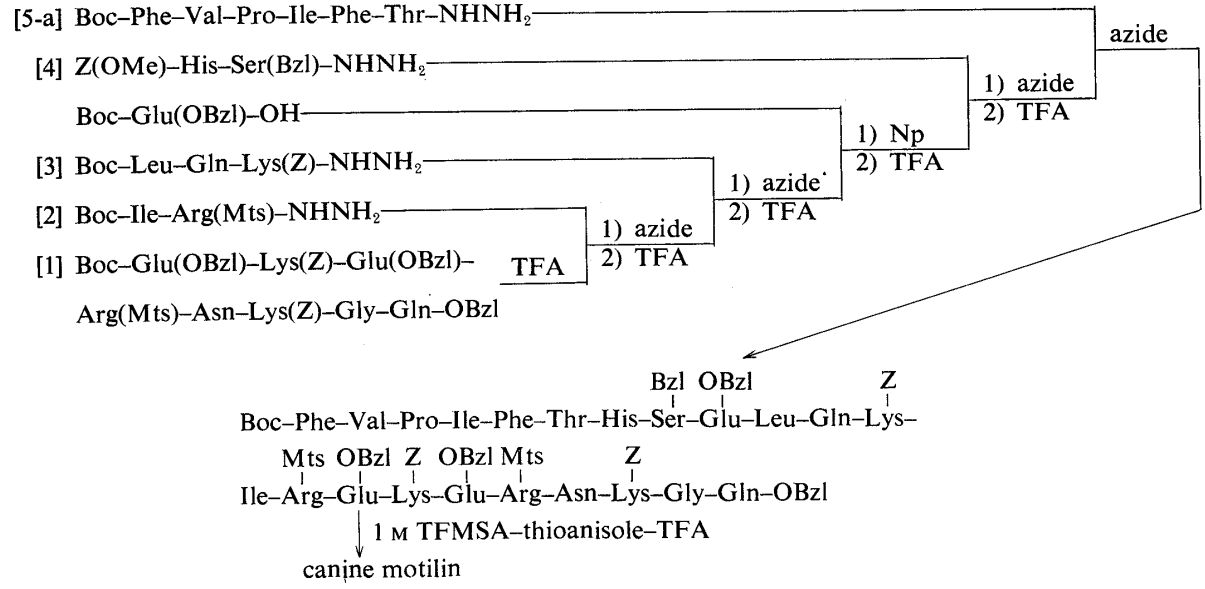

Fig. 2. Synthetic Route to Canine Motilin

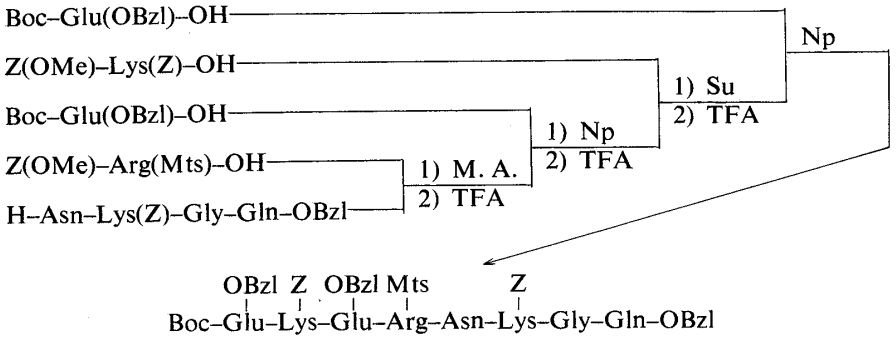

Fig. 3. Synthetic Scheme for the Protected Octapeptide Ester; Fragment [1]
M.A. = mixed anhydride method.

these, fragment [4][14] is a known compound. The Glu[9] residue was introduced as one unit in order to make the fragment synthesis easier.

First, the C-terminal octapeptide benzyl ester [1] was prepared in a stepwise manner starting with H–Gln–OBzl. The Su[15] or the Np[16] active ester procedure or the mixed anhydride method[17] was employed for elongation of the peptide chain. An intermediate, Boc–Asn–Lys(Z)–Gly–Gln–OBzl, is identical with a compound employed by Fujino *et al.* for their synthesis of porcine motilin.[8] Throughout this fragment synthesis and the later syntheses, the purity of each peptide was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis.

Fragment [2], Boc–Ile–Arg(Mts)–$NHNH_2$, was prepared by the mixed anhydride condensation of Boc–Ile–OH and H–Arg(Mts)–OMe followed by the usual hydrazine treatment of the resulting dipeptide ester. Fragment [3], Boc–Leu–Gln–Lys(Z)–$NHNH_2$, was prepared by stepwise condensation procedures *via* the Np active ester and the mixed anhydride, respectively, followed by the usual hydrazine treatment.

The N-terminal fragment [5-a], Boc–Phe–Val–Pro–Ile–Phe–Thr–$NHNH_2$, was prepared by the DCC + HOBt condensation[18] of Boc–Phe–Val–Pro–OH and a TFA-treated sample of Boc–Ile–Phe–Thr–OMe, followed by the usual hydrazinolysis, as shown in Fig. 4. The former tripeptide was prepared according to the procedure reported in the previous synthesis of

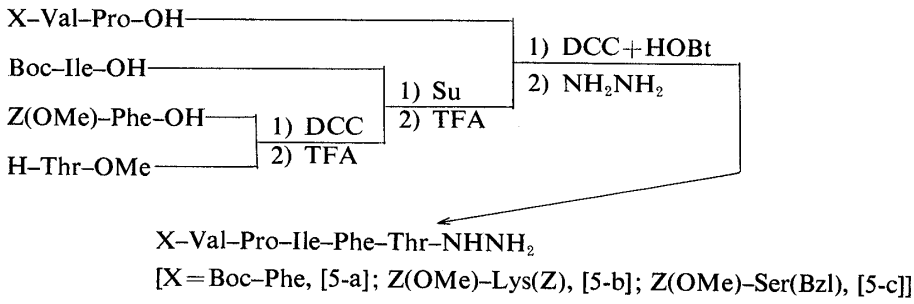

Fig. 4. Synthetic Scheme for the N-terminal Hexapeptide Hydrazides; Fragment [5-a, b, c]

TABLE I. Amino Acid Analyses of the Protected Intermediates and Synthetic Canine Motilin

	Position of the protected peptides						Synthetic motilin
	15—22	13—22	10—22	9—22	7—22	1—22	
Asp	0.95 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.96 (1)	1.19 (1)
Thr						1.01 (1)	1.02 (1)
Ser					0.95 (1)	0.91 (1)	0.95 (1)
Glu	2.85 (3)	3.16 (3)	4.25 (4)	5.08 (5)	5.18 (5)	4.54 (5)	5.10 (5)
Pro						1.15 (1)	1.08 (1)
Gly	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Val						0.90 (1)	0.96 (1)
Ile		1.00 (1)	0.95 (1)	0.87 (1)	0.92 (1)	1.82 (2)	1.87 (2)
Leu			1.12 (1)	1.08 (1)	1.08 (1)	0.85 (1)	1.07 (1)
Phe						1.85 (2)	1.75 (2)
Lys	2.05 (2)	2.09 (2)	3.19 (3)	3.05 (3)	3.13 (3)	2.86 (3)	3.02 (3)
His					1.01 (1)	0.86 (1)	1.00 (1)
Arg	0.94 (1)	1.86 (2)	1.96 (2)	1.85 (2)	2.00 (2)	1.78 (2)	2.14 (2)
Recovery (%)	87	90	95	88	89	92	87

porcine motilin.[7b] The latter tripeptide was prepared by the stepwise condensation procedure.

