

Peptide Synthesis in Aqueous Solution. II. Synthesis and Biological Activity of a Molluscan Neuropeptide, FMRFamide (Phe-Met-Arg-Phe-NH₂) Analogs for N-Terminal Moiety

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In order to elucidate the contribution of the N-terminal moiety (Phe¹ or Met²) 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)dime-thylsulfonium methyl sulfate(HODMSP·MeSO₄⁻) active ester¹⁾ 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-NH₂),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 M⁴⁾ (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 Saitoh⁶⁾ tested a number of FMRFamide analogs and other peptide with biological actions in mammals, arthropods, and molluscs. From their report, we can draw an interesting conclusion that at least the structure of Arg³-Phe⁴-NH₂ moiety of FMRFamide is necessary for the production of both contraction and relaxation. Prob-

ably, this moiety will act as the binding unit to FMRFamide receptor. However, the role of the Phe¹ or Met² residue of N-terminal moiety in FMRFamide to these productions was not fully characterized.

In order to elucidate the contribution of Phe¹ or Met² to the activity, we synthesized 16 kinds of

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

Peptide	Con- tractile effect/M	Relax- ing effect/M
Phe-Met-Arg-Phe-NH ₂ (13a) (FMRFamide)	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 Met²

Peptide	Con- tractile effect/M	Relax- ing 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.

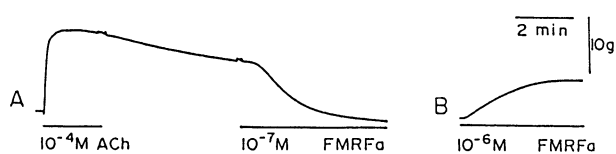


Fig. 1. Contractions and Relaxations Produced by FMRFamide. A: Relaxing action of FMRFamide on ACh-induced catch tension. B: Contractile action of FMRFamide. ACh: Acetylcholine, FMRFa: FMRFamide.

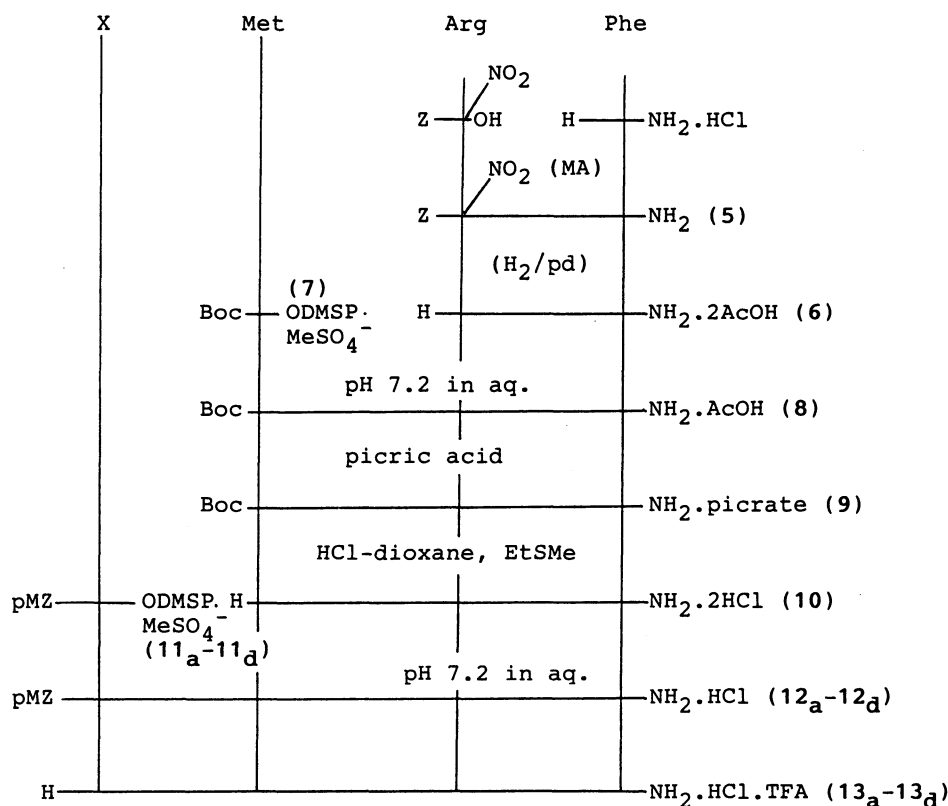
FMRFamide analogs shown in Table 1 and Table 2 and discussed their structure-activity relations of FMRFamide on a molluscan muscle.

