Amino Acids and Peptides. IX.[1)] Synthetic Studies on Leu-Enkephalin Analogues Containing a Ureylene Bond

Koichi Kawasaki,[*,a] Mitsuko Maeda,[a] Joe Watanabe,[b] and Hiroshi Kaneto[b]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a]
Ikawadani-cho, Nishi-ku, Kobe 673, Japan and Faculty of
Pharmaceutical Sciences, Nagasaki University,[b]
Bunkyo-cho, Nagasaki 852, Japan

Leu-enkephalin analogues containing a ureylene bond instead of an amide bond were synthesized. The ureylene bond was formed by a coupling reaction of the isocyanate derived from the corresponding azide by Curtius rearrangement reaction. Three analogues, each of which has a ureylene bond at the Tyr–Gly or Gly–Gly or Phe–Leu amide bond, were prepared. The ureylene bond resisted enzymatic hydrolysis, but the biological activities of the synthetic peptides on guinea pig ileum and mouse vas deferens were low compared with those of Leu-enkephalin.

Keywords——ureylene bond; Leu-enkephalin; peptide synthesis; peptide mimetic; isocyanate; Curtius rearrangement

Chemical modification of peptide bonds is a key area in studies on the structure–activity relationship of biologically active peptides. For example, the amide bond of a renin substrate at P_1-P_1' was replaced by $-CH_2-NH-$ [Szelke et al.[2)]] or by $-CH(OH)-CH_2-$ [Dann et al.[3)]] and these modified peptide analogues were reported as renin inhibitors. On the other hand, Clausen et al.[4)] and Lajoie et al.[5)] synthesized Leu–enkephalin analogues containing a thioamide bond ($-CS-NH-$) at Gly^2-Gly^3 and reported that these compounds were more effective than the original compound as regards the inhibitory effect on the ES-induced contraction of guinea pig ileum (GPI) and mouse vas deferens (MVD). Thus, modification of a peptide bond of biologically active peptides resulted in an alteration, such as enhancement, prolongation of action time, reduction or antagonism, of the original activities.

Peptide bond formation by the azide method is popular for peptide synthesis, but Curtius rearrangement[6)] is occasionally seen as a side reaction. We took advantage of this side reaction for the modification of peptide bonds.

Tyr–Gly, Gly–Gly, and Phe–Leu bonds in Leu–enkephalin (H–Tyr–Gly–Gly–Phe–Leu–OH) are hydrolyzed by enkephalin-degrading peptidases. Three Leu-enkephalin analogues, each of which has a ureylene bond ($-NHCONH-$) instead of the amide bond at one of the positions described above, were synthesized by the solution method.

