[Chem. Pharm. Bull. 25(11)2929—2934(1977)]

UDC 547.466.1.04 : 547.269.3.04

Studies on Peptides. LXXIII.[1,2] Examination of the Methanesulphonic Acid Procedure for the Synthesis of Peptides containing Methionine

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(Received March 11, 1977)

Aromatic ethers, such as anisole or phenetole, were cleaved smoothly with methanesulphonic acid (MSA) or trifluoromethanesulphonic acid in the presence of Met to form methionine-S-methyl(or ethyl)sulphonium salt (I). When the protecting group of Z–Met–OH or Boc–Met–OH was cleaved in the MSA-anisole system, (I) was found as a major product, besides a small amount of S-benzyl or S-*tert*-butyl derivative. Thus a Met-containing peptide, H–Tyr–Gly–Gly–Gly–Lys–Met–Gly–OH named endorphin, was synthesized after deprotection of Z(OMe)–Tyr–Gly–Gly–Gly–Lys(Z)–Met(O)–Gly–OH by the MSA-anisole procedure followed by reduction of the Met(O) moiety to the parent amino acid residue with dithiothreitol.

Keywords——deprotection in the methanesulphonic acid-anisole system; deprotection in the trifluoromethanesulphonic acid-trifluoroacetic acid-anisole system; formation of Lys(Bzl) from Lys(Z); methionine-S-methylsulphonium salt; methionine-S-benzylsulphonium salt; methionine-S-*tert*-butylsulphonium salt; hydrolytic cleavage of aromatic ethers; methionine sulphoxide; synthesis of endorphin; tetrachloroauric (III) acid oxidation of Met

Currently we have been examining the usefulness of the methanesulphonic acid (MSA)–anisole system[4] for the removal of all protecting groups employed at the final step of the peptide synthesis. Prior to apply such a new reagent to the synthesis of complex peptides, possible side reaction have been investigated. When protecting groups are cleaved acidolytically, one of the most troublesome side reactions is the alkylation reaction caused by cations, which are derived from protecting groups and failed to trap, despite of the presence of a cation scavenger, such as anisole.

As described in the preceding paper,[1] we confirmed that treatment of Lys (Z) with MSA-anisole regenerated Lys quantitatively without concomitant formation of a side reaction product, Lys (Bzl). This side reaction was pointed out by Mitchell and Merrifield[5] during the treatment with trifluoromethanesulphonic acid (TFMSA)–trifluoroacetic acid (TFA)–anisole[6] and seems to proceed intramolecularly, though it is a minor reaction.

In this paper, we wish to report another type of alkylation, which was observed at Met in the MSA-anisole system and in the TFMSA-anisole system as well. When various amino acid derivatives were exposed in both acid systems, recovery of Met from Z-Met-OH was very poor because of the formation of a by-product, presumably the same compound, detectable on the short column of an amino acid analysor, eluted between His and ammonia. Recovery

1) A preliminary communication of this paper has appeared in *J.C.S. Chem. Comm.*, **1976**, 922. Part LXXII: N. Fujii, S. Funakoshi, T. Sasaki, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **25**, 3096 (1977).
2) The following abbreviations are used for space conservation: Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl.
3) Location: *a) Sakyo-ku, Kyoto, 606, Japan; b) Yodogawa-ku, Osaka, 532, Japan.*
4) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
5) R.A. Mitchell and R.B. Merrifield, *J. Org. Chem.*, **41**, 2015 (1976).
6) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J.C.S. Chem. Comm.*, **1974**, 107.

of Met was improved in a certain degree by addition of sulfur compounds, but not satisfactory enough.

