Peptide Synthesis in Aqueous Solution. I. Application of *p*-Dialkylsulfoniophenols as a Water-Soluble Coupling Reagent[1)]

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(*p*-Hydroxyphenyl)dimethylsulfonium methyl sulfate(HODMSP·$MeSO_4^-$) was found to be an excellent coupling reagent having a water-soluble property and a high reactivity; it worked as satisfactory as usual active esters in regard to the reactivity, product purity, and racemization. The marked advantage of the HODMSP·$MeSO_4^-$ active ester method was the fact that bifunctional residues such as Arg, Lys, Cys, and Tyr could be selectively acylated when the pH of the reaction mixture was controlled. The molluscan neuropeptide FMRFamide(Phe-Met-Arg-Phe-NH_2) was synthesized by this new active ester method for an evaluation of this method to further applications involving peptide syntheses.

The active ester method, which allows the formation of an amide bond under a very mild condition, is known to be useful for peptide syntheses or chemical modifications of proteins. The reaction process of this active ester method proceeds by activating a carbonyl carbon atom in the carboxyl component, as shown in Fig. 1. In spite of many studies[2-5)] about the active ester method, most reactions require organic solvents since these active esters are not soluble in water due to the hydrophobic character of their molecules.

Recently, Kawasaki et al. applied *p*-trimethylammoniophenyl ester[6)] as a water-soluble active ester to peptide synthesis. They also reported that the active ester was advantageous for the removal of any unreacted active ester and resultant hydroxy compound from the reaction mixture. As a new application of the water-soluble active ester, we have considered applying it to the peptide forming reaction in an aqueous solution. The most notable effectiveness of a reaction in an aqueous solution seems to be that the selective aminolysis reaction of amino acids which have functional groups in their side chain (such as Arg, Glu, Lys, Cys, His, or Tyr) is possible only by controlling the pH of the reaction mixture. For this application, we focused on *p*-dialkylsulfoniophenols (HODASP·X^-) as water-soluble active ester reagents. These compounds generally exhibited high acidity[7)] and high solubility in water, due to the presence of the dialkylsulfonio group in the molecule which showed both hydrophilic and electron-withdrawing characteristics.

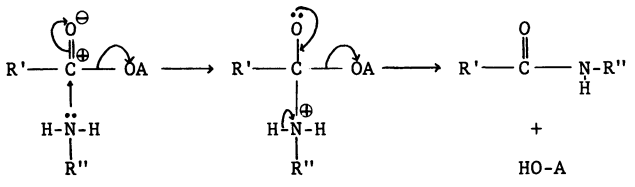
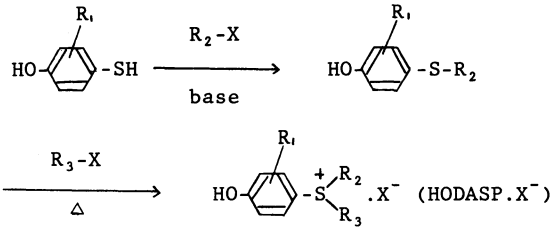


Fig. 1. Mechanism of aminolysis with active ester.

In this paper, we present such characteristic of HODASP·X^- as solubility in water or reactivity of their esters as well as fundamental information regarding applications to peptide syntheses, by making comparisons with other conventional HOSu or HONp active ester methods. Furthermore, the synthesis of FMRFamide, a molluscan neuropeptide, is also presented as an example application of the new method to a peptide synthesis in an aqueous solution.

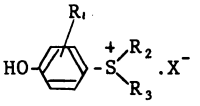
Results and Discussion

pK_a of HODASP·X^- and Solubility of Their Esters in Water. Many kinds of HODASP·X^- were prepared in order to develop a water-soluble active ester reagent according to the procedure shown in Scheme 1. The pK_a values of HODASP·X^-, which have ClO_4^- as a counter anion (**1**—**7**), were measured in 30% aqueous ethanol and compared with that of HONp or other phenols having a sulfur group at the para position. Furthermore, the solubility in water of these compounds were examined (Table 1). The pK_a values of **1** and **2** were nearly the same level as that of HONp and their solubilities in water were more than 30%. Compounds **8**$_{a-d}$ were prepared by changing the counter anion of **1** (R_1=H, R_2=Me, and R_3=Me), and examined in the same manner described above. As shown in Table 1, these compounds (**8**$_{a-d}$) exhibited the same property as **1**. Owing to the pK_a and solubility in water, we judged **8**$_{a-d}$ and **1** to be



Scheme 1.