The ureylene bond was formed by the coupling reaction of an isocyanate with an amino component. The isocyanate was prepared by Curtius rearrangement of the corresponding azide which was derived from the corresponding hydrazide with sodium nitrite and hydrochloric acid. The complete conversion of the azide to the isocyanate was necessary because isolation of a ureylene compound from a mixture containing an amide compound derived from the azide might be difficult. The conversion was done in chloroform and was checked by infrared (IR) spectroscopy. Azide and isocyanate showed each absorption bands at 2150 and 2250 cm^{-1}, respectively as shown in Fig. 1. The conversion was slow at room temperature, so it was done at the boiling point of chloroform for 4 h. The absorption band of
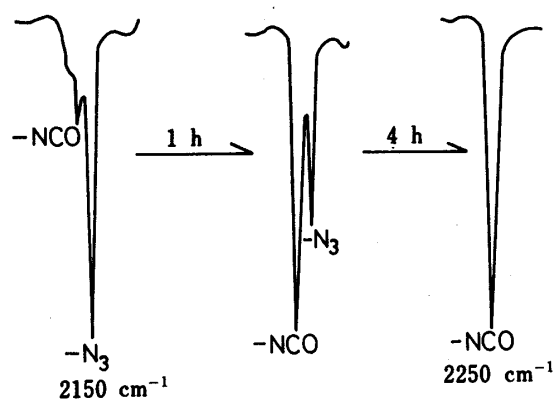
Fig. 1. IR Spectra Showing the Rearrangement of Z–Phe–N$_3$ into Z–Phe–NCO
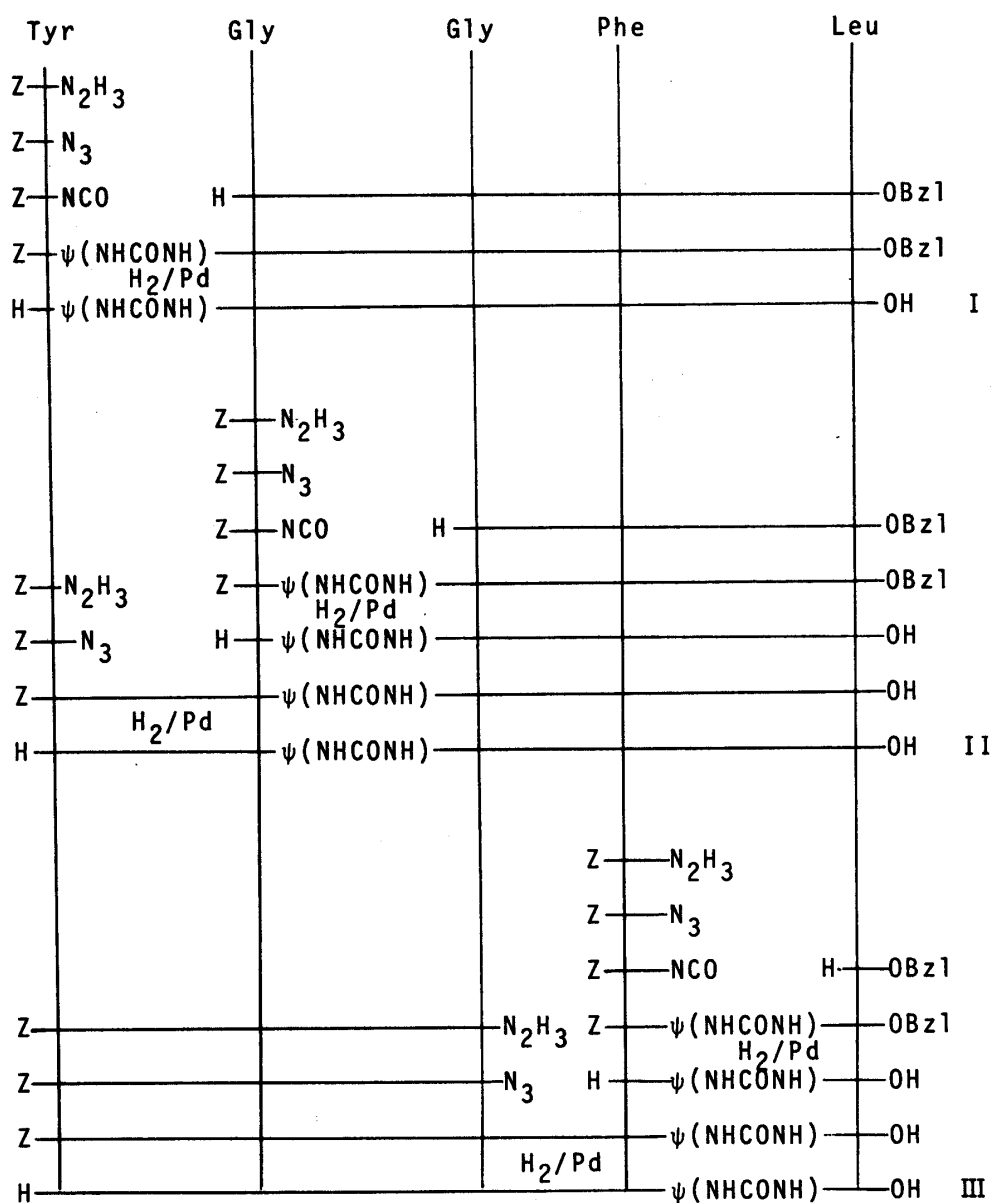

Fig. 2. Synthetic Schemes for the Ureylene Peptides

azide completely disappeared within 4 h. Judging from IR spectra (not shown in this paper), the rate of the rearrangement was in the following order; Z–Tyr–N$_3$ > Z–Phe–N$_3$ > Z–Gly–N$_3$.

