# Synthesis of Porcine Motilin by Fragment Condensation Using Three Protected Peptide Fragments[1)]

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Synthesis of porcine motilin is described. Construction of the entire molecule of this hormone was carried out by the fragment condensation method in solution employing Rudinger's azide modification, and the resulting protected docosapeptide was deblocked by sodium-liquid ammonia procedure. Purification of the final product was performed by ion exchange chromatography on CM-Sephadex using ammonium acetate as eluent and gel filtration on BiO-Gel P-6. Purity of the synthetic docosapeptide was assessed by TLC in two different solvent systems and amino acid analysis. This polypeptide showed gastric motoractivity stimulating action on 30 min intravenous infusion in dogs in 5 ng/kg/min doses, which is characteristic to porcine motilin.

Porcine motilin is a single chain peptide consisting of 22 amino acid residues.[2,3)] We have described in the preceding paper[4)] synthesis of the three protected peptide fragments 1, 2, and 3 corresponding to the amino acid sequences 1—6, 7—11, and 12—22 of the porcine motilin molecule, respectively (Chart 1). Using these fragments, our continuous synthetic studies on this gastrointestinal hormone have been focussed on its total synthesis by efficient means in regard to yield and purity of the products. The details of the synthesis are described in this communication. Syntheses of porcine motilin[5,6)] and its analogs, [13-Nle, 14-Glu]-motilin,[7)] by different approaches have been reported.

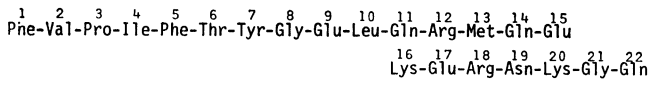
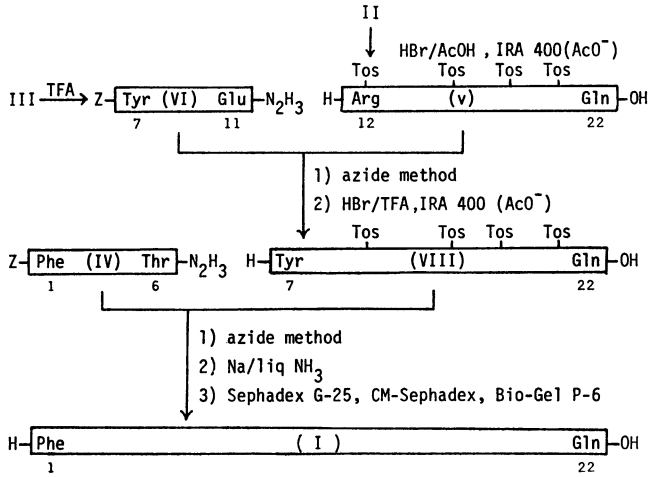



Chart 1. Primary structure of porcine motilin.

Construction of the entire molecule of porcine motilin was conducted by the fragment condensation method in solution employing Rudinger's azide modification.[8)] Scheme 1 shows the route for our synthesis of porcine motilin using the three protected peptide fragments 1, 2, and 3.

Z–Arg(Tos)–Met–Gln–Glu–Lys(Tos)–Glu–Arg(Tos)–Asn–Lys(Tos)–Gly–Gln–OH[4)] (Fragment 1) (II) was deblocked by HBr in acetic acid in the presence of anisole and ethyl methyl sulfide as scavengers. The resulting partially protected undecapeptide H–Arg(Tos)–Met–Gln–Glu–Lys(Tos)–Glu–Arg(Tos)–Asn–Lys(Tos)–Gly–Gln–OH (V) was coupled with the azide derived from Z–Tyr–Gly–Glu–Leu–Gln–NHNH–Boc[4)] (Fragment 2) (III) to give protected hexadecapeptide, Z–Tyr–Gly–Glu–Leu–Gln–Arg(Tos)–Met–Gln–Glu–Lys(Tos)–Glu–Arg(Tos)–Asn–Lys(Tos)–Gly–Gln–OH (VII). The crude product was distributed into *n*-BuOH and 10% AcOH. The *n*-BuOH layers were evaporated and the desired product was precipitated by addition of 10% AcOH. Removal of the Z-group from the protected hexadecapeptide (VII) was conducted with HBr in trifluoroacetic acid containing anisole and ethyl methyl sulfide as scavengers. The resulting partially protected hexadecapeptide hydrobromide was converted to the acetate (VIII) and coupled with the azide derived from Z–Phe–Val–Pro–Ile–Phe–Thr–$NHNH_2$[4)] (Fragment 3) (IV). The products were distributed into *n*-BuOH and 10% AcOH. Crude protected docosapeptide Z–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu–Leu–Gln–Arg(Tos)–Met–Gln–Glu–Lys(Tos)–Glu–Arg(Tos)–Asn–Lys(Tos)–Gly–Gln–OH (IX) was obtained from the *n*-BuOH layers.