Table 1. Various Phenols Prepared

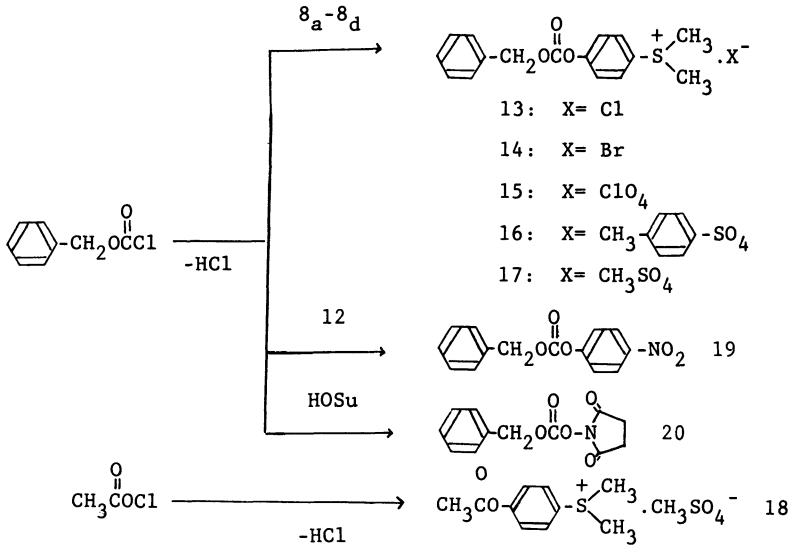


Run	R_1	R_2	R_3	X	Yield/%	Mp θ_m/°C (lit)[a]	Found(%) C	Found(%) H	Calcd(%) C	Calcd(%) H	R_{fs}[b]	pK_a	Solv.[c] (H_2O)
1	H	Me	Me	ClO_4	64	156—158 (157—159)	37.56	4.28	37.75	4.32	0.38	7.45	vs
2	H	Me	Et	ClO_4	49	79—83	40.41	4.78	40.25	4.84	0.38	7.45	vs
3	H	Et	Et	ClO_4	53	95—99	42.73	5.57	42.50	5.31	0.40	7.50	s
4	2-Me	Me	Me	ClO_4	64	111—114	40.54	4.53	40.25	4.84	0.41	7.75	s
5	3-Me	Me	Me	ClO_4	85	152—155	40.05	4.53	40.25	4.84	0.41	7.75	s
6	2,6-Me_2	Me	Me	ClO_4	73	174—176	42.17	5.47	42.50	5.31	0.45	7.85	s
7	2-Cl	Me	Me	ClO_4	74	128—131	33.54	3.23	33.24	3.46	0.38	5.80	s
8a	H	Me	Me	Cl	84	141—144	50.60	5.83	50.41	5.77	0.38	7.45	vs
8b	H	Me	Me	Br	81	145—146	40.64	4.81	40.88	4.68	0.36	7.41	vs
8c	H	Me	Me	TosO	74	77—79 (76—78)	52.78	5.94	52.33	5.81	0.42	7.43	vs
8d	H	Me	Me	$MeSO_4$	83	90—92 (92—93)	40.63	5.31	40.61	5.26	0.40	7.45	vs
9	HO-C$_6$H$_4$-SO_2Me				64	96—98 (96)	48.38	4.75	48.85	4.65	0.56	8.30	s
10	HO-C$_6$H$_4$-SCN				75	58—60 (62—63)	54.49	3.34	54.90	3.27	0.75	9.00	is
11	HO-C$_6$H$_4$-SMe				91	79—80 (76—79)	59.62	5.80	59.99	5.71	0.84	10.00	is
12	HO-C$_6$H$_4$-NO_2 (HONp)											7.40	is

Me=methyl, Et=ethyl, TosO=*p*-toluenesulfonate.

a) These literatures were described in Experimental. b) Solvent system: 1-Butanol-acetic acid-water (3 : 3 : 2).

c) Solubility in water at 25°C: vs: very soluble (above 30%), s: soluble, is: insoluble.



Scheme 2.

excellent reagents for the water-soluble active ester method. They were employed for the following investigation.

To examine the solubility of the esters derived from **1** and **8a—d** in water, we prepared their benzyl carbonates using benzyloxycarbonyl chloride as benzyloxycarbonyl donor, according to the procedure shown in Scheme 2. The benzyl carbonates from HONp and HOSu were also prepared in the same procedure. The solubilities of these compounds in water and organic solvent are summarized in Table 2. A benzyl carbonate in which a methyl sulfate anion is present as a counter anion **17** exhibited a high solubility in water (more than 30%) in spite of the hydrophobic benzyl group in its molecule. Similarly, acetyl derivative **18** of **8d**, which was prepared from acetyl chloride and **8d** was also very soluble in water. In addition, **17** and **18** were also very soluble in DMF, $CHCl_3$, and CH_3CN. These results indicate that the esters of **8d** possess sufficient solubilities to be applied to a coupling reaction in an aqueous environment.

Reactivity of the Ester of HODMSP·$MeSO_4^-$(8d) in Aminolysis. In order to compare the reaction of the benzyl carbonate (**17**) of HODMSP·$MeSO_4^-$ with that of the benzyl carbonates of HONp and HOSu, we examined their reaction rates with Gly in CH_3CN–H_2O at 25 °C (Fig. 2). As a result, it was found that **17** exhibited as a high reactivity as benzyl carbonates derived from HONp or HOSu. Furthermore, the water-soluble acylating agents derived from HODMSP·$MeSO_4^-$ were practically allowed to react with amino acids in aqueous solution (Table 3). As a result, the reaction of **17** or **18** with neutral amino acids proceeded in aqueous solution and the products were isolated in good yield. The acylation on the amino acids or peptides, which have a functional group on their side chain (such as Arg, Glu, Lys, Cys, or Gly–Tyr) was examined. The reaction with benzyl carbonate of HODMSP·$MeSO_4^-$ with Arg, Glu, His, Lys, or Gly–Tyr gave α-acylated compounds predominantly (at the controlled pH condition). Particularly, it was notable that reaction of **17** with Arg at pH 8.0 gave only an α-acylated derivative without any sub-reaction on guanidino group. However, the reaction of **17** with Cys or Lys gave an acylated product, mainly on a side functional group. This fact could explain why the reactivity of the functional group of the side chain was higher than that of the α-amino group. For the acylation of Tyr, Gly–Tyr was submitted to a test. As shown in Table 4, when the reaction proceeded at pH 6.0, the acylation was

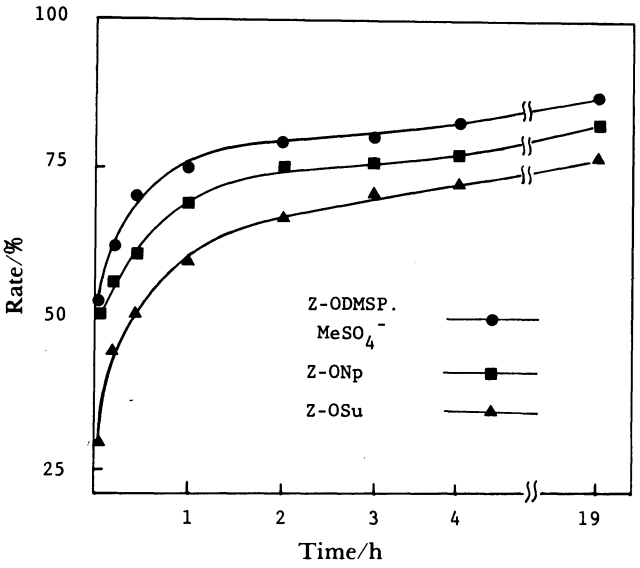
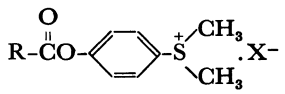


Fig. 2. Rates of aminolysis with Gly in CH_3CN–H_2O at 25 °C.