Five fragments thus prepared were assembled successively by the azide procedure[19] and Boc–Glu(OBzl)–OH was introduced by the Np method according to the route illustrated in Fig. 2. Prior to each condensation, the N^{α}-Boc or Z(OMe) group was removed by TFA in the presence of anisole. Each condensation was carried out using 1.5 to 3 equivalents of the acyl component. The protected hexadeca- and docosapeptide benzyl esters were purified by column chromatography on silica and other intermediates were purified by repeated precipitation from DMF with appropriate organic solvents, such as AcOEt or ether. Throughout this synthesis, Gly was taken as a diagnostic amino acid in acid hydrolysates. By comparison of the recovery of Gly with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation was ascertained, as shown in Table I.

In the final step of the synthesis, the protected docosapeptide benzyl ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol[20] in an ice-bath for 60 min to remove all protecting groups employed. This treatment was repeated again to ensure complete deprotection. The deprotected peptide was next treated with dil. ammonia to reverse a possible N→O shift at the Ser and Thr residues[21] and purified by gel-filtration on Sephadex G-25 using 3% AcOH as an eluant. For further purification, partition chromatography on Sephadex G-25[22] was found to be fairly effective to remove several impurities. After the removal of these impurities with the upper phase of the solvent system (*n*-BuOH–AcOH–

$H_2O = 4:1:5$), the desired peptide was eluted with the lower phase of the above solvent. In order to remove minor impurities from the partition-purified material, a part of the sample was further purified by reverse phase-high performance liquid chromatography (RP-HPLC) using gradient elution with CH_3CN (15%—90% in 75 min) in 0.1% TFA (Fig. 5). After being desalted on Sephadex G-25, the highly purified canine motilin was obtained as a fluffy powder, which exhibited a sharp single spot on TLC in two different solvent systems and a single peak on analytical HPLC. The purity of this material was further confirmed by amino acid analyses after acid hydrolysis and LAP digestion.

Next, the Lys^1 and Ser^1 derivatives were prepared by using Z(OMe)–Lys(Z)–Val–Pro–Ile–Phe–Thr–$NHNH_2$ [5-b] and Z(OMe)–Ser(Bzl)–Val–Pro–Ile–Phe–Thr–$NHNH_2$ [5-c], respectively, as N-terminal hexapeptide hydrazides. The deprotection and subsequent purifications were carried out in essentially the same manner as described above. The purities of these two derivatives were also confirmed by TLC, amino acid analyses and HPLC.

Contractile activity of the synthetic peptides was examined by using the rabbit duodenal preparation[23] and it was found that the activity of Phe^1-peptide (canine motilin) was nearly equivalent to that of synthetic porcine motilin ($EC_{50} = 6.0 \times 10^{-9}$ M). The relative potencies of Lys^1-peptide and Ser^1-peptide with respect to that of Phe^1-peptide (taken as 1) were *ca.* 1/1000 and 1/250, respectively.

From these results of biological activity tests, we can conclude that the undetermined N-terminal residue of canine motilin is Phe. In the case of porcine motilin, the N-terminal portion is regarded as important for the biological activity.[24] The bioassay results from this limited study suggest that Phe in position 1 is essential for high *in vitro* bioactivity of motilin.

Experimental

General experimental procedures employed in this study were as follows.

N^{α}-Deprotection: The N^{α}-protecting group, Z(OMe) or Boc, was treated with TFA (*ca.* 2—3 ml per 1 g of the protected peptide) in the presence of anisole (2 mol eq. or more) under ice-cooling for 60 min. After evaporation of TFA *in vacuo* at 30 °C or less, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* and used for the next coupling reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* and used for the coupling reaction.

Coupling Reactions: The DCC and the active ester couplings were carried out at room temperature. The azide coupling was carried out using isoamyl nitrite with stirring in a cold room (4 °C). Mixed anhydrides were prepared using ethyl chloroformate.

Purification: Unless otherwise mentioned, products were purified by one of the following procedures. Procedure A; for the purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized or precipitated from appropriate solvents. Procedure B; for the purification of protected peptides less soluble in AcOEt, the crude product was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, then crystallized or precipitated from appropriate solvents.

The melting points are uncorrected. Optical rotations were determined with a Union PM-201 polarimeter. Acid hydrolyses were carried out in 6 N HCl in a sealed tube, and amino acid analyses were performed on an IRICA model A-3300 amino acid analyzer. LAP (Lot. 79C-8110) was purchased from Sigma Chemical Co.

TLC was carried out on silica gel (pre-coated Silica gel 60 F_{254}, Merck) or cellulose (pre-coated Cellulose F, Merck). Solvent systems used were as follows; $Rf_1 = CHCl_3$–MeOH–H_2O (8:3:1), $Rf_2 = n$-BuOH–AcOH–pyridine–H_2O (4:1:1:2), and $Rf_3 = n$-BuOH–pyridine–AcOH–H_2O (30:20:6:24).

HPLC was conducted with a Shimadzu LC 4A instrument equipped with a Chemopak column (Nucleosil $7C_{18}$, 4.8 × 250 mm).

Z(OMe)–Gly–Gln–OBzl——A TFA-treated sample of Z(OMe)–Gln–OBzl (8.40 g, 21 mmol) was dissolved in DMF (50 ml) containing NMM (2.31 ml, 21 mmol). To this ice-chilled solution, Z(OMe)–Gly–ONp (9.08 g, 25.2 mmol), HOBt (2.80 g, 21 mmol) and NMM (2.77 ml, 25.2 mmol) were added. The mixture was stirred for 24 h, then DMF was evaporated off. H_2O and ether were added to the residue and the aqueous layer was saturated with NaCl. The resulting gelatinous precipitate solidified on standing in an ice-bath. This solid was collected, washed with ether in a batchwise manner, and recrystallized twice from MeOH with ether. Yield 8.40 g (87%), mp 130—132 °C, $[\alpha]_D^{25}$ −4.0° ($c = 0.5$, MeOH), Rf_1 0.68. *Anal.* Calcd for $C_{23}H_{27}N_3O_7$: C, 60.38; H, 5.95; N, 9.19. Found: C, 60.53; H, 6.07; N, 9.10.

