Peptide Synthesis in Aqueous Solution. II. Synthesis and Biological Activity of a Molluscan Neuropeptide, FMRFamide (Phe-Met-Arg-Phe-NH_2) Analogs for N-Terminal Moiety

Katsushige KOUGE, Haruko SOMA,† Yukari KATAKAI,† Hideo OKAI,*,†
Mayumi TAKEMOTO,†† and Yojiro MUNEOKA††
Sanshin Chemical Industrial Co. Ltd., 531, Nagahama, Hirao-cho, Kumage-gun, Yamaguchi 742-11
†Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Shitami, Saijo-cho, Higashihiroshima 724
††Physiological Laboratory, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashisenda-machi, Naka-ku, Hiroshima 730
(Received May 14, 1987)

In order to elucidate the contribution of the N-terminal moiety (Phe[1] or Met[2]) of FMRFamide to the activity, 16 kinds of FMRFamide analogs were synthesized and their structure-activity relations are discussed. From the results, it was found that hydrophobic or bulky group in N-terminal contributes to the contractile effect, while the precise length of the side chain of amino acid at 2-position is due to a relaxing effect.

Previously, we reported that (*p*-hydroxyphenyl)dimethylsulfonium methyl sulfate(HODMSP·$MeSO_4^-$) active ester[1] enabled the formation of a peptide bond in aqueous solution and was applicable for the synthesis of a molluscan neuropeptide, FMRFamide (Phe-Met-Arg-Phe-NH_2),[2] which was isolated from the ganglia of the clam *Macrocallista nimbosa* by Price and Greenberg.[3] Particularly, this active ester method was recognized as an effective process for the coupling reaction with a peptide containing Arg, which has the free guanidine group. As a further utility of this method, we next tried to apply it to a synthetic study of the structure-activity relations of FMRFamide. FMRFamide is known to have diverse effects on molluscan muscles and neurons. In the anterior byssus retractor muscle of *Mytilus*, FMRFamide relaxes catch tension induced by acetylcholine at concentrations 10^{-8}—10^{-7} M[4] (1M=1 mol dm^{-3}) and elicits a contraction by a direct action on the muscle fibers at concentrations higher than 10^{-7} M,[5] as shown in Fig. 1.

With regard to the structure-activity relations of this peptide for these actions, Muneoka and Saitoh[6] tested a number of FMRFamide analogs and other peptide with biological actions in mammals, arthropods, and molluscans. From their report, we can draw an interesting conclusion that at least the structure of Arg^3-Phe^4-NH_2 moiety of FMRFamide is necessary for the production of both contraction and relaxation. Probably, this moiety will act as the binding unit to FMRFamide receptor. However, the role of the Phe^1 or Met^2 residue of N-terminal moiety in FMRFamide to these productions was not fully characterized.

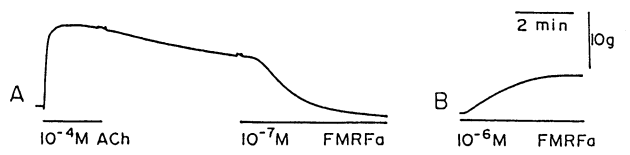


Fig. 1. Contractions and Relaxtations Produced by FMRFamide. A: Relaxing action of FMRFamide on ACh-induced catch tension. B: Contractile action of FMRFamide. ACH: Acetylcholine, FMRFa: FMRFamide.

In order to elucidate the contribution of Phe^1 or Met^2 to the activity, we synthesized 16 kinds of

Table 1. Contractile and Relaxing Effect of Analogs for N-Terminal Phe

Peptide		Contractile effect/M	Relaxing effect/M
Phe-Met-Arg-Phe-NH_2 (FMRFamide)	(**13a**)	10^{-7}	10^{-8}
Gly-Met-Arg-Phe-NH_2	(**13b**)	10^{-4}	—
Gly-Gly-Met-Arg-Phe-NH_2	(**13c**)	10^{-7}	—
Phe-Gly-Met-Arg-Phe-NH_2	(**13d**)	10^{-6}	—
Phe-Phe-Met-Arg-Phe-NH_2	(**17**)	10^{-7}	—
Phe-Phe-Gly-Met-Arg-Phe-NH_2	(**13e**)	10^{-6}	—
Ac-Phe-Met-Arg-Phe-NH_2	(**14**)	10^{-8}	—
Bz-Phe-Met-Arg-Phe-NH_2	(**15**)	10^{-8}	—

Table 2. Contractile and Relaxing Effect of Analogs for Met^2

Peptide	Contractile effect/M	Relaxing effect/M
Phe-Met-Arg-Phe-NH_2 (**13a**) (FMRFamide)	10^{-7}	10^{-8}
Phe-Gly-Arg-Phe-NH_2 (**23a**)	10^{-4}	—
Phe-Ala-Arg-Phe-NH_2 (**23b**)	10^{-5}	—
Phe-Abu-Arg-Phe-NH_2 (**23c**)	10^{-4}	—
Phe-Val-Arg-Phe-NH_2 (**23d**)	—	10^{-7}
Phe-Leu-Arg-Phe-NH_2 (**23e**)	10^{-6}	10^{-7}
Phe-Ile-Arg-Phe-NH_2 (**23f**)	10^{-5}	—
Phe-Nle-Arg-Phe-NH_2 (**23g**)	10^{-7}	10^{-8}
Phe-Phe-Arg-Phe-NH_2 (**23h**)	10^{-7} a)	—
Phe-Pro-Arg-Phe-NH_2 (**23i**)	10^{-5}	—

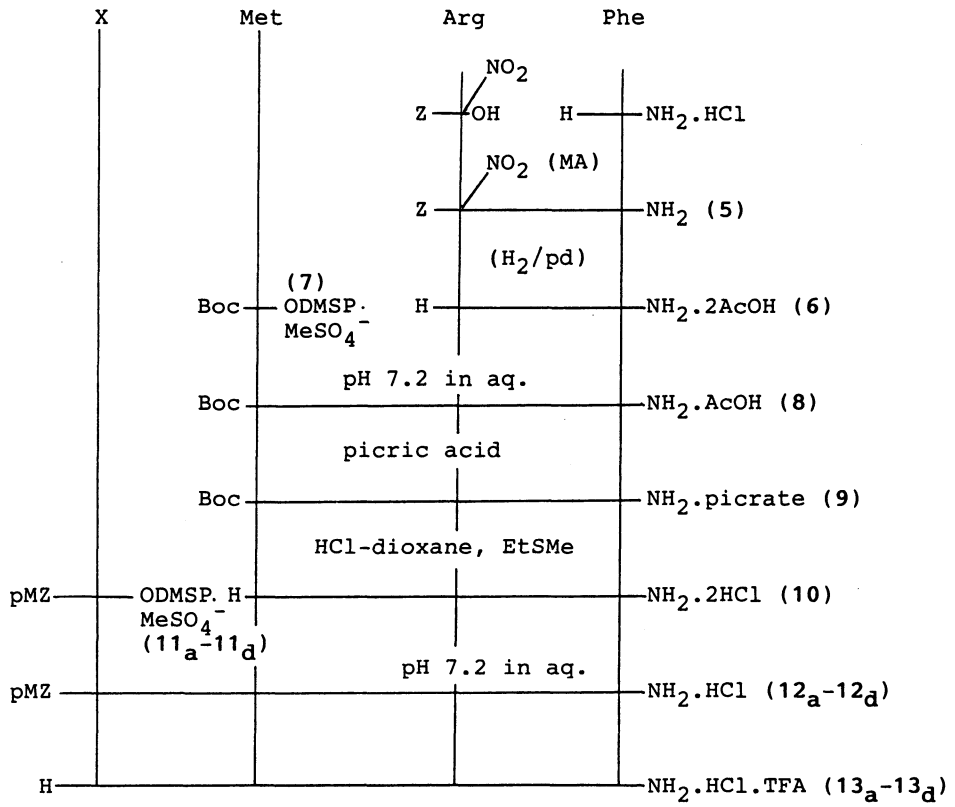
a) The contractile effect of this peptide was stronger than that of FMRFamide.