The synthetic route for the FMRFamide analogs for Phe¹ is shown in Fig. 2. Z-Arg(NO₂)-OH was coupled with H-Phe-NH₂·HCl by means of mixed anhydride (MA) method to yield Z-Arg-(NO₂)-Phe-NH₂ (5). Removal of Z-group and NO₂-group from 5 by catalytic hydrogenation afforded the corresponding dipeptide amide (6). Boc-Met-OH was esterified by DCC and HODMSP·MeSO₄⁻ to afford the water-soluble active ester (7). In aqueous solution, 7 was allowed to react with 6 in the presence of 1 M Na₂CO₃ 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·MeSO₄⁻ 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*-methoxybenzyloxy-carbonyl 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·MeSO₄⁻ or Bz-Phe-ODMSP·MeSO₄⁻ in aqueous solution at pH 7.2, respectively. In the synthesis of 17, pMZ-Phe-ODMSP·MeSO₄⁻ 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 Met² 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).

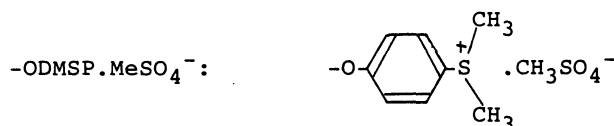


Fig. 2. Synthesis of FMRFamide Analogs for N-Terminal Phe.