This side reaction product was now isolated for identification. When a mixture of Met and anisole in MSA was kept at room temperature for 24 hr, this by-product was isolated in quantitative yield as the sole product, recrystallizable from alcohol. The structure of this compound was assigned as S-methylmethionine dimethanesulphonate (I) from infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data. Especially, in the ^{1}H NMR (D_2O) spectra, two singlet peaks (δ 2.80 and 2.97, each corresponds to 6H of two methyl groups) were observed, indicating the presence of the S-methyl sulphonium moiety in (I). Though the mass spectral data was not available because of its non-volatility, the elemental analysis matched well with the assigned formula. Confirmation of this structural assignment was next provided by direct comparison with the authentic sample of (I), derived from S-methylmethionine iodide[7] by treatment of MSA *via* the corresponding dipicrate. Next, Met was similarly treated with 33% TFMSA–TFA in the presence of anisole. It was found that the single product thus formed could be converted into the dipicrate in the same manner as the iodide mentioned above.

These experimental results suggested strongly that the sulphur atom of Met trapped the methyl group of anisole as a cation. In order to confirm further this most plausible origin of the methyl group in (I), phenetole, instead of anisole, was exposed to MSA in the presence of Met. Here, S-ethylmethionine sulphonium salt (II) was isolated as shown in Fig. 1. It

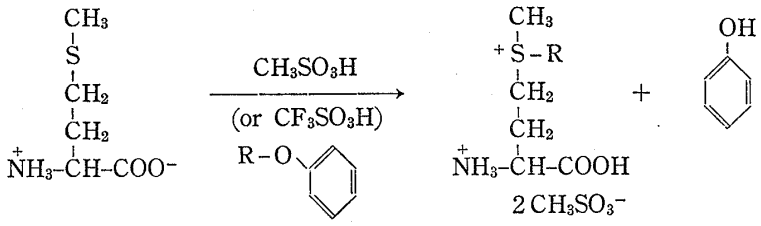


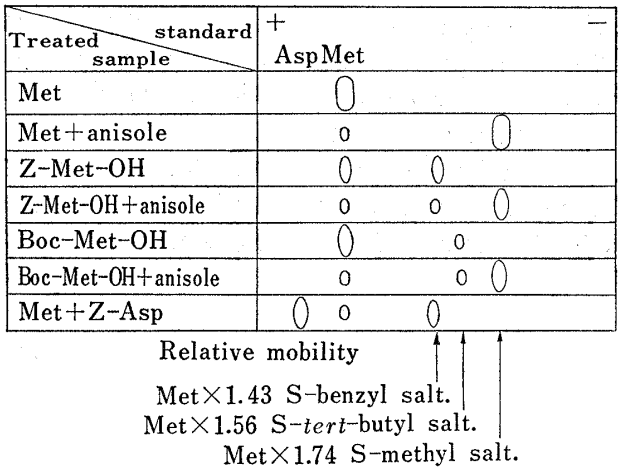
Fig. 1. Formation of S-Alkylmethionine Sulphonium Salts in the MSA-Anisole System

should be mentioned that acidic cleavage of anisole or phenetole did not take place when Met was absent and in addition, Met in these acids gave no observable side reactions in the absence of anisole or phenetole. Thus, the role of Met became evident in the facilitated cleavage of aromatic ethers by these acids. Transfer of the methyl group from anisole to the sulphur atom of Met can be explained by the Hard-Soft concept,[8] *i.e.*, CH_3^+ has an appreciable affinity with sulphur, because of its softness. This hitherto unknown reaction may possibly be applied to the hydrolytic cleavage of aromatic ethers, since anisole gave phenol with MSA-Met at room temperature in reasonable yield. These works are in progress in our laboratory and results will be reported elsewhere.

Next, protected Met derivatives were exposed to MSA in the presence or absence of anisole and products were examined qualitatively by paper electrophoresis. When Z-Met-OH and Boc-Met-OH were treated with MSA in the absence of anisole, besides the parent Met, significant amounts of the S-benzyl an S-*tert*-butyl sulphonium salts were detected respectively as illustrated in Fig. 2. They were identified by comparison of their mobilities with those of the authentic samples. Formation of the S-benzylmethionine sulphonium salt was also observed, when a mixture of Met and Z-Asp-OH was exposed to MSA in the absence of anisole. These results indicated that this alkylation reaction proceeded intermolecularly. When the

7) G. Toennies and J.J. Kolb, *J. Am. Chem. Soc.*, **67**, 849 (1945); G. Toennies, *J. Biol. Chem.*, **132**, 455 (1940).
8) R.G. Pearson and J. Songstad, *J. Am. Chem. Soc.*, **89**, 1827 (1967); R.G. Pearson, *Science*, **151**, 172 (1966).