FMRFamide analogs shown in Table 1 and Table 2 and discussed their structure-activity relations of FMRFamide on a molluscan muscle.

The synthetic route for the FMRFamide analogs for Phe[1] is shown in Fig. 2. Z-Arg(NO_2)-OH was coupled with H-Phe-NH_2·HCl by means of mixed anhydride (MA) method to yield Z-Arg-(NO_2)-Phe-NH_2 (**5**). Removal of Z-group and NO_2-group from **5** by catalytic hydrogenation afforded the corresponding dipeptide amide (**6**). Boc-Met-OH was esterified by DCC and HODMSP·$MeSO_4^-$ to afford the water-soluble active ester (**7**). In aqueous solution, **7** was allowed to react with **6** in the presence of 1 M Na_2CO_3 at pH 7.2. To the reaction mixture containing the Boc-tripeptide amide (**8**), 1% picric acid was added and then the resulting Boc-tripeptide amide picrate (**9**) was isolated by extraction with ethyl acetate. The Boc-group was removed from **9** with trifluoroacetic acid and then hydrogen chloride in the presence of ethyl methyl sulfide to yield tripeptide amide (**10**). N-protected amino acid or peptide, which was prepared by the usual method, was esterified by DCC and HODMSP·$MeSO_4^-$ to afford the water-soluble active ester (**11**$_a$—**11**$_e$), which was allowed to react with **10** in the same manner as described for **8** to yield N-protected peptide amide (**12**$_a$—**12**$_e$). Finally the *p*-methoxybenzyloxycarbonyl group (pMZ-group) was removed with trifluoroacetic acid and then hydrogen chloride respectively, to yield desired peptides (**13**$_a$—**13**$_e$).

The synthetic route to **14**, **15**, and **17** is shown in Fig. 3. **14** or **15** was prepared by the coupling reaction of **10** and Ac-Phe-ODMSP·$MeSO_4^-$ or Bz-Phe-ODMSP·$MeSO_4^-$ in aqueous solution at pH 7.2, respectively. In the synthesis of **17**, pMZ-Phe-ODMSP·$MeSO_4^-$ was coupled with FMRFamide (**13**$_e$) in aqueous solution at pH 7.2 to yield **16**. The pMZ-group was removed from **16** with trifluoroacetic acid and then hydrogen chloride in the presence of ethyl methyl sulfide to yield **17**.

FMRFamide analogs for Met[2] were synthesized by the synthetic route, as shown in Fig. 4. The desired tetrapeptides (**23**$_a$—**23**$_i$) were prepared by stepwise



X: Phe(a), Gly(b), Gly-Gly(c), Phe-Gly(d), Phe-Phe-Gly(e).

-ODMSP.$MeSO_4^-$:

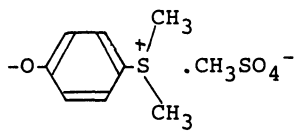


Fig. 2. Synthesis of FMRFamide Analogs for N-Terminal Phe.

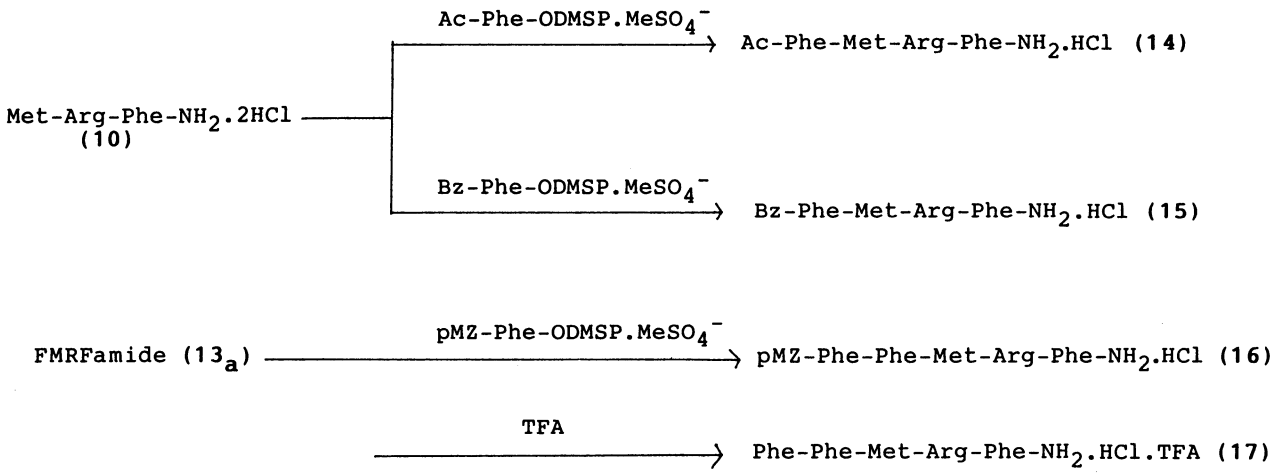
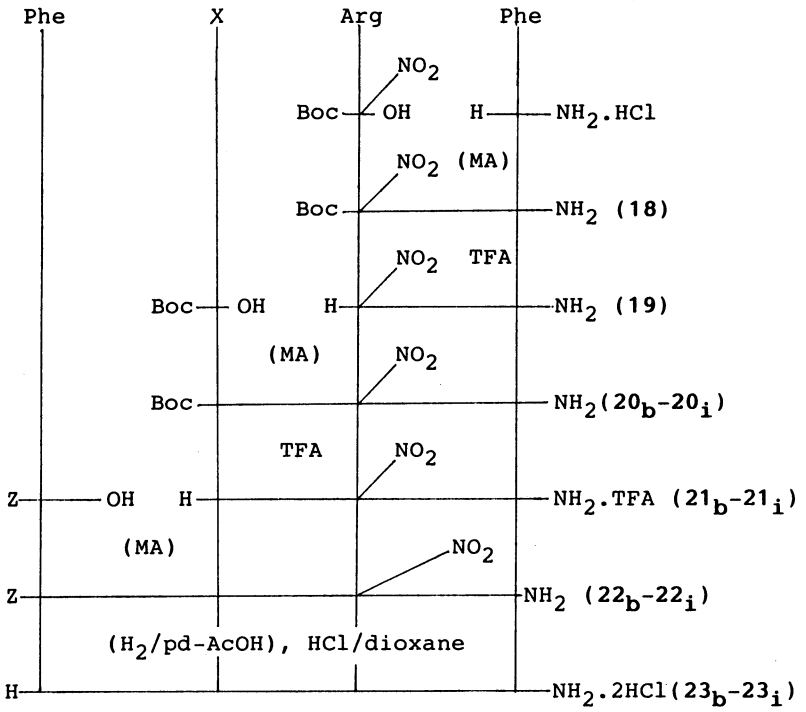


Fig. 3. Synthesis of FMRFamide Analogs for N-Terminal Phe.