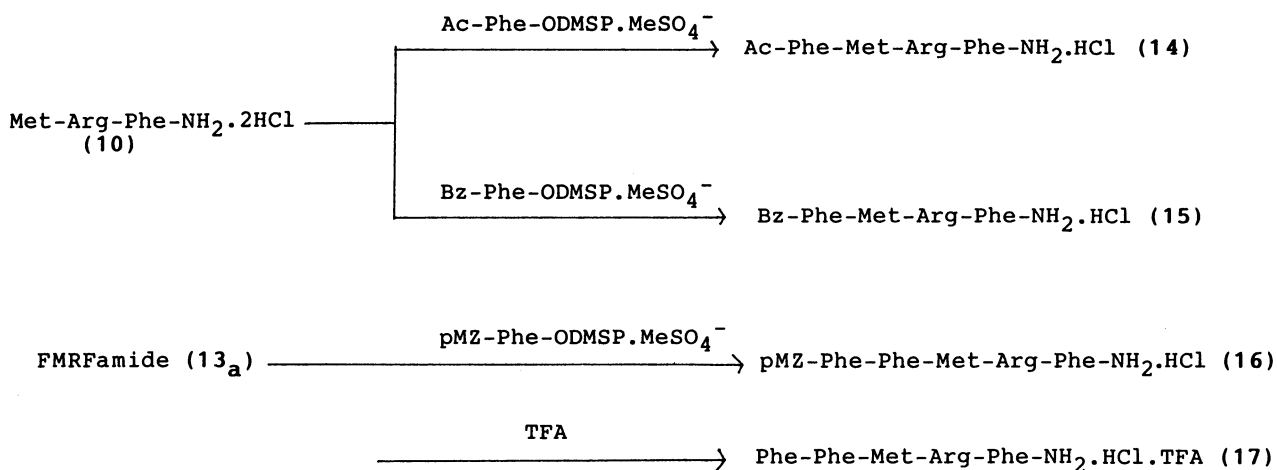
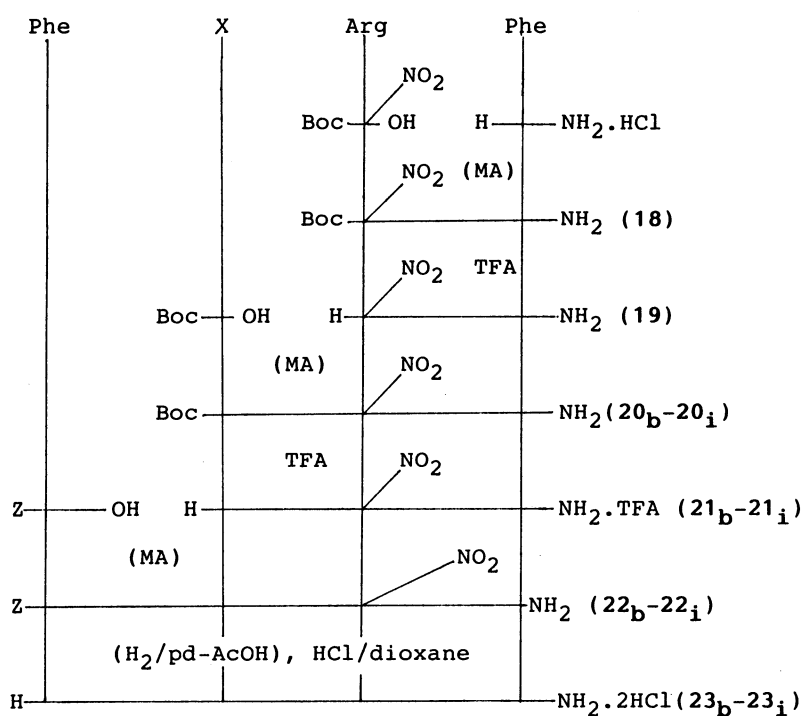
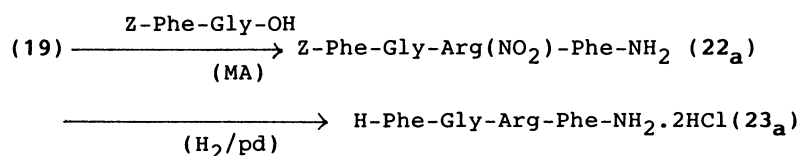


Fig. 3. Synthesis of FMRFamide Analogs for N-Terminal Phe.



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

Fig. 4. Synthesis of FMRFamide Analogs for Met².

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

In the synthesis of 23_a, Z-Phe-Gly-OH was coupled with 19 to yield 22_a. 22_a was then hydrogenated to give

23_a. 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 Phe¹ produced 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 Met² by inserting a Gly residue and was replaced with Phe-Phe or Phe-Phe-Gly residue in FMRFamide, these peptides (**13_a**, **17**, and **13_c**) 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 Met², Muneoka and Saitoh already reported that Nle can be substituted for Met².⁶⁾ 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 Met² showed a slight decrease in contractile and relaxing potency. In contrast, Gly, Ala, Abu, Ile, Phe, and Pro could not be substituted for Met²; these peptides had no relaxing activity. The most remarkable fact was that the peptide (**23_a**), in which Val was substituted for Met², 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 biologically 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-butanol-acetic acid-pyridine-water (4:1:1:2, v/v), *R_f*, CHCl₃-methanol (5:1, v/v), *R_f*, 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 UVIDECE-100V apparatus.

Synthesis of Analogs for Phe¹. Z-Arg(NO₂)-Phe-NH₂ (5): A solution of Z-Arg(NO₂)-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-NH₂·HCl (2.00 g, 10 mmol) and Et₃N (1.4 ml, 10 mmol) in CHCl₃ (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*, 0.91; *R_f*, 0.41; Found: C, 55.56; H, 5.70; N, 19.38%; Calcd for C₂₃H₂₉O₆N₇: C, 55.34; H, 5.81; N, 19.63%.

H-Arg-Phe-NH₂·2AcOH (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° (c1, H₂O); *R_f*, 0.41; *R_f*, 0.75.