MSA treatment of Z-Met-OH or Boc-Met-OH was performed in the presence of anisole, besides the above mentioned S-methyl sulphonium salt (I), S-benzyl and S-*tert*-butyl derivatives were also electrophoretically detected, though the amounts of these products became considerably less and we failed to identified these products on thin-layer chromatography (TLC) and an amino acid analysor.



Toyo filter paper No. 50. Buffer: pH 1.9 HCOOH-AcOH 500 V, 1.5 hr.

Fig. 2. Paper Electrophoretic Examination of the Products Derived from Met-derivatives in MSA

The above experiments indicated that the sulphur atom of Met has a great tendency to be alkylated with not only the benzyl or *tert*-butyl cation liberated from the protecting groups, but also the methyl of anisole, if anisole is present in the MSA or TFMSA system. The former tendency was previously pointed out by Guttmann and Boissonnas.[9] When the Z group was removed from Met-containing peptides acidolytically by hydrogen bromide, formation of the S-benzyl sulphonium salt was recorded. More recently, Noble, *et al.*[10] isolated the *tert*-butyl sulphonium form of Met-containing peptide after treatment of Boc-peptide resin with hydrogen fluoride,[11] even in the presence of anisole.

From the above experimental results and references, it can be judged that the thioether moiety of Met had better be protected, when the Z and Boc groups, possibly the benzyl and *tert*-butyl esters also, are cleaved acidolytically with hydrogen bromide or hydrogen fluoride. When MSA is applied as a deprotecting reagent, its protection is absolutely necessary. For this demand, the sulphoxide, Met (O), seems to be the only compound available in the present peptide synthesis. The use of this compound was first recommended by Iselin,[12] because of its reversible conversion to Met with thiols. Through this protection, it is possible to prevent the partial alkylation of the parent amino acid at the deprotecting stage and the partial oxidation during the peptide synthesis as well. We have first confirmed that Met (O) is stable to MSA under deprotecting conditions. Thus, Met-containing peptides could be synthesized in such a manner that protected Met (O)-peptides are exposed to MSA-anisole and then deprotected peptides are incubated with thiols to reduce Met (O) to Met.

In order to examine the usefulness of the MSA procedure in practical peptide synthesis, we applied this procedure to the synthesis of the heptapeptide, H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH, termed as endorphin. This peptide is a model opioid peptide proposed theoretically by Goldstein, *et al.*[13] As reported,[14] we synthesized the protected heptapeptide, Z (OMe)-Tyr-Gly-Gly-Gly-Lys (Z)-Met-Gly-OH, using dilute ethanesulphonic acid (ESA) as an α-deprotecting reagent and from which the Z and Z (OMe) groups were removed with hydrogen fluoride. Again, this was selected as a suitable model compound, since it contains both Lys and Met, at which two possible side reactions of the MSA procedure are concerned.

As shown in Fig. 3, Z(OMe)-Gly-Lys(Z)-Met-Gly-OH[14] was converted to the corresponding sulphoxide, Z(OMe)-Gly-Lys(Z)-Met(O)-Gly-OH, with tetrachloroaureic (III) acid accord-

9) St. Guttmann and R.A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958); *idem*, *ibid.*, **42**, 1257 (1959).
10) R.L. Noble, D. Yamashiro, and C.H. Li, *J. Am. Chem. Soc.*, **98**, 2324 (1976).
11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
12) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
13) A. Goldstein, J.S. Goldstein, and B.M. Cox, *Life Sci.*, **17**, 1643 (1975).
14) H. Yajima, H. Ogawa, N. Fujii, and S. Funakoshi, *Chem. Pharm. Bull.* (Tokyo), **25**, 740 (1977).

