N-Hydroxy Amides. Part 5.† Synthesis and Properties of *N*-Hydroxypeptides having Leucine Enkephalin Sequences

Masayasu Akiyama,* Katsuhito Iesaki, Akira Katoh, and Kazuyuki Shimizu
Department of Applied Chemistry for Resources, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, Japan

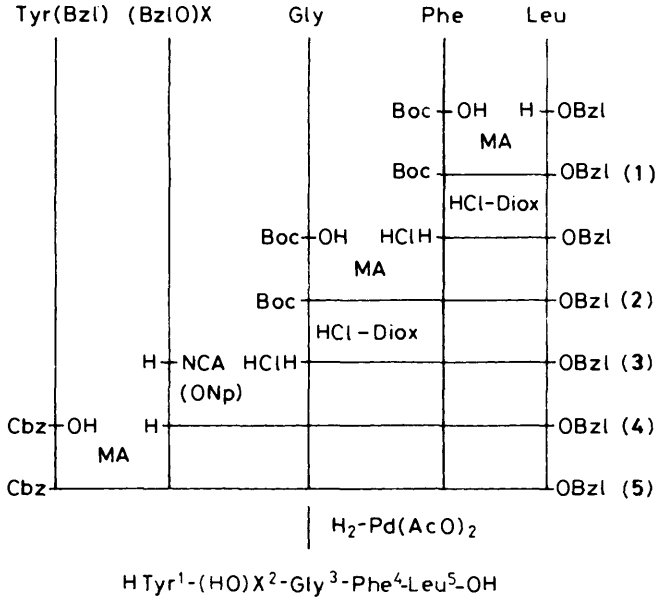
In order to study the effects of the *N*-hydroxy amide group, *N*-hydroxyenkephalin analogues with the H-Tyr-(HO)X-Gly-Phe-Leu-OH sequence, where X = Gly, L-Ala, or β-Ala, have been synthesized. A ^{1}H n.m.r. study indicates that the *N*-hydroxyenkephalins have a type I β-turn structure in dimethyl sulphoxide solution. From the interaction of (HO)Gly- and β-(HO)Ala-analogues with Cu^{II} it is suggested that the hydroxyamide function exerts a definite influence on the complexation. These *N*-hydroxy enkephalins are resistant to aminopeptidase M. A qualitative analgesia test shows that the (HO)Ala-analogue possesses a more lasting potency than that of Leu^5-enkephalin, while having a comparable activity.

Several *N*-hydroxypeptides have been found in Nature. These compounds contain one or more *N*-hydroxyamino acid residues in the chain and show unique biological activity, acting as tumour inhibitors (hadacidin), antibiotics (aspergillic acid and mycelianamide), or growth factors (rhodotorulic acid and ferrichromes).[1-5] In addition, it has been suggested that *N*-hydroxypeptides play a role in the biosynthesis of β-lactam antibiotics and dehydro- or α-functionalized peptides.[6-11] Although it is well recognized that one of the important functions of natural hydroxamic acids is to chelate iron(III) and to solubilize it for cell membrane transport,[1-3,12] its other functions[13] under physiological conditions are not yet clearly understood.

During the course of these studies on *N*-hydroxypeptides,[10,11,14-19] we were interested in discovering whether any general effects are produced when an *N*-hydroxyamino acid residue is incorporated into a peptide sequence. For our work, we chose to study the relatively simple, biologically active peptide Leu^5-enkephalin,[20] considerable information having been accumulated on the structure–activity relationships[21] and preparation of enkephalin analogues.[21-23] It appears that replacement of the residue 2 is quite effective in increasing the activity and decreasing the *in vivo* enzymatic degradation.[24] Here we describe the preparation and properties of *N*-hydroxyenkephalins containing the H-Tyr-(HO)X-Gly-Phe-Leu-OH sequence, where (HO)X stands for a small number of *N*-hydroxyamino acid residues.