Scheme 1. Synthesis of porcine motilin.

Deblocking of this material was carried out by the treatment of sodium in liquid ammonia. The tosyl groups protecting the side chain amino function of lysine and guanidino function of arginine and the Z-group attached to the N-terminal phenylalanine were simultaneously cleaved by this process. The resulting material was subjected to gel filtration on Sephadex G-25 and then to ion exchange chromatography on CM-Sephadex using as eluate $NH_4OAc$ buffer (pH 4.5) of linear gradient concentration. The desired product was eluated with 0.2—0.3 M $NH_4OAc$. The purified docosapeptide, after gel filtration on Bio-Gel P-6, gave the expected amino acid analysis with its acid hydrolysate and behaved as a single component on thin-layer chromatography in two solvent systems. Its stereohomogeneity was assessed by AP-M digestion.

This synthetic polypeptide showed gastric motoractivity stimulating action in the stomach and duodenum of dogs on 30 min intravenous infusion in 5 ng/kg/min

doses, which is characteristic to natural porcine motilin.

In the present investigation, our route for synthesis of porcine motilin using three protected peptide fragments was found to be efficient to obtain the hormone of high purity. In particular, use of sodium in liquid ammonia for deprotection of the final coupling product was effective in this synthesis.

## Experimental

The melting points were uncorrected. Optical rotations were measured on a JASCO DIP4 Automatic Polarimeter. Analytical samples were dried *in vacuo* over $P_2O_5$ at 60—70 °C for 20 h. Amino acid analysis was performed with a HITACHI Model KLA-3B amino acid analyzer. Acid hydrolysis of a sample for amino acid analysis was conducted with 6 M HCl at 110 °C for 24 h in a sealed tube. AP-M digestion[9] (400 mU AP-M/mg sample) was carried out in 0.05 M Tris-HCl buffer (pH 7.8) at 37 °C for 24 h. Designations of solvent systems for TLC on silica gel G (Merck) are: $R_f^I$ *n*-BuOH–AcOH–$H_2O$ (4 : 1 : 5) upper layer; $R_f^{II}$ *n*-BuOH–pyridine–AcOH–$H_2O$ (30 : 20 : 6 : 24). All solvents were of reagent grade and were distilled before use. Evaporations were carried out *in vacuo* at 40—45 °C in rotary evaporaters.

*Z–Tyr–Gly–Glu–Leu–Gln–Arg(Tos)–Met–Gln–Glu–Lys(Tos)–Glu–Arg(Tos)–Asn–Lys(Tos)–Gly–Gln–OH (VII).* To a solution of II (0.517 g) in glacial AcOH (2 ml) was added 25% HBr solution in AcOH (3 ml) containing anisole (0.1 ml) and ethyl methyl sulfide (0.1 ml). The solution was kept at 20 °C for 1 h and peroxide-free anhydrous ether was added. The resulting precipitate was collected, washed with ether and dried over KOH. The material was dissolved in *n*-BuOH–MeOH–$H_2O$ (1 : 1 : 1) (100 ml) and the solution was passed through IRA-400 column (acetate form). The solvents were evaporated and the residue was lyophilized to give V; yield, 0.303 g (67.2%), $R_f^I$ 0.29.

A solution of III (0.257 g) in TFA (1 ml) was kept at 20 °C for 30 min and addition of anhydrous ether gave precipitate, which was collected by filtration and dried over KOH. This material was dissolved in DMF (3 ml), and the solution cooled at −15 °C, and 6 M HCl in dioxane (0.25 ml) and 10% isopentyl nitrite in DMF (0.44 ml) were added. The mixture was left at −10 °C for 5 min and neutralized with 10% TEA in DMF (2.50 ml). To the solution was added an ice-cold solution of V (0.303 g) in DMF (5 ml) containing TEA (0.06 ml). The mixture was stirred at 4 °C for 20 h. An additional azide prepared from III (0.257 g) was added and the reaction mixture was stirred at 4 °C for further 20 h. After evaporation of solvents, the product was distributed into *n*-BuOH and 10% AcOH in 10 separatory funnels, and the *n*-BuOH layers containing the desired material was evaporated. To the residue was added 10% AcOH to give precipitate, which was collected by filtration, washed with MeOH and dried; yield, 0.190 g (46.2%); mp 120 °C (dec); $[\alpha]_D^{16}$ −21.0° (*c* 0.50 DMF); $R_f^I$ 0.40, $R_f^{II}$ 0.62. Found: C, 50.18; H, 6.03; N, 13.43%. Calcd for $C_{118}H_{166}O_{38}N_{28}S_5 \cdot 4H_2O$: C, 50.30; H, 6.22; N, 13.92%.