Z(OMe)–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of Z(OMe)–Gly–Gln–OBzl (6.40 g, 14 mmol) was dissolved in DMF (60 ml) containing Et_3N (1.96 ml, 14 mmol) and combined with a mixed anhydride prepared from 7.47 g (16.8 mmol) of Z(OMe)–Lys(Z)–OH in THF (50 ml). The mixture was stirred for 10 h, then the solvent was removed by evaporation. H_2O and ether were added to the residue, and the aqueous layer was saturated with NaCl. The mixture was allowed to stand overnight, and the resulting solid was collected, purified by procedure B, and recrystallized from MeOH. Yield 7.86 g (78%), mp 177—178 °C, $[\alpha]_D^{25}$ −1.0° (c=0.3, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{37}H_{45}N_5O_{10} \cdot 1/2\,H_2O$: C, 60.97; H, 6.37; N, 9.61. Found: C, 61.14; H, 6.17; N, 9.25.

Boc–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of Z(OMe)–Lys(Z)–Gly–Gln–OBzl (6.40 g, 8.9 mmol) was dissolved in DMF (50 ml) containing NMM (0.98 ml, 8.9 mmol). To this solution, Boc–Asn–ONp (3.78 g, 10.7 mmol) and HOBt (1.44 g, 10.7 mmol) were added. The mixture was stirred for 24 h, then DMF was removed by evaporation, and the resulting solid was purified by procedure B and recrystallized twice from EtOH with AcOEt. Yield 5.96 g (87%), mp 191—192 °C, $[\alpha]_D^{25}$ −8.0° (c=0.5, DMF), Rf_1 0.55. (lit.[8]), mp 181—183 °C, $[\alpha]_D$ −13.4° in DMF). *Anal.* Calcd for $C_{37}H_{51}N_7O_{11}$: C, 57.72; H, 6.68; N, 12.74. Found: C, 57.91; H, 6.87; N, 12.35.

Z(OMe)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of Boc–Asn–Lys(Z)–Gly–Gln–OBzl (5.00 g, 6.5 mmol) was dissolved in DMF (50 ml) containing NMM (0.72 ml, 6.5 mmol)and combined with a mixed anhydride prepared from 4.06 g (7.8 mmol) of Z(OMe)–Arg(Mts)–OH in THF (50 ml). The mixture was stirred for 15 h, then the solvent was removed by evaporation. The resulting solid was purified by procedure B and reprecipitated twice from MeOH with ether. Yield 4.60 g (61%), mp 169—171 °C, $[\alpha]_D^{25}$ −4.0 (c=0.5, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{56}H_{73}N_{11}O_{15}S \cdot H_2O$: C, 56.50; H, 6.35; N, 12.95. Found: C, 56.75; H, 6.51; N, 12.83.

Boc–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected pentapeptide benzyl ester (4.10 g, 3.5 mmol) was dissolved in DMF (50 ml) containing NMM (0.39 ml, 3.5 mmol), and Boc–Glu(OBzl)–ONp (1.92 g, 4.2 mmol) was added. The mixture was stirred for 24 h, then DMF was removed by evaporation. Addition of ether to the residue afforded a powder, which was purified by procedure B and reprecipitated from DMF with AcOEt. Yield 4.28 g (92%), mp 184—186 °C, $[\alpha]_D^{25}$ −6.7° (c=0.3, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{64}H_{86}N_{12}O_{17}S \cdot H_2O$: C, 57.13; H, 6.59; N, 12.49. Found: C, 57.26; H, 6.48; N, 12.02.

Z(OMe)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected hexapeptide benzyl ester (4.00 g, 3 mmol) was dissolved in DMF (50 ml) containing NMM (0.73 ml, 6.6 mmol), and Z(OMe)–Lys(Z)–OSu (1.95 g, 3.6 mmol) was added. The mixture was stirred for 36 h, then DMF was evaporated off. The residue was triturated with ether and the resulting solid was purified by procedure B and reprecipitated from DMF with AcOEt. Yield 4.02 g (81%), mp 183—185 °C, $[\alpha]_D^{25}$ −6.0° (c=0.5, DMF), Rf_1 0.61. *Anal.* Calcd for $C_{82}H_{104}N_{14}O_{21} \cdot H_2O$: C, 58.91; H, 6.39; N, 11.73. Found: C, 58.99; H, 6.39; N, 11.45.

Boc–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl [1]——A TFA-treated sample of the protected heptapeptide benzyl ester (3.40 g, 2 mmol) was dissolved in DMF (50 ml) containing NMM (0.46 ml, 4.2 mmol), and Boc–Glu(OBzl)–ONp (1.00 g, 2.2 mmol) was added. The mixture was stirred for 12 h, and DMF was evaporated off. The residue was triturated with ether and the resulting solid was purified by procedure B and reprecipitated from DMF with AcOEt. Yield 3.44 g (95%), mp 197—200 °C, $[\alpha]_D^{25}$ −8.0° (c=0.5, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{90}H_{117}N_{15}O_{23}S \cdot H_2O$: C, 59.16; H, 6.57; N, 11.50. Found: C, 59.17; H, 6.54; N, 11.19.

Boc–Ile–Arg(Mts)–OMe——Boc–Ile–OSu (3.94 g, 12 mmol) was added to a solution of H–Arg(Mts)–OMe [prepared from 4.10 g (10 mmol) of the hydrochloride with 3.08 ml (22 mmol) of Et_3N in DMF (60 ml)], and the reaction mixture was stirred for 24 h. DMF was evaporated off, and the residue was dissolved in AcOEt and purified by procedure A. For further purification, the oily product was purified by silica gel column chromatography with $CHCl_3$ as an eluant. Yield 5.00 g (86%), amorphous powder, $[\alpha]_D^{25}$ −20.0° (c=0.4, MeOH), Rf_1 0.58. *Anal.* Calcd for $C_{27}H_{45}N_5O_7 \cdot 1/2\,H_2O$: C, 54.71; H, 7.82; N, 11.82. Found: C, 54.59; H, 7.90; N, 11.44.

