BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 46, 2168—2174 (1973)

Synthesis and Biological Activity of Peptides Related to Eledoisin. III.[1] *C*-Terminal Hexapeptide Amides Modified in Methionine and Isoleucine Residues[2,3]

Hiroshi SUGANO

Department of Synthetic Chemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., Kashima-cho, Higashiyodogawa-ku, Osaka 532

(Received September 16, 1972)

New analogs of hexapeptide related to eledoisin containing *N*-methylmethionine and α-hydroxy-γ-methylthiobutyric acid in place of the methionine, and α-hydroxyisovaleric acid in place of the isoleucine, were synthesized in order to obtain some information about the role of the amide bond in the peptide backbone. The former two were practically inactive in the rabbit pressure assay. These results indicated that the amide bond between the leucine and the methionine residues was important for the depressor activity of eledoisin. The last one showed very little activity. The results supported the suggestion made previously that the amide bond between the phenylalanine and the isoleucine residues is important in eliciting the biological response. The role of the amide bonds in the peptide backbone of eledoisin was also discussed.

Eledoisin, Pyroglu–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2, is a potent, naturally occurring depressor substance. A number of data have accumulated on the relation between the structure of eledoisin and the biological activity. The *C*-terminal pentapeptide sequence, H–Phe–Ile–Gly–Leu–Met–NH_2, is of particular importance for the depressor activity.[4] The low depressor activity (1%) of this sequence is enhanced to almost full hormonal activity by introducing lysine to the *N*-terminus.[5] It has also been reported that the amino acid residues, phenylalanine, isoleucine, leucine, and methionine are the essential elements for the depressor activity.[4] The hydrophobic side chains in these residues may play a role in interaction with the biological receptor site.

However, there is practically no knowledge as to the part played by the amide bond which is a structural element of the polypeptide chain of eledoisin.

1) Part II of this series; H. Sugano, K. Higaki, and M. Miyoshi, This Bulletin, **46**, 231 (1973)

2) Presented in part at the 9th Symposium on Peptide Chemistry, Shizuoka, November, 1971.

3) The abbreviations recommended by the IUPAC-IUB commission on Biological Nomenclature (*J. Biol. Chem.*, **241**, 2491 (1966); **242**, 555 (1967) have been used throughout. In addition: Glyc=glycolic acid, HyMeV=α-hydroxy-β-methylvaleric acid, Phlac=β-phenyllactic acid, HyIc=α-hydroxyisocaproic acid, HyIv =α-hydroxyisovaleric acid, and HyMtb=α-hydroxy-γ-methylthiobutyric acid residues. Amino acid, α-hydroxy acid, and *N*-methylamino acid symbols except Gly, Glyc, and Sar denote the L-configuration.

4) E. Schröder and K. Lübke, "The Peptides," Academic Press, New York and London (1966), p. 127.

5) L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer A. Glaesser, and O. Goffredo, *Experientia*, **20**, 306 (1964).

In previous papers,[1,6] the present author chose the [Lys^6]-eledoisin-(6-11)-hexapeptide (I) as a model compound of eledoisin and described the synthesis and the biological activity of several analogs. In these peptides, some amide bonds in I were replaced by ester bonds or *N*-methylamide bonds by the replacement of the parent amino acids with α-hydroxy acids or *N*-methylamino acids. These studies gave us some information about the relationship between the structure of the peptide backbone of eledoisin and the activity. It was found that, in the case of the substitution of the amide group in the peptide I by an ester group or an *N*-methylamide group without any change in the amino acid side chain, the potency of the activity was dependent upon the position substituted. The replacement of the amide bond between the phenylalanine and the isoleucine residues by an ester bond or an *N*-methylamide bond led to a much less active compound. These facts suggest that the amide bond between the phenylalanine and the isoleucine residues is essential for the activity.

In order to provide further information concerning the role of the amide bond in the peptide backbone, the author will report, in the present paper, on the synthesis of [Lys^6, MeMet^{11}]-eledoisin-(6-11)-hexapeptide (XIV) and [Lys^6, HyMtb^{11}]-eledoisin-(6-11)-hexapeptide (VIII) and on their depressor activities. In these peptides the amide bond between the leucine and the methionine residues was replaced by an *N*-methylamide bond or an ester bond. In addition, to confirm the necessity of the amide bond between the phenylalanine and the isoleucine residues for the activity, [Lys^6, HyIv^8]-eledoisin-(6-11)-hexapeptide (IV) was synthesized and its activity was estimated.

A synthetic route to the [Lys^6, MeMet^{11}]-analog (XIV) is shown in Scheme 1.

Boc-Leu-OH + H-MeMet-OEt (**1**)
↓ DCC
Boc-Leu-MeMet-OEt (**2**)
↓ NH_3 in MeOH
Boc-Leu-MeMet-NH_2 (**3**)
↓ i) HCl in dioxane ii) Boc-Gly-ONp
Boc-Gly-Leu-MeMet-NH_2 (**4**)
↓ i) HCl in dioxane ii) Aoc-Phe-Ile-N_2H_3 HNO_2
Aoc-Phe-Ile-Gly-Leu-MeMet-NH_2 (**5**)
↓ i) HCl in dioxane ii) di-Boc-Lys-ONp
di-Boc-Lys-Phe-Ile-Gly-Leu-MeMet-NH_2 (**6**)
↓ HCl in AcOH
H-Lys-Phe-Ile-Gly-Leu-MeMet-NH_2·2HCl (XIV)

Scheme 1.

