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Amino Acids and Peptides. XXXI.[1-3] Phosphorus in Organic Synthesis. XVIII.[4] Synthesis of Porcine Motilin by the Solid-phase Method using Diphenyl Phosphorazidate(DPPA) and Diethyl Phosphorocyanidate(DEPC)

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Synthesis of porcine motilin, which exhibits a gastric motor stimulating activity, was accomplished by the solid-phase method with diphenyl phosphorazidate (DPPA) and diethyl phosphorocyanidate (DEPC) as coupling reagents. The purified synthetic peptide showed characteristic contractile activity towards the duodenum, colon and jejunum in the rabbit. Its potency was almost the same as that of the natural material.

Keywords——amino acid; peptide; gastrointestinal hormone; contractile activity; fragment condensation

In the preceding paper,[1,4] we reported that diphenyl phosphorazidate (DPPA, $(PhO)_2P(O)N_3$) and diethyl phosphorocyanidate (DEPC, $(EtO)_2P(O)CN$) could be efficient reagents for solid-phase peptide synthesis. In order to extend the range of usefulness of these reagents, the synthesis of porcine motilin was undertaken. Porcine motilin, exhibiting a gastric motor stimulating activity, was discovered by Brown *et al.*, and its primary structure was determined by them in 1973.[6] The proposed structure of this hormone was revised by Brown *et al.*[7] in 1974. A Gln residue instead of Glu was placed at position 14 in their revised structure (I). Wünsch and co-workers[8] synthesized the first peptide to exhibit the full activity of motilin, [13-norleucine, 14-Glu]-motilin, in 1973. After Yajima and co-workers[9] accomplished the first synthesis of motilin by the conventional method in 1975, we also succeeded in synthesizing this hormone by the solid-phase method;[3] the details of this work are the subject of this paper.

1) Part XXX: N. Ikota, T. Shioiri, and S. Yamada, *Chem. Pharm. Bull.*, **28**, 3064 (1980).
2) Unless otherwise stated, all optically active amino acids are of L-configuration. Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, *Pure Appl. Chem.*, **40**, 315 (1974). Other abbreviations used are: DMF, dimethylformamide; TFA, trifluoroacetic acid; TEA, triethylamine, chloromethyl resin, chloromethylated copolystyrene–2% divinylbenzene.
3) Part of this work was the subject of a preliminary report: S. Yamada, N. Ikota, T. Shioiri, and S. Tachibana, *J. Am. Chem. Soc.*, **97**, 7174 (1975).
4) Part XVII: N. Ikota, T. Shioiri, and S. Yamada, *Chem. Pharm. Bull.*, **28**, 3064 (1980).
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6) J.C. Brown, M.A. Cook, and J.R. Dryburgh, *Can. J. Biochem.*, **51**, 533 (1973).
7) H. Schubert and J.C. Brown, *Can. J. Biochem.*, **52**, 7 (1974).
8) E. Wünsch, J.C. Brown, K.H. Deimer, F. Dres, E. Jaeger, J. Mousiol, R. Scharr, H. Stocker, P. Thamm, and G. Wendlberger, *Z. Naturforsch*, **28c**, 235 (1973); E. Wünsch, E. Jaeger, S. Knof, R. Scharf, and P. Thamm, *Hoppe-Seyler's Z. Physiol. Chem.*, **357**, 467 (1976).
9) *a)* H. Yajima, Y. Kai, and H. Kawatani, *J. Chem. Soc. Chem. Commun.*, **1975**, 159; *b)* Y. Kai, H. Kawatani, and H. Yajima, *Chem. Pharm. Bull.*, **23**, 2339 (1975); *c)* Y. Kai, H. Kawatani, H. Yajima, and Z. Itoh, *Chem. Pharm. Bull.*, **23**, 2346 (1975).

Some other syntheses of motilin were also reported afterwards.[10,11]

For the synthesis of motilin, two peptide fragments (II and III) were condensed on a polymer support and after incorporation of the Glu residue (position 9), the fragment (IV) was introduced to construct the whole sequence of motilin, as illustrated in Chart 1. Synthesis of the peptide fragments was performed by a combination of the conventional and solid-phase methods. Since the Boc group was used for protection of the α-amino group throughout the solid-phase method, a suitable selection of side-chain protecting groups was necessary. For glutamic acid, threonine, and tyrosine, Bzl protection was used. Since Z protection for lysine is known to be unstable, the 2-Cl–Z protecting group, which was reported to be 60 times more stable,[12] was used. For arginine, nitro protection was chosen. The above protective groups were stable under TFA treatment and could be cleaved with hydrogen fluoride at the final step of synthesis.