X: Ala(b), Abu(c), Val(d), Leu(e), Ile(f), Nle(g), Phe(h), Pro(i).

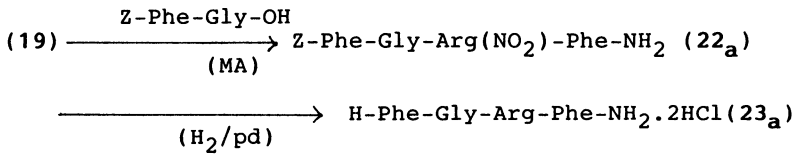


Fig. 4. Synthesis of FMRFamide Analogs for Met[2].

elongation from the C-terminal. Details of the synthetic route to those peptides is described in the experimental part.

In the synthesis of **23a**, Z-Phe-Gly-OH was coupled with **19** to yield **22a**. **22a** was then hydrogenated to give **23a**. The purity of synthetic peptides and their intermediates was confirmed by TLC with two solvent systems and by elemental analysis. The homogeneity of the final products was also confirmed by amino acid analysis.

Contractile effects and catch-relaxing effects of synthetic peptides on the anterior byssus retractor muscle of Mytilus were determined,[7] as shown in Tables 1 and 2. All the synthesized analogs of FMRFamide for Phe[1] producted only contraction and had no relaxing activity, as shown in Table 1. In the contractile activity, substitution of Gly for N-terminal Phe (**13$_b$**) showed a decrease in contractile potency. But, extension of **13$_b$** by attaching Gly (**13$_c$**) was almost equipotent to FMRFamide. When N-terminal Phe was separated from Met[2] by inserting a Gly residue and was replaced with Phe-Phe or Phe-Phe-Gly residue in FMRFamide, these peptides (**13$_d$**, **17**, and **13$_e$**) did not substantially change contractile activity. N-terminal acylation of FMRFamide with acetyl group or benzoyl group was slightly more active. These results indicate that hydrophobic or bulky group in N-terminal clearly contributes to the contractile effect of the peptide and the precise structure is not required for contractile activity.

In the analogs of FMRFamide for Met[2], Muneoka and Saitoh already reported that Nle can be substituted for Met[2].[6] We also recognized that this analog (**23$_g$**) had both contractile and relaxing activity and each activity was comparable with that of FMRFamide, as shown in Table 2. Substitution of Leu for Met[2] showed a slight decrease in contractile and relaxing potency. In contrast, Gly, Ala, Abu, Ile, Phe, and Pro could not be substituted for Met[2]; these peptides had no relaxing activity. The most remarkable fact was that the peptide (**23$_d$**), in which Val was substituted for Met[2], had only a relaxing activity. The results described above indicate that the precise length of the side chain of amino acid at 2-position, in addition to the hydrophobic group of N-terminal, contributes to the relaxing activity. In conclusion, we could biologicaly synthesize active peptides, which produced a contraction or a relaxation separately. From the viewpoint of pharmacology, we expect that these findings give an important clue to the designing effective relaxing peptides. For the structure-activity relations of FMRFamide, further studies are now in progress.

Experimental

All the melting points were uncorrected. The optical rotations were measured on a Union PM-101 polarimeter. TLC was carried out on Merck Silicagel G: R_{f_1}, 1-butanol-acetic acid-pyridine-water (4:1:1:2, v/v), R_{f_2}, $CHCl_3$-methanol (5:1, v/v), R_{f_3}, 1-butanol-acetic acid-water (3:3:2, v/v). Spots of materials possessing free amino group on TLC plate were detected by spraying with ninhydrin, and those of amino group blocked materials by spraying with 25% HBr in acetic acid and then ninhydrin. Amino acid analysis was performed by HPLC using JASCO TRI ROTAR-V and UVIDEC-100V apparatus.

Synthesis of Analogs for Phe[1]. Z-Arg(NO_2)-Phe-NH_2 (5): A solution of Z-Arg(NO_2)-OH (3.53g, 10 mmol) and NMM (1.1ml, 10 mmol) in THF (20 ml) was chilled to −5 °C, then ECF (1 ml, 10 mmol) was added to it. After 10 min, a precooled solution of H-Phe-NH_2·HCl (2.00 g, 10 mmol) and Et_3N (1.4 ml, 10 mmol) in $CHCl_3$ (20 ml) was added. The reaction mixture was refrigerated for 1 h and then allowed to stand overnight at room temperature. The mixture was concentrated in vacuo, and the solid was suspended in hot methanol (50 ml). The solid was filtered and washed with methanol; Yield 4.24g (85%); mp 218—221 °C; $[\alpha]_D^{20}$ −14° (*c* 0.5, DMF); R_{f_1} 0.91; R_{f_2} 0.41; Found: C, 55.56; H, 5.70; N, 19.38%; Calcd for $C_{23}H_{29}O_6N_7$: C, 55.34; H, 5.81; N, 19.63%.

H-Arg-Phe-NH_2·2AcOH (6): Compound **5** (2.5g, 5 mmol) in acetic acid (10 ml) was hydrogenated in the presence of palladium black. The filtrate was concentrated in vacuo and the residue was crystallized from ether as hygroscopic crystals; Yield 1.08 g (98%); $[\alpha]_D^{20}$ +9° (*c*1, H_2O); R_{f_1} 0.41; R_{f_3} 0.75.

Boc-Met-ODMSP·$MeSO_4^-$ (7) (Water-Soluble Active Ester): To a chilled solution of Boc-Met-OH (2.49g, 10 mmol) and HODMSP·$MeSO_4^-$ (2.66 g, 10 mmol) in CH_3CN (80 ml), DCC (2.06 g. 10 mmol) was added. The reaction mixture was held overnight at 0 °C and the DCurea which formed was filtered off. The filtrate was concentrated in vacuo, and the residual oil was washed with ether by decantation; Yield 3.93 g (80%); $[\alpha]_D^{20}$ −30° (*c*1, MeOH); R_{f_1} 0.82; R_{f_2} 0.34.