Boc-Met-ODMSP·MeSO₄⁻ (7) (Water-Soluble Active Ester): To a chilled solution of Boc-Met-OH (2.49g, 10 mmol) and HODMSP·MeSO₄⁻ (2.66 g, 10 mmol) in CH₃CN (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° (c1, MeOH); *R_f*, 0.82; *R_f*, 0.34.

Boc-Met-Arg-Phe-NH₂·Picrate (9): Compound **6** (0.88 g, 2 mmol) was dissolved in H₂O and the pH of the solution was adjusted to 7.2 by addition of Et₃N. Boc-Met-ODMSP·MeSO₄⁻ (**7**) (0.99 g, 2 mmol) was added to the stirred solution below 20 °C, the pH being maintained automatically at 7.2 with Et₃N. 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 CHCl₃; Yield 0.75 g (45%); mp 110 °C (decomp); $[\alpha]_D^{20}$ -14° (c 0.5, DMF); *R_f*, 0.83; *R_f*, 0.32; Found C, 46.84; H, 5.81; N, 17.95%; Calcd for C₂₅H₄₁O₅N₇S·C₆H₃N₃O₇·1/2H₂O; 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-NH₂·2HCl (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, H₂O); *R_f*, 0.52; *R_f*, 0.74; Found: C, 49.03; H, 6.87; N, 20.01%; Calcd for C₂₀H₃₃O₃N₇S·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·MeSO₄⁻ (11_a–11_d) (Water-Soluble Active Ester): pMZ-Phe-ODMSP·MeSO₄⁻ (**11_a**), pMZ-Gly-ODMSP·MeSO₄⁻ (**11_b**), pMZ-Gly-Gly-ODMSP·MeSO₄⁻ (**11_c**), pMZ-Phe-Gly-ODMSP·MeSO₄⁻ (**11_d**), pMZ-Phe-Phe-Gly-ODMSP·MeSO₄⁻ (**11_e**), Ac-Phe-ODMSP·MeSO₄⁻, and Bz-Phe-ODMSP·MeSO₄⁻ were also prepared from the corresponding carboxyl components and HODMSP·MeSO₄⁻ by the same method as **7**.

11_a: Yield 90%; *R_f*, 0.85; *R_f*, 0.51.

11_b: Yield 85%; *R_f*, 0.70; *R_f*, 0.39.

11_c: Yield 90%; *R_f*, 0.72; *R_f*, 0.38.

11_d : Yield 88%; R_f 0.82; R_i 0.45.

11_e : Yield 80%; R_f 0.85; R_i 0.51.

Ac-Phe-ODMSP·MeSO₄⁻: Yield 82%; R_f 0.81; R_i 0.42.

Bz-Phe-ODMSP·MeSO₄⁻: Yield 88%; R_f 0.88; R_i 0.55.