Optically active *N*-methylmethionine was prepared by the method reported by Izumiya *et al.*[7] Boc-leucine was condensed with *N*-methylmethionine ethyl ester with dicyclohexylcarbodiimide (DCC) to yield the Boc-dipeptide ester, **2**, which was then converted to the amide, **3**. After the removal of the protecting group, the resulting dipeptide amide was coupled with Boc-glycine *p*-nitrophenyl ester to form the Boc-tripeptide amide, **4**. The condensation of the azide derived from *t*-amyloxycarbonylphenylalanylisoleucine hydrazide, Aoc-Phe-Ile-N_2H_3, with the tripeptide amide gave an acylpentapeptide amide, **5**. The di-Boc-hexapeptide amide, **6**, was obtained *via* the *p*-nitrophenyl ester method. Since all attempts to crystallize the intermediates **2**—**6** were unsuccessful, the products were purified by column chromatography on silica gel after each coupling step.

The [Lys^6, HyMtb^{11}]-analog (VIII) was synthesized by the method shown in Scheme 2.

H-HyMtb-OH (**7**) ⟶ H-HyMtb-OMe (**8**)
⟶ H-HyMtb-NH_2 (**9**)
Boc-Leu-OH + **9**
↓ CDI
Boc-Leu-HyMtb-NH_2 (**10**)
↓ i) HCl in dioxane ii) Boc-Ile-Gly-ONp
Boc-Ile-Gly-Leu-HyMtb-NH_2 (**11**)
↓ i) HCl in dioxane ii) Boc-Phe-ONp
Boc-Phe-Ile-Gly-Leu-HyMtb-NH_2 (**12**)
↓ i) HCl in dioxane ii) di-Boc-Lys-ONp
di-Boc-Lys-Phe-Ile-Gly-Leu-HyMtb-NH_2 (**13**)
↓ HCl in AcOH
H-Lys-Phe-Ile-Gly-Leu-HyMtb-NH_2·2HCl (VIII)

Scheme 2.

L-α-Hydroxy-γ-methylthiobutyric acid (**7**) was prepared from L-methionine by the procedure of Winitz *et al.*[8] The α-hydroxy acid, **7**, was converted to the methyl ester, **8**; subsequent amidation yielded the corresponding amide, **9**. Boc-leucine was coupled with the free hydroxyl by the use of *N*,*N*′-carbonyldiimidazole (CDI)[9] to yield the Boc-didepsipeptide amide, **10**, in a good yield. After the removal of the Boc-group of **10**, the resulting didepsiptide amide was condensed with Boc-isoleucylglycine *p*-nitrophenyl ester to yield the Boc-tetradepsipeptide amide, **11**. The di-Boc-hexapeptide amide, **13**, was prepared by two successive stepwise elongations *via* the *p*-nitrophenyl ester method.

In the synthesis of [Lys^6, HyIv^8]-analog (IV), benzenesulfonyl chloride and pyridine were used for the formation of the depsipeptide bond, as had been reported previously for the synthesis of [Lys^6, HyMeV^8]-analog (III)[6] (Scheme 3).

The peptides, XIV, VIII, and IV, were obtained by the removal of the Boc group of the di-Boc-hexapeptide amides with hydrogen chloride in acetic acid.

6) H. Sugano, K. Higaki, and M. Miyoshi, This Bulletin, **46**, 226 (1973).
7) N. Izumiya, A. Nagamatsu, and S. Ota, *Kyushu Mem. Med. Sci.*, **4**, 1 (1953).
8) M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker, and J. P. Greenstein, *J. Amer. Chem. Soc.*, **78**, 2423 (1956).
9) H. A. Staab, *Angew. Chem.*, **71**, 194 (1959).

Boc-Phe-OH + H-HyIv-OBzl
↓ $C_6H_5SO_2Cl$/pyridine
Boc-Phe-HyIv-OBzl
↓ H_2/Pd
Boc-Phe-HyIv-OH (**14**) + H-Gly-Leu-Met-NH_2
↓ EtOCOCl/Et_3N
Boc-Phe-HyIv-Gly-Leu-Met-NH_2 (**15**)
↓ ii) HCl in dioxane; ii) di-Boc-Lys-ONp
di-Boc-Lys-Phe-HyIv-Gly-Leu-Met-NH_2 (**16**)
↓ HCl in AcOH
H-Lys-Phe-HyIv-Gly-Leu-Met-NH_2·2HCl (IV)

Scheme 3.

They were found to be homogeneous by the criteria of paper chromatography, paper electrophoresis, and elementary analysis. The presence of the depsipeptide bond was confirmed by IR, which showed an ester carbonyl band near 1750 cm^{-1}.

