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Highly Efficient and Enantiomer-Selective Hydrolysis of α-Amino Acid Active Ester Hydrochlorides by a Cyclic Hexapeptide Containing Histidines

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ABSTRACT: A cyclic hexapeptide, cyclo(D-Leu-L-Glu-L-His)$_2$ was synthesized and its catalytic activity for the hydrolysis of various carboxylic acid *p*-nitrophenyl esters was investigated. Cyclo(D-Leu-L-Glu-L-His)$_2$ was 3 to 20 times as effective as imidazole as a catalyst for the hydrolysis of $CH_3(CH_2)_8COOPh(NO_2)$ and $CH_3(CH_2)_{10}COOPh(NO_2)$ in 20% dioxane/H_2O mixture at pH 7.8 (phosphate buffer). Cyclo(D-Leu-L-Glu-L-His)$_2$ showed neither higher catalytic activity than imidazole nor enantiomer-selective catalysis in the hydrolysis of Val-OPh$(NO_2)\cdot$HCl and Leu-OPh$(NO_2)\cdot$HCl in aqueous solution at pH 6.95 (phosphate buffer). When $Cu(ClO_4)_2$ was added to an aqueous solution of cyclo(D-Leu-L-Glu-L-His)$_2$ at pH 6.05 (phosphate buffer), a 40—150 time increase in the second-order rate constant of both the above α-amino acid active ester hydrochlorides was observed and the L-enantiomers were hydrolyzed slightly faster than the D-enantiomers. The addition of $Cu(ClO_4)_2$ to an aqueous solution containing the cyclic hexapeptide at pH 6.01 (citrate buffer) did not lead to peptide-metal ion interaction and an enhanced rate of enantiomer-selective hydrolysis. It was made clear that cyclo(D-Leu-L-Glu-L-His)$_2$ is a highly efficient and weakly enantiomer-selective catalyst in the hydrolysis of α-amino acid active ester hydrochlorides only when it forms a complex with copper ion.

KEY WORDS Cyclic Hexapeptide / Hydrolytic Catalyst / Hydrophobic Interaction / Enantiomer-Selective Catalysis / Cu^{2+}–Peptide Complex /

We investigated the construction of an efficient and specific hydrolytic catalyst by controlling the spatial arrangement, that is, the intramolecular cooperation, of functional groups by incorporating them into the side chains of a relatively rigid cyclic peptide.

Cyclic dipeptides consisting of α-amino acid residues having hydrophobic and nucleophilic side chains have been found efficient catalysts for the hydrolyses of *p*-nitrophenyl esters of long-chain carboxylic acids[1,2] and *p*-nitrophenyl ester hydrochlorides of hydrophobic α-amino acids.[3] Cyclic dipeptides having the D-L configuration were more efficient as hydrolytic catalysts than imidazole and diastereomeric dipeptides having the L-L configuration. These findings have been discussed in terms of the effect of the spatial arrangement of functional groups on the hydrophobic binding of a substrate and intramolecular nucleophilic or general-base catalysis.[3,4]

Cyclic dipeptides consisting of α-amino acid residues having anionic and nucleophilic side chains have been found efficient catalysts for the hydrolyses of positively charged carboxylic acid esters and *p*-nitrophenyl ester hydrochlorides of α-amino acids.[5] Cyclic dipeptides having the L-L configuration were more efficient as hydrolytic catalysts than imidazole which is more basic and the diastereomeric cyclic dipeptides having the D-L configuration. These findings have been discussed in terms of the effect of the spatial arrangement of functional groups on the electrostatic binding of a substrate and an intramolecular nucleophilic or a general-base catalysis.[5]

A series of cyclic dipeptide catalysts have been found efficient as catalysts for the hydrolysis of α-amino acid active ester hydrochlorides under suitable conditions, but an enantiomer-selective catalysis

has yet to be discovered. As one possible way for obtaining an enantiomer-selective catalysis, the introduction of an additional steric barrier into cyclic dipeptide was considered and tripeptides having cyclic dipeptide backbone were constructed.[6] These tripeptide catalysts were enantiomer-selective in the hydrolysis of *p*-nitrophenyl leucinate hydrochloride, and the selectivity for the D-ester was induced by a conformational change of the tripeptide catalysts on binding of the D-ester resulting in enhanced catalysis.[7]

In the present investigation, as an alternative means for constructing an enantiomer-selective catalyst, the ring expansion of cyclic peptide catalysts was considered. A cyclic hexapeptide consisting of hydrophobic and anionic α-amino acids as well as nucleophilic histidine was synthesized. Using this cyclic hexapeptide as a hydrolytic catalyst for long-chain carboxylic acid esters and α-amino acid active ester hydrochlorides, the stereochemistry participating in substrate binding and intramolecular cooperative catalysis was investigated.