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

pMZ-X-Met-Arg-Phe-NH₂·HCl (12_a—12_d and 14—16): The synthesis of pMZ-Gly-Met-Arg-Phe-NH₂·HCl (**12_b**) is as follows. Compound **10** (0.52 g, 1 mmol) was dissolved in H₂O (10 ml) and the pH of the solution was adjusted to 7.2 by addition of 1M Na₂CO₃. pMZ-Gly-ODMSP·MeSO₄⁻ (**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 Na₂CO₃. 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 0.75; R_i 0.81; Found: C, 50.72; H, 6.53; N, 15.21%; Calcd for C₃₁H₄₄O₇N₈S·HCl·H₂O: 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-NH₂ (**12_a**), pMZ-Gly-Gly-Met-Arg-Phe-NH₂·HCl (**12_c**), pMZ-Phe-Gly-Met-Arg-Phe-NH₂·HCl (**12_d**), pMZ-Phe-Phe-Gly-Met-Arg-Phe-NH₂·HCl (**12_e**), pMZ-Phe-Phe-Met-Arg-Phe-NH₂·HCl (**16**), Ac-Phe-Met-Arg-Phe-NH₂·HCl (**14**), Bz-Phe-Met-Arg-Phe-NH₂·HCl (**15**) were prepared from the corresponding HODMSP·MeSO₄⁻ 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 0.80; R_i 0.91; Found: C, 55.91; H, 6.31; N, 13.47%; Calcd for C₃₈H₅₀O₇N₈S·HCl·H₂O: 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 0.74; R_i 0.80; Found: C, 49.87; H, 6.40; N, 15.61%; Calcd for C₃₃H₄₄O₈N₉S·HCl·3/2H₂O: 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 0.74; R_i 0.82; Found: C, 54.76; H, 6.38; N, 14.21%; Calcd for C₄₀H₅₃O₈N₉S·HCl·H₂O: 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 0.83; R_i 0.91; Found: C, 55.41; H, 6.39; N, 13.22%; Calcd for C₄₉H₆₂O₉N₁₀S·HCl·2H₂O: 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 0.81; R_i 0.91; Found: C, 53.28; H, 6.72; N, 15.93%; Calcd for C₃₁H₄₄O₅N₈S·HCl·H₂O: 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 0.79; R_i 0.83; Found: C, 56.01; H, 6.36; N, 13.59%; Calcd for C₃₈H₅₀O₇N₈S·HCl·2/3H₂O: 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 0.80; R_i 0.85; Found: C, 57.21; H, 6.34; N, 12.67%; Calcd for C₄₇H₅₉O₈N₉S·HCl·2H₂O: 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-NH₂·HCl·TFA (13_a—13_d and 17): The synthesis of H-Gly-Met-Arg-Phe-NH₂·HCl·TFA (**13_b**) is as follows. Compound **12_b** (0.36 g, 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 0.76; R_i 0.85; Found: C, 42.87; H, 5.80; N, 16.65%; Calcd for C₂₂H₃₆O₄N₈S·CF₃COOH·HCl·1/2H₂O: 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-NH₂·HCl·TFA (**13_a**), H-Gly-Gly-Met-Arg-Phe-NH₂·HCl·TFA (**13_c**), H-Phe-Gly-Met-Arg-Phe-NH₂·HCl·TFA (**13_d**), H-Phe-Phe-Gly-Met-Phe-NH₂·HCl·TFA (**13_e**), H-Phe-Phe-Met-Arg-Phe-NH₂·HCl·TFA (**17**) were prepared from the corresponding pMZ-tetrapeptide amide by the same method described for the preparation of **13_b**.

13_a: Yield 92%; mp 164 °C (decomp); $[\alpha]_D^{20}$ +8° (c 0.5, H₂O); R_f 0.76; R_i 0.85; Found: C, 51.38; H, 5.94; N, 15.32%; Calcd for C₂₉H₄₂O₄N₈S·CF₃COOH·HCl·1/2H₂O: 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 0.52; R_i 0.85; Found: C, 41.27; H, 5.82; N, 16.61%; Calcd for C₂₄H₃₉O₅N₉S·CF₃COOH·HCl·2H₂O: 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 0.50; R_i 0.88; Found: C, 45.87; H, 6.09; N, 14.48%; Calcd for C₃₁H₄₅O₅N₉S·CF₃COOH·HCl·3H₂O: 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 0.83; R_i 0.91; Found: C, 51.19; H, 6.01; N, 14.01%; Calcd for C₄₀H₅₄O₆N₁₀S·CF₃COOH·HCl·3/2H₂O: 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 0.77; R_i 0.90; Found: C, 51.32; H, 6.08; N, 13.38%; Calcd for C₃₈H₅₁O₅N₉S·CF₃COOH·HCl·2H₂O: 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 Met². Boc-Arg-(NO₂)-Phe-NH₂ (18**)**: Boc-Arg(NO₂)-OH (1.58 g, 5 mmol) and H-Phe-NH₂·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 CHCl₃; Yield 1.97 g (84%); mp 115 °C (decomp); $[\alpha]_D^{20}$ -14° (c 0.5, DMF); R_f 0.86; R_i 0.56; Found: C, 51.37; H, 6.39; N, 20.96%; Calcd for C₂₀H₃₁O₆N₇: C, 51.56; H, 6.66; N, 21.05%.