ing to the procedure of Bordignon, *et al.*[15] Stereospecific oxidation of the Met residue to (S)-Met-(S)-sulphoxide was reported to proceed with this particular oxidant. It was possible to follow the two stage reactions, probably the fast formation of the sulphide complex and subsequent oxidation of the Met residue,[15] by monitoring the reaction mixture with TLC. The reaction completed at 40° for 3 hours and the desired peptide was isolated in 76% yield. Elongation of this tetrapeptide to the heptapeptide, Z(OMe)-Tyr-Gly-Gly-Gly-Lys(Z)-Met(O)-Gly-OH, was performed in essentially the same manner as described previously[14] by depro-

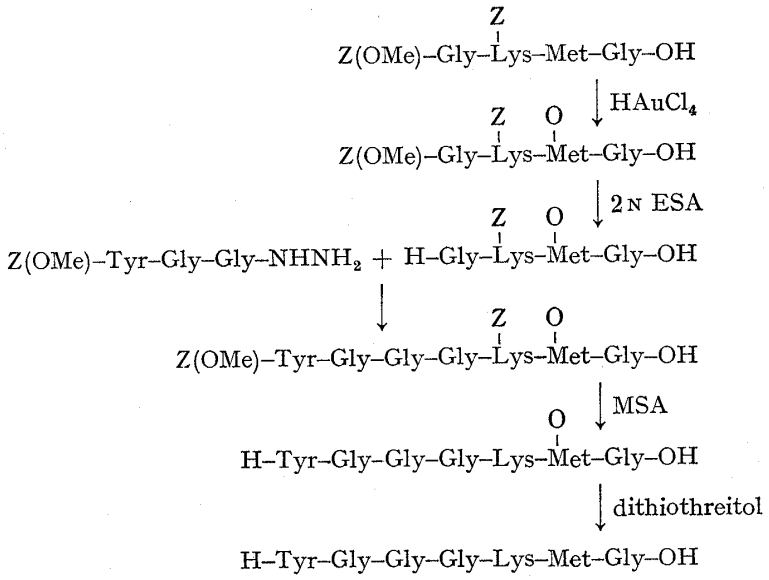


Fig. 3. Synthetic Scheme of Endorphin

tection of the Z(OMe) group with 2N ESA–acetic acid followed by condensation of Z(OMe)-Tyr-Gly-Gly-$NHNH_2$ *via* the modified azide procedure.[16] The protected heptapeptide thus obtained was exposed to MSA in the presence of anisole at room temperature for 60 minutes to remove the protecting groups, Z and Z(OMe). After conversion to the corresponding acetate with Amberlite IR-4B, the thin-layer chromatographically pure looking heptapeptide, H-Tyr-Gly-Gly-Gly-Lys-Met(O)-Gly-OH, was incubated with dithiothreitol at 50° for 24 hours. During this period, reduction proceeded quantitatively and the desired synthetic endorphin was isolated by column chromatography on CM-cellulose using ammoium bicarbonate buffers as eluents.

Recently, one of our research groups (M.F. and S.S.) was able to demonstrate that the MSA procedure could be successfully applied to the synthesis of the Met-containing docosapeptide named motilin.[17] Further evaluation of this procedure will be reported in the future.

Experimental

Chemical shifts in ^{13}C-nuclear magnetic resonance (CMR) were measured with respect to internal dioxane and converted to the Me_4Si scale using the formula $\delta_{Me_4Si} = \delta_{(dioxane)} + 67.4$ ppm. TLC was performed on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf^2 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf^3 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1).