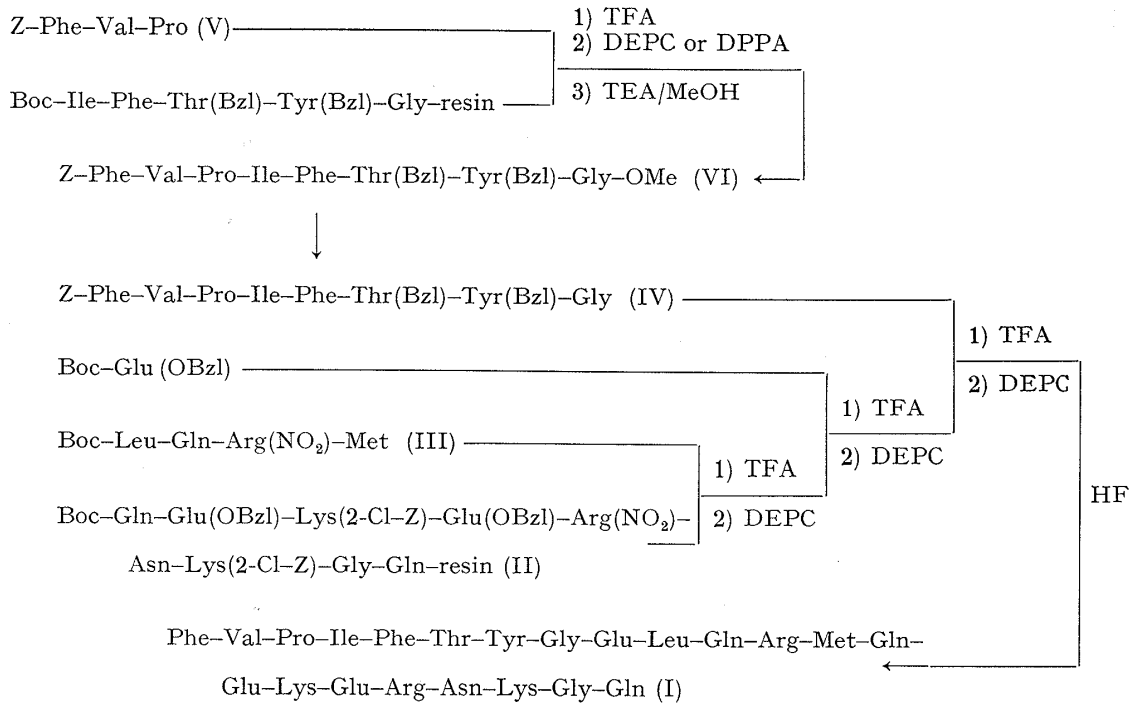

Chart 1

For the preparation of the protected octapeptide resin (Z–Phe–Val–Pro–Ile–Phe–Thr-(Bzl)-Tyr(Bzl)-Gly-resin), Z–Phe–Val–Pro was condensed with the Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly-resin. The synthesis of Z–Phe–Val–Pro (V) started from Pro–OMe, which was coupled with Z–Val using DPPA or DEPC in DMF. The resulting Z–Val–Pro–OMe obtained in 90% (DPPA) or 86% (DEPC) yield was hydrogenated over 5% palladium on charcoal to afford Val–Pro–OMe. Next, Z–Phe was coupled to this with DPPA or DEPC to give Z–Phe–Val–Pro–OMe in 85% (DPPA) or 84% (DEPC) yield. Z–Phe–Val–Pro–OMe was hydrolyzed to yield Z–Phe–Val–Pro (V).

The preparation of Boc–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly–resin was obtained by stepwise elongation from Boc–Gly-resin using DPPA or DEPC. The protected octapeptide resin was prepared by fragment condensation of Z–Phe–Val–Pro with the pentapeptide resin. Boc–Gly-

10) S. Mihara, F. Shimizu, and S. Miyamoto, *Bull. Chem. Soc. Japan*, **49**, 3589 (1976); F. Shimizu, K. Imagawa, S. Mihara, and N. Yanaihara, *ibid.*, **49**, 3594 (1976).
11) M. Fujino, S. Shinagawa, M. Wakimasu, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 101 (1978).
12) B. Erickson and R. Merrifield, *J. Am. Chem. Soc.*, **95**, 3757 (1973).

resin, which was prepared by the reaction of Boc-Gly cesium salt and chloromethyl resin in DMF,[13] was deprotected by treatment with TFA–CH_2Cl_2 (1 : 2) and converted to the glycyl resin with TEA–DMF according to the general procedure[1] for the solid-phase method.[14] To this resin were successively coupled Boc–Tyr(Bzl), Boc–Thr(Bzl), Boc–Phe, Boc–Ile, and Z–Phe–Val–Pro·$1/2C_6H_6$ using DPPA under the conditions given in Table I. The finished peptide resin was treated with methanol in the presence of TEA to give Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly–OMe (VI) in 48% yield after purification by preparative thin layer chromatography. The homogeneity of the protected octapeptide obtained was assessed by thin layer chromatogrophy, acid hydrolysis, and elemental analysis.

Table I. Conditions for DPPA Coupling in the Solid–Phase Synthesis of Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly–resin[a]

Reactant[b,c]	Equivalent	Reaction time[d] (hr)
Boc–Tyr(Bzl)	3	2
Boc–Thr(Bzl)	3	4[e]
Boc–Phe	3	2
Boc–Ile·$1/2H_2O$	4	4[e]
Z–Phe–Val–Pro·$1/2C_6H_6$	3	6

a) 1.0 g of Boc–Gly–resin was used (Boc–Gly: 0.4 mM/g).
b) Equimolar amounts of DPPA and TEA were used.
c) After addition of Boc-amino acid or peptide fragment in DMF (5 ml), a solution of DPPA in DMF (0.5 ml) was added with ice-cooling, followed by the addition of TEA in DMF (0.5 ml).
d) The coupling was carried out at 0° for 30 min, then at room temperature.
e) After the coupling, the resin was washed with DMF (×3) and CH_2Cl_2(×3), then treated with acetic anhydride (0.5 ml) in CH_2Cl_2 (5 ml) at room temperature for 10 min.

Table II. Conditions for DEPC Coupling in the Solid–Phase Synthesis of Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly–resin[a]

Reactant[b,c]	Equivalent	Reaction time (hr)
Boc–Tyr(Bzl)	3	2
Boc–Thr(Bzl)	3	4
Boc–Phe	3	2
Boc–Ile·$1/2H_2O$	4	4
Z–Phe–Val–Pro·$1/2C_6H_6$	3	4

a) 2.0 g of Boc–Gly–resin (Boc–Gly: 0.8 mM) was used.
b) Equimolar amounts of DEPC and TEA were used.
c) After addition of Boc-amino acid or peptide fragment in DMF (8 ml), a solution of DEPC in DMF (2 ml) was added, followed by the addition of TEA in DMF (2 ml).
d) The coupling was conducted at 0° for 30 min, then at room temperature.

The protected octapeptide (VI) was similarly prepared in 68% yield by the solid-phase method with DEPC under the conditions given in Table II. Compound VI was hydrolyzed with sodium hydroxide in aqueous dioxane to give Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly (IV) in 70% yield.

For the synthesis of the protected tetrapeptide (Boc–Leu–Gln–Arg(NO_2)–Met–OMe (VII)), Boc–Arg(NO_2) was coupled with Met–OMe·HCl using DPPA in DMF to give Boc–Arg(NO_2)–Met–OMe in 84% yield. The Boc group was removed by treatment with methanolic hydrogen

13) B.F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
14) Solid-phase peptide synthesis was carried out using a reaction apparatus with a jacket to control the temperature, designed by the Tanabe research group: T. Mizoguchi, K. Shigezane, and N. Takamura, *Chem. Pharm. Bull.*, **18**, 1465 (1970).

chloride and the resulting hydrochloride of Arg(NO_2)–Met–OMe was coupled with Boc–Gln using DEPC in DMF to afford Boc–Gln–Arg(NO_2)–Met–OMe in 70% yield. Removal of the Boc group was done by treatment with TFA or HCl-dioxane. Similar attachment of Boc–Leu·H_2O to the TFA salt or the hydrochloride of Gln–Arg(NO_2)–Met–OMe using DPPA in DMF gave Boc–Leu–Gln–Arg(NO_2)–Met–OMe (VII) in 60—67% yield. Boc–Leu–Gln–Arg(NO_2)–Met (III) was obtained in 70% yield by hydrolysis of the corresponding methyl ester.