Boc-Met-Arg-Phe-NH_2·Picrate (9): Compound **6** (0.88 g, 2 mmol) was dissolved in H_2O and the pH of the solution was adjusted to 7.2 by addition of Et_3N. Boc-Met-ODMSP·$MeSO_4^-$ (**7**) (0.99 g, 2 mmol) was added to the stirred solution below 20 °C, the pH being maintained automatically at 7.2 with Et_3N. After 12 h, 1% picric acid was added to the solution and the pH of the solution was adjusted to 5.6. A yellow precipitate was filtered off and it was dissolved in ethyl acetate (100 ml). The solution was washed successively with 4% sodium hydrogencarbonate, 4% citric acid and water, and then dried over anhydrous sodium sulfate. The solution was concentrated to an oily residue which was crystallized by addition of ether. It was recrystallized from $CHCl_3$; Yield 0.75 g (45%); mp 110 °C (decomp); $[\alpha]_D^{20}$ −14° (*c* 0.5, DMF); R_{f_1} 0.83; R_{f_2} 0.32; Found C, 46.84; H, 5.81; N, 17.95%; Calcd for $C_{25}H_{41}O_5N_7S \cdot C_6H_3N_3O_7 \cdot 1/2H_2O$; C, 47.17; H, 5.70; N, 17.74%; Amino acid ratios in acid hydrolyzate: Arg 0.94, Met 0.89, Phe 1.00.

H-Met-Arg-Phe-NH_2·2HCl (10): To a solution of **27** (0.78g, 1 mmol) and ethyl methyl sulfide, 4M HCl in dioxane (5 ml) was added. The solution was allowed to stand for 1 h at room temperature, and then concentrated in vacuo. The residue was crystallized with ether; Yield 0.47 g (90%); mp 140 °C (decomp); $[\alpha]_D^{20}$ +10° (*c* 0.5, H_2O); R_{f_1} 0.52; R_{f_3} 0.74; Found: C, 49.03; H, 6.87; N, 20.01%; Calcd for $C_{20}H_{33}O_3N_7S \cdot 2HCl$; C, 49.25; H, 6.97; N, 20.09%; Amino acid ratios in acid hydrolyzate: Arg 0.96, Met 0.84, Phe 1.00.

pMZ-X-ODMSP·$MeSO_4^-$ (11$_a$—11$_d$) (Water-Soluble Active Ester): pMZ-Phe-ODMSP·$MeSO_4^-$ (**11$_a$**), pMZ-Gly-ODMSP·$MeSO_4^-$ (**11$_b$**), pMZ-Gly-Gly-ODMSP·$MeSO_4^-$ (**11$_c$**), pMZ-Phe-Gly-ODMSP·$MeSO_4^-$ (**11$_d$**), pMZ-Phe-Phe-Gly-ODMSP·$MeSO_4^-$ (**11$_e$**), Ac-Phe-ODMSP·$MeSO_4^-$, and Bz-Phe-ODMSP·$MeSO_4^-$ were also prepared from the corresponding carboxyl components and HODMSP·$MeSO_4^-$ by the same method as **7**.

11$_a$: Yield 90%; R_{f_1} 0.85; R_{f_2} 0.51.

11$_b$: Yield 85%; R_{f_1} 0.70; R_{f_2} 0.39.

11$_c$: Yield 90%; R_{f_1} 0.72; R_{f_2} 0.38.

11_d : Yield 88%; R_{f_1} 0.82; R_{f_2} 0.45.

11_e : Yield 80%; R_{f_1} 0.85; R_{f_2} 0.51.

Ac-Phe-ODMSP·$MeSO_4^-$: Yield 82%; R_{f_1} 0.81; R_{f_2} 0.42.

Bz-Phe-ODMSP·$MeSO_4^-$: Yield 88%; R_{f_1} 0.88; R_{f_2} 0.55.

Since these compounds were obtained as the oily products, they were soon employed for the next reactions.

pMZ-X-Met-Arg-Phe-NH_2·HCl (12_a—12_d and 14—16): The synthesis of pMZ-Gly-Met-Arg-Phe-NH_2·HCl (**12_b**) is as follows. Compound **10** (0.52 g, 1 mmol) was dissolved in H_2O (10 ml) and the pH of the solution was adjusted to 7.2 by addition of 1M Na_2CO_3. pMZ-Gly-ODMSP·$MeSO_4^-$ (**11_b**) (0.59 g, 1.2 mmol) was added to the stirred solution below 20 °C, the pH being maintained automatically at 7.2 with 1M Na_2CO_3. After 12 h, the crude product was precipitated from the reaction mixture. It was filtered and then washed with 4% sodium hydrogencarbonate and water. It was recrystallized from methanol; Yield 0.23 g (32%); mp 146 °C (decomp); $[\alpha]_D^{20}$ −20° (*c* 0.5, DMF); R_{f_1} 0.75; R_{f_3} 0.81; Found: C, 50.72; H, 6.53; N, 15.21%; Calcd for $C_{31}H_{44}O_7N_8S$·HCl·H_2O: C, 51.23; H, 6.47; N, 15.41%; Amino acid ratios in acid hydrolyzate: Arg 0.95, Gly 0.93, Met 0.87, Phe 1.00.

pMZ-Phe-Met-Arg-Phe-NH_2 (**12_a**), pMZ-Gly-Gly-Met-Arg-Phe-NH_2·HCl (**12_c**), pMZ-Phe-Gly-Met-Arg-Phe-NH_2·HCl (**12_d**), pMZ-Phe-Phe-Gly-Met-Arg-Phe-NH_2·HCl (**12_e**), pMZ-Phe-Phe-Met-Arg-Phe-NH_2·HCl (**16**), Ac-Phe-Met-Arg-Phe-NH_2·HCl (**14**), Bz-Phe-Met-Arg-Phe-NH_2·HCl (**15**) were prepared from the corresponding HODMSP.$MeSO_4^-$ active ester and **28** by the same method described for the preparation of **12_b**.

12_a: Yield 41%; mp 190 °C (decomp); $[\alpha]_D^{20}$ −20° (*c* 0.5, DMF); R_{f_1} 0.80; R_{f_3} 0.91; Found: C, 55.91; H, 6.31; N, 13.47%; Calcd for $C_{38}H_{50}O_7N_8S$·HCl·H_2O: C, 55.87; H, 6.49; N, 13.71%; Amino acid ratios in acid hydrolyzate: Arg 0.95, Met 0.87, Phe 2.00.

12_c: Yield 31%; mp 136 °C (decomp); $[\alpha]_D^{20}$ −10° (*c* 0.5, DMF); R_{f_1} 0.74; R_{f_3} 0.80; Found: C, 49.87; H, 6.40; N, 15.61%; Calcd for $C_{33}H_{47}O_8N_9S$·HCl·3/2H_2O: C, 49.99; H, 6.43; N, 15.89%; Amino acid ratios in acid hydrolyzate: Arg 0.93, Gly 1.92, Met 0.89, Phe 1.00.