TABLE 1. PHARMACOLOGICAL RESULTS ON THE BLOOD PRESSURE IN RABBITS

Peptide		Relative[a] potency
H-Lys-Phe-Ile-Gly-Leu-Met-NH_2	(I)	100
H-Lys-Phlac-Ile-Gly-Leu-Met-NH_2	(II)	90[b]
H-Lys-Phe-HyMeV-Gly-Leu-Met-NH_2	(III)	10[b]
H-Lys-Phe-HyIv-Gly-Leu-Met-NH_2	(IV)	6
H-Lys-Phe-Ile-Glyc-Leu-Met-NH_2	(V)	30[b]
H-Lys-Phe-Ile-Gly-HyIc-Met-NH_2	(VI)	120[b]
H-Lys-Phlac-Ile-Gly-HyIc-Met-NH_2	(VII)	30[b]
H-Lys-Phe-Ile-Gly-Leu-HyMtb-NH_2	(VIII)	0.1
H-Lys-MePhe-Ile-Gly-Leu-Met-NH_2	(IX)	10[b]
H-Lys-Phe-MeIle-Gly-Leu-Met-NH_2	(X)	0.1[b]
H-Lys-Phe-Ile-Sar-Leu-Met-NH_2	(XI)	200[b]
H-Lys-Phe-Ile-Gly-MeLeu-Met-NH_2	(XII)	120[b]
H-Lys-MePhe-Ile-Gly-MeLeu-Met-NH_2	(XIII)	10[b]
H-Lys-Phe-Ile-Gly-Leu-MeMet-NH_2	(XIV)	0.1

a) The activity of the reference standard (I) is taken as 100. The potency of the other peptides is compared on a weight basis which causes fall of 20 mmHg of the blood pressure and expressed in per cent.
b) Taken from the previous papers.[1,6]

Results and Discussion

Table 1 shows the biological activities of the analogs synthesized here, together with those reported previously. In the rabbit blood pressure assay, the peptides VIII and XIV possessed 0.1% and the peptide IV possessed 6% of the depressor activity of the reference standard (I). The inactivity of the former two analogs strongly suggests the necessity of the amide bond between the leucine and the methionine residues for the biological activity. Although IV still had an appreciable activity, its potency was more decreased than those of II, V, VI, and VII, in which the phenylalanine, the glycine, or the leucine was replaced by the α-hydroxy acid. This low activity may be due not to the change in the side chain, but to the change in the backbone, because α-hydroxyisovaleric acid has a β-branched side chain like isoleucine, and because a [Val[8]]-eledoisin-(6-11)-hexapeptide, in which the isoleucine is replaced by valine, retains the same activity as the parent *C*-terminal hexapeptide of eledoisin.[10] These results support the suggestion made previously that the amide bond between the phenylalanine and the isoleucine residues is probably important for the depressor activity.[6]

Summarizing the results obtained in this series of studies, both the amide bond between the phenylalanine and the isoleucine residues and that between the leucine and the methionine residues are indispensable in manifesting the depressor activity of eledoisin. The analogs which are lacking in either of the essential amide bonds show much less activity.

Apart from minor differences in geometry, such as the steric effect of the *N*-methyl group, the amide bond differs fundamentally from the ester bond or the *N*-methylamide bond in its ability of hydrogen-bond formation. This loss of activity may indicate that the hydrogen bonding is required to maintain a favorable conformation of the peptide for the activity or to bind the molecule with the biological receptor site.

To gain some insight into conformational changes in solution following chemical alteration, circular dichroism (CD) spectroscopy was applied. Figures 1 and 2 show representative CD spectra in water between 250 and 210 nm. The biologically active hexapeptide analog of eledoisin (I) shows a trough at 233 nm ($[\theta]=-2800$). The CD curves of the biologically active analogs, such as [Lys[6], Phlac[7]]-analog (II), [Lys[6], Glyc[9]]-analog (V), [Lys[6], HyIc[10]]-analog (VI),

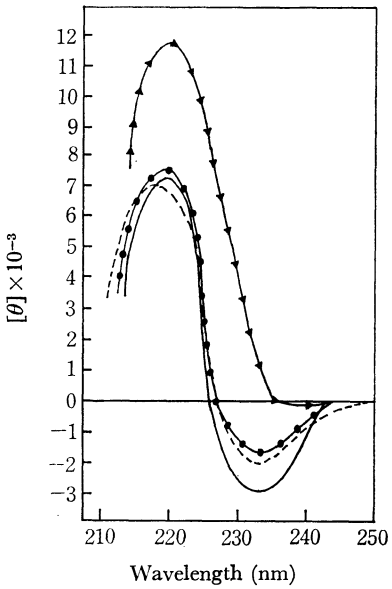

Fig. 1. Circular dichroism of depsipeptide analogs in the region 210—250 nm in water; concentration 1 mg/ml.
———: [Lys[6]]-eledoisin-(6-11)-hexapeptide (I)
– – – – –: [Lys[6], Phlac[7]]-analog (II)
▶—▶: [Lys[6], HyMeV[8]]-analog (III)
●—●: [Lys[6], HyMtb[11]]-analog (VIII)

10) K. Lübke, E. Schröder. R. Hempel, and R. Schmiechen, Japan, 26004 (1967).

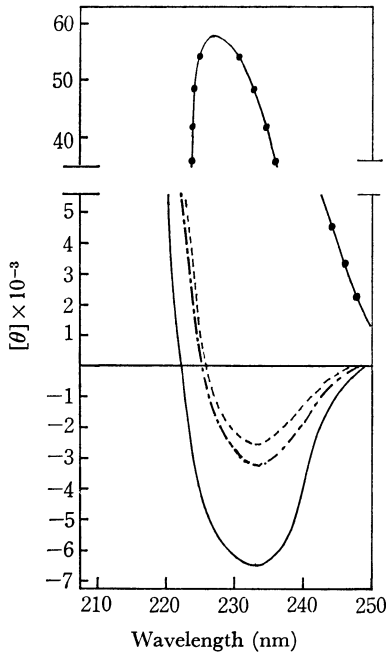

Fig. 2. Circular dichroism of *N*-methylpeptide analogs in the region 210—250 nm in water; concentration 1 mg/ml.
———: [Lys^6, MePhe^7]-analog (IX)
●—●: [Lys^6, MeIle^8]-analog (X)
— — —: [Lys^6, Sar^9]-analog (XI)
- - - - -: [Lys^6, MeMet^{11}]-analog (XIV)