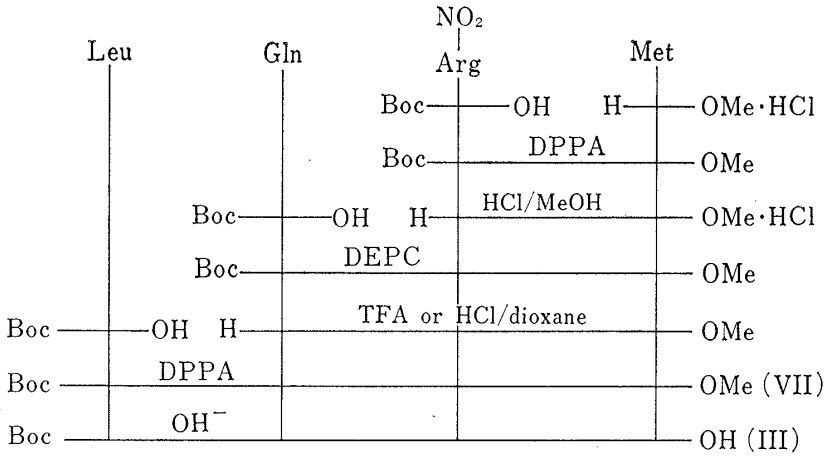

Chart 2. Synthetic Scheme for Boc–Leu–Gln–Arg(NO_2)–Met

For the preparation of the protected nonapeptide resin, attachment of Boc–Gln to the solid support was done by the reaction of Boc–Gln cesium salt and chloromethyl resin in DMF. Boc–Gln–resin (1.5 g Boc–Gln: 0.24 mM/g) was deprotected and converted to the glutaminyl resin as usual. Boc–Gly, Boc–Lys(2-Cl–Z), Boc–Asn, Boc–Arg(NO_2), Boc–Glu(OBzl), Boc–Lys(2-Cl–Z), Boc–Glu(OBzl), and Boc–Gln were successively coupled to the glutaminyl resin with DEPC under the conditions given in Table III. After the coupling of Boc–Gln(position 14), the resin was collected and dried *in vacuo*. Amino acid analysis of this protected nonapeptide resin after HCl-propionic acid (1: 1) hydrolysis at 130° for 4 hr[15] showed the following ratios: Glu 4.24, Arg 0.78, Lys 1.89, Asp 1.00, Gly 1.18 (average peptide content 0.15 mM/g based on Asp).

The protected nonapeptide resin (1.0 g) was placed in the synthesis apparatus and Boc–Leu–Gln–Arg(NO_2)–Met (III), Boc–Glu(OBzl), and Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr-(Bzl)–Gly(IV) were successively condensed with the nonapeptide resin using DEPC under the conditions given in Table III.

Amino acid analysis of the protected docosapeptide resin after HCl-propionic acid (1: 1) hydrolysis at 130° for 4 hr[15] showed the following ratios: Phe 1.03, Val 0.44, Pro 0.49, Ile 0.5, Thr 0.45, Tyr 0.13, Gly 2.03, Leu 0.54, Glu 5.00, Arg 1.19, Met 0.28, Lys 1.70, Asp 1.00, average peptide content 0.12 mM/g). The average content of fragments III and IV was about half with respect to the C-terminal nonapeptide.

The finished peptide resin (1.04 g) was treated with hydrogen fluoride[16] in the presence of anisole for removal of all the protecting groups and the polymer support. The resulting material was passed through Dowex 1-X4 (acetate form) and lyophilized to afford a crude powder (190 mg). After treatment of the crude product with thioglycolic acid to convert any methionine sulfoxide to methionine, the material was chromatographed on SP-Sephadex

15) J. Scotchler, R. Lozier, and A.B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
16) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

Table III. Conditions for DEPC Coupling in the Solid-Phase Synthesis of Motilin[a]

Reactant[b]	Equivalent	Reaction time (hr)[d]
Boc–Gly[c]	3	4
Boc–Lys(2-Cl–Z)[c]	3	6
Boc–Asn[c]	4(2)[e]	6(2)[e]
Boc–Arg(NO_2)[c]	4(2)	6(3)
Boc–Glu(OBzl)[c]	4(2)	6(3)
Boc–Lys(2-Cl–Z)[c]	4(2)	6(3)
Boc–Glu(OBzl)[c]	4(2)	6(3)
Boc–Gln[c]	4(2)	6(3)
Boc–Leu–Gln–Arg(NO_2)–Met (III)[f,g]	4(2)	30[h] (30)[h]
Boc–Glu(OBzl)[g]	4(2)	6(6)
IV[g]	3	49

a) 1.5 g of Boc–Gln–resin (Boc–Gln: 0.36 mM) was used.
b) Equimolar amounts of DEPC and TEA were used.
c) After addition of Boc-amino acid in DMF (7 ml), a solution of DEPC in DMF (0.5 ml) was added, followed by the addition of TEA in DMF (0.5 ml).
d) The coupling was conducted at 0° for 30 min, then at room temperature.
e) Double coupling and treatment with acetic anhydride were carried out. Numbers in parentheses represent equivalents of reactant and reaction time for the second coupling. After the coupling, the resin was treated with acetic anhydride (10 eq) and TEA (3 eq) in DMF at room temperature for 1 hr.
f) 1.0 g of the protected nonapeptide resin (peptide : 0.15 mM) was used.
g) After addition of Boc-amino acid or peptide fragment in DMF (4 ml), a solution of DEPC in DMF (0.5 ml) was added, followed by the addition of TEA in DMF (0.5 ml).
h) The coupling was conducted at 0°.