H-Arg(NO₂)-Phe-NH₂·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 0.66; R_i 0.00; Found: C, 41.54; H, 5.21; N, 19.71%; Calcd for C₁₅H₂₃O₄N₇·CF₃COOH·1/2H₂O: C, 41.83; H, 5.12; N, 20.08%.

Boc-X-Arg(NO₂)-Phe-NH₂ (20_b—20_i): Boc-Ala-Arg(NO₂)-Phe-NH₂ (20_b), Boc-Abu-Arg(NO₂)-Phe-NH₂ (20_c), Boc-Val-Arg(NO₂)-Phe-NH₂ (20_d), Boc-Leu-Arg(NO₂)-Phe-NH₂ (20_e), Boc-Ile-Arg(NO₂)-Phe-NH₂ (20_f), Boc-Nle-Arg(NO₂)-Phe-NH₂ (20_g), Boc-Phe-Arg(NO₂)-Phe-NH₂ (20_h), and Boc-Pro-Arg(NO₂)-Phe-NH₂ (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 0.81; *R*_i 0.64; Found: C, 50.32; H, 6.81; N, 20.21%; Calcd for C₂₃H₃₆O₇N₈ · 1/3H₂O: 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 0.92; *R*_i 0.39; Found: C, 51.09; H, 6.94; N, 19.74%; Calcd for C₂₄H₃₈O₇N₈ · 2/3H₂O: 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 0.90; *R*_i 0.64; Found: C, 51.79; H, 7.03; N, 19.17%; Calcd for C₂₅H₄₀O₇N₈ · H₂O: 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 0.88; *R*_i 0.64; Found: C, 52.83; H, 7.32; N, 19.16%; Calcd for C₂₆H₄₂O₇N₈ · 1/2H₂O: 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 0.91; *R*_i 0.62; Found: C, 53.71; H, 7.34; N, 19.51%; Calcd for C₂₆H₄₂O₇N₈: 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 0.87; *R*_i 0.64; Found: C, 53.68; H, 7.26; N, 19.20%; Calcd for C₂₆H₄₂O₇N₈: 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 0.85; *R*_i 0.59; Found: C, 55.71; H, 6.53; N, 18.27%; Calcd for C₂₅H₄₀O₇N₈: 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 0.92; *R*_i 0.64; Found: C, 51.81; H, 6.76; N, 19.35%; Calcd for C₂₅H₃₈O₇N₈ · H₂O: C, 51.71; H, 6.89; N, 19.29%.

H-Ala-Arg(NO₂)-Phe-NH₂ · TFA (21_b—21_i): H-Ala-Arg(NO₂)-Phe-NH₂ (21_b), H-Abu-Arg(NO₂)-Phe-NH₂ · TFA (21_c), H-Val-Arg(NO₂)-Phe-NH₂ · TFA (21_d), H-Leu-Arg(NO₂)-Phe-NH₂ · TFA (21_e), H-Ile-Arg(NO₂)-Phe-NH₂ · TFA (21_f), H-Nle-Arg(NO₂)-Phe-NH₂ · TFA (21_g), H-Phe-Arg(NO₂)-Phe-NH₂ · TFA (21_h), and H-Pro-Arg(NO₂)-Phe-NH₂ · TFA (21_i) were prepared by the same method described for the preparation of **19**.

21_b: Yield 91%; mp 117 °C (decomp); $[\alpha]_D^{20}$ $+6^\circ$ (*c* 0.5, DMF); *R*_f 0.68; *R*_i 0.21; Found: C, 54.78; H, 6.89; N, 25.30%; Calcd for C₁₈H₂₈O₅N₈ · CF₃COOH · 2/3H₂O: C, 54.65; H, 6.90; N, 25.48%.