Table 2. Solubility of Esters Prepared



Run	R	X	Solubility	
			Water[a]	Organic solvent[b]
13	Benzyloxy	Cl	is	DMF, CH_3CN
14	Benzyloxy	Br	is	DMF, CH_3CN
15	Benzyloxy	ClO_4	is	DMF, CH_3CN
16	Benzyloxy	TosO	s(3%)	DMF, CH_3CN, $CHCl_3$
17	Benzyloxy	$MeSO_4$	vs(>30%)	DMF, CH_3CN, $CHCl_3$
18	Methyl	$MeSO_4$	vs(>30%)	DMF, CH_3CN, $CHCl_3$
19			is	Ether, AcOEt, THF
20			is	Ether, AcOEt, THF

TosO = *p*-toluensulfonate, Me = methyl, AcOEt = ethyl acetate.

a) Solubility in water at 25°C, vs = very soluble, s = soluble, is = insoluble (<1%). b) The solvents having solubility above 10% were listed.

predominantly inclined on the α-amino group of Gly moiety; while at pH 8.0, the acylation toward the phenolic hydroxy group of the Tyr moiety was increased. Because of the results described above, the esters of HODMSP·$MeSO_4^-$ were subjected to aminolysis in an aqueous solution to easily form an amide bond. In addition to this fact, a selective acylation was carried out at the suitable pH. We considered that the esters of HODMSP·$MeSO_4^-$ are quite useful as amino-protecting reagents.

Application of HODMSP·$MeSO_4^-$ Active Ester for Peptide Synthesis. In order to know whether the

Table 4. Acylation of Gly-Tyr with **17**

17 —(H-Gly-Tyr-OH; in 0.4 M phosphate buffer (pH 6 or 8) at 15°C, 10 h)→ Z-Gly-Tyr-OH **21** / Z-Gly-Tyr(Z)-OH **22**

pH	Product/% (HPLC)[a]		Yield of **21**/%
	21	**22**	
6	92.3	7.7	41
8	72.2	27.8	64

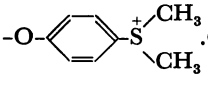
a) Product(%) means the ratio of **21** and **22** by HPLC.

Table 3. Preparation of Acylamino Acids

Acylating agent	Amino acid	Product	Reaction pH	Yield/%	Mpθ_m/°C(lit)	$[\alpha]_D$/°(c,solvent)(lit)	Ref.
17	Gly	Z-Gly-OH	10.0	72	118—120(120)	—	15
17	Ala	Z-Ala-OH	10.0	70	85—86(87)	−15(c2, AcOH) (−13.9)	16
18	Phe	Ac-Phe-OH	10.0	63	166—168(168)	+45(c2, AcOH) (+47)	17
17	Ser	Z-Ser-OH	9.0	85	118—119(119)	+6(c2, AcOH) (+5.9)	18
17	Arg	Z-Arg-OH	8.0	82	170—173(175)	−11(c2, 1M HCl) (−9.3)	19
17	Glu	Z-Glu-OH	9.0	62	120—122(120—121)	−10(c2, AcOH) (−7.9)	20
17	His	Z-His-OH	8.5	51	163—167(167)	−22(c2, 6M HCl) (−25)	21
17	Lys	H-Lys(Z)-OH	8.0	48	250—253(251—254)	+16(c1, 6M HCl) (+14.4)	22
17	Cys	H-Cys(Z)-OH	6.3	38	162—164(165)	−122(c1, DMF) (−134)	23

Table 5. Preparation of Protected Peptides

Active ester	Amine component	Product	Solvent (pH)	Yield/%	Mpθ_m/°C(lit)	$[\alpha]_D$/°(c,solvent)(lit)	Ref.
Z-Ala-ODMSP	Gly-OMe	Z-Ala-Gly-OMe	$CHCl_3$	74	99—100 (98—99)	−26(c1, MeOH) (−25)	24
Z-Phe-ODMSP	Leu-OMe	Z-Phe-Leu-OMe	$CHCl_3$	81	108—111 (110—111)	−23(c1, MeOH) (−25)	25
Boc-Leu-ODMSP	Gly-OMe	Boc-Leu-Gly-OMe	$CHCl_3$	63	128—130 (131)	−23(c1, DMF) (−21)	26
Z-Ala-ODMSP	Gly	Z-Ala-Gly-OH	H_2O(10.0)	62	132—134 (134—135)	−16(c1, EtOH) (−17)	27
Z-Ala-ONp	Gly	Z-Ala-Gly-OH	THF-H_2O	63	130—134 (134—135)	−16(c1, EtOH) (−17)	27
Z-Ala-OSu	Gly	Z-Ala-Gly-OH	THF-H_2O	47	132—134 (134—135)	−15(c1, EtOH) (−17)	27
Z-Phe-ODMSP	Pro	Z-Phe-Pro-OH	H_2O(10.0)	40	104—106 (105—106)	−63(c1, py) (−64)	28
Z-Phe-ONp	Pro	Z-Phe-Pro-OH	THF-H_2O	78	102—104 (105—106)	−58(c1, py) (−64)	28
Z-Phe-OSu	Pro	Z-Phe-Pro-OH	THF-H_2O	56	104—106 (105—106)	−70(c1, py) (−64)	28
Z-Ala-ODMSP	Ser	Z-Ala-Ser-OH	H_2O(9.0)	63	202—205 (205)	+27(c1, DMF) (+28)	29
Z-Ile-ODMSP	Glu	Z-Ile-Glu-OH	H_2O(9.0)	47	186—188 (189—190)	−23(c1, 0.5 M $KHCO_3$) (−21)	30
Z-Phe-ODMSP	His	Z-Phe-His-OH	H_2O(7.0)	43	181—184 (183—185)	−7(c1, DMF)	31
Z-Pro-ODMSP	Gly-Tyr	Z-Pro-Gly-Tyr-OH	H_2O(6.8)	41	102—103 (102)	−12(c1, EtOH) (−12)	32

-ODMSP=  , py= pyridine.

active esters of HODMSP·$MeSO_4^-$ are applicable to peptide synthesis, some esters of the N-protected amino acids were prepared by direct condensation using DCC in CH_3CN. These esters, thus obtained, were very soluble in water, and were able to react with amine components for the formation of the amide bond (Table 5). When they were allowed to react with amine components in $CHCl_3$, the yield of the reaction products was always good and their purities were also quite satisfactory. In addition, the removal of unreacted active ester and the hydroxy compound resulting from the reaction was easy by an extraction procedure. Furthermore, these esters were also able to react with amino acids in aqueous solution in the presence of Et_3N at room temperature. The reaction proceeded smoothly, while the reaction of the HONp or HOSu active ester required an addition of THF. The products were isolated in good yield, and were comparable with those of HONp or HOSu active ester method. When HODMSP·$MeSO_4^-$ active esters were treated with Ser, His, Glu, and Gly-Tyr as amine components in aqueous solution, every reaction afforded the *N*-α-acylpeptides selectively.