Results and Discussion

The synthetic route to the *N*-hydroxyenkephalins is illustrated in the Scheme. Benzyl protective groups were used where protection was desirable. *N*-Benzyloxy-L-alanine and -valine obtained by reaction of appropriate D-α-bromoalkanoic acids with *O*-benzylhydroxylamine, had optical rotations larger than reported values.[25] The protected tripeptide (**2**) was prepared by a standard procedure. Acylation of the deprotected tripeptide (**3**) with *N*-benzyloxy-*N*-carboxy α-amino acid anhydrides (BzlO-NCA) of Gly, Ala, and Val[17,18] or with *p*-nitrophenyl 3-(*N*-benzyloxy)aminopropanoate[26] afforded the corresponding *N*-benzyloxytetrapeptides (**4**) in good yields (Table 1).



Scheme.

Acylation of the *N*-benzyloxyamino terminal tetrapeptide (**4**) with Cbz-Tyr(Bzl)-OH by the mixed anhydride procedure gave the protected pentapeptides (**5**) (Table 2). A bulky Tyr unit is able to couple only with *N*-benzyloxyamino acid residues with small side-chains. Although in the case of the *N*-benzyloxyvalyltripeptide, repeated acylation was ineffective, an alternative route for such a reaction has been described.[10] Hydrogenolysis[19,25] of the pentapeptide (**5**), followed by purification by chromatography, afforded the desired *N*-hydroxypentapeptides. The synthetic results are summarized in Table 3. Homogeneity of the products was determined by t.l.c. and h.p.l.c. and the *N*-hydroxy amide unit was detected by a characteristic colour test with $FeCl_3$ solution. Amide i.r. absorption was observed at 1 670sh and 1 635 cm^{-1}, the latter being assigned to a *cis* orientated –N(OH)CO– group.[27]

200 MHz ^{1}H n.m.r. spectra were recorded in $(CD_3)_2SO$ solution at 23 °C. The conformational study of Leu^5- and Met^5-enkephalins has been made by several groups,[28,29] particularly by Stimson *et al.*[30] using ^{13}C enrichment experiments. These

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Table 1. Preparation of *N*-benzyloxytetrapeptides, H-(BzlO)X-Gly-Phe-Leu-OBzl

X	Yield (%)	M.p. (°C)	T.l.c.[a] R_F^1	T.l.c.[a] R_F^2	$[\alpha]_D$[b] (°)	Found (%) C	Found (%) H	Found (%) N	Requires (%) C	Requires (%) H	Requires (%) N
Gly	92	130—131	0.22	0.87[c]	−19.0	67.2	6.9	9.4	67.3	6.9	9.5
Ala	88	140—141	0.31	0.60	−26.6	67.5	7.1	9.3	67.8	7.0	9.3
Val	86	127—128	0.60	0.71	2.4	68.5	7.4	8.9	68.4	7.4	8.9
β-Ala	82	126—127	0.21	0.75	−21.8	66.9	7.0	9.2	66.7	7.1	9.2[d]

[a] R_F^1, $CHCl_3$–AcOEt (1:1); R_F^2, $CHCl_3$–MeOH–C_6H_6 (6:1:3). [b] THF (*c* 1). [c] $CHCl_3$–MeOH (5:1). [d] Water of crystallization (0.5 H_2O) is included.

Table 2. Acylation[a] of H-(BzlO)X-Gly-Phe-Leu-OBzl with Cbz-Tyr(Bzl)-OH

X	Solvent	Acylation run[b]	Yield (%)
Gly	THF	1	85
β-Ala	DMF	1	85
Ala	DMF	2	35
Val	DMF	3	*c*

[a] Bu^iOCOCl, −17 °C, 15 min activation. [b] The amino component was checked by t.l.c. [c] No product isolated.