Paper Electrophoretic Examination of the Products——Each sample (4 mmol of Met, Z–Met–OH or Boc–Met–OH) was treated with MSA (40 equiv.) in the absence or presence of anisole (2 equiv.) at room temperature (22°) for 60 min. Dry ether was added and the resulting oily precipitate was washed with

15) E. Bordignon, L. Cattalini, G. Natile, and A. Scatturin, *J.C.S. Chem. Comm.*, **1973**, 878.
16) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, **26**, 2333 (1961).
17) M. Fujino, S. Shinagawa, M. Wakimasu, C. Kitada, and H. Yajima, Proceeding of the 14th Symposium on Peptide Chemistry, Ed. T. Nakajima, 1976, p. 61.

ether and then dissolved in H_2O (20 ml). A part of the solution was examined by paper electrophoresis on Toyo Filter Paper (No. 50) in pH 1.9 formic acid–acetic acid buffer at 500 V for 1.5 hr. Products were revealed by ninhydrin and the results were shown in Fig. 2.

S-Methylmethionine Dimethanesulphonate——(a) Treatment of Met: A mixture of Met (0.5 g), anisole (1 ml, 2.8 equiv.) and MSA (3 ml, 14 equiv.) was kept on standing at room temperature (22°). When monitored by TLC Met (Rf^2 0.48) disappeared completely within 24 hr and a new single spot (Rf^2 0.13) was detected. Dry ether was added and the resulting oily precipitate was washed three times with ether. Treatment of the residue with EtOH afforded a crystalline compound, which was recrystallized from EtOH; yield 851 mg (72%). mp 196—201°. $[\alpha]_D^{24}$ +16.6° (c=1, 5% AcOH). IR ν_{max} cm^{-1} 1715 (CO), 3400 (NH). Proton-magnetic resonance (PMR): (D_2O): δ 2.80 (6H, s., Me), 2.97 (6H, s., Me), 3.89 (1H, t., α-CH), 2.40 (2H, m., CH_2), 3.48 (2H, m., CH_2). CMR (dioxane): δ 170.9 (CO), 51.9 (CH), 39.9 (CH_2), 39.4 (CH_2), 25.5 (Me), 25.2 (Me). *Anal.* Calcd. for $C_6H_{14}NO_2S \cdot CH_3SO_3 \cdot CH_3SO_3H$: C, 27.03; H, 5.96; N, 3.94; S, 27.06. Found: C, 27.19; H, 5.88; N, 3.97; S, 26.76. This compound emerged from the short column of an amino acid analysor between His and ammonia peaks (retention time 27 min) and can be converted to homoserine by treatment with 1 N NaOH at 80° for 3 hr.

(b) Treatment of Z-Met-OH: A mixture of Z–Met–OH (540 mg), anisole (1 ml) and MSA (3 ml) was kept on standing at room temperature for 24 hr. The product, mp 192—197°, isolated similarly was found identical with the methanesulphonate obtained in (a) by comparison with their IR spectra; yield 467 mg (69%). Isolation of the S-benzylsulphonium salt was unsuccessful and its formation could not be identified by thin-layer chromatography.

(c) Preparation from S-Methylmethionine Iodide: To a solution of S-methylmethionine iodide[7] (200 mg) in H_2O (2 ml) was added a saturated aqueous solution of picric acid to give a yellow precipitate, which was washed with a small amount of H_2O and recrystallized from 50% aqueous EtOH; yield 264 mg (62%), mp 154—156°. *Anal.* Calcd. for $C_6H_{14}NO_2S \cdot C_6H_2N_3O_7 \cdot C_6H_3N_3O_7$: C, 34.79; H, 3.08; N, 15.78; S, 5.16. Found: C, 34.81; H, 2.79; N, 15.73; S, 5.23. The above dipicrate (510 mg) was dissolved in 30% aqueous MSA and the solution, after washing with ether until the ether layer became colorless, was concentrated to dryness under reduced pressure to leave the dimethanesulphonate (I), which was recrystallized from EtOH; yield 231 mg (79%), mp 199—201°. *Anal.* Found: C, 27.12; H, 6.11; N, 3.69.