[Lys^6, Phlac^7, HyIc^{10}]-analog (VII), [Lys^6, Sar^9]-analog (XI), and [Lys^6, MeLeu^{10}]-analog (XII), are very similar to that of the standard peptide (I). On the other hand, in the spectra of the less active peptides, such as [Lys^6, HyMeV^8]-analog (III) and [Lys^6, HyIv^8]-analog (IV), the positions of the troughs are shifted to longer wavelengths and the troughs are shallower. In the inactive compound, [Lys^6, MeIle^8]-analog (X), an anomalous spectrum is observed. However, [Lys^6, MeMet^{11}]-analog (XIV) and [Lys^6, HyMtb^{11}]-analog (VIII), which are devoid of biological activity, give spectra like that of I. These findings suggest that all the active peptides have very similar molecular conformations, and that the less active ones, such as III, IV, and X, are different from the active ones in backbone conformation. Furthermore, in the spectra of the less active compounds, such as [Lys^6, MePhe^7]-analog (IX) and [Lys^6, MePhe^7, MeLeu^{10}]-analog (XIII), the shapes and the positions of the troughs are almost identical with that of I, but the depths of the troughs are much enhanced ($[\theta] = -6500$ and -5900 respectively). Although the origin of the Cotton effect at about 233 nm is considered to be the n-π^* peptide transition, the contribution from the absorption of the aromatic group cannot be disregarded. From this point of view, the author assumes that these two peptides (IX and XIII) are different from I in the aromatic side chain conformation due to the steric effect of the *N*-methyl group in the *N*-methylphenylalanine residue.

Judging from these observations, the amide bond between the phenylalanine and the isoleucine residues probably restricts the favorable conformation of eledoisin. Such a conformation would facilitate an orientation of the side chains of the phenylalanine, the isoleucine, the leucine, and the methionine to a position in space in which a productive interaction with the biological receptor could occur. When *N*-methylisoleucine was introduced in place of the isoleucine, the conformation would be destroyed because of the lack of hydrogen bonding and also because of the steric effect of the *N*-methyl group. This is a factor which accounts for the much lower activity of the peptide, X, and for the anomalous spectrum. The exchange of the amide bond between the phenylalanine and the isoleucine residues by the ester bond appears to change the conformation to a considerable degree, but it does not completely destroy the molecular shape in spite of the lack of hydrogen bonding. In addition, the replacement of the amide bond between the lysine and the phenylalanine residues by the *N*-methylamide bond would cause a change in the orientation of the aromatic side chain and thereby affect the interaction with the receptor site.

From the fact that the peptides, VIII and XIV, were inactive even though they had favorable conformations, one can also guess that the amide bond between the leucine and the methionine residues plays an important role in exhibiting the biological activity. The possibility of binding *via* hydrogen bonding between the amide bond and the receptor site may be considered.

Experimental

The melting points are uncorrected. All the intermediates containing *N*-methylmethionine were purified by column chromatography on silica gel (Merck, 70-325 mesh). Each fraction was checked by thin-layer chromatography (tlc) on silica gel G(Merck); the products were determined by NMR in $CDCl_3$, which showed *N*-methyl protons (2.8—3.1 ppm) and *S*-methyl protons (2.0—2.1 ppm). The CD spectra were recorded on a JASCO Model ORD/UV-5 spectrophotometer. The amino acid analysis was performed as had been described previously.[1]

Pharmacological Assay. White male rabbits (2—3 kg) were anesthetized subcutaneously with 1.2 g/kg of urethane. Anesthesia was maintained with additional doses as needed. Arterial blood pressure was measured from a canulated carotid artery and recorded on a Kymograph by mercury manometer. All the peptides were dissolved in physiological saline and were injected into the femoral vein. Each dose was tested on five animals and was always checked against the reference standard material, I. The activity of the peptides was compared on a weight basis. The data were obtained with five animals per group.

H-MeMet-OEt·HCl (1). Thionyl chloride (22 g, 180 mmol) was added to absolute ethanol (200 ml) at 0—2 °C. To this solution, *N*-methylmethionine[7] (9.8 g, 60 mmol) was added, after which the solution was stirred at room temperature for 5 hr. After the removal of the solvent, this procedure was repeated two times. The oily residue thus obtained was dissolved in water, and the solution was filtered with activated charcoal and made alkaline by the addition of sodium bicarbonate. The oil thus separated was extracted repeatedly with ether, and the extracts were washed with saturated sodium chloride solution and dried over magnesium sulfate. To the filtered solution, dry hydrogen chloride was introduced, and the precipitated white crystals were collected

and recrystallized from ethanol–ether; yield, 6.7 g (29%); mp 95—97 °C; $[\alpha]_D^{25}$ +35.7° (*c* 1, EtOH). Found: C, 42.40; H, 7.78; N, 5.80; S, 13.98; Cl, 15.78%. Calcd for $C_8H_{18}NO_2SCl$: C, 42.19; H, 7.91; N, 6.15; S, 14.06; Cl, 15.60%.