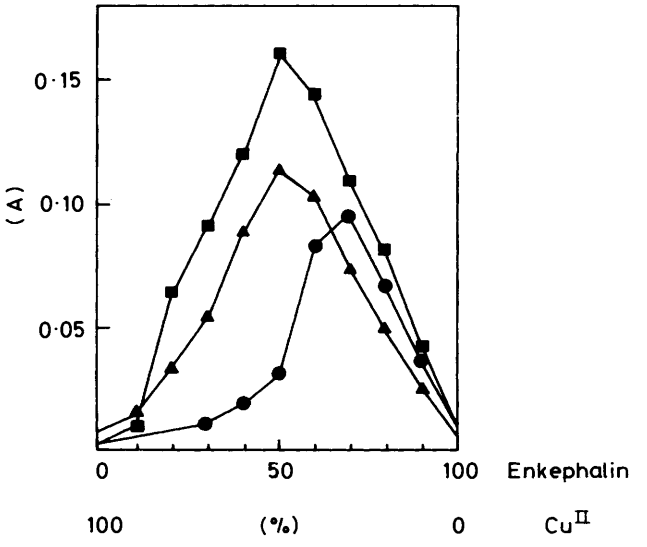

Figure. Job plots for complex formation between *N*-hydroxyenkephalins and copper(II) in comparison with Leu[5]-enkephalin. Absorbance (A) *versus* mol % of Cu(II) and enkephalins: Leu[5]-enkephalin at 580 nm (pH 7.6), — ▲ —; (HO)Gly[2]-enkephalin at 560 nm (pH 7.8), — ■ —; β-(HO)Ala[2]-enkephalin at 600 nm (pH 7.8), — ● —.

investigations have pointed to the presence of a well-defined type I β-turn structure for the two enkephalins; this would involve a loop of the -CO-Gly[3]-Phe[4]-NH- sequence connected by NH···O=C hydrogen bonding. No data are available concerning the influence of the *N*-hydroxy amide group upon peptide conformations. Although the ^1H n.m.r. spectra of *N*-hydroxyenkephalins are different from those of Leu[5]-enkephalin, peak assignment was possible using the homonuclear decoupling technique and by comparison of data with previously reported results.[28–30] ^1H Chemical shifts and coupling constants are summarized in Table 4. The downfield shifts for the α-H and β-H of Tyr and the C–H of Gly[3] as well as the protons of the residue X[2], attributable to the inductive effect of the *N*-hydroxy group, are worthy of note. The other peaks appear at positions close to those of enkephalin itself. The absence of phenolic and *N*-hydroxy protons peaks implies that rapid proton exchange is occurring between these groups and the solvent molecules. The temperature dependence of the amide proton chemical shifts was also determined (see Table 4). In all the analogues, the Gly[3] and Phe[4] amide protons showed much larger temperature coefficients than that of Leu[5]; this was taken to indicate the existence of a hydrogen bond involving the NH of Leu[5]. The dihedral angle (θ) of the H–N–C^α–H plane was calculated from the coupling constant $^3J_{C^\alpha H\text{-}NH}$ using Neel's modification,[31] and the torsional angle (φ) was obtained from these θ values. Among the values calculated there are a set of values suitable to a type I β-turn structure (−60° for Gly[3] and −90° for Phe[4]) as shown in Table 5. The Leu[5]-OH unit is in a conformation common to the three analogues. The proton chemical shifts, temperature dependence of the NH protons, and calculated φ values, strongly support the theory that the *N*-hydroxyenkephalins assume a type I β-turn conformation in Me_2SO solution similar to the parent enkephalins.[28]

Copper(II) Complexes.—In relation to the hydroxamate group, the complexation of *N*-hydroxyenkephalins with metal ions is of interest: the Cu^{II} complexes of enkephalins have already been studied[32] for aqueous solutions at physiological pH. Job plots indicate that with Cu^{II}, (HO)Gly[2]-enkephalin forms a 1:1 complex as does enkephalin itself, while β-(HO)Ala[2]-enkephalin forms a 2:1 complex (Figure.). The 1:1 complex formation coupled with an increase in absorbance for (HO)Gly[2]-enkephalin suggests that in this complex the position of the Cu^{II} is influenced by the presence of the hydroxamate group. The formation of a 1:2 complex in the case of the β-(HO)Ala[2]-enkephalin is explained in terms of complexation of the hydroxamate group, the increased size of the β-methylene unit giving the latter greater flexibility and allowing it to act as a powerful ligand.

In support of this view, in solutions of high pH, although absorbances are much smaller, visible spectral maxima appear at longer wavelengths for (HO)Gly[2]- and β-(HO)Ala[2]-enkephalins (560 and 600 nm), compared with that (520 nm) for the parent enkephalin.