21_c: Yield 96%; mp 116 °C (decomp); $[\alpha]_D^{20}$ $+14^\circ$ (*c* 0.5, DMF); *R*_f 0.86; *R*_i 0.20; Found: C, 43.08; H, 5.65; N, 18.98%; Calcd for C₁₉H₃₀O₅N₈ · CF₃COOH · H₂O: C, 43.33; H, 5.67; N, 19.24%.

21_d: Yield 96%; mp 140 °C (decomp); $[\alpha]_D^{20}$ $+22^\circ$ (*c* 0.5, DMF); *R*_f 0.84; *R*_i 0.24; Found: C, 44.73; H, 5.77; N, 18.88%; Calcd for C₂₀H₃₂O₅N₈ · CF₃COOH · 1/2H₂O: C, 45.00; H, 5.79; N, 19.08%.

21_e: Yield 94%; mp 120 °C (decomp); $[\alpha]_D^{20}$ $+16^\circ$ (*c* 0.5, DMF); *R*_f 0.80; *R*_i 0.15; Found: C, 45.68; H, 5.90; N, 18.38%; Calcd for C₂₁H₃₄O₅N₈ · CF₃COOH · 1/2H₂O: C, 45.95; H, 5.99; N, 18.63%.

21_f: Yield 95%; mp 125 °C (decomp); $[\alpha]_D^{20}$ $+24^\circ$ (*c* 0.5, DMF); *R*_f 0.72; *R*_i 0.22; Found: C, 44.83; H, 6.01; N, 18.01%; Calcd for C₂₁H₃₄O₅N₈ · CF₃COOH · H₂O: C, 45.27; H, 6.06; N, 18.36%.

21_g: Yield 100%; mp 105 °C (decomp); $[\alpha]_D^{20}$ $+16^\circ$ (*c* 0.5, DMF); *R*_f 0.78; *R*_i 0.25; Found: C, 45.72; H, 5.83; N, 18.39%; Calcd for C₂₁H₃₄O₅N₈ · CF₃COOH · 1/2H₂O: C, 45.95; H,

5.99; N, 18.63%.

21_h: Yield 99%; mp 121 °C (decomp); $[\alpha]_D^{20}$ $+4^\circ$ (*c* 0.5, DMF); *R*_f 0.82; *R*_i 0.24; Found: C, 49.55; H, 5.21; N, 17.67%; Calcd for C₂₄H₃₂O₅N₈ · CF₃COOH: 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 0.63; *R*_i 0.27; Found: C, 44.18; H, 5.50; N, 18.56%; Calcd for C₂₀H₃₀O₅N₈ · CF₃COOH · H₂O: C, 44.47; H, 5.55; N, 18.85%.

Z-Phe-X-Arg(NO₂)-Phe-NH₂ (22_b—22_i): Z-Phe-Ala-Arg(NO₂)-Phe-NH₂ (22_b), Z-Phe-Abu-Arg(NO₂)-Phe-NH₂ (22_c), Z-Phe-Val-Arg(NO₂)-Phe-NH₂ (22_d), Z-Phe-Leu-Arg(NO₂)-Phe-NH₂ (22_e), Z-Phe-Ile-Arg(NO₂)-Phe-NH₂ (22_f), Z-Phe-Nle-Arg(NO₂)-Phe-NH₂ (22_g), Z-Phe-Phe-Arg(NO₂)-Phe-NH₂ (22_h), and Z-Phe-Pro-Arg(NO₂)-Phe-NH₂ (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 0.91; *R*_i 0.66; Found: C, 56.61; H, 6.10; N, 16.75%; Calcd for C₃₅H₄₃O₈N₉ · 3/2H₂O: 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 0.90; *R*_i 0.53; Found: C, 57.59; H, 6.19; N, 16.63%; Calcd for C₃₆H₄₅O₈N₉ · H₂O: 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 0.85; *R*_i 0.35; Found: C, 58.21; H, 6.48; N, 16.31%; Calcd for C₃₇H₄₇O₈N₉ · H₂O: 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 0.88; *R*_i 0.47; Found: C, 58.87; H, 6.55; N, 16.37%; Calcd for C₃₈H₄₉O₈N₉ · H₂O: 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 0.90; *R*_i 0.56; Found: C, 60.28; H, 6.57; N, 16.23%; Calcd for C₃₈H₄₉O₈N₉: 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 0.92; *R*_i 0.53; Found: C, 57.76; H, 6.32; N, 15.76%; Calcd for C₃₈H₄₉O₈N₉ · 3/2H₂O: 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 0.93; *R*_i 0.62; Found: C, 60.99; H, 6.00; N, 15.74%; Calcd for C₄₁H₄₇O₈N₉ · 2/3H₂O: C, 61.10; H, 5.92; N, 15.64%.