Boc–Leu–MeMet–OEt (2). To a solution of Boc–Leu–OH·H_2O (8.3 g, 33 mmol) in chloroform (100 ml), DCC (7.2 g, 35 mmol) was added with stirring at −5—0 °C. After 1 hr, there was added a solution of **1** (6.7 g, 30 mmol) and triethylamine (4.2 ml, 30 mmol) in chloroform (50 ml). After the mixture had been stirred overnight at room temperature, the dicyclohexylurea was filtered off. The filtrate was washed successively with 4% sodium bicarbonate, 1 M hydrochloric acid, and water, and dried over magnesium sulfate. The filtrate was evaporated *in vacuo* to yield an oil (11 g), which was then chromatographed on silica gel, with elution by a mixture of benzene and ethyl acetate (9: 1). In this solvent system, the by-products (R_f 0.8 and 0.9) were eluted. The desired product (R_f 0.4) was then removed by a mixture of ethyl acetate and methanol (8: 2). The fractions containing the product, as detected by tlc, were combined and evaporated *in vacuo* to yield a colorless oil; yield, 6.5 g (51%); $[\alpha]_D^{25}$ −64.8° (*c* 2.6, MeOH). NMR δ ($CDCl_3$): 3.03 (s, 3H, $-NCH_3$), 2.08 (s, 3H, $-SCH_3$), 1.43 (s, 9H, $-C(CH_3)_3$), 4.16 (q, 2H, $-OCH_2-$), 0.95 (dd, 6H, $-CH(CH_3)_2$).

Boc–Leu–MeMet–NH_2 (3). A solution of compound **2** (6.5 g) in methanol (100 ml) was saturated with dry ammonia gas at 0 °C, and the solution was allowed to stand for 40 hr at room temperature. The reaction mixture was then concentrated to dryness under reduced pressure, and the residual oil (6 g) was chromatographed on silica gel, with elution by a mixture of benzene and ethyl acetate (6: 4). After removal of the unreacted ester **2** (R_f 0.9), the product was eluted by a mixture of benzene and ethyl acetate (4: 6). The fractions containing the product (R_f 0.7, benzene–AcOEt (3: 7)) were pooled and evaporated, yield of oil, 5.45 g (86%); $[\alpha]_D^{25}$ −26.4° (*c* 0.7, MeOH). NMR δ ($CDCl_3$): 3.08 (s, 3H, $-NCH_3$), 2.10 (s, 3H, $-SCH_3$), 1.43 (s, 9H, $-C(CH_3)_3$), 6.45 (s, 2H, $-NH_2$).

Boc–Gly–Leu–MeMet–NH_2 (4). Compound **3** (5.6 g, 15 mmol) was dissolved in 3M hydrogen chloride in dioxane (450 ml), and the solution was allowed to stand at room temperature for 30 min. The solvent was distilled off *in vacuo*, and the residue was triturated with ether. The H–Leu–MeMet–NH_2·HCl thus obtained was dissolved in chloroform (30 ml), neutralized with triethylamine (2.1 ml), and then subjected to a reaction with Boc–Gly–ONp[11] (6 g, 20 mmol). After standing overnight at room temperature, the solution was diluted with ethyl acetate (350 ml), washed successively with 1M hydrochloric acid, 4% sodium bicarbonate, and water, and dried over magnesium sulfate. The solvent was distilled off *in vacuo*, and the remaining oil was chromatographed on silica gel, with elution by a mixture of benzene and ethyl acetate (9: 1). In this solvent system, the unreacted Boc–Gly–ONp (R_f 0.8) was eluted, while the product was not eluted ($R_f < 0.1$). The product was removed from the column by a mixture of ethyl acetate and benzene (9: 1). The fractions containing the product, as determined by tlc (R_f 0.35, $CHCl_3$–MeOH–AcOH (95: 5: 3)) was pooled and evaporated *in vacuo*; yield of oil, 4.3 g (66%); $[\alpha]_D^{25}$ −25.0° (*c* 1, MeOH). Found: C, 52.41; H, 8.27; N, 12.00; S, 7.01%. Calcd for $C_{19}H_{36}N_4O_5S$: C, 52.77; H, 8.33; N, 12.96; S, 7.40%.

Aoc–Phe–Ile–Gly–Leu–MeMet–NH_2 (5). Compound **4** (2.2 g, 5 mmol) was treated with 2M hydrogen chloride in dioxane (30 ml) for 30 min at room temperature. After evaporation, the residue was triturated with ether; the remaining oil, corresponding to H–Gly–Leu–MeMet–NH_2·HCl, was used without further purification. To a solution of Aoc–Phe–Ile–$NHNH_2$[6] (2 g, 5 mmol) in a mixture of 2 M hydrochloric acid (10 ml) and dimethylformamide (100 ml), was added with stirring a chilled solution of sodium nitrite (350 mg, 5 mmol) in water (1 ml) over a period of 15 min at −15—−13 °C. After 20 min, a solution of previously prepared tripeptide amide hydrochloride, dissolved in dimethylformamide (20 ml), was added to the reaction mixture, and then the pH was adjusted to 7 with triethylamine. Stirring was continued for 3 hr at 0 °C and the mixture was then poured into cold water (500 ml). The oil thus separated was extracted with ethyl acetate, and the extract was washed successively with 0.5M sulfuric acid, 4% sodium bicarbonate, and water, and dried over magnesium sulfate. The dried solution was concentrated to an oil, which was subsequently chromatographed with elution by a mixture of ethyl acetate and methanol (95: 5). After the undesired products had been eluted (R_f 0.9, 0.8, and 0.6, AcOEt), the desired product (R_f 0.1, AcOEt; 0.35, $CHCl_3$–MeOH–AcOH (95: 5: 3)) was eluted with methanol; yield of oil, 700 mg (20%); $[\alpha]_D^{25}$ −24.8° (*c* 1, MeOH). Found: C, 58.34; H, 8.41; N, 11.29; S, 4.28%. Calcd for $C_{35}H_{58}N_6O_7S \cdot H_2O$: C, 58.01; H, 8.28; N, 11.60; S, 4.42%.