12_d: Yield 32%; mp 146°C (decomp); $[\alpha]_D^{20}$ −18° (*c* 0.5 DMF); R_{f_1} 0.74; R_{f_3} 0.82; Found: C, 54.76; H, 6.38; N, 14.21%; Calcd for $C_{40}H_{53}O_8N_9S$·HCl·H_2O: C, 54.97; H, 6.41; N, 14.47%; Amino acid ratios in acid hydrolyzate: Arg 0.91, Gly 0.96, Met 0.87, Phe 2.00.

12_e: Yield 25%; mp 151 °C (decomp); $[\alpha]_D^{20}$ −30° (*c* 0.5, DMF); R_{f_1} 0.83; R_{f_3} 0.91; Found: C, 55.41; H, 6.39; N, 13.22%; Calcd for $C_{49}H_{62}O_9N_{10}S$·HCl·2H_2O: C, 55.65; H, 6.45; N, 13.48%; Amino acid ratios in acid hydrolyzate; Arg 0.95, Gly 0.93, Met 0.89, Phe 3.00.

14: Yield 30%; mp 72°C (decomp); $[\alpha]_D^{20}$ +35° (*c* 0.5, DMF); R_{f_1} 0.81; R_{f_3} 0.91; Found: C, 53.28; H, 6.72; N, 15.93%; Calcd for $C_{31}H_{44}O_5N_8S$·HCl·H_2O: C, 53.93; H, 6.76; N, 16.12%; Amino acid ratios in acid hydrolyzate: Arg 0.93, Met 0.87, Phe 2.00.

15: Yield 35%; mp 118°C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, DMF); R_{f_1} 0.79; R_{f_3} 0.83; Found: C, 56.01; H, 6.36; N, 13.59%; Calcd for $C_{38}H_{50}O_7N_8S$·HCl·2/3H_2O: C, 56.29; H, 6.45; N, 13.81%; Amino acid ratios in acid hydrolyzate: Arg 0.95, Met 0.84, Phe 2.00.

16: Yield 33%; mp 146 °C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, DMF); R_{f_1} 0.80; R_{f_3} 0.85; Found: C, 57.21; H, 6.34; N, 12.67%; Calcd for $C_{47}H_{59}O_8N_9S$·HCl·2H_2O: C, 57.49; H, 6.52; N, 12.83%; Amino acid ratios in acid hydrolyzate: Arg 0.97, Met 0.88, Phe 3.00.

H-X-Met-Arg-Phe-NH_2·HCl·TFA (13_a—13_d and 17): The synthesis of H-Gly-Met-Arg-Phe-NH_2·HCl·TFA (**13_b**) is as follows. Compound **12_b** (0.36g, 0.5 mmol) and ethyl methyl sulfide (0.5 ml) were dissolved in TFA (5 ml). The reaction mixture was allowed to stand at room temperature. After 2 h, the solution was concentrated in vacuo. The residue was crystallized with ether; Yield 0.17 g (52%); mp 90 °C (decomp); $[\alpha]_D^{20}$ −6° (*c* 0.5, MeOH); R_{f_1} 0.76; R_{f_3} 0.85; Found: C, 42.87; H, 5.80; N, 16.65%; Calcd for $C_{22}H_{36}O_4N_8S$·CF_3COOH·HCl·1/2H_2O: C, 43.17; H, 5.84; N, 16.77%; Amino acid ratios in acid hydrolyzate: Arg 0.92, Gly 0.92, Met 0.88, Phe 1.00.

H-Phe-Met-Arg-Phe-NH_2·HCl·TFA (**13_a**), H-Gly-Gly-Met-Arg-Phe-NH_2·HCl·TFA (**13_c**), H-Phe-Gly-Met-Arg-Phe-NH_2·HCl·TFA (**13_d**), H-Phe-Phe-Gly-Met-Phe-NH_2·HCl·TFA (**13_e**), H-Phe-Phe-Met-Arg-Phe-NH_2·HCl·TFA (**17**) were prepared from the corresponding pMZ-tetrapeptide amide by the same method described for the preparation of **13_b**.

13a: Yield 92%; mp 164 °C (decomp); $[\alpha]_D^{20}$ +8° (*c* 0.5, H_2O); R_{f_1} 0.76; R_{f_3} 0.85; Found: C, 51.38; H, 5.94; N, 15.32%; Calcd for $C_{29}H_{42}O_4N_8S$·CF_3COOH·HCl·1/2H_2O: C, 51.62; H, 6.10; N, 15.53%; Amino acid ratios in acid hydrolyzate: Arg 0.92, Met 0.90, Phe 2.00.

13_c: Yield 90%; mp 175 °C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, MeOH); R_{f_1} 0.52; R_{f_3} 0.85; Found : C, 41.27; H, 5.82; N, 16.61%; Calcd for $C_{24}H_{39}O_5N_9S$·CF_3COOH·HCl·2H_2O: C, 41.54; H, 5.99; N, 16.76%; Amino acid ratios in acid hydrolyzate: Arg 0.91, Gly 1.95, Met 0.83, Phe 1.00.

13_d: Yield 100%; mp 110 °C (decomp); $[\alpha]_D^{20}$ +6° (*c* 0.5, MeOH); R_{f_1} 0.50; R_{f_3} 0.88; Found: C, 45.87; H, 6.09; N, 14.48%; Calcd for $C_{31}H_{45}O_5N_9S$·CF_3COOH·HCl·3H_2O: C, 46.10; H, 6.16; N, 14.66%; Amino acid ratios in acid hydrolyzate: Arg 0.95, Gly 0.92, Met 0.87, Phe 2.00.

13_e: Yield 87%; mp 121°C (decomp); $[\alpha]_D^{20}$ +4° (*c* 0.5, MeOH); R_{f_1} 0.83; R_{f_3} 0.91; Found: C, 51.19; H, 6.01; N, 14.01%; Calcd for $C_{40}H_{54}O_6N_{10}S$·CF_3COOH·HCl·3/2H_2O: C, 51.48; H, 6.02; N, 14.29%; Amino acid ratios in acid hydrolyzate: Arg 0.97, Gly 0.92, Met 0.84, Phe 3.00.

17: Yield 91%; mp 182 °C (decomp); $[\alpha]_D^{20}$ +2° (*c* 0.3, MeOH); R_{f_1} 0.77; R_{f_3} 0.90; Found: C, 51.32; H, 6.08; N, 13.38%; Calcd for $C_{38}H_{51}O_5N_9S$·CF_3COOH·HCl·2H_2O: C, 51.55; H, 6.12; N,13.52; Amino acid ratios in acid hydrolyzate: Arg 0.91, Met 0.83, Phe 3.00.