Boc–Ile–Arg(Mts)–NHNH$_2$ [2]——The above-prepared methyl ester (5.00 g, 8.6 mmol) was dissolved in EtOH (60 ml) and treated with 80% hydrazine hydrate (4.2 ml, 86 mmol) for 48 h. EtOH was evaporated off and H_2O was added to the residue. The resulting precipitate was collected and washed with H_2O. Yield 2.70 g (54%), mp 114—117 °C, $[\alpha]_D^{25}$ −16.7° (c=0.3, MeOH), Rf_1 0.60. *Anal.* Calcd for $C_{26}H_{45}N_7O_6S \cdot 1/2\,H_2O$: C, 52.68; H, 7.82; N, 16.54. Found: C, 52.86; H, 8.07; N, 16.62.

Z(OMe)–Gln–Lys(Z)–OMe——Z(OMe)–Gln–ONp (24.0 g, 55.6 mmol) was added to a solution of H–Lys(Z)–OMe [prepared from 20.0 g (60.5 mmol) of the hydrochloride and Et_3N (8.47 ml, 61 mmol) in DMF (100 ml)] and the reaction mixture was stirred for 24 h. DMF was removed by evaporation, and ether was added to the residue. The resulting solid product was purified by procedure B and recrystallized from DMF with AcOEt. Yield 27.7 g (85%), mp 168—170 °C, $[\alpha]_D^{25}$ −4.0° (c=1.0, MeOH), Rf_1 0.64. *Anal.* Calcd for $C_{29}H_{38}N_4O_9$: C, 59.37; H, 6.53; N, 9.55. Found: C, 59.12; H, 6.56; N, 9.40.

Boc–Leu–Gln–Lys(Z)–OMe——A TFA-treated sample of Z(OMe)–Gln–Lys(Z)–OMe (8.80 g, 15 mmol) was dissolved in DMF (50 ml) containing Et_3N (2.10 ml, 15 mmol) and combined with a mixed anhydride prepared from 5.20 g (22.5 mmol) of Boc–Leu–OH in THF (75 ml). After being stirred for 2 h, the mixture was concentrated. Addition of ether to the residue afforded a solid, which was purified by procedure B and recrystallized from EtOH with ether. Yield 7.06 g (74%), mp 124—126 °C, $[\alpha]_D^{25}$ −30.0° (c=1.0, MeOH), Rf_1 0.54. *Anal.* Calcd for $C_{31}H_{49}N_5O_9$: C, 58.56; H, 7.77; N, 11.02. Found: C, 58.24; H, 7.88; N, 10.98.

Boc–Leu–Gln–Lys(Z)–NHNH$_2$ [3]——The above-prepared methyl ester (1.59 g, 2.5 mmol) was dissolved in DMF (30 ml) and treated with 80% hydrazine hydrate (1.25 ml, 25 mmol). After 48 h, DMF was evaporated off and EtOH was added to the residue. The resulting precipitate was collected and washed with H_2O. Yield 1.50 g (94%), mp 197—199 °C, $[\alpha]_D^{25}$ −6.0° (c=1.0, DMF), Rf_1 0.33. *Anal.* Calcd for $C_{30}H_{49}N_7O_8 \cdot 1/2\,H_2O$: C, 55.88; H, 7.82; N, 15.21. Found: C, 56.04; H, 7.83; N, 15.38.

Boc–Phe–Val–OMe——The title compound was prepared by the DCC condensation of Boc–Phe–OH (8.00 g, 30 mmol) and H–Val–OMe hydrochloride (5.00 g, 30 mmol) in the same manner as described for Z–Phe–Val–OMe,[7b] and was recrystallized from AcOEt with petroleum ether. Yield 5.20 g (49%), mp 122—124 °C, $[\alpha]_D^{25}$ −18.0° (c=0.5, MeOH), Rf_1 0.84. *Anal.* Calcd for $C_{20}H_{30}N_2O_5$: C, 63.49; H, 7.99; N, 7.40. Found: C, 63.54; H, 8.19; N, 7.21.

Boc–Phe–Val–NHNH$_2$——The above-prepared methyl ester (9.50 g, 25 mmol) was converted to its hydrazide in the usual manner. The crystalline hydrazide was collected and washed with cold EtOH. Yield 5.38 g (62%), mp 182—185 °C, $[\alpha]_D^{25}$ −26.0° (c=0.5, MeOH), Rf_1 0.68. *Anal.* Calcd for $C_{19}H_{30}N_4O_4$: C, 60.29; H, 7.99; N, 14.81. Found: C, 60.01; H, 8.15; N, 15.07.

Boc–Phe–Val–Pro–OH——Boc–Phe–Val–NHNH$_2$ (2.65 g, 7 mmol) was converted to its azide in the usual manner and mixed with an aqueous solution (50 ml) of H–Pro–OH (1.60 g, 14 mmol) containing Et_3N (3.92 ml, 28 mmol). After being stirred for 24 h at 4 °C, the mixture was evaporated to dryness. The residue was purified in the same manner as described for the preparation of Z–Phe–Val–Pro–OH[7b] and recrystallized twice from AcOEt with ether. Yield 2.70 g (84%), mp 116—118 °C, $[\alpha]_D^{25}$ −68.0° (c=0.5, MeOH), Rf_1 0.47. *Anal.* Calcd for $C_{24}H_{35}N_3O_6$: C, 62.45; H, 7.64; N, 9.11. Found: C, 62.45; H, 7.91; N, 9.10.

Z(OMe)–Phe–Thr–OMe——DCC (24.8 g, 0.12 mol) was added to a solution of Z(OMe)–Phe–OH (39.5 g, 0.12 mol) and H–Thr–OMe [prepared from 15.8 g (0.12 mol) of the hydrochloride and Et_3N (16.8 ml, 0.12 mol)] in THF–DMF (150 ml–150 ml) with ice-cooling. The mixture was stirred for 24 h, and the formed urea derivative was removed by filtration. The filtrate was concentrated. The residue was dissolved in AcOEt and purified by procedure A, followed by recrystallization from AcOEt. Yield 42.0 g (79%), mp 134—136.5 °C, $[\alpha]_D^{25}$ −13.3° (c=0.3, MeOH), Rf_1 0.83. *Anal.* Calcd for $C_{23}H_{28}N_2O_7$: C, 61.73; H, 6.26; N, 6.31. Found: C, 62.23; H, 6.39; N, 6.30.

Boc–Ile–Phe–Thr–OMe——A TFA-treated sample of Z(OMe)–Phe–Thr–OMe (4.40 g, 10 mmol) was dissolved in THF (50 ml) containing Et_3N (1.40 ml, 10 mmol) and combined with a mixed anhydride prepared from 3.00 g (13 mmol) of Boc–Ile–OH in THF (50 ml). The mixture was stirred for 2 h, then the solvent was removed by evaporation. The residue was dissolved in AcOEt and purified by procedure A, followed by recrystallization from MeOH with ether. Yield 3.70 g (75%), mp 179—181.5 °C, $[\alpha]_D^{25}$ −36.7° (c=0.3, MeOH), Rf_1 0.80. *Anal.* Calcd for $C_{25}H_{38}N_3O_7$: C, 60.83; H, 7.76; N, 8.51. Found: C, 60.54; H, 7.94; N, 8.34.