Racemization Test. Izumiya et al. established the racemization test by ion-exchange chromatography.[33] We tried to determine the exact degree of racemization in the HODMSP·$MeSO_4^-$ active ester method by the same principle as their report. As a model peptide, L-Leu-L-Asp was prepared by this method and the ratio of diastereomeric dipeptide was measured by HPLC. The result was compared with that of the HONp or HOSu active ester method (Fig. 3). Figure 3 shows that racemization in our method, if any, was not detectable as well as in other methods. A further examination of the racemization was carried out in the coupling reaction of Z-Gly-L-Ala-OH and L-Leu (Table 6). The results suggested that the HODMSP·$MeSO_4^-$ active ester method is as useful as the existing active ester method in stepwise condensation using an N-protected amino acid with a urethane-type protecting group.

Application of HODMSP·$MeSO_4^-$ Active Ester for Synthesis of Biologically Active Peptide (Synthesis of FMRFamide). In order to evaluate the utility of the HODMSP·$MeSO_4^-$ active ester method, we tried to apply it to a synthesis of a tetra peptide amide, Phe-Met-Arg-Phe-NH_2 (FMRFamide) as an example of biologically active peptides.

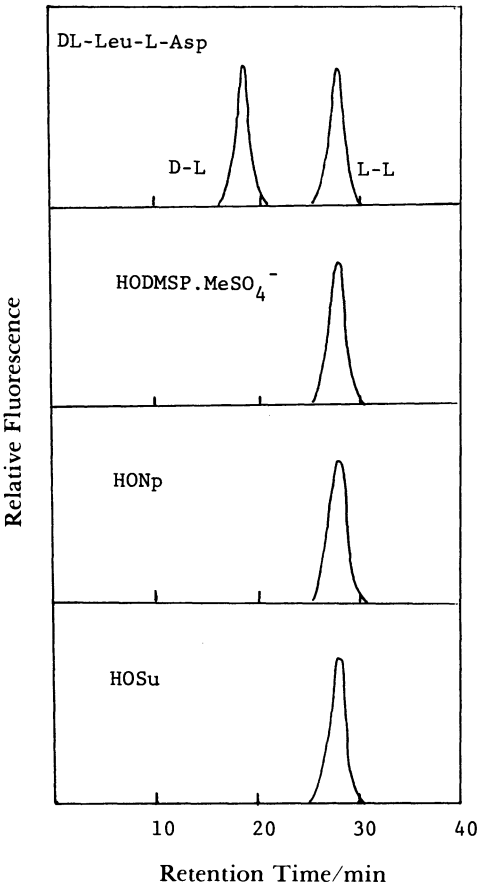


Fig. 3. Racemization test in Leu-Asp.

Table 6. Racemization Test in Gly-Ala-Leu

I. Z-Gly-L-Ala-OH + HO-A $\xrightarrow{DCC}$ Z-Gly-Ala-OA
(L or D)

II. Z-Gly-Ala-OA + L-Leu ⟶ Z-Gly-Ala-Leu-OH $\xrightarrow{H_2/Pd}$ H-Gly-Ala-L-Leu-OH
(L or D) (L or D) (L or D)

HO-A	I		II		D-L / L-L + D-L (%)
	Solv.	Temp/°C	Solv.	Temp/°C	
HOSu	THF	5	THF-H_2O	RT	16.4
HONp	THF	5	THF-H_2O	RT	50.9
HOPcp	THF	5	THF-H_2O	RT	40.7
HODMSP	CH_3CN	5	H_2O	RT	39.3

HODMSP= HO-C$_6$H$_4$-S$^+$(CH$_3$)$_2$.$CH_3SO_4^-$, Solv.= solvent, RT= room temperature.

FMRFamide was isolated from the ganglia of the clam *Macrocallista nimbosa* by Prince and Greenberg.[34] It has diverse effects on molluscan muscles and neurones.[35] For the synthesis of FMRFamide, the method of selective acylation to the Arg residue was employed. The synthetic route is shown in Fig. 4. Boc-Met-ODMSP·$MeSO_4^-$ (**25**), which was obtained by the esterification of Boc-Met-OH with HODMSP·$MeSO_4^-$, was allowed to react with H-Arg-Phe-NH_2·2AcOH (**24**), which was obtained

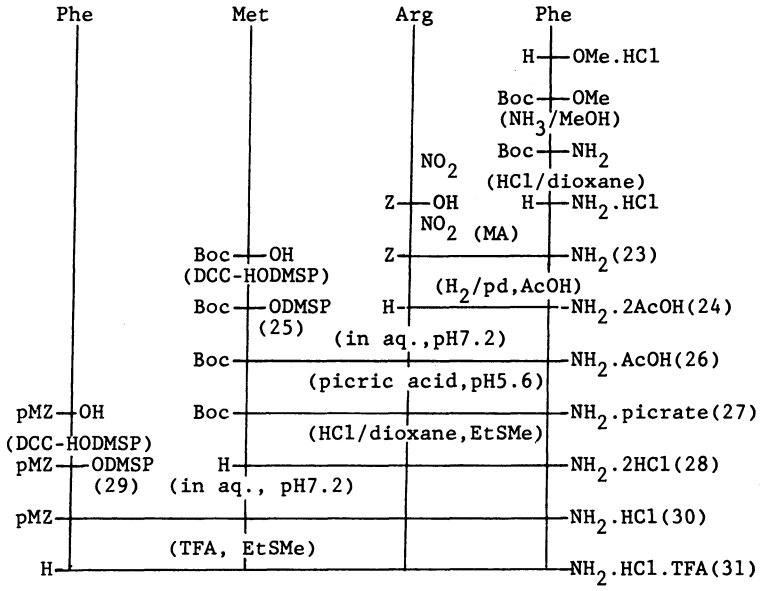


Fig. 4. Synthetic route of FMRFamide.

