Amino Acids and Peptides. XXXVI. Synthesis of Enkephalin Chloromethyl Ketone and Evaluation of Its Inhibitory Activity against Endopeptidase 22.19[1,2]

Hiroaki TAGUCHI,[a] Yasuhiro NISHIYAMA,[a] Antonio C. M. CAMARGO,[b] and Yoshio OKADA*,[a]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Nishi-ku, Kobe 651-21, Japan and Department of Pharmacology, Institute of Biomedical Sciences, University of San Paulo,[b] San Paulo, Brazil. Received April 14, 1993

Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl was synthesized by the conventional solution method. During the course of acid hydrolysis (6 N HCl, 110 °C, 18 h) of Boc–Phe–Leu–CH_2Cl, side reaction occurred, resulting in low recovery of Phe residue on amino acid analysis. The inhibitory activity of the synthesized Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl against endopeptidase 22.19, an enzyme related to the metabolism of opioid peptides, was examined.

Keywords peptide chloromethyl ketone; enkephalin; inhibitor; chemical synthesis; endopeptidase 22.19 (EC 3.4.22.19); side reaction

Endopeptidase 22.19 (EC 3.4.22.19), which has been isolated from rabbit brain by Camargo *et al.*, is a member of the thiol-proteinase family.[3] This enzyme releases enkephalin from endogenous enkephalin precursor peptides, and presumably plays an important role in the metabolism of opioid peptides in the brain.[4] However, the enzymatic properties and the actions of endopeptidase 22.19 have not been investigated in detail yet. For such studies, a specific inhibitor would be a useful tool. Since it has been well established that C-terminal chloromethyl ketone derivation of the specific substrate produces a specific irreversible inhibitor in the cases of serine- and thiol-proteinases,[5,6] it is expected that enkephalin chloromethyl ketone would inhibit endopeptidase 22.19 specifically and irreversibly. This report deals with the synthesis of Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl[7] and the examination of its inhibitory activity against endopeptidase 22.19.

The desired peptidyl chloromethyl ketone was synthesized by the conventional solution method according to the scheme shown in Fig. 1. Boc–Tyr–OH was coupled with H–Gly–Gly–OBzl, which was prepared by DCC-HOBt coupling of Boc–Gly–OH and H–Gly–OBzl followed by HCl treatment, to give Boc–Tyr–Gly–Gly–OBzl.[8] On the other hand, Boc–Leu–CH_2Cl was obtained by the mixed anhydride coupling of Boc–Leu–OH and diazomethane, followed by the addition of HCl/dioxane[9] instead of HCl gas. After removal of the Boc group, Boc–Phe–OH was coupled by a mixed anhydride method to give Boc–Phe–Leu–CH_2Cl. Although the same compound, Boc–Phe–Leu–CH_2Cl was prepared previously[10,11] by coupling Boc–Phe–Leu–OH and diazomethane, followed by addition of HCl gas, our procedure is more suitable from the viewpoint of avoiding racemization of the Leu residue. Boc–Phe–Leu–CH_2Cl, thus obtained, gave satisfactory TLC, ^{1}H-NMR and elemental analysis results, but the recovery of Phe residue on amino acid analysis after acid hydrolysis (6 N HCl, 110 °C, 18 h) was remarkably low. This phenomenon is presumably due to the occurrence of side reaction during the acid hydrolysis of the dipeptidyl chloromethyl ketone. This side reaction is now under investigation in our laboratory and the results will be presented elsewhere. After removal of the Boc group, Boc–Tyr–Gly–Gly–OH, which was prepared from the corresponding benzyl ester[8] *via* catalytic hydrogenation, was condensed by the Bop-mediated coupling method[12] to give Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl. This compound gave a satisfactory result on amino acid analysis after acid hydrolysis, in addition to TLC and elemental analysis.

Next, we examined the inhibitory activity of Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl by the method described previously.[13] This compound inhibited endopeptidase 22.19 with an IC_{50} value of 0.13 mM. Since enkephalin chloromethyl ketone exhibited low affinity for endopeptidase 22.19, the synthesis of large chloromethyl ketone derivatives containing enkephalin peptide is in progress in our laboratory with the aim of increasing the specificity of the enzyme inhibition. We believe that the development of a more specific irreversible inhibitor for endopeptidase 22.19 will contribute to investigations on the role of this enzyme in neuropeptide metabolism in brain, including the biotransformation of opioid peptides.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Amino acid compositions of an hydrolysate (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitu Co.). ^{1}H-NMR spectra were measured with a Bruker AM400 spectrometer operating at a frequency of 400 MHz and controlled by an Aspect 3000 computer. Chemical shift values are expressed as ppm downfield from tetramethylsilane used as an internal standard (δ-value). Mass spectra (MS) were determined on a Hitachi M-2000 mass spectrometer. On TLC (Kieselgel G, Merck), *Rf* values refer to the system of $CHCl_3$, MeOH and H_2O (90 : 8 : 2).