Boc–Phe–Val–Pro–Ile–Phe–Thr–OMe——A TFA-treated sample of Boc–Ile–Phe–Thr–OMe (2.60 g, 5.3 mmol) was dissolved in DMF (15 ml) and treated with 3 N HCl–dioxane (5.20 ml, 16 mmol). After being stirred for 30 min, the solution was concentrated. Addition of ether to the residue afforded a crystalline HCl salt, which was collected and dried *in vacuo*. This HCl salt was dissolved in DMF (30 ml) containing Et_3N (0.74 ml, 5.3 mmol) and mixed with a DMF (25 ml) solution of Boc–Phe–Val–Pro–OH (2.03 g, 4.4 mmol). To this solution, DCC (0.91 g, 4.4 mmol) and HOBt (0.60 g, 4.4 mmol) were added. The mixture was stirred for 24 h, then the formed urea derivative was removed by filtration and the filtrate was concentrated. Addition of ether to the residue afforded a solid product, which was purified by procedure B and recrystallized from MeOH with ether. Yield 4.26 g (96%), mp 167—170 °C, $[\alpha]_D^{25}$ −74.0° (c=0.5, MeOH), Rf_1 0.80. *Anal.* Calcd for $C_{44}H_{64}N_6O_{10}$: C, 63.14; H, 7.71; N, 10.04. Found: C, 63.23; H, 8.10; N, 10.10.

Boc–Phe–Val–Pro–Ile–Phe–Thr–NHNH$_2$ [5-a]——The above-prepared methyl ester (2.68 g, 3.2 mmol) was dissolved in MeOH (50 ml) and treated with 80% hydrazine hydrate (1.60 ml, 30 mmol). The mixture was stirred for 24 h, then the precipitate was collected and washed with cold H_2O. Yield 2.12 g (79%), mp 214—217 °C, $[\alpha]_D^{25}$ −28.0° (c=0.3, DMF), Rf_1 0.54. *Anal.* Calcd for $C_{43}H_{64}N_8O_9$: C, 61.70; H, 7.71; N, 13.39. Found: C, 61.47; H, 7.91; N, 13.33. Amino acid ratios in 6 N HCl hydrolysate: Phe 2.00, Val 0.93, Pro 1.03, Ile 0.92, Thr 1.10 (recovery of Phe, 92%).

Boc–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of Boc–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl (2.71 g, 1.5 mmol) was dissolved in DMF (50 ml) containing Et_3N (0.21 ml, 1.5 mmol). To this ice-chilled solution, the azide [prepared from 1.30 g (2.2 mmol) of Boc–Ile–Arg(Mts)–NHNH$_2$] and Et_3N (0.31 ml, 2.2 mmol) were added. The mixture was stirred for 24 h at 4 °C, then the solvent was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B and repeatedly reprecipitated from DMF with ether. Yield 3.07 g (91%), mp 210—212 °C, $[\alpha]_D^{25}$ +12.0° (c=0.5, DMF), Rf_1 0.65. *Anal.* Calcd for $C_{111}H_{150}N_{20}O_{27}S_2$: C, 58.97; H, 6.69; N, 12.39. Found: C, 58.70; H, 6.79; N, 12.41.

Boc–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected decapeptide benzyl ester (2.26 g, 1 mmol) was dissolved in DMF (40 ml) containing Et_3N (0.14 ml, 1 mmol). To this ice-chilled solution, the azide [prepared from 0.96 g (1.5 mmol) of Boc–Leu–Gln–Lys(Z)–NHNH$_2$] and Et_3N (0.21 ml, 1.5 mmol) were added. The mixture was stirred for 48 h at 4 °C, then the solvent was removed by evaporation. The residue was triturated with ether, and the resulting solid was purified by

procedure B and reprecipitated twice from DMF with ether. Yield 2.46 g (89%), mp 236—239 °C, $[\alpha]_D^{25}$ +4.0° (c=0.5, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{136}H_{187}N_{25}O_{33}S_2$: C, 59.08; H, 6.82; N, 12.67. Found: C, 58.87; H, 7.01; N, 12.67.

Boc–Glu(OBzl)–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected tridecapeptide benzyl ester (1.10 g, 0.4 mmol) was dissolved in DMF (20 ml) containing Et_3N (0.17 ml, 1.2 mmol), and Boc–Glu(OBzl)–ONp (0.22 g, 0.48 mmol) was added. The mixture was stirred for 48 h, then DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B and reprecipitated twice from DMF with ether. Yield 0.94 g (79%), mp 104—107 °C, $[\alpha]_D^{25}$ −6.7° (c=0.3, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{148}H_{200}N_{26}O_{36}S_2 \cdot H_2O$: C, 59.22; H, 6.78; N, 12.13. Found: C, 59.09; H, 6.77; N, 12.25.

Z(OMe)–His–Ser(Bzl)–Glu(OBzl)–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected tetradecapeptide benzyl ester (3.00 g, 1 mmol) was dissolved in DMF (50 ml) containing Et_3N (0.14 ml, 1 mmol). To this solution, the azide [prepared from 1.04 g (2 mmol) of Z(OMe)–His–Ser(Bzl)–$NHNH_2$[14]] and Et_3N (0.28 ml, 2 mmol) were added. The mixture was stirred for 48 h at 4 °C, then DMF was removed by evaporation. Trituration with ether afforded a solid, which was collected and dried. This sample was dissolved in $CHCl_3$–MeOH–H_2O (8 : 3 : 1, 5 ml) and purified by silica gel column chromatography using the same solvent system as an eluant. Fractions containing the desired material (Rf_1=0.67) were pooled and the solvent was removed by evaporation. The residue was triturated with ether and the resulting powder was recrystallized from MeOH with ether. Yield 2.00 g (60%), mp 98—107 °C, $[\alpha]_D^{25}$ −10.0° (c=0.3, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{168}H_{218}N_{30}O_{40}S_2 \cdot 3H_2O$: C, 59.07; H, 6.61; N, 12.30. Found: C, 59.06; H, 6.54; N, 12.15.