(d) Treatment of Met with TFMSA: A mixture of Met (504 mg), anisole (1 ml) in 33% TFMSA-TFA (3 ml) was kept at room temperature for 24 hr. Dry ether was added to form an oily precipitate, which was washed with ether and then dissolved in a small amount of H_2O. A saturated aqueous solution of picric acid was added and the resulting precipitate was crystallized from 50% aqueous EtOH; yield 1.22 g (58%), mp 154—156°. Its IR spectra were identical with those of the authentic dipicrate obtained above.

S-Ethylmethionine Sulphonium Bromide Salt——Met (1.50 g) was treated with MSA (15 ml) in the presence of phenetole (1.5 ml) at room temperature overnight. Dry ether was added and the resulting oily precipitate was dissolved in H_2O (50 ml). The solution was applied to a column of Amberlite CG-120 (H^+ form, 2×10 cm), which after washing with H_2O (100 ml), was eluted with 1 N NH_4OH. The latter eluates, after addition of 47% HBr (1 ml), was passed through a column of Amberlite IRA-410 (acetate form, 2×10 cm). Eluates were then lyophilized and the residue was treated with EtOH to give a solid; yield 0.90 g (35%), $[\alpha]_D^{26}$ +23.3° (c=1.0, AcOH). *Anal.* Calcd. for $C_7H_{16}BrNO_2S$: C, 32.56; H, 6.24; N, 5.42; S, 12.47; Br, 30.95. Found: C, 32.98; H, 6.07; N, 5.47; S, 12.89; Br, 31.71.

S-Benzyl and *tert*-Butylmethionine Sulphonium Bromides——The title compounds were prepared according to the procedure described for the synthesis of the corresponding iodide[7] with benzyl bromide and *tert*-butyl bromide respectively. Attempt to crystallize these compounds has been unsuccessful. Rf^3 S-benzyl-bromide 0.37; S-*tert*-butylbromide 0.20. They were used as markers in the paper electrophoresis stated above.

Identification of Phenol from Anisole——A mixture of anisole (1.08 g) and Met (2.24 g, 1.5 equiv.) in MSA (5.8 ml, 9 equiv.) was kept at room temperature overnight and *n*-hexane and ice were added. The organic phase was washed with 1% NaOH. The washing was acidified with 6 N HCl, saturated with NaCl and extracted with ether. The ether extract was dried over Na_2SO_4 and concentrated to dryness to give needles; yield 0.55 g (59%). The product was identified as phenol by comparison of their IR spectra and gas chromatographic examination.

Synthesis of Endorphin——Z(OMe)–Gly–Lys–Met(O)–Gly–OH: Z(OMe)–Gly–Lys(Z)–Met–Gly–OH[14] (478 mg) was dissolved in a mixture of MeOH (5 ml), dimethyl formamide (DMF) (2 ml) and H_2O (1.5 ml). To this solution, $NaHCO_3$ (174 mg) and $HAuCl_4 \cdot 4H_2O$ (285 mg) were added. The mixture was stirred at 40° for 3 hr, while the starting material (Rf^1 0.34) disappeared on TLC and a new spot Rf^1 0.22 was detected. The solvent was evaporated and the residue was treated with EtOH. The resulting mass was washed with H_2O and then recrystallized twice from MeOH and EtOH; yield 370 mg (76%), mp 128—134°, $[\alpha]_D^{18}$ −19.8° (c=0.8, DMF). Rf^1 0.22. IR $_{max}$ cm^{-1} 1110 (S–O). *Anal.* Calcd. for $C_{32}H_{43}N_5O_{11}S$: C, 54.45; H, 6.14; N, 9.92. Found: C, 54.16; H, 6.02; N, 9.77.