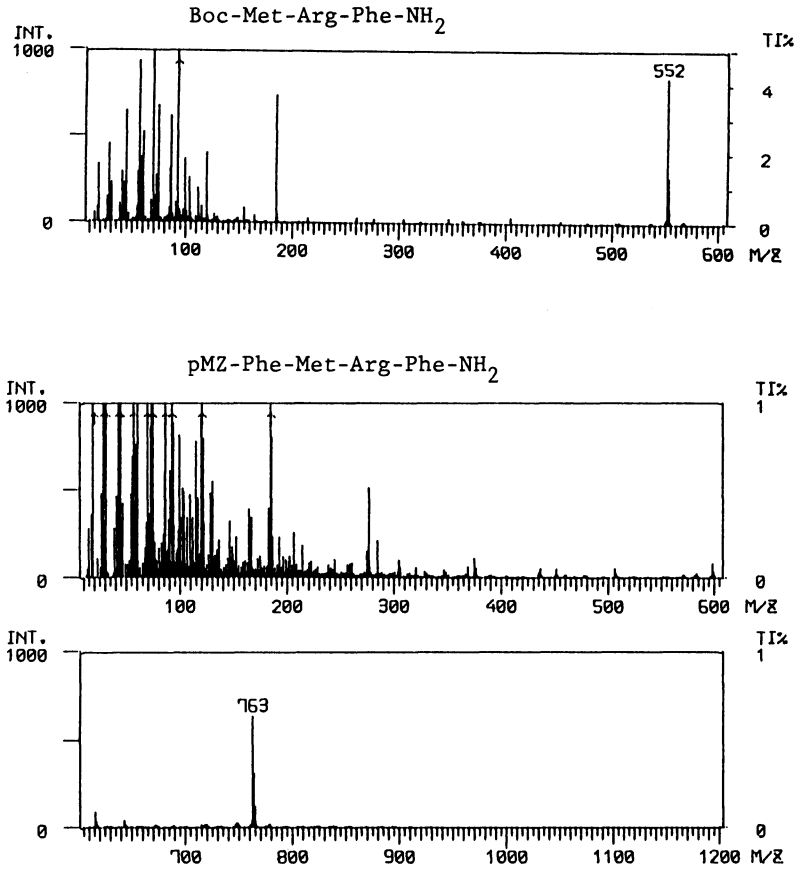


Fig. 5. Mass spectra in intermediates of FMRFamide.

from Z-Arg(NO_2)-OH and H-Phe-NH_2·2HCl by the conventional mixed anhydride method, followed by catalytic hydrogenation. The reaction proceeded on an aqueous solution with the presence of Et_3N at pH 7.2. After the isolation of Boc-Met-Arg-Phe-NH_2 as a picrate **27** by extraction with ethyl acetate from the reaction mixture, the Boc-group was removed by HCl in the presence of ethyl methyl sulfide, to yield tripeptide amide·2HCl (**28**). Then pMZ-Phe-ODMSP (**29**), which was obtained by the esterification of pMZ-Phe-OH with HODMSP·$MeSO_4^-$, was allowed to react with the tripeptide amide **28** (in the same manner as described regarding compound **26**) to yield pMZ-FMRFamide·HCl(**30**). After removing the pMZ-group of pMZ-FMRFamide (**30**) with trifluoroacetic acid in the presence of ethyl methyl sulfide, FMRFamide (**31**) was obtained. In the process of synthesizing **26** in aqueous solution, the product was not isolated, as such, owing to its high water-solubility. For this reason, this peptide was isolated and purified as a picrate. In the case of **30**, it could be extracted with ethyl acetate and purified smoothly. The homogeneity of these intermediates and the product was ascertained by TLC, elemental analysis, amino acid analysis, and mass spectra (Fig. 5).

The biological activity was measured on the anterior byssus retractor muscle of *Mytilus*, FMRFamide causes a relaxation or a contraction depending on its concentration.[36] At 10^{-8}—10^{-7} M (1 M=1 mol dm^{-3}), FMRFamide relaxes catch tension induced by acetylcholine (Ach). However, at a concentration higher than 10^{-7} M, it elicits a contraction by a direct action on the muscle fibers. From measurements of biological activity,[37] this synthesized FMRFamide was shown to possess the same activity as a natural one (Fig. 6).

All these results indicate that the HODMSP active ester method is promising for peptide syntheses in aqueous solution. We expect that our new active ester method will also be applicable for the chemical modifications of proteins.

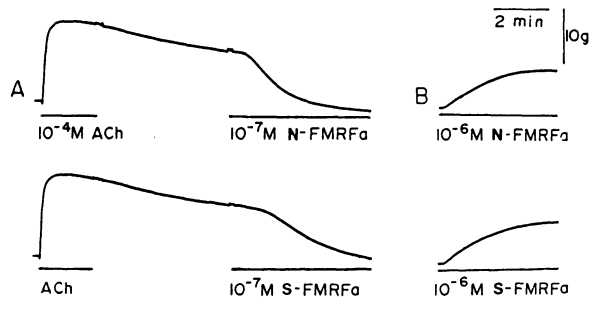


Fig. 6. Contractions and relaxations produced by synthesized FMRFamide and natural FMRFamide. A: Contractile effect, B: relaxing effect, N-FMRFa: natural FMRFamide, S-FMRFa: synthesized FMRFamide.

Experimental

All melting points were uncorrected. IR spectra were recorded on a Hitachi 269-30 infrared spectrometer. NMR spectra were measured on a Hitachi R-24B (60 MHz) spectrometer. The chemical shifts are given in δ (ppm) scale with tetramethylsilane as an internal standard. Mass spectra were obtained on a JEOL DX-300 spectrometer. The optical rotations were measured on a Union PM-101 polarimeter. TLC was carried out on Merck Silicagel G and Eastman chromagram sheet with solvent systems: 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 a free amino group on the TLC plate were detected by spraying with ninhydrin; those of amino group blocked materials were detected by spraying with 25% HBr in acetic acid and then ninhydrin. R_f spots of HODASP·X^- and its derivatives were visible with UV light. Amino acid analysis, detection of racemization, and other detection of substances were performed by HPLC using JASCO TRI ROTAR-V and UVIDEC-100V apparatus.

Preparation of HODASP·X^- and Other Phenols. **1**,[8] **8_c**,[9] **8_d**,[9] **9**,[10] **10**,[11] and **11**[12] were prepared by methods described in the literature. **2**—**7** and **8_a**—**$_b$** were prepared by reacting the corresponding thioether with a slight excess of dimethyl sulfate or diethyl sulfate. The reaction mixture was dissolved in ethanol; then, the ethanol solution was treated with an equimolar 70% $HClO_4$ below 5 °C. The resulting crystals were removed from the reaction mixture by filtration. The filter cake was washed with precooled ethanol and then recrystallized from ethanol or ethanol-ether. In the case of **8_a** or **8_d**, the ethanol solution was treated with 35% HCl or 15% HBr in place of 70% $HClO_4$. A white crystalline product was obtained. The precursor thioethers were prepared by the alkylation of the 4-mercaptophenols.

Preparation of Acylating Agents. Benzyl Carbonates: **19**[13] and **20**[14] were prepared by the methods described in the literature. **13**—**17** were prepared as follows. A mixture of the corresponding phenols and equimolar benzyloxycarbonyl chloride in CH_3CN was stirred at 5 °C. After 10 min, equimolar Et_3N was added. The reaction mixture was stirred at 5 °C for 1 h and then the precipitated triethylammonium chloride was removed from the reaction mixture by filtration. The filter cake was washed with CH_3CN, and combined filtrates were concentrated in vacuo to give the product either as a crystalline solid or as an oily residue, which was readily crystallized from ethyl acetate or ether.