Enzymatic Cleavage.—*N*-Hydroxyenkephalins were incubated with an enzyme[33] such as aminopeptidase M or carboxypeptidase Y in neutral aqueous solution; substrate disappearance was monitored by h.p.l.c. (Table 6). Enzymatic cleavage is responsible for the limited *in vivo* activity of enkephalins, and the cleavage is possible at each of the four peptide bonds.[34] The h.p.l.c. procedure detected any intact substrate remaining in the solution; a pseudo-first order rate was noted for most of the reactions. Although *N*-hydroxyenkephalins showed a resistance to degradation by aminopeptidases, they were smoothly digested by carboxypeptidase. Presumably, in the former the *N*-hydroxy amide bond resists

Table 3. Synthesis of *N*-hydroxyenkephalin analogues, H-Tyr-(HO)X-Gly-Phe-Leu-OH

X	Yield[a] (%)	M.p. (°C)	T.l.c. R_F[c]	$[\alpha]_D$[b] (°)	Formula	Found (Requires) C	H	N (%)
Gly	72	160—161	0.72	−7.7 (*c* 0.9)	$C_{28}H_{37}N_5O_8 \cdot H_2O$	57.0 (57.0)	6.5 (6.7)	11.9 (11.9)
β-Ala	48	166—167[d]	0.72	−11.4 (*c* 1.0)	$C_{29}H_{39}N_5O_8 \cdot H_2O$	57.6 (57.7)	6.7 (6.9)	11.6 (11.6)
Ala	44	164—165[d]	0.75	−13.0 (*c* 0.9)	$C_{29}H_{39}N_5O_8 \cdot H_2O$	58.0 (57.7)	6.7 (6.9)	11.6 (11.6)

[a] From deprotection and chromatography. [b] DMF. [c] BuOH–AcOH–water (4:1:2). [d] (decomp.).

Table 4. Proton chemical shifts, amide proton temperature coefficients, and coupling constants for *N*-hydroxyenkephalins[a]

Residue	Chemical shift (p.p.m.) NH	C(α)H	C(β)H	Others[b]	Coupling constants (Hz) $^3J_{NH-C(\alpha)H}$	$^3J_{C(\alpha)H-C(\beta)H}$	^2J	^2J	Others[b]
				H-Tyr-(HO)Gly-Gly-Phe-Leu-OH					
Tyr	*c*	4.31	3.06	6.66 (*o*) 7.02 (*m*)		4.4		15.7	9.7 (*o*, *m*)
(HO)Gly		4.07							
Gly	8.24 −7.4[d]	3.66			4.3, 5.6		15.4		
Phe	8.37 −7.2[d]	4.43	2.73	7.24	8.6	3.4, 2.1		*e*	
Leu	7.85 −1.2[d]	4.23	1.51	1.53 [C(γ)H] 0.86 (Me)	7.6	*e*		*e*	2.9 [C(γ)H-Me]
				H-Tyr-(HO)Ala-Gly-Phe-Leu-OH					
Tyr	*c*	4.23	2.67 2.80	6.60 (*o*) 7.03 (*m*)		6.4 7.0		12.0	8.5 (*o*, *m*)
(HO)Ala		4.72	1.30			7.6			
Gly	7.77 −4.6[d]	3.65			5.7, 6.3		19.4		
Phe	8.17 −4.8[d]	4.46	3.04	7.22	8.6	2.9		13.1	
Leu	7.91 −1.9[d]	4.04	1.52	1.55 [C(γ)H] 0.86 (Me)	6.9	7.2		*e*	5.4 [C(γ)H-Me]
				H-Tyr-β(HO)Ala-Gly-Phe-Leu-OH					
Tyr	*c*	4.20	2.72	6.66 (*o*) 6.99 (*m*)		4.4		*e*	8.0 (*o*, *m*)
β(HO)Ala		2.93	3.71			6.3			
Gly	8.26 −6.7[d]	3.60			5.1		16.0		
Phe	8.20 −5.7[d]	4.48	3.01	7.20	8.8	10.1		*e*	
Leu	7.87 −2.1[d]	4.08	1.53	1.56 [C(γ)H] 0.86 (Me)	8.0	7.3		*e*	5.6 [C(γ)H-Me]