Synthesis of FMRFamide Analogs for Met^2. Boc-Arg(NO_2)-Phe-NH_2 (18): Boc-Arg(NO_2)-OH (1.58 g, 5 mmol) and H-Phe-NH_2·HCl (1.00 g, 5 mmol) were coupled by the same method as described for the preparation of **5**. The reaction mixture was evaporated in vacuo and then poured into ice water. The resulting precipitate was filtered and washed with 4% sodium hydrogencarbonate, 4% citric acid and water successively. The purified product thus obtained was recrystallized from $CHCl_3$; Yield 1.97 g (84%); mp 115 °C (decomp); $[\alpha]_D^{20}$ −14° (*c* 0.5, DMF); R_{f_1} 0.86; R_{f_2} 0.56; Found: C, 51.37; H, 6.39; N, 20.96%; Calcd for $C_{20}H_{31}O_6N_7$: C, 51.56; H, 6.66; N, 21.05%.

H-Arg(NO_2)-Phe-NH_2·TFA (19): This compound was prepared by the same method described for the preparation of **13_b**. Yield 1.49 g (89%); mp 103 °C (decomp); $[\alpha]_D^{20}$ +28° (*c* 0.5, DMF); R_{f_1} 0.66; R_{f_2} 0.00; Found: C, 41.54; H, 5.21; N, 19.71%; Calcd for $C_{15}H_{23}O_4N_7$·CF_3COOH·1/2H_2O: C, 41.83; H, 5.12; N, 20.08%.

Boc-X-Arg(NO_2)-Phe-NH_2 (20_b—20_i): Boc-Ala-Arg(NO_2)-Phe-NH_2 (**20_b**), Boc-Abu-Arg(NO_2)-Phe-NH_2 (**20_c**), Boc-Val-Arg(NO_2)-Phe-NH_2 (**20_d**), Boc-Leu-Arg(NO_2)-Phe-NH_2 (**20_e**), Boc-Ile-Arg(NO_2)-Phe-NH_2 (**20_f**), Boc-Nle-Arg(NO_2)-Phe-NH_2 (**20_g**), Boc-Phe-Arg(NO_2)-Phe-NH_2 (**20_h**), and Boc-Pro-Arg(NO_2)-Phe-NH_2 (**20_i**) were prepared from the corresponding Boc-amino acid and **19** by the same method described for the preparation of **18**.

20_b: Yield 63%; mp 115 °C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, DMF); R_{f_1} 0.81; R_{f_2} 0.64; Found: C, 50.32; H, 6.81; N, 20.21%; Calcd for $C_{23}H_{36}O_7N_8 \cdot 1/3H_2O$: C, 50.63; H, 6.78; N, 20.53%.

20_c: Yield 59% ; mp 120 °C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, DMF); R_{f_1} 0.92; R_{f_2} 0.39; Found: C, 51.09; H, 6.94; N, 19.74%; Calcd for $C_{24}H_{38}O_7N_8 \cdot 2/3H_2O$: C, 51.23; H, 6.99; N, 19.91%.

20_d: Yield 63%; mp 135 °C (decomp) $[\alpha]_D^{20}$ −12° (*c* 0.5, DMF); R_{f_1} 0.90; R_{f_2} 0.64; Found: C, 51.79; H, 7.03; N, 19.17%; Calcd for $C_{25}H_{40}O_7N_8 \cdot H_2O$: C, 51.53; H, 7.21; N, 9.22%.

20_e: Yield 70%; mp 122 °C (decomp); $[\alpha]_D^{20}$ −26° (*c* 0.5, DMF); R_{f_1} 0.88; R_{f_2} 0.64; Found: C, 52.83; H, 7.32; N, 19.16%; Calcd for $C_{26}H_{42}O_7N_8 \cdot 1/2H_2O$: C, 53.13; H, 7.32; N, 19.06%.

20_f: Yield 38%; mp 120 °C (decomp); $[\alpha]_D^{20}$ −20° (*c* 0.5, DMF); R_{f_1} 0.91; R_{f_2} 0.62; Found: C, 53.71; H, 7.34; N, 19.51%; Calcd for $C_{26}H_{42}O_7N_8$: C, 53.92; H, 7.26; N, 19.36%.

20_g: Yield 67%; mp 102 °C (decomp); $[\alpha]_D^{20}$ −26° (*c* 0.5, DMF); R_{f_1} 0.87; R_{f_2} 0.64; Found: C, 53.68; H, 7.26; N, 19.20%; Calcd for $C_{26}H_{42}O_7N_8$: C, 53.92; H, 7.26; N, 19.36%.

20_h: Yield 74%; mp 124 °C (decomp); $[\alpha]_D^{20}$ −8° (*c* 0.5, DMF); R_{f_1} 0.85; R_{f_2} 0.59; Found: C, 55.71; H, 6.53; N, 18.27%; Calcd for $C_{29}H_{40}O_7N_8$: C, 56.80; H, 6.53; N, 18.38%.

20_i: Yield 50%; mp 115 °C (decomp); $[\alpha]_D^{20}$ −24° (*c* 0.5, DMF); R_{f_1} 0.92; R_{f_2} 0.64; Found: C, 51.81; H, 6.76; N, 19.35%; Calcd for $C_{25}H_{38}O_7N_8 \cdot H_2O$: C, 51.71; H, 6.89; N, 19.29%.

H-Ala-Arg(NO_2)-Phe-NH_2 · TFA (21_b—21_i): H-Ala-Arg(NO_2)-Phe-NH_2 (**21_b**), H-Abu-Arg(NO_2)-Phe-NH_2 · TFA (**21_c**), H-Val-Arg(NO_2)-Phe-NH_2 · TFA (**21_d**), H-Leu-Arg(NO_2)-Phe-NH_2 · TFA (**21_e**), H-Ile-Arg(NO_2)-Phe-NH_2 · TFA (**21_f**), H-Nle-Arg(NO_2)-Phe-NH_2 · TFA (**21_g**), H-Phe-Arg(NO_2)-Phe-NH_2 · TFA (**21_h**), and H-Pro-Arg(NO_2)-Phe-NH_2 · TFA (**21_i**) were prepared by the same method described for the preparation of **19**.

21_b: Yield 91%; mp 117 °C (decomp); $[\alpha]_D^{20}$ +6° (*c* 0.5, DMF); R_{f_1} 0.68; R_{f_2} 0.21; Found: C, 54.78; H, 6.89; N, 25.30%; Calcd for $C_{18}H_{28}O_5N_8 \cdot CF_3COOH \cdot 2/3H_2O$: C, 54.65; H, 6.90; N, 25.48%.

21_c: Yield 96%; mp 116 °C (decomp); $[\alpha]_D^{20}$ +14° (*c* 0.5, DMF); R_{f_1} 0.86; R_{f_2} 0.20; Found: C, 43.08; H, 5.65; N, 18.98%; Calcd for $C_{19}H_{30}O_5N_8 \cdot CF_3COOH \cdot H_2O$: C, 43.33; H, 5.67; N, 19.24%.

21_d: Yield 96%; mp 140°C (decomp); $[\alpha]_D^{20}$ +22° (*c* 0.5, DMF); R_{f_1} 0.84; R_{f_2} 0.24; Found: C, 44.73; H, 5.77; N, 18.88%; Calcd for $C_{20}H_{32}O_5N_8 \cdot CF_3COOH \cdot 1/2H_2O$: C, 45.00; H, 5.79; N, 19.08%.