Di-Boc–Lys–Phe–Ile–Gly–Leu–MeMet–NH_2 (6). Compound **5** (700 mg, 1 mmol) was treated with 3M hydrogen chloride in dioxane (7 ml) for 40 min at room temperature. After evaporation, the residue was dissolved in dimethylformamide (5 ml), neutralized with triethylamine (0.13 ml), and subjected to a reaction with di-Boc–Lys–ONp[12] (700 mg) for 24 hr at room temperature. Ethyl acetate (100 ml) was then added to the reaction mixture, and the solution was washed with 1% hydrochloric acid, 4% sodium bicarbonate, and water, and dried over magnesium sulfate. The solution was concentrated to dryness, and remaining oil was purified by silica gel column chromatography, by elution with a mixture of ethyl acetate and methanol (9: 1). After the unreacted di-Boc–Lys–ONp ($R_f \fallingdotseq 1.0$, AcOEt–MeOH (9: 1)) had been eluted, the product ($R_f < 0.1$, AcOEt–MeOH (9: 1); 0.3, $CHCl_3$–MeOH–AcOH (95: 5: 3)) was eluted with methanol; yield of oil, 660 mg (70%); $[\alpha]_D^{25}$ −34.0° (*c* 1, MeOH). Found: C, 57.39; H, 8.28; N, 11.20; S, 3.14%. Calcd for $C_{45}H_{76}N_8O_{10}S \cdot H_2O$: C, 57.50; H, 8.30; N, 11.92; S, 3.40%.

H–Lys–Phe–Ile–Gly–Leu–MeMet–NH_2·2HCl (XIV). Compound **6** (200 mg) was dissolved in 2M hydrogen chloride in acetic acid (5 ml), and the solution was allowed to react at room temperature for 20 min. The product was precipitated by adding ice-cold dry ether (20 ml), and the precipitate was collected by filtration, washed well with ether, and dried over sodium hydroxide *in vacuo*. The product was dissolved in 60% methanol (4 ml), and an insoluble material was filtered off. The filtrate was concentrated to dryness over phosphorus pentoxide *in vacuo* to afford the final product; yield, 170 mg; mp 80—120 °C; $[\alpha]_D^{25}$ −34.0° (*c* 0.5, H_2O); R_f, 0.74;[13] amino acid ratios in the acid hydrolysate: Lys, 1.14; Phe, 1.03; Ile, 1.02; Gly, 0.95; Leu, 1.00; MeMet, 1.09. Found: C, 51.26; H, 7.88; N, 12.92; S, 3.68%. Calcd for $C_{35}H_{60}N_8O_6S \cdot 2HCl \cdot 2H_2O$: C, 50.66; H, 7.96; N, 13.51; S, 3.86%.

11) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

12) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).

13) Paper chromatography was carried out on Toyo Roshi No. 50 with *n*-butanol–acetic acid–water (4: 1: 1).

H–HyMtb–OH (7). This was prepared by the nitrous acid deamination of L-methionine (150 g) by the procedure reported by Winitz *et al.* for L-α-hydroxy-β-methylvaleric acid;[8] yield of oil, 27 g (16.7%). A sample for analysis was dissolved in ether; the subsequent addition of dicyclohexylamine to the solution gave crystalline dicyclohexylammonium salt, which was then recrystallized from ethyl acetate–petroleum ether; mp 130—131 °C; $[\alpha]_D^{25}$ −20.3° (*c* 1, EtOH). Found: C, 61.49; H, 10.15; N, 4.28; S, 9.92%. Calcd for $C_5H_{10}O_3S \cdot C_{12}H_{23}N$: C, 61.63; H, 9.96; N, 4.22; S, 9.66%.

H–HyMtb–OMe (8). Thionyl chloride (28 g, 230 mmol) was added with stirring to methanol (150 ml) at −2—2 °C. To the solution, compound **7** (33 g, 220 mmol) was added and the mixture was stirred for 1 hr at 0 °C and 3 hr at room temperature. After the solution had then been concentrated *in vacuo*, the remaining oil was distilled to give the ester (**8**); yield, 22 g (62%); bp 91—93 °C/3 mmHg; $[\alpha]_D^{25}$ −16.4° (*c* 1, MeOH). IR cm^{-1} (liq. film): 3450 (OH), 1740 (C=O). NMR δ ($CDCl_3$): 4.30—4.50 (dd, 1H, –CH–), 3.82 (s, 3H, $-OCH_3$), 3.15 (s, 1H, –OH), 2.54—2.82 (m, 2H, $-CH_2S-$), 2.15 (s, 3H, $-SCH_3$), 1.82—2.20 (m, 2H, $-CH_2-$).