[a] Determined in $(CD_3)_2SO$ at 23 °C. [b] (*o*) and (*m*) for the *ortho* and *meta* positions of the phenolic group. [c] Not observed. [d] Temperature coefficients (10^3 p.p.m. K^{-1}). [e] Indeterminable.

digestion whilst in the latter it is remote from the carboxy terminal. Under comparable conditions, neither enzyme cleaved H-Tyr-(HO)Gly-OH.* These results† suggest that *N*-hydroxy amide bonds are useful substitutes for peptide linkages.

* The observed slow degradation of (HO)Gly^2- and (HO)Ala^2-analogues by aminopeptidase M is supposed to be due to contaminating diaminopeptidases: commercial aminopeptidase M contained a small amount of such enzymes.[35]

† A preliminary analgesia test with mice through intracerebroventricular administration of enkephalins in dimethyl sulphoxide solution showed that the duration of an analgesic state was continued in the following order; (HO)Ala^2-analogue > Leu^5-enkephalin > (HO)Gly^2-analogue > β-(HO)Ala^2-analogue; the last of which had a small effect.

Experimental

M.p.s are uncorrected. I.r. spectra were obtained with a JASCO A-302 spectrophotometer and ^{1}H n.m.r. spectra were recorded on a JEOL JNM-FX 200 spectrometer with $SiMe_4$ as an internal standard. Optical rotations were measured with a JASCO ORD/UV-5 spectrometer. H.p.l.c. was carried out with a JASCO model Twincle apparatus using a column packed with Finepak SIL C_{18}. T.l.c. was carried out using Merck precoated silica gel $60F_{254}$ plates. Aminopeptidase M (L-6007) and carboxypeptidase Y (42F-8150) were obtained from Sigma Chem. and used as received.

*Benzyloxy-*L-α-*amino Acids and Their NCAs.*—The acids were prepared from *O*-benzylhydroxylamine and D-bromoalkanoic

Table 5. Dihedral angles (φ/°) consistent with $^3J_{NH-C(\alpha)H}$ for H-Tyr-(HO)X^2-Gly^3-Phe^4-Leu^5-OH in dimethyl sulphoxide[a]

Residue	(HO)Gly^2	(HO)Ala^2	β-(HO)Ala^2
Gly^3	88, 32,	70, 50,	66, 54,
	−170, −70,	−170, −70,	−173, −67
	−177, *−63*,	65, 55,	
	72, 48	−166, −74	
Phe^4	*−92*, −148	*−92*, −148	*−92*, −148
Leu^5	−82, −157	−78, −162	−84, −156

[a] 180° ≥ φ > −180°. Italicized figures correspond to a type I β-turn.

Table 6. Enzymatic degradation of *N*-hydroxyenkephalins

Substrate	Aminopeptidase M[a] k^c/min^{-1} (Relative)	Carboxypeptidase Y[b] k^c/min^{-1} (Relative)
Leu^5-enkephalin	6.1×10^{-2} (1.00)	2.1×10^{-3} (1.00)
(HO)Gly^2 derivative	3.9×10^{-3} (0.064)[d]	1.1×10^{-2} (5.2)
(HO)Ala^2 derivative	1.5×10^{-3} (0.041)[d]	8.3×10^{-3} (4.9)
β-(HO)Ala^2 derivative	No reaction	1.2×10^{-2} (5.7)

[a] Substrate (2.8×10^{-3} mmol) was incubated with the enzyme (15 μg) at 35 °C in phosphate buffer (2 ml) at pH 7.2. [b] Substrate (2.7×10^{-3} mmol) was incubated with the enzyme (21.5 μg) at 25 °C in phosphate buffer (2 ml) at pH 6.7. [c] Pseudo-first order rate constants. [d] The values were estimated at initial 5% conversion.

acids, and purified by repeated crystallization: H-(BzlO)Ala-OH, from EtOH–water, m.p. 118—119 °C (lit.,[25] 113—114 °C); $[\alpha]_D$ −42° (EtOH) (lit.,[25] −25°): H-(BzlO)Val-OH, from EtOH–water, m.p. 126—127 °C (lit.,[25] 117—118 °C); $[\alpha]_D$ −51° (EtOH) (lit.,[25] −22°). The NCAs were prepared by the reported procedure:[18] BzlO-L-Ala NCA, m.p. 94—95 °C; $[\alpha]_D$ +49°, and BzlO-Val NCA, m.p. 74—75 °C; $[\alpha]_D$ +14°.