22_i: Yield 63%; mp 108 °C (decomp) $[\alpha]_D^{20}$ -24° (*c* 0.5, DMF); *R*_f 0.94; *R*_i 0.48; Found: C, 58.50; H, 6.06; N, 16.56%; Calcd for C₃₇H₄₅O₈N₉ · H₂O: C, 58.33; H, 6.17; N, 16.54%.

Z-Phe-Gly-Arg(NO₂)-Phe-NH₂ (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 0.88; *R*_i 0.66; Found: C, 56.82; H, 5.95; N, 17.21%; Calcd for C₃₄H₄₁O₈N₉ · H₂O: C, 56.58; H, 5.96; N, 17.46%.

H-Phe-X-Arg-Phe-NH₂ · 2HCl (23_a—23_i): H-Phe-Gly-Arg-Phe-NH₂ · 2HCl (23_a), H-Phe-Ala-Arg-Phe-NH₂ · 2HCl (23_b), H-Phe-Abu-Arg-Phe-NH₂ · 2HCl (23_c), H-Phe-Val-Arg-Phe-NH₂ · 2HCl (23_d), H-Phe-Leu-Arg-Phe-NH₂ · 2HCl (23_e), H-Phe-Ile-Arg-Phe-NH₂ · 2HCl (23_f), H-Phe-Nle-Arg-Phe-NH₂ · 2HCl (23_g), H-Phe-Phe-Arg-Phe-NH₂ · 2HCl (23_h) and H-Phe-Pro-Arg-Phe-NH₂ · 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); $[\alpha]_D^{20}$ $+16^\circ$ (*c* 0.5,

MeOH); R_{f1} 0.59; R_{f2} 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.

23_b: Yield 99%; mp 129 °C (decomp); $[\alpha]_D^{20}$ -8° (c 0.5, MeOH); R_{f1} 0.67; R_{f2} 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.

23_c: Yield 96%; mp 140 °C (decomp); $[\alpha]_D^{20}$ -6° (c 0.5, MeOH); R_{f1} 0.63; R_{f2} 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.

23_d: Yield 93%; mp 174 °C (decomp); $[\alpha]_D^{20}$ -19° (c 0.5, MeOH); R_{f1} 0.72; R_{f2} 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.

23_e: Yield 94%; mp 172 °C (decomp); $[\alpha]_D^{20}$ -14° (c 0.5, MeOH); R_{f1} 0.76; R_{f2} 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.

23_f: Yield 88%; mp 170 °C (decomp); $[\alpha]_D^{20}$ -16° (c 0.5, MeOH); R_{f1} 0.77; R_{f2} 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.

23_g: Yield 94%; mp 102 °C (decomp); $[\alpha]_D^{20}$ -6° (c 0.5, MeOH); R_{f1} 0.71; R_{f2} 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.

23_h: Yield 93%; mp 181 °C (decomp); $[\alpha]_D^{20}$ -2° (c 0.5,

MeOH); R_{f1} 0.69; R_{f2} 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.

23_i: Yield 98%; mp 92 °C (decomp); $[\alpha]_D^{20}$ -18° (c 0.5, MeOH); R_{f1} 0.59; R_{f2} 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.

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References

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