H–Phe–ψ(NHCONH)–Leu–OH was prepared before synthesis of the enkephalin analogues. Z–Phe–N_3 was converted to Z–Phe–NCO, which was reacted with H–Leu–OBzl followed by hydrogenation to give H–Phe–ψ(NHCONH)–Leu–OH. The product and H–Phe–Leu–OH[7] were treated with chymotrypsin for 24 h. H–Phe–Leu–OH gave H–Phe–OH and H–Leu–OH in the ratio of 1.00 and 0.92, while H–Phe–ψ(NHCONH)–Leu–OH was not hydrolyzed at all. The ureylene bond was quite stable to enzymatic hydrolysis.

Synthetic schemes for H–Tyr–ψ(NHCONH)–Gly–Gly–Phe–Leu–OH (I), H–Tyr–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH (II) and H–Tyr–Gly–Gly–Phe–ψ(NHCONH)–Leu–OH (III) are shown in Fig. 2. The Z group was used as an N^α-protecting group to be removed afterward by hydrogenation, since the ureylene bond is labile to acidic conditions,[8] such as trifluoroacetic acid treatment. Compound I was synthesized by the coupling of Z–Tyr–NCO with H–Gly–Gly–Phe–Leu–OBzl[9] followed by hydrogenation. For the preparation of II, Z–Tyr–N_3 was reacted with H–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH, which was prepared from Z–Gly–NCO and H–Gly–Phe–Leu–OBzl[9] followed by hydrogenation. The resulting protected peptide was hydrogenated to give II. Compound III was prepared by the coupling of Z–Tyr–Gly–Gly–N_3[10] and H–Phe–(NHCONH)–Leu–OH followed by hydrogenation.

Compounds I, II, III and Leu–enkephalin were treated with aminopeptidase M (AP-M), and the results are summarized in Table I. Leu–enkephalin was hydrolyzed completely, while the ureylene analogues were not hydrolyzed at the ureylene bond. The ureylene bond is not cleaved by the enzyme.

The synthetic compounds were tested for opioid activity in the isolated GPI and MVD preparations and the results are summarized in Table II. Replacement of the amide bond of Leu–enkephalin with a ureylene bond resulted in the decrease or loss of the opioid activity in vitro. Clausen et al.[4] and Lajoie et al.[5] reported respectively that the opioid activities of [$^2\psi^3$, CSNH]–Leu–enkephalin were greater than those of Leu–enkephalin in the MVD and GPI tests. H–Tyr–D-Ala–Phe–phenylpropylamide[11] and H–Tyr–D-Met(O)–Phe–phenethylamide[12] were reported as potent opiates and we also reported that the distance between the Tyr residue and phenyl ring might not be crucial for the opioid activity.[13] Furthermore, we reported that the Gly^2–Gly^3 amide bond in Met–enkephalin was not essential for the production of the analgesic activity.[14] Thus, the poor opioid activities of the

TABLE I.   Amino Acid Ratios in AP-M Digests of the Urelene Peptides

	Tyr	Gly	Phe	Leu
Leu-enkephalin	1.00	2.25	1.13	1.12
I	1.00	1.97	—	—
II	1.00	—	—	0.03
III	—	—	—	—

TABLE II.   Opioid Activity of the Ureylene Peptides on GPI and MVD

Compounds	GPI		MVD	
	IC_{50}	Activity ratio	IC_{50}	Activity ratio
Morphine	72.3 ± 8.2 nM	100		
Leu-enkephalin			8.4 ± 0.9 µM	100
I	inactive (20 µM)		47.2 ± 2.3 µM	0.02
II	inactive (20 µM)		56.4 ± 6.7 µM	0.02
III	33.7 ± 4.4 µM	0.4	48.3 ± 0.9 µM	0.02

newly synthesized compounds were contrary to our expectation. However, the difference in the activities in GPI and MVD preparations may suggest the possibility of selectivity of the compounds for the opioid receptor subtypes. Further studies on the biological activities of the ureylene-containing enkephalin analogues will be reported elsewhere. The ureylene bond analogues of Leu–enkephalin were synthesized successfully and the ureylene bond was not hydrolyzed by enzymes. Peptides containing a ureylene bond will be interesting as enzymatically non-hydrolyzable analogues, like other peptide mimetics.