H–HyMtb–NH₂ (9). A solution of compound **8** (18 g, 110 mmol) in methanol (300 ml) was saturated with dry ammonia gas at 0 °C. After standing for 48 hr at room temperature, the solution was concentrated to dryness; yield of oil, 16 g (100%); $[\alpha]_D^{25}$ −55.0° (*c* 0.65, H_2O). NMR δ ($CDCl_3$): 6.88 (d, 2H, $-NH_2$), 5.34 (s, 1H, –OH), 4.13—4.34 (dd, 1H, –CH–), 2.45—2.75 (m, 2H, $-CH_2S-$), 2.10 (s, 3H, $-SCH_3$), 1.60—2.18 (m, 2H, $-CH_2-$).

Boc–Leu–HyMtb–NH₂ (10). To a solution of carbonyldiimidazole[9] (13 g, 80 mmol) in tetrahydrofuran (80 ml) was added a solution of Boc–Leu–OH·H_2O (20 g, 80 mmol) in tetrahydrofuran (50 ml) at 0 °C over a period of 30 min. Then there was added a solution of compound **9** (11.5 g, 80 mmol) in tetrahydrofuran (20 ml); the reaction mixture was stirred at 0 °C for 1 hr and then at room temperature for 2 days. Water (20 ml) was added to the solution, and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate, and the ethyl acetate solution was washed with 1% hydrochloric acid, 4% sodium bicarbonate, and water, and dried over magnesium sulfate. The evaporation of the solvent gave white crystals, which were then recrystallized from ethyl acetate–petroleum ether; yield, 20 g (69%); mp 97—99 °C; $[\alpha]_D^{25}$ −56.5° (*c* 1, EtOH). Found: C, 52.98; H, 8.32; N, 7.61; S, 8.77%. Calcd for $C_{16}H_{30}N_2O_5S$: C, 53.02; H, 8.34; N, 7.73; S, 8.82%.

Boc–Ile–Gly–Leu–HyMtb–NH₂ (11). Compound **10** (4 g, 11 mmol) was treated with 3M hydrogen chloride in dioxane for 30 min. After evaporation, the residue, corresponding to didepsipeptide amide hydrochloride, was dissolved in dimethylformamide (50 ml), neutralized with triethylamine (1.5 ml), and subjected to a reaction with Boc–Ile–Gly–ONp[14] (3.6 g, 9 mmol) for 24 hr at room temperature. Ethyl acetate (500 ml) was then added to the reaction mixture, and the solution was washed thoroughly with 1M ammonia, 1% hydrochloric acid, and water, and dried over magnesium sulfate. On removal of the solvent a crystalline residue was obtained; this was recrystallized from ethyl acetate–petroleum ether; yield, 2.8 g (60%); mp 114—115 °C; $[\alpha]_D^{25}$ −39.1° (*c* 1, MeOH). Found: C, 54.47; H, 8.41; N, 10.43; S, 5.88%. Calcd for $C_{24}H_{44}N_4O_2S$: C, 54.12; H, 8.33; N, 10.52; S, 6.00%.

Boc–Phe–Ile–Gly–Leu–HyMtb–NH₂ (12). Compound **11** (2.7 g, 5 mmol) was treated with 3M hydrogen chloride in dioxane for 40 min. The solvent was evaporated under reduced pressure, and the residue was dissolved in dimethylformamide (25 ml), neutralized with triethylamine, and subjected to a reaction with Boc–Phe–ONp[11] (2 g, 5 mmol) for 24 hr at room temperature. Ethyl acetate (200 ml) was then added to the reaction mixture, and the solution was treated as described for the preparation of **11**. The crude product was recrystallized from ethyl acetate–methanol–petroleum ether; yield, 3.1 g (92%); mp 179—183 °C; $[\alpha]_D^{25}$ −35.5° (*c* 1, MeOH). Found: C, 58.81; H, 7.84; N, 10.13; S, 4.50%. Calcd for $C_{33}H_{53}N_5O_8S$: C, 58.32; H, 7.80; N, 10.30; S, 4.71%.

Di-Boc–Lys–Phe–Ile–Gly–Leu–HyMtb–NH₂ (13). Compound **12** (700 mg, 1 mmol) was treated with 3M hydrogen chloride in dioxane for 30 min. After evaporation, the residue was dissolved in dimethylformamide (5 ml), neutralized with triethylamine, and subjected to a reaction with di-Boc–Lys–ONp (450 mg, 0.97 mmol) for 48 hr. Ethyl acetate (100 ml) was then added to the reaction mixture, and the solution was treated as described for the preparation of **11**. The crude product was recrystallized from ethyl acetate–methanol–petroleum ether; yield, 700 mg (75%); mp 172—175 °C; $[\alpha]_D^{25}$ −47.4° (*c* 1, MeOH). Found: C, 58.30; H, 8.21; N, 10.69; S, 3.23%. Calcd for $C_{44}H_{93}N_7O_{11}S$: C, 58.19; H, 8.10; N, 10.79; S, 3.53%.

H–Lys–Phe–Ile–Gly–Leu–HyMtb–NH₂·2HCl (VIII). This was obtained from compound **13** (200 mg) as described for the preparation of XIV; yield, 165 mg; mp 225—227 °C; $[\alpha]_D^{25}$ −36.6° (*c* 0.21, H_2O); R_f, 0.76;[13] amino acid ratios in the acid hydrolysate: Lys, 1.01; Phe, 0.98; Ile, 1.10; Gly, 0.95; Leu, 1.00. Found: C, 50.81; H, 7.48; N, 12.08; S, 3.80%. Calcd for $C_{34}H_{57}N_7O_7S \cdot 2HCl \cdot 2H_2O$: C, 51.12; H, 7.64; N, 12.28; S, 4.01%.