Boc–Phe–Val–Pro–Ile–Phe–Thr–His–Ser(Bzl)–Glu(OBzl)–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——A TFA-treated sample of the protected hexadecapeptide benzyl ester (1.01 g, 0.3 mmol) was dissolved in DMF (30 ml) containing Et_3N (42 μl, 0.3 mmol). To this ice-chilled solution, the azide [prepared from 0.50 g (0.6 mmol) of Boc–Phe–Val–Pro–Ile–Phe–Thr–$NHNH_2$] and Et_3N (84 μl, 0.6 mmol) were added. The mixture was stirred for 48 h at 4 °C, then DMF was removed by evaporation. The residue was triturated with ether and the resulting powder was collected and dried. This sample was dissolved in $CHCl_3$–MeOH (10 : 1, 5 ml) and purified by silica gel column chromatography using the same solvent system as an eluant. The fractions containing the desired material (Rf_1=0.58) were pooled and the solvent was removed by evaporation. The residue was triturated with ether and the resulting powder was reprecipitated twice from MeOH with ether. Yield 0.83 g (69%), mp 136—138 °C, $[\alpha]_D^{25}$ −30.0° (c=0.3, MeOH), Rf_1 0.58. *Anal.* Calcd for $C_{202}H_{270}N_{36}O_{46}S_2 \cdot 5H_2O$: C, 59.28; H, 6.90; N, 12.32. Found: C, 59.46; H, 6.88; N, 12.18.

H–Phe–Val–Pro–Ile–Phe–Thr–His–Ser–Glu–Leu–Gln–Lys–Ile–Arg–Glu–Lys–Glu–Arg–Asn–Lys–Gly–Gln–OH; Canine Motilin——The fully protected docosapeptide benzyl ester (200 mg, 50 μmol) was treated with 1 M TFMSA–thioanisole–TFA (4.14 ml) in the presence of *m*-cresol (0.22 ml) in an ice-bath for 60 min, then washed with *n*-hexane, and dry ether was added. The resulting precipitate was collected by centrifugation, washed with ether and again treated with 1 M TFMSA–thioanisole–TFA under the same conditions as described above. The deprotected peptide, isolated by addition of dry ether, was dissolved in H_2O (30 ml) and the pH of the solution was adjusted to 8.6 with 1 N NH_4OH, then after 30 min, to 6.8 with 1 N AcOH, and the solution was lyophilized.

The lyophilized material was dissolved in 3% AcOH (3 ml) and insoluble material was removed by centrifugation, then the solution was applied to a column of Sephadex G-25 (2.6 × 44 cm), which was eluted with the same solvent. Each fraction (5 ml) was checked for ultraviolet (UV) absorption at 275 nm. The fractions corresponding to the main peak (tube nos. 33—46) were pooled and the solvent was removed by lyophilization to give a fluffy powder; 150 mg (≒100%).

The crude material (50 mg) thus obtained was dissolved in the upper phase of *n*-BuOH–AcOH–H_2O (4 : 1 : 5, 3 ml) and the solution was applied to a column of Sephadex G-25 (2.4 × 31 cm), which was equilibrated with the same solvent system. After 150 ml of the upper phase of the solvent system had been passed, the lower phase of the solvent system was used as an eluant. Each fraction (5 ml) of the lower phase eluate was checked by TLC with ninhydrin reagent. The fractions which exhibited a single ninhydrin-positive spot on TLC were combined and the solvent was removed by evaporation at 35 °C. The rest of the Sephadex G-25 purified material was purified in the same manner in two batchs, and a total of 47 mg (31%) of the purified peptide was obtained after repeated lyophilization.

This sample exhibited the single spot on TLC, but on RP-HPLC some minor peaks were detected. To remove these impurities, a part of this sample (25 mg, *ca.* 1 mg each) was purified by RP-HPLC on Chemopak column using the solvent system of CH_3CN (from 15% to 90% in 75 min) in 0.1% TFA. The eluate corresponding to the main peak was pooled (Fig. 5). The rest of the sample was similarly purified and the solvent of the combined eluates was removed by evaporation *in vacuo* at 35 °C. The residue was dissolved in 0.1 M NaOAc (1 ml) and the solution was desalted on Sephadex G-25 (2.6 × 44 cm) using 3% AcOH as an eluant. The desired fractions were pooled and the solvent was removed by lyophilization to give a white fluffy powder; 12.5 mg (63%). $[\alpha]_D^{25}$ −58.0° (c=0.5, 3% AcOH), Rf_2 (cellulose) 0.29, Rf_3 (cellulose) 0.45. HPLC: retention time, 24.80 min (Fig. 6). *Anal.* Calcd for $C_{120}H_{194}N_{36}O_{34} \cdot 5CH_3COOH \cdot 13H_2O$: C, 48.49; H, 7.51; N, 15.66. Found: C, 48.25; H, 6.88; N, 15.85. Amino acid ratios in 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in LAP digest (numbers in parentheses are theoretical values): Thr 1.07(1), Ser 0.95(1), Glu 2.92(3), Pro 1.00(1), Gly 1.00(1), Val 0.88(1), Ile 1.91(2), Leu 0.98(1),

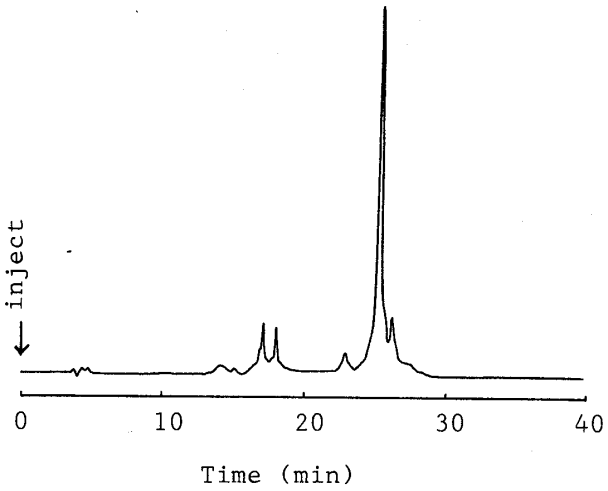

Fig. 5. Purification of Partition-Purified [Phe^1]Canine Motilin by HPLC

HPLC was performed on a reverse-phase column (Chemopak, Nucleosil 7C_{18}, 4.8 × 250 mm). Isocratic elution with (A) (2 min) was followed by linear gradient elution from (A) to (B) (75 min) at a flow rate of 1 ml/min. (A): 15% CH_3CN (0.1% TFA). (B): 90% CH_3CN (0.1% TFA). O.D. at 215 nm.