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2 = BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf^4 = AcOEt–benzene (1:1), Rf^5 = $CHCl_3$–AcOH–MeOH (90:2:8). Amino acid compositions of acid and enzymatic hydrolysates were determined with a Kyowa K-101 AS amino acid analyzer and a Hitachi 835 amino acid analyzer.

Z–Tyr–ψ(NHCONH)–Gly–Gly–Phe–Leu–OBzl——NaNO$_2$ (0.42 g, 6 mM) dissolved in H_2O (5 ml) was added to a solution of Z–Tyr–N$_2$H$_3$ (2 g, 6 mM) in a mixture of AcOH (12 ml), 1 N HCl (12 ml) and $CHCl_3$ (100 ml) at 4 °C and the whole was stirred for 10 min. The $CHCl_3$ layer was washed with 10% Na_2CO_3, dried over Na_2SO_4 and refluxed for 4 h. After cooling, the solution was combined with a solution of H–Gly–Gly–Phe–Leu–OBzl (0.8 g, 1.66 mM) in $CHCl_3$ (30 ml). The mixture was stirred overnight and the solvent was evaporated off. The residue was washed successively with cold 5% AcOH and H_2O, and precipitated from DMF–ether. Yield 1.03 g (71%), mp 160 °C, $[\alpha]_D^{25}$ −15.9° (c = 1.0, DMF), Rf^3 0.76, Rf^5 0.40. Anal. Calcd for $C_{43}H_{50}N_6O_9$: C, 65.0; H, 6.4; N, 10.6. Found: C, 64.7; H, 6.2; N, 10.5. Amino acid ratios in an acid hydrolysate: Gly, 2.00; Phe, 1.12; Leu, 1.02 (recovery of Gly, 79%).

H–Tyr–ψ(NHCONH)–Gly–Gly–Phe–Leu–OH (I)——Z–Tyr–ψ(NHCONH)–Gly–Gly–Phe–Leu–OBzl (0.5 g, 0.63 mM) was hydrogenated over Pd catalyst in a mixture of MeOH (50 ml) and dioxane (20 ml) for 14 h. The solvent was evaporated off and the residue was lyophilized from dioxane. Yield 331 mg (92%), mp 150—157°C, $[\alpha]_D^{25}$ −12.9° (c = 1.0, 30% AcOH), Rf^1 0.74, Rf^2 0.79. Anal. Calcd for $C_{28}H_{38}N_6O_7 \cdot H_2O$: C, 57.1; H, 6.9; N, 14.3. Found: C, 57.5; H, 6.8; N, 14.3. Amino acid ratios in an acid hydrolysate: Gly, 2.00; Phe, 1.03; Leu, 1.07 (recovery of Gly, 72%). Tyr was not detected in the acid hydrolysate.

Z–Gly–ψ(NHCONH)–Gly–Phe–Leu–OBzl——NaNO$_2$ (0.5 g, 7.2 mM) dissolved in H_2O (5 ml) was added to a mixture of Z–Gly–N$_2$H$_3$ (1.6 g, 7.2 mM), 1 N HCl (22 ml) and $CHCl_3$ (50 ml) and the whole was stirred at 4 °C for 15 min. The $CHCl_3$ layer was washed with 5% NaHCO$_3$, dried over Na_2SO_4 and refluxed for 6 h. After cooling, the solution was combined with a solution of H–Gly–Phe–Leu–OBzl (4.2 g, prepared from its TFA salt[9]) and 10% Na_2CO_3) in $CHCl_3$ (50 ml) and the whole was stirred overnight at room temperature. The solvent was evaporated off and the residue was precipitated from DMF–ether. The material was washed successively with cold 5% AcOH and H_2O, and dried. Yield 1.76 g (39%), mp 170—175 °C, $[\alpha]_D^{25}$ −18.0° (c = 1.0, DMF), Rf^3 0.88, Rf^5 0.92. Anal. Calcd for $C_{34}H_{41}N_5O_7$: C, 63.6; H, 6.6; N, 11.4. Found: C, 63.4; H, 6.5; N, 11.4. Amino acid ratios in an acid hydrolysate: Gly, 1.00; Phe, 1.01; Leu, 0.98 (recovery of Gly, 96%).