13: Yield 84%; mp 68—69 °C; R_{f_3} 0.67; IR (KBr) 1762 cm^{-1} (ester C=O); ^{1}H NMR (DMSO-d_6) δ=3.15 (6H,s,-$\overset{+}{S}(CH_3)_2$), 5.28 (2H, s, -CH_2-), 7.44 (5H, s, C_6H_5), 7.63 (2H, d, J=8Hz, C_6H_4), 8.16 (2H, d, J=8Hz, C_6H_4); Found: C, 59.40; H, 5.19%; Calcd for $C_{16}H_{17}O_3SCl$: C, 59.19; H, 5.24%.

14: Yield 87%; mp 71—73 °C; R_{f_3} 0.65; IR (KBr) 1762 cm^{-1} (ester C=O); ^{1}H NMR (DMSO-d_6) δ=3.25 (6H, s, -$\overset{+}{S}(CH_3)_2$), 5.30 (2H, s, -CH_2-), 7.40 (5H, s, C_6H_5), 7.61 (2H, d, J=8Hz, C_6H_4), 8.12 (2H, d, J=8Hz, C_6H_4); Found: C, 52.21; H, 4.53%; Calcd for $C_{16}H_{17}O_3SBr$: C, 52.06; H, 4.61%.

15: Yield 72%; mp 141—143 °C; R_{f_3}, 0.54; IR (KBr) 1764 cm^{-1} (ester C=O); ^{1}H NMR (DMSO-d_6) δ=3.30 (6H, s, -$\overset{+}{S}(CH_3)_2$), 5.34 (2H, s, -CH_2-), 7.44 (5H, s, C_6H_5), 7.65 (2H, d, J=9Hz, C_6H_4), 8.15 (2H, d, J=8Hz, C_6H_4); Found: C, 49.21; H, 4.45%; Calcd for $C_{16}H_{17}O_7SCl$: C, 49.45; H, 4.37%.

16: Yield 80%; mp 155—157 °C; R_{f_3} 0.71; IR (KBr) 1770 cm^{-1} (ester C=O); ^1H NMR (DMSO-d_6) δ=2.20 (3H, s, CH_3-C_6H_5), 3.24 (6H, s, $-\overset{+}{S}(CH_3)_2$), 5.44 (2H, s, $-CH_2-$), 7.05—8.30 (13H, m, aromatic); Found: C, 60.31; H, 5.34%; Calcd for $C_{23}H_{24}O_6S_2$: C, 60.01; H; 5.21%.

17: Yield 81%; mp 78—80 °C; R_{f_3} 0.55; IR (KBr) 1760 cm^{-1} (ester C=O); ^1H NMR (DMSO-d_6) δ=3.30 (6H, s, $-\overset{+}{S}(CH_3)_2$), 3.42 (3H, s, CH_3SO_4), 5.35 (2H, s, $-CH_2-$), 7.41 (5H, s, C_6H_5), 7.60 (2H, d, J=9Hz, C_6H_4), 8.08 (2H, d, J=8Hz, C_6H_4); Found: C, 51.42; H, 4.58%; Calcd for $C_{17}H_{20}O_7S_2$: C, 51.51; H, 4.99%.

4-Acetoxyphenyldimethylsulfonium Methylsulfate: A mixture of **8**$_d$ and equimolar acetyl chloride in CH_3CN was stirred at 5 °C. After 10 min, equimolar Et_3N was added to it. A white crystalline product was obtained (as described for the preparation of benzyl carbonates). Yield 65%; mp 91—93 °C; R_{f_3} 0.56; IR (KBr) 1750 cm^{-1} (ester C=O); ^1H NMR (DMSO-d_6) δ=2.40 (3H, s, CH_3CO), 3.30 (6H, s, $-\overset{+}{S}(CH_3)_2$), 3.41 (3H, s, CH_3SO_4), 7.45 (2H, d, J=8Hz, C_6H_4), 8.10 (2H, d, J=8Hz, C_6H_4); Found: C, 42.43; H, 5.24%; Calcd for $C_{11}H_{16}O_6S_2$: C, 42.87; H, 5.19%.

Preparation of Active Ester of N-Protected Amino Acids. A typical example is as follows. **Z-Ala-ODMSP·$MeSO_4^-$**: Z-Ala-OH (2.23 g, 10 mmol) and **8**$_d$ (2.66 g, 10 mmol) were dissolved in CH_3CN and DCC (2.66 g, 10 mmol) was added to the solution below 0 °C with stirring. After stirring overnight DCurea was filtered off and the filtrate was concentrated in vacuo to give an oily residue. The oily residue was washed with ether by decantation. Yield 87%; R_{f_1} 0.82; R_{f_2} 0.25; $[\alpha]_D^{20}$ −18° (c1, MeOH); IR (neat) 1760 cm^{-1} (ester C=O), 1700 cm^{-1} (amide C=O). **Z-Phe-ODMSP·$MeSO_4^-$**: Yield 90%; R_{f_1} 0.77; R_{f_2} 0.41; $[\alpha]_D^{20}$ −16° (c1, MeOH); IR (neat) 1764 cm^{-1} (ester C=O), 1700 cm^{-1} (amide C=O). **Boc-Leu-ODMSP·$MeSO_4^-$**: Yield 88%; R_{f_1} 0.77; R_{f_2} 0.38; $[\alpha]_D^{20}$ −29° (c1, MeOH); IR (neat) 1762 cm^{-1} (ester C=O), 1698 cm^{-1} (amide C=O). **Z-Pro-ODMSP·$MeSO_4^-$**: Yield 90%; R_{f_1} 0.61; R_{f_2} 0.30; $[\alpha]_D^{20}$ −62° (c1, MeOH); IR (neat) 1760 cm^{-1} (ester C=O), 1700 cm^{-1} (amide C=O). All these active esters were obtained as an oily product.