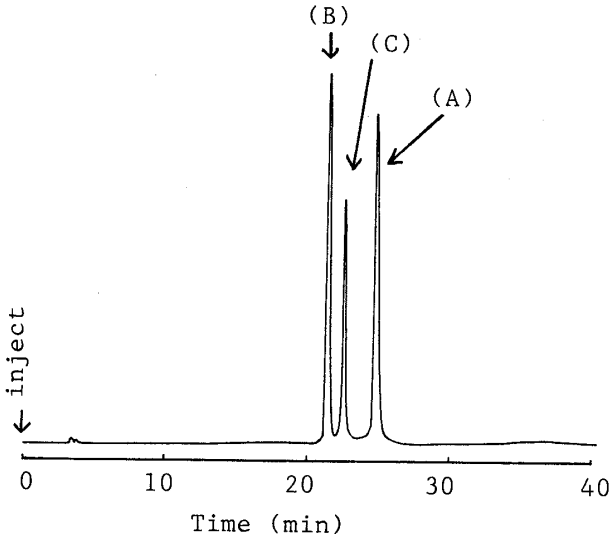

Fig. 6. HPLC of a Mixture of Synthetic Canine Motilin (A) and Its Lys^1 (B) and Ser^1 (C) Derivatives

Purified samples (*ca.* 150 μg each) were injected and HPLC was performed under the same conditions as described in Fig. 5. O.D. at 215 nm.

Phe 1.76(2), Lys 2.81(3), His 0.87(1), Arg 1.92(2), Asn(1)+Gln(2) were not determined (recovery of Gly, 97%).

Synthesis of [Lys^1]Canine Motilin

Z(OMe)–Lys(Z)–Val–OMe——The title compound was prepared by the DCC condensation of Z(OMe)–Lys(Z)–OH (13.30 g, 30 mmol) and H–Val–OMe hydrochloride (6.10 g, 36 mmol) and purified by procedure A, followed by recrystallization from AcOEt with ether. Yield 14.70 g (88%), mp 117—119 °C, $[\alpha]_D^{25}$ −13.0° (c=1.0, DMF), Rf_1 0.72. *Anal.* Calcd for $C_{29}H_{39}N_3O_8$: C, 62.46; H, 7.05; N, 7.54. Found: C, 62.61; H, 7.20; N, 7.57.

Z(OMe)–Lys(Z)–Val–NHNH$_2$——The above-prepared methyl ester (14.40 g, 25 mmol) was converted to its hydrazide by the usual hydrazine treatment. Yield 11.20 g (78%), mp 195—196 °C, $[\alpha]_D^{25}$ +4.0° (c=0.5, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{28}H_{39}N_5O_7$: C, 60.31; H, 7.05; N, 12.56. Found: C, 60.27; H, 7.10; N, 12.52.

Z(OMe)–Lys(Z)–Val–Pro–OH——The title compound was prepared by the azide condensation of Z(OMe)–Lys(Z)–Val–NHNH$_2$ (5.60 g, 10 mmol) and H–Pro–OH (2.30 g, 20 mmol) and purified in the same manner as described for Boc–Phe–Val–Pro–OH, followed by recrystallization from EtOH with *n*-hexane. Yield 5.30 g (83%), mp 81—83 °C, $[\alpha]_D^{25}$ −57.0° (c=1.0, MeOH), Rf_1 0.50. *Anal.* Calcd for $C_{33}H_{44}N_4O_9$: C, 61.86; H, 6.92; N, 8.75. Found: C, 61.86; H, 7.10; N, 8.72.

Z(OMe)–Lys(Z)–Val–Pro–Ile–Phe–Thr–OMe——The title compound was prepared by the DCC+HOBt condensation of Z(OMe)–Lys(Z)–Val–Pro–OH (2.56 g, 4 mmol) and the HCl salt of H–Ile–Phe–Thr–OMe [prepared from 2.43 g (4 mmol) of Boc–Ile–Phe–Thr–OMe], and purified by procedure B, followed by recrystallization from MeOH with ether. Yield 3.50 g (87%), mp 156—159 °C, $[\alpha]_D^{25}$ −40.0° (c=1.0, DMF), Rf_1 0.80. *Anal.* Calcd for $C_{53}H_{73}N_7O_{10}$: C, 62.64; H, 7.26; N, 9.45. Found: C, 62.27; H, 7.43; N, 9.66.

Z(OMe)–Lys(Z)–Val–Pro–Ile–Phe–Thr–NHNH$_2$ [5-b]——The above-prepared methyl ester (5.10 g, 5 mmol) was converted to its hydrazide by the usual hydrazine treatment. Yield 4.50 g (88%), mp 184—186 °C, $[\alpha]_D^{25}$ −34.0° (c=1.0, DMF), Rf_1 0.55. *Anal.* Calcd for $C_{52}H_{73}N_9O_{12}\cdot 1/2\,H_2O$: C, 60.92; H, 7.28; N, 12.30. Found: C, 60.89; H, 7.40; N, 12.20. Amino acid ratios in 6 N HCl hydrolysate: Lys 1.00, Val 0.98, Pro 1.09, Ile 1.16, Phe 1.89, Thr 0.98 (recovery of Lys, 86%).

Z(OMe)–Lys(Z)–Val–Pro–Ile–Phe–Thr–His–Ser(Bzl)–Glu(OBzl)–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——The title compound was prepared by the azide condensation of [5-b] (0.50 g, 0.5 mmol) and a TFA-treated sample of the protected hexadecapeptide benzyl ester (0.84 g, 0.25 mmol). The product was purified by silica gel column chromatography using $CHCl_3$: MeOH (10 : 1) as an eluant, followed by gel-filtration on Sephadex LH-20 (3.1 × 113 cm) using DMF as an eluant. The fractions corresponding to the main peak were combined and DMF was evaporated off. The residue was triturated with ether and the resulting powder was collected and dried. Yield 0.56 g (54%), mp 104—108 °C, $[\alpha]_D^{25}$ −10.0° (c=0.4, MeOH), Rf_1 0.71. *Anal.* Calcd for $C_{211}H_{279}N_{37}O_{49}S_2\cdot 3H_2O$: C, 59.83; H, 6.78; N, 12.24. Found: C, 59.73; H, 6.75; N, 11.84. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.04, Thr 0.84, Ser 0.73, Glu 5.41, Gly 1.00, Pro 1.13, Val

1.05, Ile 2.05, Leu 0.87, Phe 0.94, Lys 4.38, His 0.92, Arg 2.15 (recovery of Gly, 85%).

[Lys^1]Canine Motilin——The fully protected Lys^1-docosapeptide benzyl ester (100 mg, 24 μmol) was treated with 1 M TFMSA–thioanisole–TFA (2.10 ml) in the presence of *m*-cresol (0.11 ml) in essentially the same manner as described for the synthesis of the [Phe^1]peptide. Purification was carried out successively by gel-filtration on Sephadex G-25, partition chromatography and RP-HPLC under the same conditions as described above. Yield 12.8 mg (17.6% from deprotection). $[\alpha]_D^{25}$ $-72.5°$ ($c=0.4$, 3% AcOH), Rf_2 (cellulose) 0.16, Rf_3 (cellulose) 0.32. HPLC: retention time, 21.25 min (Fig. 6). Amino acid ratios in 6 N HCl hydrolysate: Asp 0.99(1), Thr 0.93(1), Ser 0.94(1), Glu 4.54(5), Pro 1.12(1), Gly 1.00(1), Val 0.92(1), Ile 1.59(2), Leu 0.92(1), Phe 0.93(1), Lys 3.81(4), His 0.92(1), Arg 1.94(2) (recovery of Gly, 91%).