H–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH——Z–Gly–ψ(NHCONH)–Gly–Phe–Leu–OBzl (0.5 g, 0.8 mM) dissolved in a mixture of MeOH (2 ml), dioxane (10 ml) and DMF (40 ml) was hydrogenated over Pd catalyst for 13 h. The solvent was evaporated off and the residue was lyophilized from dioxane. Yield 300 mg (92%), mp 230—234 °C, $[\alpha]_D^{25}$ −22.8° (c = 1.0, 30% AcOH), Rf^1 0.76, Rf^2 0.78. Anal. Calcd for $C_{19}H_{29}N_5O_5$: C, 56.0; H, 7.2; N, 17.2. Found: C, 56.4; H, 6.9; N, 17.4. Amino acid ratios in an acid hydrolysate: Gly, 1.00; Phe, 1.00; Leu, 1.00 (recovery of Gly 91%).

Z–Tyr–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH——Isoamyl nitrite (0.28 ml, 2.1 mM) was added to a mixture of Z–Tyr–N$_2$H$_3$ (0.7 g, 2.1 mM), 6 N HCl–dioxane (1.05 ml) and DMF (20 ml) at −20 °C and the mixture was stirred for 10 min. Et$_3$N (0.88 ml) was added and the mixture was combined with a solution of H–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH (300 mg, 0.7 mM) in a mixture of DMF (10 ml) and Et$_3$N (0.1 ml, 0.7 mM). The whole was stirred for 24 h in a cold room and the solvent was evaporated off. The residue was precipitated from DMF–ether and washed successively with cold 5% AcOH and H_2O. Yield 181 mg (37%), mp 151—153 °C, $[\alpha]_D^{25}$ −13.8° (c = 1.0, DMF), Rf^5 0.28. Anal. Calcd for $C_{36}H_{44}N_6O_9$: C, 61.3; H, 6.3; N, 11.9. Found: C, 61.4; H, 6.3; N, 11.9. Found: C, 61.4; H, 6.3; N, 12.1. Amino acid ratios in an acid hydrolysate: Tyr, 0.82; Gly, 1.00; Phe, 1.06; Leu, 0.93 (recovery of Gly, 77%).

H–Tyr–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH (II)——Z–Tyr–Gly–ψ(NHCONH)–Gly–Phe–Leu–OH (136 mg, 0.19 mM) was hydrogenated over Pd catalyst in DMF (50 ml) for 12 h. The solvent was evaporated off and the residue was lyophilized from dioxane. Yield 108 mg (99%), mp 156—160 °C, $[\alpha]_D^{25}$ −16.4° (c = 1.0, 30% AcOH), Rf^1 0.30, Rf^2 0.53. Anal. Calcd for $C_{28}H_{38}N_6O_7 \cdot 2H_2O$: C, 55.4; H, 7.0; N, 13.9. Found: C, 55.4; H, 6.7; N, 13.8. Amino acid ratios in an acid hydrolysate: Tyr, 0.81; Gly, 1.00; Phe, 1.11; Leu, 0.92 (recovery of Gly, 72%).