21_e: Yield 94%; mp 120°C (decomp); $[\alpha]_D^{20}$ +16° (*c* 0.5, DMF); R_{f_1} 0.80; R_{f_2} 0.15; Found: C, 45.68; H, 5.90; N, 18.38%; Calcd for $C_{21}H_{34}O_5N_8 \cdot CF_3COOH \cdot 1/2H_2O$: C, 45.95; H, 5.99; N, 18.63%

21_f: Yield 95%; mp 125 °C (decomp); $[\alpha]_D^{20}$ +24° (*c* 0.5, DMF); R_{f_1} 0.72; R_{f_2} 0.22; Found: C, 44.83; H, 6.01; N, 18.01%; Calcd for $C_{21}H_{34}O_5N_8 \cdot CF_3COOH \cdot H_2O$: C, 45.27; H, 6.06; N, 18.36%.

21_g: Yield 100%; mp 105 °C (decomp); $[\alpha]_D^{20}$ +16° (*c* 0.5, DMF); R_{f_1} 0.78; R_{f_2} 0.25; Found: C, 45.72; H, 5.83; N, 18.39%; Calcd for $C_{21}H_{34}O_5N_8 \cdot CF_3COOH \cdot 1/2H_2O$: C, 45.95; H, 5.99; N, 18.63%.

21_h: Yield 99%; mp 121 °C (decomp); $[\alpha]_D^{20}$ +4° (*c* 0.5, DMF); R_{f_1} 0.82; R_{f_2} 0.24; Found: C, 49.55; H, 5.21; N, 17.67%; Calcd for $C_{24}H_{32}O_5N_8 \cdot CF_3COOH$: C, 49.87; H, 5.27; N, 17.89%.

21_i: Yield 94%; mp 92 °C (decomp); $[\alpha]_D^{20}$ −8° (*c* 0.5, DMF); R_{f_1} 0.63; R_{f_2} 0.27; Found: C, 44.18; H, 5.50; N, 18.56%; Calcd for $C_{20}H_{30}O_5N_8 \cdot CF_3COOH \cdot H_2O$: C, 44.47; H, 5.55; N, 18.85%.

Z-Phe-X-Arg(NO_2)-Phe-NH_2 (22_b—22_i): Z-Phe-Ala-Arg(NO_2)-Phe-NH_2 (**22_b**), Z-Phe-Abu-Arg(NO_2)-Phe-NH_2 (**22_c**), Z-Phe-Val-Arg(NO_2)-Phe-NH_2 (**22_d**), Z-Phe-Leu-Arg(NO_2)-Phe-NH_2 (**22_e**), Z-Phe-Ile-Arg(NO_2)-Phe-NH_2 (**22_f**), Z-Phe-Nle-Arg(NO_2)-Phe-NH_2 (**22_g**), Z-Phe-Phe-Arg(NO_2)-Phe-NH_2 (**22_h**), and Z-Phe-Pro-Arg(NO_2)-Phe-NH_2 (**22_i**) were also prepared from the coupling reaction of the corresponding Z-amino acid and tripeptide amide by the same method described for the preparation of **18**. These protected peptides were recrystallized from ethyl acetate.

22_b: Yield 90%; mp 165 °C (decomp); $[\alpha]_D^{20}$ −6° (*c* 0.5, DMF); R_{f_1} 0.91; R_{f_2} 0.66; Found: C, 56.61; H, 6.10; N, 16.75%; Calcd for $C_{35}H_{43}O_8N_9 \cdot 3/2H_2O$: C, 56.44; H, 6.18; N, 16.92%.

22_c: Yield 85%; mp 174 °C (decomp); $[\alpha]_D^{20}$ −12° (*c* 0.5, DMF); R_{f_1} 0.90; R_{f_2} 0.53; Found: C, 57.59; H, 6.19; N, 16.63%; Calcd for $C_{36}H_{45}O_8N_9 \cdot H_2O$: C, 57.66; H, 6.27; N,16. 80%.

22_d: Yield 91%; mp 192 °C (decomp); $[\alpha]_D^{20}$ −6° (*c* 0.5, DMF); R_{f_1} 0.85; R_{f_2} 0.35; Found: C, 58.21; H, 6.48; N, 16.31%; Calcd for $C_{37}H_{47}O_8N_9 \cdot H_2O$: C, 58.17; H, 6.42; N, 16.50%.

22_e: Yield 79%; mp 196 °C (decomp); $[\alpha]_D^{20}$ −14° (*c* 0.5, DMF); R_{f_1} 0.88; R_{f_2} 0.47; Found: C, 58.87; H, 6.55; N, 16.37%; Calcd for $C_{38}H_{49}O_8N_9 \cdot H_2O$: C, 58.67; H, 6.56; N, 16.20%.

22_f: Yield 89%; mp 200 °C (decomp); $[\alpha]_D^{20}$ −10° (*c* 0.5, DMF); R_{f_1} 0.90; R_{f_2} 0.56; Found: C, 60.28; H, 6.57; N, 16.23%; Calcd for $C_{38}H_{49}O_8N_9$: C, 60.01; H, 6.45; N, 16.58%.

22_g: Yield 75%; mp 192 °C (decomp); $[\alpha]_D^{20}$ −12° (*c* 0.5, DMF); R_{f_1} 0.92; R_{f_2} 0.53. Found: C, 57.76; H, 6.32; N, 15.76%; Calcd for $C_{38}H_{49}O_8N_9 \cdot 3/2H_2O$: C, 58.00; H, 6.60; N, 16.01%.

22_h: Yield 95%; mp 186 °C (decomp); $[\alpha]_D^{20}$ −18° (*c* 0.5, DMF); R_{f_1} 0.93; R_{f_2} 0.62. Found: C, 60.99; H, 6.00; N, 15.74%; Calcd for $C_{41}H_{47}O_8N_9 \cdot 2/3H_2O$: C, 61.10; H, 5.92; N, 15.64%.

22_i: Yield 63%; mp 108 °C (decomp) $[\alpha]_D^{20}$ −24° (*c* 5.0, DMF); R_{f_1} 0.94; R_{f_1} 0.48; Found: C, 58.50; H, 6.06; N, 16.56%; Calcd for $C_{37}H_{45}O_8N_9 \cdot H_2O$: C, 58.33; H, 6.17; N, 16.54%.

Z-Phe-Gly-Arg(NO_2)-Phe-NH_2 (22_a): Z-Phe-Gly-OH (0.53 g, 1.5 mmol) and **19** (0.72 g, 1.5 mmol) were coupled by the same method as described for the preparation of **18**. It was recrystallized from ethyl acetate; Yield 0.85 g (80%); mp 183 °C (decomp); $[\alpha]_D^{20}$ −38° (*c* 0.5, DMF); R_{f_1} 0.88; R_{f_2} 0.66; Found: C, 56.82; H, 5.95; N, 17.21%; Calcd for $C_{34}H_{41}O_8N_9 \cdot H_2O$: C, 56.58; H, 5.96; N, 17.46%.