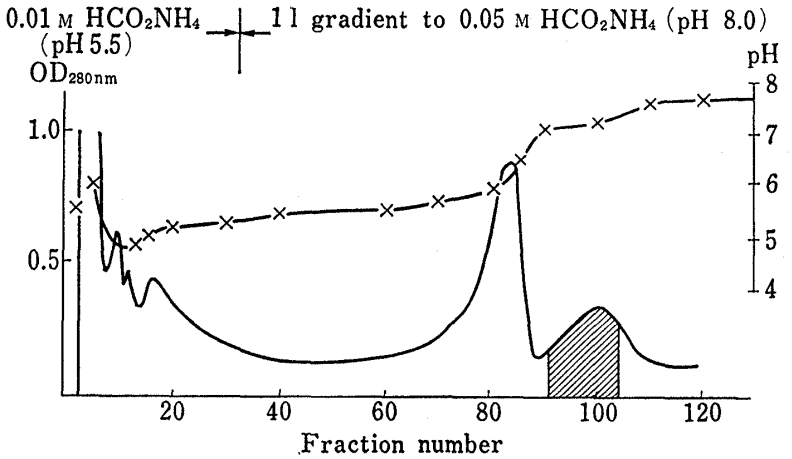

Fig. 1. Chromatography on SP-Sephadex C-25
Column, 1.5×30 cm; 6 ml fractions.

C-25 by a gradient elution with ammonium formate buffer. The UV absorbance of the fractions at 280 nm is shown in Fig. 1. The relative biological activity of individual fractions was examined using rabbit duodenum[17] throughout the purification process. The shaded area (tube Nos. 91—104) was collected and lyophilized to give a powder, which was applied to a column of Sephadex G-25 in 0.1 N acetic acid (Fig. 2). The shaded area (tube Nos. 32—39) was collected and lyophilized to furnish a white powder (22 mg). This material was chromato-

17) W. Domschke, U. Strunz, P. Mitznegg, H. Ruppin, S. Domschke, E. Schubert, E. Wünsch, E. Jaeger, and L. Demling, *Naturwissenschaften*, **61**, 370 (1974); U. Strunz, W. Domschke, P. Mitznegg, S. Domschke, E. Schubert, E. Wünsch, E. Jaeger, and L. Demling, *Gastroenterology*, **68**, 1485 (1975).

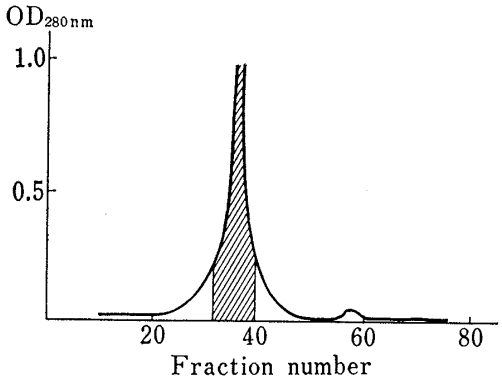

Fig. 2. Chromatography on Sephadex G-25

Column, 2.5×40 cm; 0.1 N AcOH; 4 ml fractions.

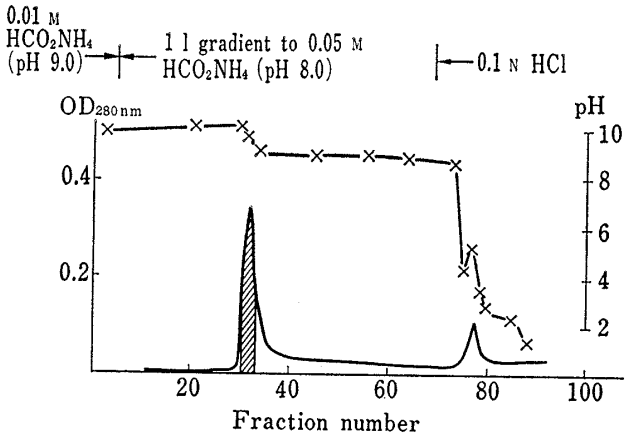

Fig. 3. Chromatography on QAE-Sephadex A-25

Column, 0.9×25 cm; 4 ml fractions.

graphed on QAE-Sephadex A-25 by a gradient elution with ammonium formate buffer (Fig. 3). The shaded area was collected and lyophilized to give a powder (8.1 mg) after desalting with Biogel P4 in 0.1 N acetic acid.

Rechromatography of this material on SP-Sephadex C-25 with ammonium formate buffer gave the results shown in Fig. 4. The shaded area was collected and lyophilized to give a powder, which was applied to a column of Sephadex G-25 in 0.1 N acetic acid (Fig. 5). The shaded area was collected and lyophilized to furnish purified motilin (3.05 mg) as a powder.

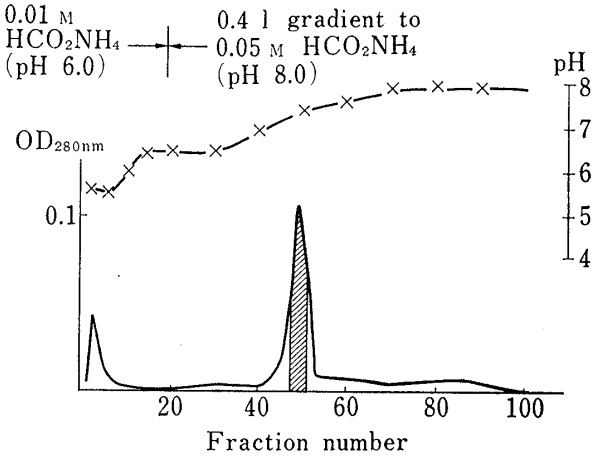

Fig. 4. Chromatography on SP-Sephadex C-25

Column, 0.9×10 cm; 3 ml fractions.

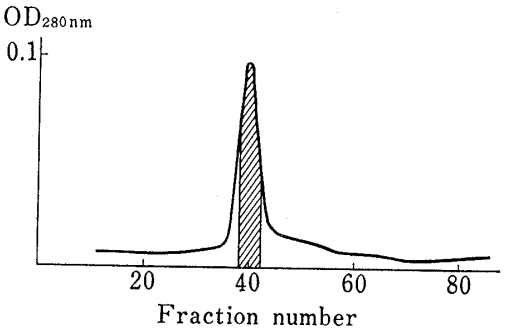

Fig. 5. Chromatography on Sephadex G-25

Column, 2.5×40 cm; 0.1 N AcOH; 3 ml fractions.