Z-Phe-ψ(NHCONH)-Leu-OBzl——NaNO$_2$ (0.75 g, 10.8 mM) dissolved in H$_2$O (5 ml) was added to a mixture of Z-Phe-N$_2$H$_3$ (3.36 g, 10.7 mM), 1 N HCl (32 ml) and CHCl$_3$ (50 ml), and the mixture was stirred at 4 °C for 10 min. The CHCl$_3$ layer was washed with 5% NaHCO$_3$, dried over Na$_2$SO$_4$ and refluxed for 6 h. Then the CHCl$_3$ layer was combined with H-Leu-OBzl (3 g, 13.6 mM) dissolved in CHCl$_3$ (50 ml) and the whole was stirred at room temperature for 15 h. The solvent was evaporated off and the residue was washed successively with chilled 5% AcOH and ether in a mortar. The material was recrystallized from MeOH. Yield 3.61 g (60%), mp 160—162 °C. $[\alpha]_D^{26}$ −7.3° (c = 1.0, DMF), Rf^3 0.98, Rf^5 0.62. Anal. Calcd for C$_{30}$H$_{35}$N$_3$O$_5$: C, 69.6; H, 6.8; N, 8.1. Found: C, 69.6; H, 6.8; N, 8.3. Phe was not detected in the acid hydrolysate.

H-Phe-ψ(NHCONH)-Leu-OH——Z-Phe-ψ(NHCONH)-Leu-OBzl (3 g, 5.8 mM) was hydrogenated over Pd catalyst in MeOH (200 ml) for 6 h. The solvent was evaporated off and the residue was washed with ether in a mortar. Yield 1.54 g (90%), mp 130—133 °C, $[\alpha]_D^{25}$ −2.4° (c = 1.0, 30% AcOH), Rf^1 0.82, Rf^2 0.87. Anal. Calcd for C$_{15}$H$_{23}$N$_3$O$_3$: C, 61.4; H, 7.9; N, 14.3. Found: C, 61.7; H, 8.3; N, 14.7. Phe was not detected in an acid hydrolysate.

Chymotrypsin Treatment of H-Phe-ψ(NHCONH)-Leu-OH and H-Phe-Leu-OH——α-Chymotrypsin (1 ml, 2.5 units. Sigma) was added to a solution of H-Phe-ψ(NHCONH)-Leu-OH (or H-Phe-Leu-OH, 1 μM) in 0.1 M phosphate buffer (1 ml, pH 8.0). The mixture was stirred for 24 h at 37 °C, diluted with citrate buffer (pH 2.0), and analyzed. The results were as follows; H-Phe-ψ(NHCONH)-Leu-OH [Phe 0.00, Leu 0.00], H-Phe-Leu-OH [Phe 1.00, Leu 0.92; average recovery, 76%].

Z-Tyr-Gly-Gly-Phe-ψ(NHCONH)-Leu-OH——Isoamyl nitrite (0.3 ml, 2.4 mM) was added to a mixture of Z-Tyr-Gly-Gly-N$_2$H$_3$ (925 mg, 2.1 mM), 6 N HCl-dioxane (1.05 ml) and DMF (8 ml) at −15 °C. The mixture was stirred for 10 min, neutralized with Et$_3$N (0.15 ml) and combined with H-Phe-ψ(NHCONH)-Leu-OH (306 mg, 1.04 mM) dissolved in a mixture of DMF (10 ml) and Et$_3$N (0.15 ml, 1.04 mM). The whole was stirred overnight in a cold room and the solvent was evaporated off. The residue was washed with 5% AcOH and precipitated from DMF-ether. Yield 651 mg (44%), mp 188—192 °C, $[\alpha]_D^{25}$ −12.7° (c = 1.0, DMF), Rf^5 0.83. Anal. Calcd for C$_{36}$H$_{44}$N$_6$O$_9$: C, 61.3; H, 6.3; N, 11.9. Found: C, 61.6; H, 6.2; N, 11.6. Amino acid ratios in an acid hydrolysate: Tyr, 0.86; Gly, 2.00; Leu, 0.98 (recovery of Gly, 89%).