Reaction of 17 or 18 with Amino Acids or Peptide in Aqueous Solution. In the Case of Neutral Amino Acids: Amino acids and equimolar Et_3N were dissolved in water and equimolar **17** or **18** was added to the solution at room temperature with stirring. After 4 h, 20% citric acid was added to the solution and the pH of the solution was adjusted to 3.0. The mixture was extracted with ethyl acetate. The solution was washed with water and then dried over anhydrous sodium sulfate. The filtrate was concentrated in vacuo. The oily residue was crystallized with petroleum ether. **In the Case of Arg, Ser, His, Glu, Lys, Cys, and Gly-Tyr:** Each amino acid or peptide was dissolved in water and the pH of the solution was adjusted to 6.0—9.0 by addition of Et_3N. An equimolar amount **17** was added to a stirred solution below 20 °C, the pH being maintained automatically at the starting pH with Et_3N for 7 h. In the case of Ser or Glu, 20% citric acid was added to the solution and the pH of the solution was adjusted to 3.0. The mixture was extracted with ethyl acetate. The solution was washed with water and then dried over anhydrous sodium sulfate. The filtrate was concentrated in vacuo. The oily residue was crystallized with ether and petroleum ether. In the case of Arg, His, Lys, and Cys, each product was precipitated from the solution. The crystalline product was removed from the reaction mixture by filtration. Gly-Tyr was acylated using **17** in 0.4 M phosphate buffer (Na_2HPO_4-NaH_2PO_4) at pH 6.0 and 8.0 in a similar manner to the acylation of amino acids. The product which resulted from the reaction at each pH was isolated by extraction with ethyl acetate, respectively; then, the ratio of **21** and **22** in the product was determined by HPLC (column: Lichrosob PR-18; eluent: 85% MeOH). The results of acylation of amino acids and peptide in aqueous solution are shown in Tables 3 and 4.

Preparation of Protected Peptide Using HODMSP·$MeSO_4^-$ Active Ester. Reaction in $CHCl_3$: To a solution of HODMSP·$MeSO_4^-$ active ester of N-protected amino acid in $CHCl_3$, a solution of equimolar amino acid ester and Et_3N in $CHCl_3$ was added. The reaction mixture was stirred for 7 h at room temperature. After removing the solvent in vacuo, the residue was dissolved in ethyl acetate, and the solution was washed, successively, with water and 4% citric acid. The organic solution was dried over anhydrous sodium sulfate. The solution was concentrated to an oily residue which was crystallized by addition of ether and petroleum ether. **Reaction in H_2O: (A) In the Case of Neutral Amino Acid as Amine Component:** To a solution of HODMSP·$MeSO_4^-$ active ester of N-protected amino acid in water, a solution of equimolar amino acid and Et_3N in water was added. The reaction mixture was stirred at room temperature. After 12 h, 20% citric acid was added to the solution and the pH of the solution was increased to 3.0—4.0. The mixture was extracted with ethyl acetate. The solution was washed with water and dried over anhydrous sodium sulfate. The filtrate was concentrated in vacuo. The oily residue was crystallized with ether. **(B) In the Case of Ser, Glu, His, and Gly-Tyr as Amine Component:** These peptide were prepared with HODMSP·$MeSO_4^-$ active ester of N-protected amino acid in a similar manner to the acylation of amino acid having functional group in the side chain. Z-Ala-Ser-OH, Z-Ile-Glu-OH, and Z-Pro-Gly-Tyr-OH were obtained after extraction with ethyl acetate from the reaction mixture and crystallized from ether and petroleum ether. They were recrystallized from ethyl acetate. Z-Phe-His-OH was obtained after filtration from the reaction mixture. The results of these preparations are shown in Table 5.

Preparation of Protected Peptides Using HONp or HOSu Active Ester. To a solution of HONp or HOSu active ester of N-protected amino acid in THF, a solution of equimolar amino acid and Et_3N in water was added. The reaction mixture was stirred for 7 h at room temperature. After removing THF in vacuo, the aqueous solution was extracted with ethyl acetate to remove the unreacted active ester. 20% citric acid was added to the aqueous layer and the pH was adjusted to 3.0. The resulting oily product was extracted with ethyl acetate, and then washed with water. The organic solution was dried over anhydrous sodium sulfate. The filtrate was concentrated to an oily residue which was crystallized by the addition of ether.

Determination of the Rate of Aminolysis. Into a 50 ml volumetric flask was added a solution containing 2.0 mmol of an appropriate derivative **17**, **19**, or **20** in CH_3CN. To the solution having been heated at 25 °C was added a solution of 2.4 mmol of Gly in H_2O (50 ml). At predetermined time intervals, 1 ml aliquotes of the solution

were withdrawn and diluted with distilled water (total volume, 100 ml). The consumption of Gly was measured using HPLC (column: AApack Na; eluent: 0.1 M citrate buffer, pH 3.05, 60 °C). Each point in Fig. 3 represents a mean value of three runs.

Detection of Racemization. Leu-Asp: Boc-Lue-Asp-OH was prepared using HODMSP·$MeSO_4^-$ active ester, HONp active ester, or HOSu active ester. Each Boc-Leu-Asp-OH obtained was then treated with 4 M HCl in dioxane to afford Leu-Asp. A chromatographic detection of this diastereomeric dipeptide was carried out with HPLC (column: AApack Na; eluent: 0.1 M citrate buffer, pH 3.25, 60 °C). The detection of the dipeptide was performed by OPA detection system. Authentic samples of L-Leu-L-Asp and D-Leu-L-Asp were prepared by the usual manner. **Gly-Ala-Leu:** Z-Gly-D-Ala-OH and authentic samples of H-Gly-L-Ala-L-Leu-OH and H-Gly-D-Ala-L-Leu-OH were prepared by the methods described in the literature.[33] The dipeptide (Z-Gly-L-Ala-OH) was esterified with HODMSP·$MeSO_4^-$, HONp, HOSu, or HOPcp and DCC to afford the corresponding active ester. The conditions of esterification are summarized in Table 6. Each active ester, thus obtained, was allowed to react with L-Leu at room temperature overnight to give protected tripeptide. After catalytic hydrogenation to remove the protecting group, the tripeptide was analyzed according to the procedure in Leu-Asp.