Synthesis of [Ser^1]Canine Motilin

Z(OMe)–Ser(Bzl)–Val–OMe——The title compound was prepared by the DCC condensation of Z(OMe)–Ser(Bzl)–OH (10.8 g, 30 mmol) and H–Val–OMe hydrochloride (5.00 g, 30 mmol), and purified by procedure A, followed by recrystallization from EtOH with ether. Yield 7.80 g (57%), mp 75—78 °C, $[\alpha]_D^{25}$ $-6.0°$ ($c=1.0$, MeOH), Rf_1 0.83. *Anal.* Calcd for $C_{25}H_{32}N_2O_7$: C, 63.54; H, 6.83; N, 5.93. Found: C, 63.86; H, 7.01; N, 5.88.

Z(OMe)–Ser(Bzl)–Val–NHNH$_2$——The above-prepared methyl ester (6.86 g, 15 mmol) was converted to its hydrazide by the usual hydrazine treatment. Yield 6.40 g (93%), mp 201—203 °C, $[\alpha]_D^{25}$ $+11.0°$ ($c=1.0$, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{24}H_{32}N_4O_6$: C, 61.00; H, 6.83; N, 11.86. Found: C, 61.06; H, 6.92; N, 11.63.

Z(OMe)–Ser(Bzl)–Val–Pro–OH——The title compound was prepared by the azide condensation of Z(OMe)–Ser(Bzl)–Val–NHNH$_2$ (4.60 g, 10 mmol) and H–Pro–OH (2.30 g, 20 mmol) and purified in the same manner as described for Boc–Phe–Val–Pro–OH. The oily product was analyzed as the cyclohexylamine salt. Yield 4.11 g (74%), mp 164—170 °C, $[\alpha]_D^{25}$ $-45.0°$ ($c=1.0$, MeOH), Rf_1 0.62. *Anal.* Calcd for $C_{29}H_{37}N_3O_8 \cdot C_6H_{13}N$: C, 64.20; H, 7.70; N, 8.56. Found: C, 63.96; H, 7.88; N, 8.37.

Z(OMe)–Ser(Bzl)–Val–Pro–Ile–Phe–Thr–OMe——The title compound was prepared by the DCC+HOBt condensation of Z(OMe)–Ser(Bzl)–Val–Pro–OH (2.80 g, 5 mmol) and the HCl salt of H–Ile–Phe–Thr–OMe [prepared from 2.50 g (5 mmol) of Boc–Ile–Phe–Thr–OMe] and purified by procedure B, followed by reprecipitation from DMF with AcOEt. Yield 3.50 g (75%), mp 214—216 °C, $[\alpha]_D^{25}$ $-36.0°$ ($c=1.0$, DMF), Rf_1 0.74. *Anal.* Calcd for $C_{49}H_{66}N_6O_{12}$: C, 63.21; H, 7.15; N, 9.03. Found: C, 63.25; H, 7.24; N, 9.04.

Z(OMe)–Ser(Bzl)–Val–Pro–Ile–Phe–Thr–NHNH$_2$ [5-c]——The above-prepared methyl ester (1.58 g, 1.7 mmol) was converted to its hydrazide by the usual hydrazine treatment. Yield 1.46 g (93%), mp 237—240 °C, $[\alpha]_D^{25}$ $-26.0°$ ($c=1.0$, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{48}H_{66}N_8O_{11}$: C, 61.92; H, 7.15; N, 12.04. Found: C, 62.07; H, 7.18; N, 11.59. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.93, Val 0.85, Pro 0.90, Ile 1.00, Phe 1.76, Thr 1.00 (recovery of Ile, 89%).

Z(OMe)–Ser(Bzl)–Val–Pro–Ile–Phe–Thr–His–Ser(Bzl)–Glu(OBzl)–Leu–Gln–Lys(Z)–Ile–Arg(Mts)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Arg(Mts)–Asn–Lys(Z)–Gly–Gln–OBzl——The title compound was prepared by the azide condensation of [5-c] (0.47 g, 0.5 mmol) and a TFA-treated sample of the protected hexadecapeptide benzyl ester (0.84 g, 0.25 mmol). The product was purified by silica gel column chromatography using $CHCl_3$–MeOH (10:1) as an eluant, followed by Sephadex LH-20 chromatography using DMF as an eluant. Yield 0.66 g (64%), mp 113—116 °C, $[\alpha]_D^{25}$ $-5.0°$ ($c=0.4$, MeOH), Rf_1 0.66. *Anal.* Calcd for $C_{207}H_{272}N_{36}O_{48}S_2 \cdot 10H_2O$: C, 57.86; H, 6.88; N, 11.83. Found: C, 57.84; H, 6.57; N, 11.74. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.12, Thr 0.87, Ser 1.86, Glu 5.35, Pro 0.96, Gly 1.00, Val 0.91, Ile 2.07, Leu 1.05, Phe 0.83, Lys 3.04, His 0.89, Arg 1.96 (recovery of Gly, 88%).

[Ser^1]Canine Motilin——The fully protected Ser^1-docosapeptide benzyl ester (100 mg, 24 μmol) was treated with 1 M TFMSA–thioanisole–TFA in the presence of *m*-cresol and purified in the same manner as described for the Phe^1 and Lys^1 peptides. Yield 15.2 mg (20.4% from deprotection), $[\alpha]_D^{25}$ $-82.5°$ ($c=0.4$, 3% AcOH), Rf_2 (cellulose) 0.20, Rf_3 (cellulose) 0.33. HPLC: retention time, 22.60 min (Fig. 6). Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00(1), Thr 0.97(1), Ser 1.82(2), Glu 4.59(5), Pro 1.12(1), Gly 1.00(1), Val 0.98(1), Ile 1.73(2), Leu 0.97(1), Phe 0.94(1), Lys 3.02(3), His 0.93(1), Arg 2.13(2) (recovery of Gly, 92%).

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature: *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, Et_3N = triethylamine, NMM = *N*-methylmorpholine, DMF = dimethylformamide, DMSO = dimethylsulfoxide, MeOH = methanol, EtOH = ethanol, THF = tetrahydrofuran, AcOEt = ethyl

acetate, AcOH = acetic acid, LAP = leucine aminopeptidase.
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