H-Phe-X-Arg-Phe-NH_2 · 2HCl (23_a—23_i): H-Phe-Gly-Arg-Phe-NH_2 · 2HCl (**23_a**), H-Phe-Ala-Arg-Phe-NH_2 · 2HCl (**23_b**), H-Phe-Abu-Arg-Phe-NH_2 · 2HCl (**23_c**), H-Phe-Val-Arg-Phe-NH_2 · 2HCl (**23_d**), H-Phe-Leu-Arg-Phe-NH_2 · 2HCl (**23_e**), H-Phe-Ile-Arg-Phe-NH_2 · 2HCl (**23_f**), H-Phe-Nle-Arg-Phe-NH_2 · 2HCl (**23_g**), H-Phe-Phe-Arg-Phe-NH_2 · 2HCl (**23_h**) and H-Phe-Pro-Arg-Phe-NH_2 · 2HCl (**23_i**) were also prepared from the corresponding protected tetrapeptides by the catalytic hydrogenation, according to the preparation of **6**. These peptides were obtained as hydrochlorides.

23_a: Yield 90%; mp 134 °C (decomp); $[\alpha]_D^{20}$ +16° (*c* 0.5,

MeOH); R_{f_1} 0.59; R_{f_2} 0.15; Found; C, 49.83; H, 6.55; N, 17.67%; Calcd for $C_{26}H_{36}O_4N_8 \cdot 2HCl \cdot 3/2H_2O$: C, 50.00; H, 6.57; N, 17.93%; Amino acid ratios in acid hydrolyzate: Arg 0.94, Gly 0.91, Phe 2.00.

23b: Yield 99%; mp 129 °C (decomp); $[\alpha]_D^{20}$ −8° (*c* 0.5, MeOH); R_{f_1} 0.67; R_{f_2} 0.21; Found; C, 51.22; H, 6.65; N, 17.91%; Calcd for $C_{27}H_{38}O_4N_8 \cdot 2HCl \cdot H_2O$: C, 51.51; H, 6.67; N, 17.79%; Amino acid ratios in acid hydrolyzate: Arg 0.93, Ala 0.94, Phe 2.00.

23c: Yield 96%; mp 140 °C (decomp); $[\alpha]_D^{20}$ −6° (*c* 0.5, MeOH); R_{f_1} 0.63; R_{f_2} 0.18; Found: C, 50.63; H, 6.86; N, 16.70%; Calcd for $C_{28}H_{40}O_4N_8 \cdot 2HCl \cdot 2H_2O$: C, 50.82; H, 6.95; N, 16.93%; Amino acid ratios in acid hydrolyzate: Arg 0.94, Phe 2.00.

23d: Yield 93%; mp 174 °C (decomp); $[\alpha]_D^{20}$ −19° (*c* 0.5, MeOH); R_{f_1} 0.72; R_{f_2} 0.19; Found: C, 51.37; H, 6.80; N, 16.53%; Calcd for $C_{29}H_{42}O_4N_8 \cdot 2HCl \cdot 2H_2O$: C, 51.55; H, 7.10; N, 16.58%; Amino acid ratios in acid hydrolyzate: Arg 0.94, Val 0.91, Phe 2.00.

23e: Yield 94%; mp 172 °C (decomp); $[\alpha]_D^{20}$ −14° (*c* 0.5, MeOH); R_{f_1} 0.76; R_{f_2} 0.21; Found: C, 54.30; H, 7.06; N, 16.67%; Calcd for $C_{30}H_{44}O_4N_8 \cdot 2HCl \cdot 2/3H_2O$: C, 54.13; H, 7.11; N, 16.83%; Amino acid ratios in acid hydrolyzate: Arg 0.90, Leu 0.93, Phe 2.00.

23f: Yield 88%; mp 170 °C (decomp); $[\alpha]_D^{20}$ −16° (*c* 0.5, MeOH); R_{f_1} 0.77; R_{f_1} 0.22; Found: C, 51.96; H, 7.21; N, 16.31%; Calcd for $C_{30}H_{44}O_4N_8 \cdot 2HCl \cdot 2H_2O$: C, 52.25; H, 7.25; N, 16.24%; Amino acid ratios in acid hydrolyzate: Arg 0.92, Ile 0.88, Phe 2.00.

23g: Yield 94%; mp 102 °C (decomp); $[\alpha]_D^{20}$ −6° (*c* 0.5, MeOH); R_{f_1} 0.71; R_{f_2} 0.21; Found: C, 52.01; H, 7.23; N, 16.01%; Calcd for $C_{30}H_{44}O_4N_8 \cdot 2HCl \cdot 2H_2O$: C, 52.25; H, 7.25; N, 16.24%; Amino acid ratios in acid hydrolyzate: Arg 0.99, Phe 2.00.

23h: Yield 93%; mp 181 °C (decomp): $[\alpha]_D^{20}$ −2° (*c* 0.5, MeOH); R_{f_1} 0.69; R_{f_2} 0.22; Found: C, 55.21; H, 6.30; N, 15.51%; Calcd for $C_{33}H_{42}O_4N_8 \cdot 3/2H_2O$: C, 55.46; H, 6.30; N, 15.67%; Amino acid ratios in acid hydrolyzate: Arg 0.92, Phe 3.00.

23i: Yield 98%; mp 92 °C (decomp); $[\alpha]_D^{20}$ −18° (*c* 0.5, MeOH); R_{f_1} 0.59; R_{f_2} 0.23; Found: C, 51.54; H, 6.95; N, 16.60%; Calcd for $C_{29}H_{41}O_4N_8 \cdot 2HCl \cdot 2H_2O$: C, 51.71; H, 6.98; N, 16.63%; Amino acid ratios in acid hydrolyzate: Arg 0.90, Pro 0.85, Phe 2.00.

We wish to thank Professor Yojiro Muneoka of Hiroshima University for biological assays and helpful discussions.

References

1) K. Kouge, T. Koizumi, H. Okai, and T. Kato, *Bull. Chem. Soc. Jpn.*, **60**, 2409 (1987).

2) Abbreviations used are according to IUPAC-IUB Commissions, *Eur, J. Biochem.*, **138**, 9 (1984). Other abbreviations: DCC, dicyclohexylcarbodiimide; DCurea, *N,N'*-dicyclohexylurea; ECF, ethyl chloroformate; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; Bz-, benzoyl-.

3) A. D. Price and J. M. Greenberg, *Science*, **197**, 670 (1977).

4) Y. Muneoka and M. Matsuura, *Comp. Biochem. Physiol.*, **81c**, 61 (1985)

5) A. D. Price and J. M. Greenberg, *Am. Zool*, **19**, 163 (1979).

6) Y. Muneoka and H. Saitoh, *Comp. Biochem. Physiol.*, **85c**, 207 (1986).

7) We asked Professor Yojiro Muneoka of Hiroshima University to measure the biological assays. He measured these assays according to the Ref. 6.