Amino acid analysis data for the purified synthetic preparation after both 6 N hydrochloric acid hydrolysis and enzymic digestion with amino peptidase (AP-M)[18] were in agreement with the expected values (Table IV). The purified material gave a single spot on thin layer chromatography (silica gel, BuOH: AcOH: pyridine: water=15: 10: 3: 12) with *Rf* 0.70 (positive to ninhydrin and the Sakaguchi test) and was essentially identical with the natural material, which was kindly provided by Professor J.C. Brown. Paper chromatogrophy and paper electrophoresis each gave a single ninhydrin-positive spot.

18) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966). AP-M of Rohm and Haas (Darmstadt), P.R.F. (191226), was employed.

Table IV. Amino Acid Analyses of Synthetic Motilin

Amino acid	Calcd	Found	
		Acid hydrolysis	AM–P digestion
Phe	2	1.77	1.95
Val	1	0.84	1.22
Pro	1	0.98	1.04
Ile	1	0.87	1.01
Glu	3	5.83	3.33
Gln	3	—	6.08[a]
Thr	1	0.92	
Asn	1	—	
Asp	0	1.05	0
Tyr	1	0.78	0.83
Gly	2	2.00	1.91
Leu	1	1.00	1.00
Arg	2	2.13	1.88
Met	1	0.95	0.99
Lys	2	2.22	1.82

a) Peaks of these amino acids were not completely separated.

Biological activity was determined by measuring rabbit duodenum, jejunum, and colon contractile activity *in vitro*.[17] Synthetic motilin showed a marked contractile activity on these organs (the threshold value, less than 5 ng/ml). In a preliminary experiment, it showed a potency similar to that of the natural hormone towards rabbit colon.

Experimental[19]

Z-Val-Pro-OMe——To a stirred mixture of Z–Val (1.25 g, 5 mM), Pro–OMe (0.97 g, 7.5 mM), and DPPA (1.375 g, 5 mM) in DMF (10 ml) was added TEA (0.505 g, 5 mM) in DMF (2 ml) at 0°. The mixture was stirred under ice-cooling for 4 hr, then at room temperature overnight. After dilution with AcOEt–benzene (4: 1, 300 ml), the mixture was successively washed with 10% aq. citric acid (×2), water (×1), sat. aq. NaCl (×1), sat. aq. $NaHCO_3$ (×2), water (×2), and sat. aq. NaCl (×2), then dried over Na_2SO_4 (this washing and drying process being described as "treated as usual"). Removal of the solvent by evaporation gave an oil, which was purified by column chromatography over silica gel (ether–hexane=4: 1) to give Z–Val–Pro–OMe as an oil (1.58 g, 90%), $[\alpha]_D^{20}$ −83° (c=1.6, MeOH). NMR (in $CDCl_3$): 1.0 (6H, t, $CH(C\underline{H}_3)_2$, J=6 Hz), 2.1 (5H, m), 3.7 (5H, $-OCH_3$, $-CH_2-$), 4.2—4.7 (2H, m, CH×2), 5.1 (2H, s, $C_6H_5C\underline{H}_2$), 5.75 (1H, NH), 7.3 (5H, s, C_6H_5). IR ν_{max}^{Nujol}, cm^{-1}: 3300, 1730, 1650. Z–Val–Pro–OMe was prepared analogously by the DEPC method, yield 86%, $[\alpha]_D^{20}$ −84° (c=1.6, MeOH).

Z-Phe-Val-Pro-OMe——A stirred solution of Z–Val–Pro–OMe (1.56 g, 4.3 mM) in MeOH (30 ml) was hydrogenated for 4 hr with 5% palladium on charcoal (0.4 g) in the presence of 29% methanolic hydrogen chloride (0.9 ml). After removal of the catalyst, the solvent was evaporated off *in vacuo* to give an oil, which was dried over KOH pellets *in vacuo*, and dissolved in DMF (10 ml). DPPA (1.3 g, 4.75 mM) and Z–Phe (1.42 g, 4.75 mM) were then added with ice-cooling, followed by the addition of TEA (0.91 g, 9.1 mM) in DMF. After stirring at 0° for 4 hr, then at room temperature overnight, the mixture was diluted with AcOEt–benzene (4: 1, 300 ml) and treated as usual. The solvent was evaporated off *in vacuo* to give a solid, which was purified by recrystallization from AcOEt–hexane to give Z–Phe–Val–Pro–OMe (1.84 g, 85%) as colorless crystals, mp 158—160°, $[\alpha]_D^{20}$ −72° (c=0.8, MeOH). *Anal.* Calcd for $C_{28}H_{35}N_3O_6$: C, 65.99; H, 6.92; N, 8.25. Found: C, 65.87; H, 6.99; N, 8.13.

Z–Phe–Val–Pro–OMe was prepared analogously by the DEPC method, yield 84%, mp 158—160°, $[\alpha]_D^{20}$ −73° (c=0.84, MeOH).

19) All melting points are uncorrected. IR spectral measurements were performed with a JASCO DS-402G infrared spectrometer and a JASCO IRA-1 grating infrared spectrometer. NMR spectra were measured with a JNM-PS 100 spectrometer and a Hitachi R-24 high resolution NMR spectrometer. All signals are expressed as ppm downfield from tetramethylsilane used as an internal standard. Optical rotations were measured with a YANACO OR-50 automatic polarimeter. UV spectra were measured with a Hitachi 124 spectrophotometer and a Hitachi EPS-3T spectrometer.

Z-Phe-Val-Pro-OH (V)——To a suspension of Z–Phe–Val–Pro–OMe (2.0 g, 3.9 mM) in acetone (20 ml) was added 1 N NaOH (4.7 ml). After stirring at room temperature for 5 hr, most of the solvent was removed *in vacuo* and the mixture was washed with AcOEt, then acidified with 10% aq. citric acid and extracted with AcOEt (×3). The AcOEt extracts were washed with 10% aq. citric acid and water. Drying followed by concentration gave an oil, which was crystallized from a small volume of benzene. Recrystallization from AcOEt–benzene gave V (1.68 g, 81%) as colorless crystals, mp 99—102°, $[\alpha]_D^{20}$ −49° (c=0.74, DMF) (lit.[9c] mp 93—95°, $[\alpha]_D^{20}$ −62° (c=1, MeOH)). *Anal.* Calcd for $C_{27}H_{33}N_3O_6 \cdot 1/2C_6H_6$: C, 67.39; H, 6.79; N, 7.86. Found: C, 67.48; H, 6.81; N, 7.74.