Boc–Phe–HyIv–OH (14). To a solution of Boc–Phe–OH (15 g, 57 mmol) in a mixture of pyridine (100 ml) and tetrahydrofuran (100 ml), was added with stirring benzenesulfonyl chloride (8.8 g, 50 mmol) at −10 °C over a period of 10 min. After 10 min, a solution of benzyl L-α-hydroxyisovalerate[15] (10 g, 48 mmol) in pyridine (30 ml) was added to the solution. The mixture was then allowed to stand at 0 °C for 1 hr and at room temperature for 8 hr, and subsequently poured into water (1000 ml). The oil thus separated was extracted with ether. The extract was washed repeatedly with 2% hydrochloric acid, 4% sodium bicarbonate, and water, and dried over magnesium sulfate. The solvent was distilled off *in vacuo* to leave an oil (19.6 g); this oil was then dissolved in methanol (100 ml) and hydrogenated in the presence of 10% palladium on charcoal. The filtrate from the catalyst was evaporated to dryness, and the crude acid was dissolved in 4% sodium bicarbonate, washed with ether, and acidified with 1% hydrochloric acid. The oil thus separated was extracted with ether. The ethereal solution was washed with water and dried over magnesium sulfate. The ether was removed under reduced pressure; yield of oil, 15 g (88%). IR cm^{-1} (liq. film): 1740, 1720, 1705 (C=O).

Boc–Phe–HyIv–Gly–Leu–Met–NH₂ (15). To a solution of **14** (7.3 g, 20 mmol) and triethylamine (2.8 ml) in chloroform (200 ml), was added ethyl chloroformate (2.2 g, 20 mmol) at −15—−10 °C. After 15 min, there was added a solution of H–Gly–Leu–Met–NH_2·HCl[16] (7.3 g, 20 mmol)

14) H. Niedrich, *Chem. Ber.*, **100**, 3273 (1967).

15) G. Losse and G. Bachmann, *ibid.*, **97**, 2671 (1964). P. Quitt, R. O. Studer, and K. Volger, *Helv. Chim. Acta*, **47**, 166 (1964).

16) E. Schröder, R. Schmiechen, and H. Gibian, *Ann. Chem.*, **679**, 195 (1964).

and triethylamine (2.8 ml) in dimethylformamide (50 ml) at −15—−10 °C, and this mixture was allowed to stand at room temperature overnight. The mixture was poured into cold water (1000 ml), and the resulting solid material was extracted with chloroform. The extract was washed successively with 2% hydrochloric acid, 4% sodium bicarbonate, and water. The solvent was then evaporated *in vacuo*, and the crude product thus obtained was recrystallized from ethyl acetate–methanol–petroleum ether; yield, 9.4 g (71%); mp 160—164 °C; $[\alpha]_D^{25}$ −43.3° (*c* 1, MeOH). Found: C, 57.32; H, 7.80; N, 10.26; S, 4.75%. Calcd for $C_{32}H_{51}N_5O_8S$: C, 57.73; H, 7.72; N, 10.52; S, 4.80%.

*Di-Boc–Lys–Phe–HyIv–Gly–Leu–Met–NH$_2$ (**16**).* Compound **15** (5.32 g, 8 mmol) was dissolved in 3M hydrogen chloride in dioxane (70 ml) at room temperature. After 20 min, the solution was evaporated to dryness *in vacuo*. The pentadepsipeptide amide hydrochloride thus obtained was dissolved in dimethylformamide (40 ml) and then neutralized with triethylamine (1.2 ml). Di-Boc–Lys–ONp (4.2 g, 9 mmol) was added to the solution, and the mixture was allowed to stand at room temperature for 20 hr. Water was added to the reaction mixture to precipitate the product, which was then collected by filtration, washed well with 2% hydrochloric acid, 1M ammonia, and water, and dried. Recrystallization from ethyl acetate–petroleum ether gave 4.2 g (58%) of a product; mp 198—201 °C; $[\alpha]_D^{25}$ −40.2° (*c* 1, MeOH). Found: C, 57.53; H, 8.08; N, 11.05; S, 3.85%. Calcd for $C_{43}H_{71}N_7O_{11}S$: C, 57.76; H, 8.00; N, 10.96; S, 3.58%.

H–Lys–Phe–HyIv–Gly–Leu–Met–NH$_2$·2HCl (IV). This was obtained from **16** (200 mg) as described for the preparation of XIV; yield, 180 mg; mp 95—100 °C; $[\alpha]_D^{25}$ −42.2° (*c* 0.265, H_2O); R_f, 0.68;[13] amino acid ratios in the acid hydrolysate: Lys, 0.96; Phe, 0.95; Gly, 0.98; Leu, 1.00; Met, 0.81. Found: C, 50.25; H, 7.66; N, 12.21; S, 4.11; Cl, 8.69%. Calcd for $C_{33}H_{55}N_7O_7S\cdot H_2O$: C, 50.51; H, 7.52; N, 12.50; S, 4.08; Cl, 9.05%.

The author wishes to express his thanks to Professor N. Izumiya of Kyushu University and Director Dr. I. Chibata of this laboratory for their encouragement in the course of this study. Thanks are also due to Associate Professor Y. Mukohata of Osaka University for his helpful discussions and to Dr. K. Higaki for his biological assay.