H-Tyr-Gly-Gly-Phe-ψ(NHCONH)-Leu-OH (III)——Z-Tyr-Gly-Gly-Phe-ψ(NHCONH)-Leu-OH (0.5 g, 0.7 mM) was hydrogenated over Pd catalyst in a mixture of MeOH (10 ml) and dioxane (20 ml) for 12 h. The solvent was evaporated off and the residue was lyophilized from dioxane. Yield 338 mg (85%), mp 186—190 °C, $[\alpha]_D^{25}$ −12.9° (c = 1.0, 30% AcOH), Rf^1 0.30, Rf^2 0.53. Anal. Calcd for C$_{28}$H$_{38}$N$_6$O$_7$·1.5H$_2$O: C, 58.0; H, 6.8; N, 14.5. Found: C, 57.9; H, 6.9; N, 14.9. Amino acid ratios in an acid hydrolysate: Tyr, 0.84; Gly, 2.00; Leu, 0.91 (recovery of Gly, 63%).

AP-M Treatment of the Ureylene Peptide——AP-M (0.1 ml of 5 mg/ml suspension; Boehringer Mannheim) was added to a solution of the ureylene peptide (1 μM) in 0.01 M Tris buffer (1 ml, pH 7.5). The mixture was stirred for 24 h. at 37 °C, diluted with citrate buffer (pH 2.0), and analyzed. The results are shown in Table I and the recoveries of Tyr were as follows: I (0%), II (75%), III (72%), Leu-enkephalin (84%).

Opioid Activities of the Ureylene Peptides——Inhibitory effect of the synthetic compounds on the electrically induced contraction of GPI and MVD were determined according to the method reported in the preceding paper.[13b] The results are given in Table II.

References and Notes

1) Standard abbreviations for amino acids, protecting groups, and peptides are used [Eur. J. Biochem., 138, 9 (1984)]. Other abbreviations include: DMF = dimethylformamide, TFA = trifluoroacetic acid.

2) M. Szelke, B. Leckie, A. Hallett, D. M. Jones, J. Sueiras, B. Atrash, and A. F. Lever, Nature (London), 299, 555 (1982).

3) J. G. Dann, D. K. Stammers, C. J. Harris, R. J. Arrowsmith, D. E. Davies, G. W. Hardy, and J. A. Morton, Biochem. Biophys. Res. Commun., 134, 71 (1986).

4) K. Clausen, M. K. Anwer, A. L. Bettag, D. E. Benovitz, J. V. Edwards, S. O. Lawesson, A. F. Spatola, D. Winkler, B. Brown, P. Rowell, P. Schiller, and C. Lemieux, "Peptides. Structure and Function. Proceedings of the 8th American Peptide Symposium," ed. by V. J. Hruby and D. H. Rich, Pierce Chemical Company, Rockford, Illinois, 1984, p. 307.

5) G. Lajoie, F. Lepine, S. Lemaire, F. Jolicoeur, C. Aube, A. Turcotte, and B. Belleau, Int. J. Peptide Protein Res., 24, 316 (1984).

6) T. Curtius, J. Prakt. Chem., 50, 275 (1894).

7) A. Cosmatos, I. Photaki, and L. Zervas, Chem. Ber., 94, 2644 (1961); Z. Pravda, K. Poduska, and K. Blaha, Collect. Czech. Chem. Commun., 29, 2626 (1964).

8) K. Inouye and K. Watanabe, "Peptide Chemistry 1973," ed. by K. Kotake, Protein Research Foundation, Osaka, 1974, p.35.

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

10) K. Nagren, B. Langstroem, and U. Ragnarsson, Acta Chem. Scand., Ser. B, 34, 157 (1985).

11) R. J. Vavrek, L.-H. Hsi, E. J. York, M. E. Hall, and J. M. Stewart, *Peptides*, **2**, 303 (1981).

12) Y. Kiso, T. Miyazaki, M. Inai, K. Kitagawa, T. Akita, H. Moritoki, and H. Nakamura, "Peptide Chemistry 1982," ed. by S. Sakakibara, Protein Research Foundation, Osaka, 1983, p. 245.

13) *a*) M. Maeda, S. Okusada, and K. Kawasaki, *Chem. Pharm. Bull.*, **32**, 4157 (1984); *b*) J. Watanabe, H. Kaneto, M. Maeda, T. Tsuji, and K. Kawasaki, *J. Pharmacobio-Dyn.*, **8**, 975 (1985).

14) K. Kawasaki and M. Maeda, *Biochem. Biophys. Res. Commun.*, **106**, 113 (1982).