Z(OMe)–Tyr–Gly–Gly–Gly–Lys(Z)–Met(O)–Gly–OH: The above protected tetrapeptide (370 mg) was treated with 2 N ESA–AcOH (1 ml) in the presence of anisole (0.3 ml) in an ice-bath for 15 min and then at room temperature for 60 min. Dry ether was added to give an oily precipitate, which was washed with

ether, dried over KOH pellets *in vacuo* for 2 hr and then dissolved in DMF (3 ml). To this ice-chilled solution, Et_3N (0.25 ml) and the azide (prepared from 298 mg of Z(OMe)–Tyr–Gly–Gly–$NHNH_2$[14] with 0.42 ml of 3.3 N HCl–DMF, 0.09 ml of isoamylnitrite and 0.19 ml of Et_3N) in DMF (3 ml) were combined. After stirring at 4° for 48 hr, the solvent was condensed and the residue was dissolved in 5% NH_4OH. The aqueous phase was washed with AcOEt and then acidified with citric acid. The resulting powder was washed batchwisely with ether, 5% citric acid and H_2O and then recrystallized from MeOH and AcOEt; yield 273 mg (53%), mp 149—154°, $[\alpha]_D^{18}$ −15.8° (c=1.0, DMF), Rf^1 0.16. Amino acid ratios in an acid hydrolysate: Tyr 1.02, Gly 4.00, Lys 0.99, Met 0.74 plus some homocysteic acid (not identified).[18] (average recovery 92%). *Anal.* Calcd. for $C_{45}H_{58}N_8O_{15}S \cdot H_2O$: C, 53.99; H, 6.04; N, 11.19. Found: C, 54.02; H, 6.08; N, 10.92.

H–Tyr–Gly–Gly–Gly–Lys–Met–Gly–OH: The above protected heptapeptide (250 mg) was treated with MSA (1 ml) in the presence of anisole (0.25 ml) in an ice-bath for 15 min and then at room temperature for 60 min. Dry ether was added to form an oily precipitate, which was washed with ether and then dissolved in H_2O (4 ml). Amberlite IR-4B (acetate form, approximately 1 g) was added and the solution, after stirring for 30 min, was filtered. The filtrate was lyophilized to give a powder; yield 180 mg (97%). Rf^1 0.05, Rf^2 0.12, Rf^3 0.34. H–Tyr–Gly–Gly–Gly–Lys–Met(O)–Gly–OH thus obtained was dissolved in H_2O (1.8 ml) containing dithiothreitol (300 mg) and the solution was incubated at 50° for 24 hr, while the starting material, Rf^3 0.34, was disappeared and a new spot Rf^3 0.45 was detected on TLC. The solution was applied to a column of Sephadex G-10 (3×125 cm), which was eluted with 3% AcOH. Individual fractions (6 ml each) were collected and absorbancy at 275 nm was determined. Fractions of the main peak (tube No. 78—92) were collected and the solvent was removed by lyophilization; yield 123 mg (70%). This powder was dissolved in H_2O (3 ml) and the solution was next applied to a column of CM-cellulose (2.5×2.5 cm), which was eluted first with H_2O (230 ml) and then with 0.01 M NH_4HCO_3 buffer (pH 7.8, 600 ml) through a mixing flask containing H_2O (150 ml). Individual fractions (7.6 ml each) were collected and absorbancy at 275 nm was determined. A single peak (tube No. 53—64) was detected. These fractions were collected and the solvent and NH_4HCO_3 were removed by repeated lyophilization to give a fluffy white powder; yield 87 mg (70%). $[\alpha]_D^{18}$ −6.3° (c=0.5, 3% AcOH). Rf^2 0.49, Rf^3 0.45. Amino acid ratios in an aminopeptidase (AP-M)[19] digest: Tyr 0.88, Gly 4.00, Lys 1.09, Met 0.85 (average recovery 90%). *Anal.* Calcd. for $C_{28}H_{44}N_8O_9S \cdot 2H_2O$: C, 47.71; H, 6.87; N, 15.90. Found: C, 47.85; H, 6.80; N, 15.59.

Acknowledgement We wish to express our sincere thanks to Miss Makiko Sugiura of Kobe Women's College of Pharmacy for C^{13}-NMR measurement. Technical assistance of Mr. Masanori Sugiura is also gratefully acknowledged.

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19) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963).