Synthesis of FMRFamide. Z-Arg(NO_2)-Phe-NH_2 (23): A solution of Z-Arg(NO_2)-OH (3.53g, 10 mmol) and NMM (1.1 ml, 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 reaction mixture was concentrated in vacuo, and the residue was suspended in hot methanol (50 ml). The solid was filtered and washed with methanol; yield 4.24 g (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 (24):** Compound **23** (2.50 g, 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·$MeSO_4^-$ (25):** To a chilled solution of Boc-Met-OH (2.49 g, 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 kept 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 (27):** Compound **24** (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·$MeSO_4^-$ (0.99 g, 2 mmol) was added to the stirred solution below 20 °C, the pH being automatically maintained 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 successively washed 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 the 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 $C_{25}H_{41}O_5N_7S·C_6H_3N_3O_7·1/2H_2O$: C, 47.17; H, 5.70; N, 17.74%; MS *m/z* 552 (M^+H); Amino acid ratios in acid hydrolyzate: Arg 0.94, Met 0.89, Phe 1.00. **H-Met-Arg-Phe-NH_2·2HCl (28):** To a solution of **27** (0.78 g, 1 mmol) and ethyl methyl sulfide (1 ml) in dioxane (5 ml), 4.1 M HCl in dioxane (5 ml) was added. 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 0.47 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 $C_{20}H_{33}O_3N_7S.2HCl$: 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-Phe-ODMSP·$MeSO_4^-$ (29):** pMZ-Phe-OH (3.29 g, 10 mmol) and HODMSP·$MeSO_4^-$ (2.66 g, 10 mmol) were coupled by the same method as **25**. The oily product was obtained; yield of the oily product 5.20 g (90%); $[\alpha]_D^{20}$ −7° (*c* 1, MeOH); R_{f_1} 0.85; R_{f_2} 0.51. **pMZ-Phe-Met-Arg-Phe-NH_2 (30):** Compound **28** (0.52 g, 1 mmol) was dissolved in H_2O (10 mmol) and the solution was adjusted to 7.2 by addition of Et_3N. pMZ-Phe-ODMSP.$MeSO_4^-$ (0.69 g, 1.2 mmol) was added to the stirred solution below 20 °C, the pH being maintained automatically at 7.2 with Et_3N. After 12 h, the mixture was extracted with ethyl acetate (100 ml). The solution was successively washed with 4% sodium hydrogencarbonate, 4% citric acid, and water, and then dried over anhydrous sodium sulfate. The solution was concentrated to a crystalline residue. It was recrystallized from methanol; yield 0.33 g (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%. MS *m/z* 763 (M^+H); amino acid ratios in acid hydrolyzate: Arg 0.95, Met 0.87, Phe 2.00. **H-Phe-Met-Arg-Phe-NH_2 (FMRFamide)·HCl·TFA (31):** Compound **30** (0.4 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 0.37 g (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.

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References

1) The abbreviations recommended by the IUPAC-IUB commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Amino acid symbols except

glycine denote the L-configuration. Additional abbreviations: DCC, dicyclohexylcarbodiimide; DCurea, *N,N'*-dicyclohexylurea; HONp, *p*-nitrophenol; HOSu, *N*-hydroxysuccinimide; ECF, ethyl chloroformate; THF, tetrahydrofuran; MeOH, methanol; Et_3N, triethylamine; NMM, *N*-methylmorpholine; MA,mixed anhydride; TFA, trifluoroacetic acid; py, pyridine.

2) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

3) J. Kovace, M. Q. Ceperini, C. A. Dupraz, and G. N. Schmit, *J. Org. Chem.*, **32**, 3696 (1967).

4) G. W. Anderson, J. E. Zimmerman, and F. Callahar, *J. Am. Chem. Soc.*, **85**, 3039 (1967).

5) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).

6) T. Tsuji, S. Okusada, M. Maeda, and K. Kawasaki, *Chem. Pharm. Bull.*, **34**, 2214 (1986).

7) S. Oae and C. C. Price, *J. Am. Chem. Soc.*, **80**, 3425 (1958).

8) J. K. Coward and W. D. Sweet, *J. Org. Chem.*, **36**, 2337 (1971).

9) F. G. Bordwell and P. J. Boutan, *J. Am. Chem. Soc.*, **78**, 87 (1956).

10) J. Yates and E. Haddock, Brit. Patent, 1128217 (1958).

11) F. G Bordwell and P. J. Bautan, *J. Am. Chem. Soc.*, **78**, 854 (1956).

12) Nippon Kayaku Co. Ltd., Jpn. Patent, 9134 (1967).

13) M. C. Khosla, F. M. Bumpus, and R. R. Smery, *Indian J. Chem.*, **5**, 279 (1967).

14) M. Frankel, D. Ladkany, C. Gilon, and Y. Wolman, *Tetrahedron Lett.*, **1966**, 4765.

15) M. Bergman and L. Zervas, *Ber.*, **65**, 1192 (1932).

16) M. Hunt and V. du Vigneaud, *J. Biol. Chem.*, **124**,699 (1938).

17) B. Weinstein and A. E. Pritchard, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 1015.

18) E. Wünch and J. Jentsch, *Chem. Ber.*, **97**, 2490 (1964).

19) R. A. Boissonnas, S. Guttman, R. L. Huguenin, P. A. Jaquenoud, and E. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).

20) E. Wünch, and A. Zwick, *Chem. Ber.*, **97**, 2497 (1964).

21) A. Patchornik, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, **79**, 6416 (1957).

22) S. Kuwata, and H. Watanabe, *Bull. Chem. Soc. Jpn.*, **38**, 676 (1965).

23) E. Scoffone, R. Rocchi, G. Vidal, A. Scatturine, and F. Marchiori, *Gazz. Chim. Ital.*, **94**, 743 (1964).

24) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

25) Z. Pravda, K. Poduska, and K. Blaha, *Collect. Czech. Chem. Commun.*, **29**, 2626 (1964).

26) A. S. Dutta and J. S. Morley, *J. Chem. Soc. C*, **1971**, 2896.

27) F. H. C. Stewart, *Aust. J. Chem.*, **21**, 2543 (1968).

28) S. Sakakibara and M. Itoh, *Bull. Chem. Soc. Jpn.*, **40**, 656 (1967).

29) N. Yanaihara, C. Yanaihara, M. Sakagami, T. Nakajima, T. Nakayama, and K. Matsumoto, *Chem. Pharm. Bull.*, **21**, 616 (1973).

30) D. S. Kemp, S. J. Worbel, Jr., S. W. Wang, Z. Bernstein, and J. Rebek, Jr., *Tetrahedron*, **30**, 3677 (1974).

31) G. Losse and G. Muler, *Chem. Ber.*, **94**, 2768 (1961).

32) I. Z. Siemion, *Rcczniki Chem.*, **38**, 811 (1964).

33) N. Izumiya, M. Muraoka, and H.Aoyagi, *Bull. Chem. Soc. Jpn.*, **44**, 3391 (1971).

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

35) S. D. Painter and J. M. Greenberg, *Biol. Bull.*, **162**, 311 (1982).

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

37) We asked Professor Yojiro Muneoka of Hiroshima University to measured the biological activity. He measured these activities according to his report. Y. Muneoka and H. Saitoh, *Comp. Biochem. Physiol.*, **85c**, 207 (1986).