Preparation of Z-Phe-Val-Pro-Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly-OMe (VI). Boc-Gly-resin——Boc–Gly-resin was prepared from the cesium salt of Boc–Gly (0.55 g, 3.1 mM) and chloromethyl resin[20] (6.0 g, Cl: 1.7 mM/g) in DMF at 50° for 24 hr[13] (Boc–Gly: 0.4 mM/g, determined by the Porath method[1]).

a) The DPPA Method: Boc–Gly-resin (1.0 g, Boc–Gly: 0.4 mM) was placed in the apparatus, deprotected by treatment with TFA–CH_2Cl_2 (1: 2, 6 ml room temp., 30 min) and converted to the glycyl resin with TEA–DMF (0.4 g/6 ml, room temp., 10 min). To this resin were successively coupled Boc–Tyr (Bzl) (0.45 g, 1.2 mM), Boc–Thr(Bzl) (0.37 g, 1.2 mM), Boc–Phe (0.32 g, 1.2 mM), Boc–Ile·$1/2H_2O$ (0.39 g, 1.6 mM), and Z–Phe–Val–Pro·$1/2C_6H_6$ (0.64 g, 1.2 mM) with equimolar amounts of DPPA and TEA under the conditions given in Table I. After the coupling of Boc–Thr(Bzl) and Boc–Ile·$1/2H_2O$, the resin was treated with acetic anhydride (0.5 ml) in CH_2Cl_2 (5 ml) at room temperature for 10 min. The completed peptide resin was treated with MeOH (70 ml) in the presence of TEA (7 g) at room temperature for 24 hr. The resin was filtered and washed thoroughly with MeOH and $CHCl_3$. The solvent was removed *in vacuo* to give a solid (130 mg). After repeated treatment of the resin with MeOH (70 ml) containing TEA (7 g), 335 mg of solid was obtained. This crude material was purified by preparative TLC (silica gel, $CHCl_3$: MeOH: AcOEt= 15: 1: 4) to give VI as a crystalline powder (0.24 g, 48%), mp 206—210°, $[\alpha]_D^{20}$ −34.8° (c=0.5, $CHCl_3$), Rf 0.35 (TLC on silica gel, $CHCl_3$: MeOH: AcOEt=15: 1: 4). *Anal.* Calcd for $C_{72}H_{80}N_8O_{13} \cdot H_2O$: C, 67.06; H, 6.88; N, 8.69. Found: C, 67.09; H, 6.87; N, 8.69. Amino acid analysis of an acid hydrolysate; Phe 2.06, Val 0.97, Pro 1.00, Ile 0.97, Thr 0.95, Tyr 0.78, Gly 1.06. Average recovery 91%.

b) The DEPC Method: Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly-resin was similarly prepared from Boc–Gly-resin (2.0 g, Boc–Gly: 0.8 mM). To the glycyl resin were coupled Boc–Tyr(Bzl) (0.9 g, 2.4 mM), Boc–Thr(Bzl) (0.74 g, 2.4 mM), Boc–Phe (0.64 g, 2.4 mM), Boc–Ile·$1/2H_2O$ (0.77 g, 3.2 mM), and Z–Phe–Val–Pro·$1/2C_6H_6$ (1.28 g, 2.4 mM) with equimolar amounts of DEPC and TEA. After repeated treatment of the completed peptide resin with MeOH (70 ml) in the presence of TEA (7 g), the crude solid was reprecipitated from DMF–AcOEt–hexane to afford VI (0.7 g, 68%) as a crystalline powder, mp 208—211°. $[\alpha]_D^{20}$ −33.2° (c=0.55, $CHCl_3$). *Anal.* Calcd for $C_{72}H_{80}N_8O_{13} \cdot H_2O$: C, 67.06; H, 6.88; N, 8.69. Found: C, 67.12; H, 6.78; N, 8.70.

Z-Phe-Val-Pro-Ile-Phe-Thr(Bzl)-Tyr(Bzl)-Gly-OH (IV)——To a solution of Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly–OMe (VI, 1.0 g, 0.78 mM) in dioxane (17 ml) was added 1 N NaOH (0.94 ml) and the mixture was stirred at room temperature for 3 hr [after 1 hr, MeOH (5 ml) was added to dissolve the precipitate], then acidified with 10% citric acid. After removal of most of the dioxane *in vacuo*, 300 ml of chloroform was added and the whole was washed with 10% citric acid, H_2O, and sat. aq. NaCl, then dried over $MgSO_4$. Removal of the solvent by evaporation gave a white solid, which was reprecipitated from $CHCl_3$–AcOEt–hexane to afford IV (0.69 g, 70%) as a crystalline powder, mp 198—201°, $[\alpha]_D^{20}$ −23° (c=0.6, $CHCl_3$). Rf 0.45 (TLC on silica gel, $CHCl_3$: MeOH: H_2O=8: 3: 1). *Anal.* Calcd for $C_{71}H_{78}N_8O_{13} \cdot 1/2H_2O$: C, 67.33; H, 6.77; N, 8.85. Found: C, 67.14; H, 6.72; N, 8.89. Amino acid analysis of an acid hydrolysate: Phe 2.05, Val 0.91, Pro 0.92, Ile 0.90, Thr 0.94, Tyr 0.91, Gly 1.00. Average recovery 80%.

Boc-Arg(NO_2)-Met-OMe——To a stirred mixture of Boc–Arg(NO_2) (1.85 g, 5.65 mM), Met–OMe·HCl (1.35 g, 6.78 mM) and DPPA (1.55 g, 5.65 mM) in DMF (12 ml) was added TEA (1.25 g, 12.4 mM) in DMF (3 ml) with ice-cooling. The mixture was stirred with ice-cooling for 4 hr, and then at room temperature overnight. The mixture was diluted with AcOEt–benzene (4: 1, 500 ml) and treated as usual. After removal of the solvent, the resulting oil was kept in AcOEt–hexane to give Boc–Arg(NO_2)–Met–OMe (2.2 g, 84%) as colorless crystals, mp 121—122°, $[\alpha]_D^{20}$ −22° (c=1.1, MeOH). *Anal.* Calcd for $C_{17}H_{32}N_6O_7S$: C, 43.95; H, 6.94; N, 18.09. Found: C, 43.96; H, 9.03; N, 17.94.