Boc–Leu–CH_2Cl Diazomethane [prepared from nitrosomethylurea (6.1 g, 60 mmol)] in ether (50 ml) was added to a mixed anhydride [prepared

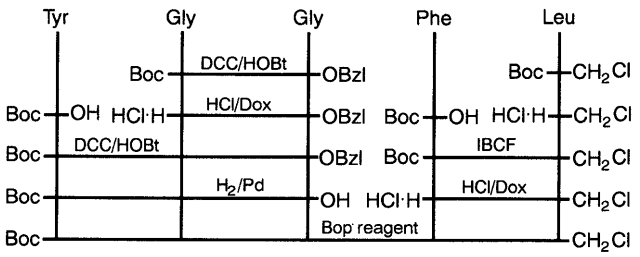

Fig. 1. Synthetic Scheme for Enkephalin Chloromethyl Ketone
Dox, dioxane; IBCF, isobutyl chloroformate.

from Boc–Leu–OH (6.9 g, 30 mmol), Et_3N (4.2 ml, 30 mmol) and ethyl chloroformate (2.8 ml, 30 mmol) as usual] in THF (100 ml) at −15 °C and the reaction mixture was stirred at 4 °C overnight. Then 7.6 N HCl/dioxane (7.8 ml, 66 mmol) was added to the reaction mixture at 0 °C, and the resultant solution was stirred at 0 °C for 3 h. After neutralization of the solution with Et_3N and removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. *n*-Hexane was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 5.0 g (63.3%), mp 60—62 °C, $[\alpha]_D^{25}$ −42.6 °C ($c=1.0$, MeOH), *Rf* 0.84. MS *m/z*: 264 $(M+H)^+$. ^{1}H-NMR ($CDCl_3$) δ: 5.0 (1H, d, $J=7.7$ Hz), 4.54—4.48 (1H, m), 4.3 (2H, d, $J=7.5$ Hz), 1.62—1.53 (1H, m), 1.44 (9H, s), 1.42—1.39 (2H, m), 0.97 (3H, d, $J=6.6$ Hz), 0.96 (3H, d, $J=6.6$ Hz). *Anal.* Calcd for $C_{12}H_{22}ClNO_3$: C, 54.6; H, 8.41; N, 5.31. Found: C, 54.3; H, 8.49; N, 5.33.

Boc–Phe–Leu–CH_2Cl A mixted anhydride [prepared from Boc–Phe–OH (1.68 g, 6.75 mmol), Et_3N (1.0 ml, 7.42 mmol) and isobutyl chloroformate (0.9 ml, 6.75 mmol) as usual] in THF (100 ml) was added to an ice-cold solution of H–Leu–CH_2Cl·HCl [prepared from Boc–Leu–CH_2Cl (2.0 g, 7.6 mmol) and 7.6 N HCl–dioxane (6.0 ml, 45.6 mmol) as usual] in DMF (100 ml) containing Et_3N (1.0 ml, 7.42 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 1.4 g (51.8%), mp 146—150 °C, $[\alpha]_D^{25}$ −62.6° ($c=1.0$, MeOH), *Rf* 0.83. MS *m/z*: 411 $(M+H)^+$. ^{1}H-NMR ($CDCl_3$) δ: 7.32—7.19 (5H, m), 6.33 (1H, s), 5.0 (1H, s), 4.72 (1H, s), 4.43 (1H, s), 4.06 (2H, s), 3.06 (2H, d, $J=7.3$ Hz), 1.63—1.51 (3H, m), 1.42 (9H, s), 0.91 (3H, d, $J=7.3$ Hz), 0.89 (3H, d, $J=6.5$ Hz) *Anal.* Calcd for $C_{21}H_{31}ClN_2O_4$: C, 61.4; H, 7.60; N, 6.81. Found: C, 61.5; H, 7.54; N, 6.85. Amino acid analysis: recovery of Phe was 13%.

Boc–Tyr–Gly–Gly–Phe–Leu–CH_2Cl BOP (150 mg, 0.34 mmol) and Boc–Tyr–Gly–Gly–OH (130 mg, 0.34 mmol) [prepared from Boc–Tyr–Gly–Gly–OBzl[8] (550 mg, 1.1 mmol) by catalytic hydrogenation over 5% Pd/C] were added to a solution of H–Phe–Leu–CH_2Cl·HCl (120 mg, 0.34 mmol) [prepared from Boc–Phe–Leu–CH_2Cl (200 mg, 0.48 mmol) and 5.9 N HCl/dioxane (0.4 ml, 2.4 mmol) as usual] in CH_3CN (10 ml) containing Et_3N (0.04 ml) under cooling with ice. Coupling was initiated by addition of DIEA (0.08 ml, 0.45 mmol). The reaction mixture was stirred at room temperature for 1 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residual oil in $CHCl_3$ (1 ml) was applied to a silica gel column (1.5 × 28 cm), equilibrated with 50% (v/v) AcOEt–$CHCl_3$ and eluted with 50% (v/v) AcOEt–$CHCl_3$ (300 ml) and 75% (v/v) AcOEt–$CHCl_3$ (500 ml). After removal of the solvent of the eluate (550—750 ml), petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 25 mg (11%), mp 134—136 °C, $[\alpha]_D^{25}$ −40.8° ($c=0.5$, DMF), *Rf* 0.34. *Anal.* Calcd for $C_{34}H_{46}ClN_5O_8\cdot 0.5H_2O$: C, 58.6; H, 6.79; N, 10.0. Found: C, 58.6; H, 6.70; N, 9.93. Amino acid analysis: Tyr (1) 0.8; Gly (2) 2.6; Phe (1) 1.0; Leu (−) (average recovery 74.0%).

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References and Notes

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