H-(BzlO)X-Gly-Phe-Leu-OBzl (**4**): *General Procedure.*—Boc-Gly-Phe-Leu-OBzl (**2**) was obtained *via* Boc-Phe-Leu-OBzl (**1**). The tripeptide (**2**) was treated with HCl–dioxane to give HCl·H-Gly-Phe-Leu-OBzl (**3**).[36] The tripeptide (**3**) (1.63 g, 3.53 mmol) and Et_3N (0.36 g, 3.56 mmol) in $CHCl_3$ (20 ml) was mixed with the appropriate BzlO-NCA (3.53 mmol) or H-β(BzlO)Ala-ONp[26] in $CHCl_3$ (15 ml), and stirred for 12 h at 5 °C. The solution was washed with aqueous $NaHCO_3$ and water, and dried (Na_2SO_4). Evaporation of the solvent gave a residue, which was recrystallized from THF–Et_2O–light petroleum.

Cbz-Tyr(Bzl)-(BzlO)X-Gly-Phe-Leu-OBzl (**5**): *General Procedure.*—A mixture of Cbz-Tyr(Bzl)-OH (1.34 g, 3.3 mmol) and Et_3N (0.34 g, 3.3 mmol) in DMF (15 ml) was chilled (−17 °C). To this was added isobutyl chloroformate (0.41 g, 3 mmol) in DMF (5 ml) and the mixture was kept for 15 min. H-(BzlO)X-Gly-Phe-Leu-OBzl (**4**) (3 mmol) in DMF (15 ml) was added at −17 °C with stirring and the resulting mixture kept at −17 °C for 3 h, and then for 40 h in a refrigerator. The solvent was evaporated and the residue was extracted with AcOEt and the AcOEt layer was washed with aqueous $NaHCO_3$. When unchanged tetrapeptide was detected by t.l.c. in the extract, the peptide was further acylated by the above procedure. The AcOEt solution was washed with dilute hydrochloric acid and water, and then dried. The product was purified by column chromatography on Sephadex LH-20 eluting with MeOH and then on Toyopearl HW-40 eluting with DMF.

H-Tyr-(HO)X-Gly-Phe-Leu-OH: *General Procedure.*—The Cbz-Tyr(Bzl)-tetrapeptide benzyl ester was hydrogenated in MeOH or DMF at 30 °C for 6 h with H_2 in the presence of $Pd(AcO)_2$. The Pd was filtered off and the product was chromatographed on Sephadex LH-20 with MeOH as the eluant, and recrystallized from MeOH.

Copper(II) Complex Formation.—Aqueous sample solutions (3.0mM) and aqueous cupric nitrate solution (3.0mM) were prepared. The solutions were mixed at appropriate volume ratios and the pH of the resulting mixture was adjusted with aqueous KOH (0.01M). After 1 h, the spectrum of the solution was measured with a Hitachi 320 S spectrophotometer.

Enzymatic Degradation.—A sample of the enkephalin analogue (2.8 μmol) was incubated with aminopeptidase M (0.015 mg) in phosphate buffer (2 ml; pH 7.2) at 36 °C. At appropriate time intervals, 8 μl aliquots of the reaction mixture were analysed for the original peptide using h.p.l.c. with MeCN–water (65:35; v/v) containing H_3PO_4 (0.1% v/v) as the eluant. The peptide was detected at 265 nm. In the case of carboxypeptidase Y, the substrate (2.7 μmol) and the enzyme (0.022 mg) were incubated at 25 °C at pH 6.7.

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