Boc-Gln-Arg(NO_2)-Met-OMe——Boc–Arg(NO_2)–Met–OMe was treated with 18% methanolic hydrogen chloride at room temperature for 1 hr. After repeated evaporation to dryness with MeOH *in vacuo*, the residual oil was dried *in vacuo* over KOH pellets and then dissolved in DMF (30 ml). To this solution were added Boc–Gln (2.93 g, 11.9 mM) and DEPC (1.94 g, 11.9 mM) with ice-cooling, followed by the addition of TEA (2.16 g, 21.4 mM). The mixture was stirred with ice-cooling for 6 hr, and then at room temperature overnight. After removal of DMF *in vacuo* below 40°, the residue was diluted with AcOEt (1500 ml) and treated as usual. Removal of the solvent by evaporation gave a white solid, which was recrystallized from DMF–AcOEt to give Boc–Gln–Arg(NO_2)–Met–OMe (3.94 g, 70%) as colorless crystals, mp 120—123°, $[\alpha]_D^{20}$ −34° (c=0.65, MeOH). *Anal.* Calcd for $C_{22}H_{40}N_8O_9S \cdot 1/2H_2O$: C, 43.91; H, 6.86; N, 18.62. Found: C,

20) Chloromethyl resin (100—200 mesh) was purchased from the Protein Research Foundation.

44.18, H, 6.69; N, 18.44.

Boc-Leu-Gln-Arg(NO_2)-Met-OMe (VII)——a) Boc–Gln–Arg(NO_2)–Met–OMe (0.59 g, 1.0 mM) was treated with 4 N HCl–dioxane (3 ml) at room temperature for 1 hr. The solvent was removed *in vacuo*. After repeated evaporation with AcOEt, the resulting solid was dried *in vacuo* over KOH pellets, and dissolved in DMF (4 ml). To this solution were added Boc–Leu·H_2O (0.275 g, 1.1 mM) and DPPA (0.3 g, 1.1 mM) with ice-cooling, followed by the addition of TEA (0.21 g, 2.1 mM) in DMF (1 ml). The mixture was stirred with ice-cooling for 4 hr, and then at room temperature overnight. The mixture was diluted with AcOEt–benzene (4: 1, 800 ml) and treated as usual. Removal of the solvent by evaporation gave a solid, which was reprecipitated from MeOH–AcOEt–hexane to give VII (0.47 g, 67%) as a crystalline powder, mp 174—177°, $[\alpha]_D^{20}$ −43° (c=0.6, MeOH). *Anal.* Calcd for $C_{28}H_{51}N_9O_{10}S$: C, 47.64; H, 7.28; N, 17.86. Found: C, 47.35; H, 7.21; N, 17.61.

b) Boc–Gln–Arg(NO_2)–Met–OMe (1.78 g, 3 mM) was treated with TFA (7 ml) for 45 min with ice-cooling, and then diluted with dry ether (50 ml). The resulting precipitate was collected, washed with dry ether, dried *in vacuo* over KOH pellets and dissolved in DMF (12 ml). To this solution were added Boc–Leu·H_2O (0.81 g, 3.3 mM) and DPPA (0.91 g, 3.3 mM) with ice-cooling, followed by the addition of TEA (0.66 g, 6.6 mM) in DMF (1 ml). The mixture was stirred with ice-cooling for 6 hr, and then at room temperature overnight. After dilution with AcOEt–benzene (5: 1, 1200 ml) followed by treatment as usual, removal of the solvent gave a solid, which was reprecipitated from MeOH–AcOEt–hexane to give VII (1.28 g, 60%) as a crystalline powder, mp 174—177°, $[\alpha]_D^{20}$ −42° (c=0.8, MeOH).

Boc-Leu-Gln-Arg(NO_2)-Met-OH (III)——To a suspension of Boc–Leu–Gln–Arg(NO_2)–Met–OMe (1.0 g, 1.42 mM) in MeOH (10 ml) was added 1 N NaOH (1.7 ml). After stirring at room temperature for 5 hr, most of the MeOH was evaporated off below room temperature, and 4 ml of H_2O was added. The mixture was washed with AcOEt, then acidified with 10% citric acid, and extracted with AcOEt (200 ml, 100 ml, and 100 ml). The AcOEt extracts were washed with 10% citric acid and sat. NaCl, then dried over Na_2SO_4. Removal of the solvent by evaporation afforded a solid which was reprecipitated from MeOH–AcOEt–hexane to give III (0.705 g, 70%) as a crystalline powder, mp 161—164°, $[\alpha]_D^{20}$ −33° (c=0.56, MeOH). *Rf* 0.1 (TLC on silica gel, $CHCl_3$: MeOH: H_2O=8: 3: 1). *Anal.* Calcd for $C_{27}H_{49}N_9O_{10}S\cdot H_2O$: C, 45.68; H, 7.24, N, 17.76. Found: C, 45.91; H, 7.01; N, 17.53. Amino acid analysis of an acid hydrolysate: Leu 1.06, Glu 1.00, Arg 0.8, Met 0.51. Average recovery 81%.

Protected Peptide Resin of Porcine Motilin——Boc–Gln-resin was prepared by reaction of the cesium salt of Boc–Gln (0.64 g, 2.6 mM) and chloromethyl resin[20] (5.0 g, Cl: 1.7 mM/g) in DMF (35 ml) at 30° for 24 hr[13] (Boc–Gln: 0.24 mM/g, determined by the Porath method[1]). Boc–Gln–resin (1.5 g, Boc–Gln: 0.36 mM) was placed in the solid-phase synthesis apparatus and deprotected by treatment with TFA–CH_2Cl_2 (1: 1, 8 ml, room temperature, 30 min). It was converted to the glutaminyl resin with TEA–DMF (0.36 g/8 ml, at room temperature for 7 min, then same treatment at room temperature for 3 min). Protected amino acids were successively coupled to this resin with DEPC and TEA under the conditions given in Table III. In every coupling process involving the incorporation of Asn residue (position 20), a double coupling procedure followed by treatment with acetic anhydride (Ac_2O (10 eq), TEA (3 eq) in DMF (8 ml), room temperature, 1 hr) was carried out. After the coupling of the Gln residue (position 14), the resin was collected and dried *in vacuo* to furnish the protected nonapeptide resin (1.6 g). A portion of this resin was subjected to acid hydrolysis (12 N HCl: propionic acid=1: 1, 130°, 4 hr) and the ratio of amino acids was calculated by means of an amino acid analyzer.