*H–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu–Leu–Gln–Arg–Met–Gln–Glu–Lys–Glu–Arg–Asn–Lys–Gly–Gln–OH (I) (Porcine motilin).* Compound VII (0.170 g) was dissolved in TFA (5 ml) containing anisole (0.1 ml) and ethyl methyl sulfide (0.1 ml), which was bubbled with HBr gas at 0 °C for 15 min and then at 20 °C for 15 min. The reaction mixture was allowed to stand at 20 °C for 30 min, and peroxide-free anhydrous ether was added. The resulting precipitate was collected by filtration and dried. The amorphous material was dissolved in *n*-BuOH–MeOH–$H_2O$ (1 : 1 : 1) (50 ml) and the solution was passed through IRA-400 column (acetate form). The solvents were evaporated and the residue was lyophilized to yield VIII as acetate, $R_f^I$ 0.38. A solution of IV (0.160 g) in DMF (3 ml) was cooled to −15 °C and 6 M HCl in dioxane (0.16 ml) and 10% isopentyl nitrite in DMF (0.28 ml) were added. After 1 min, the solution was neutralized with 10% TEA in DMF (1.28 ml) and mixed with an ice-cold solution of the above partially deblocked material VIII in DMF (4 ml) containing TEA (0.04 ml). The mixture was stirred at 4 °C for 20 h. An additional azide prepared from IV (0.120 g) was added and the reaction mixture was stirred at 4 °C for further 20 h. After evaporation of solvents, the product was distributed into *n*-BuOH and 10% AcOH in 5 separated funnels and the *n*-BuOH layers containing the desired material were evaporated. Ethyl acetate was added to the residual solution to give amorphous material, which was reprecipitated from MeOH with ethyl acetate to give IX.

The protected docosapeptide, IX, was dissolved in liquid ammonia (20 ml) and small pieces of Na were added until the blue color of the reaction mixture was maintained for 2 min. Ammonium chloride (1.0 g) was added and ammonia was evaporated off. The residue was dissolved in 50% AcOH and applied to a Sephadex G-25 column (3.1×190 cm) which was eluted with the same solvent. Fractions of 15 g each were collected. The desired material was located in the tubes No. 36—46. The solvents of these fractions were evaporated and the residue was dissolved in 0.01 M $NH_4OAc$ (pH 4.5) (5 ml). The solutions were applied to a column of CM-Sephadex C-25 (2.0×5 cm) equilibrated with the buffer. The column was washed with 0.01 M $NH_4OAc$ (pH 4.5) (200 ml) and then eluted with linear gradient of $NH_4OAc$ (pH 4.5) from 0.01 M to 0.5 M using 0.01 M (500 ml) and 0.5 M (500 ml) of the buffer. Fractions of 10 g each were collected. Each fraction was examined by TLC in two different solvent systems and UV absorption at 278 nm. The desired material with minor contamination was eluted in the tubes No. 65—85. The eluates were collected and the solvent was evaporated. After repeated lyophilization, the product was dissolved in 50% AcOH and applied on Bio-Gel P-6 column (3.2×190 cm), which was eluted with the same solvent. Fractions of 10 ml each were collected. The desired peptide behaving as a single component on TLC was located in tubes No. 64—75. These were pooled and lyophilized; yield, 49 mg. $[\alpha]_D^{20}$ −53.7° (*c* 0.49 $H_2O$); $R_f^I$ 0.15, $R_f^{II}$ 0.43; amino acid ratios in acid hydrolysate: Phe, 1.87; Val, 0.96; Pro, 0.96; Ile, 1.04; Thr, 0.94; Tyr, 0.97; Gly, 2.00; Glu, 5.60; Leu, 1.07; Arg, 2.00; Met, 0.77; Lys, 2.13; Asp, 1.00 (average recovery 88%); amino acid ratios in AP-M digest[10]: Phe, 2.18; Ile, 1.22; Tyr, 0.97; Gly, 2.08; Glu, 2.90; Leu, 1.01; Arg, 1.69; Met, 0.84; Lys, 2.10 (recovery 81%) (Asn, Gln and Thr were not estimated and Val and Pro were found in trace).[11,12] Found: C, 47.45; H, 7.39; N, 15.38%. Calcd for $C_{120}H_{188}O_{35}N_{34}S \cdot CH_3COOH \cdot 18H_2O$: C, 47.52; H, 7.45; N, 15.44%.

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## References

1) The amino acids except glycine are L-configuration.

Abbreviations used are: Z, benzyloxycarbonyl; Tos, *p*-toluenesulfonyl; HBr, hydrogen bromide; Boc, *t*-butoxycarbonyl; *n*-BuOH, 1-butanol; HCl, hydrogen chloride; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; TEA, triethylamine; AP-M, aminopeptidase-M.

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