The protected nonapeptide resin (1.0 g; average peptide content 0.15 mM/g based on amino acid analysis) was used for the synthesis of motilin. After deprotection (TFA–CH_2Cl_2=1: 1, 6 ml, at room temperature for 30 min) and neutralization (TEA–DMF=0.3 g/ 6 ml, at room temperature for 7 min, then the same treatment for 3 min), Boc–Leu–Gln–Arg(NO_2)–Met·H_2O (III), Boc–Glu(OBzl), and Z–Phe–Val–Pro–Ile–Phe–Thr(Bzl)–Tyr(Bzl)–Gly·H_2O (IV) were successively coupled to the peptide resin with DEPC and TEA in DMF according to the schedule shown in Table III. The completed peptide resin was collected and dried *in vacuo* (yield, 1.1 g). A portion of this resin was used for amino acid analysis after HCl-propionic acid (1: 1) hydrolysis.

Motilin——The completed peptide resin (1.04 g) was treated with HF (16 ml) at −20° for 40 min, and then at 0° for 40 min in the presence of anisole (1 ml). After removal of HF *in vacuo*, the resin was extracted with 10% AcOH (10 ml), filtered off and washed with 10% AcOH. The combined filtrates were washed with ether, and then placed on a 40 ml column of Dowex 1-X4 (acetate form) and eluted with water. The first 200 ml of eluate was collected and lyophilized to give a crude powder (190 mg). This powder was dissolved in H_2O (8 ml) and the pH of this solution was adjusted to 7.0 with 0.1 N NH_4OH. Two drops of thioglycolic acid were added and the mixture was kept at 40° for 2 hr. After adjusting the pH of the solution to 5.5, the solution was applied to a column of SP-Sephadex C-25 (1.5×30 cm) which had previously been equilibrated with 0.01 M ammonium formate buffer (pH 5.5). Elution was carried out first with the same buffer, and then with a gradient formed from 500 ml of 0.01 M ammonium formate, pH 5.5, and 500 ml of 0.05 M ammonium formate, pH 8.0. An elution profile is shown in Fig. 1. The eluate was collected in 6.0 ml fractions at a flow rate of 24 ml/hr. The absorbance of the fractions was measured at 280 nm and the relative contractive activity of each fraction was examined using rabbit duodenum *in vitro*[17] throughout the purifica-

tion process. Fractions 91—104 were collected and lyophilized to give a powder, which was dissolved in 0.1 N AcOH (3 ml). This solution was applied to a column (2.5 × 40 cm) of Sephadex G-25 (superfine) and developed with 0.1 N AcOH. Fractions of 4 ml were collected at a flow rate of 16 ml/hr. An elution profile is shown in Fig. 2. Fractions 32—39 were pooled and lyophilized to give a powder (22 mg). This powder was dissolved in H_2O (3 ml) and the pH of the solution was adjusted to 9.7 with 0.1 N NH_4OH. This solution was applied to a column (0.9 × 25 cm) of QAE-Sephadex A-25 which had been equilibrated with 0.01 M ammonium formate buffer, pH 9.0. Elution was carried out with the same buffer, followed by a gradient formed from 500 ml of 0.01 M ammonium formate, pH 9.0, and 500 ml of 0.05 M ammonium formate, pH 8.0. Fractions of 4 ml were collected at a flow rate of 16 ml/hr. An elution profile is shown in Fig. 3. Fractions 31—33 were pooled and lyophilized to give a powder (8.1 mg) after desalting with a column (2.5 × 40 cm) of Biogel P4 in 0.1 N AcOH. This material was dissolved in 3 ml of H_2O and the pH of the solution was adjusted to 5.0. This solution was rechromatographed on SP-Sephadex C-25 (0.9 × 10 cm) in 0.01 M ammonium formate (pH 6.0), followed by elution with a gradient formed from 200 ml of 0.01 M ammonium formate, pH 6.0, and 200 ml of 0.05 M ammonium formate, pH 8.0. Fractions of 3 ml were collected a flow rate of 12 ml/hr (Fig. 4). Fractions 48—51 were pooled and lyophilized to give a powder, which was dissolved in 0.1 N AcOH (2 ml). This solution was applied to a column (2.5 × 40 cm) of Sephadex G-25 (superfine) in 0.1 N AcOH, and fractions of 3 ml were collected at a flow rate of 12 ml/hr (Fig. 5). Fractions 39—42 were pooled, and repeated lyophilization of these fractions gave a white powder (3.05 mg, 1.8% in the purification step). *Rf* 0.70 (TLC on silica gel (Merck), BuOH: AcOH: pyridine: water = 15: 10: 3: 12). *Rf* 0.69 (TLC on cellulose powder (Eastmann), BuOH: AcOH: pyridine: water = 15: 6: 10: 12). *Rf* 0.46 (paper chromatography, Toyo filter paper 51A, BuOH: AcOH: pyridine: water = 15: 3: 10: 12). On paper electrophoresis (Toyo No. 51) in pyridine acetate buffer (pH 5.3, 350 V, 60 min) or formic-aetic acid buffer (0.8 N formic acid–1 N acetic acid = 1: 1, pH 2.0, 500 V, 30 min), the synthetic material gave a single ninhydrin-positive spot with *Rf* 0.21 or *Rf* 0.58 relative to arginine, respectively. Amino acid ratios in an acid hydrolysate (110°, 24 hr) and an AP-M digest are shown in Table IV. *Anal.* Calcd for $C_{120}H_{188}N_{34}O_{35}S \cdot 8CH_3COOH \cdot 14H_2O$: C, 47.60; H, 7.28; N, 13.88. Found: C, 47.67; H, 7.21; N, 13.66.

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