EXPERIMENTAL

Catalyst

The cyclic hexapeptide used in the present investigation was cyclo(D-Leu-L-Glu-L-His)$_2$. In this cyclic hexapeptide, hydrophobic α-amino acid residues (Leu) and nucleophilic α-amino acid residues (His) are in a D-L sequence which has been found effective for the hydrolysis of hydrophobic substrate.[1,2] Anionic α-amino acid residues (Glu) and nucleophilic α-amino acid residues (His) are in an L-L sequence which has been found effective for the hydrolysis of cationic substrate.[5] The synthesis of the cyclic hexapeptide is illustrated in Figure 1.

N-t-Butyloxycarbonyl-γ-benzyl-L-glutamic Acid Succinimide Ester [*Boc-L-Glu(OBzl)-OSu*] (**I**). This was synthesized from L-glutamic acid according to the literature.[8] Yield *ca.* 30%; mp 102°C [lit.[8] 101°C].

L-Histidine Methyl Ester Dihydrochloride (L-His-OMe·2HCl) (**II**). This was synthesized from L-histidine according to the literature.[9] Yield *ca.* 85%; mp 203°C [lit.[9] 200—201°C].

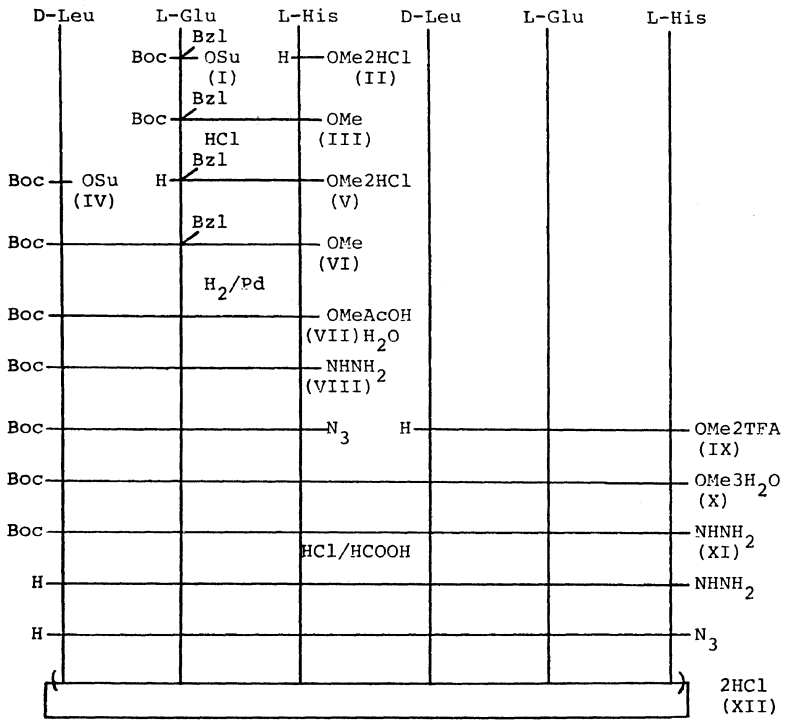

Figure 1. Synthetic route to cyclo(D-Leu-L-Glu-L-His)$_2$.

N-t-Butyloxycarbonyl-γ-benzyl-L-glutamyl-L-histidine Methyl Ester [*Boc-L-Glu(OBzl)-L-His-OMe*] (**III**). Et_3N (5.4 cm^3, 0.04 mol) and **I** (8.70 g, 0.02 mol) were added to a solution of **II** (4.84 g, 0.02 mol) in CH_2Cl_2 at 0°C. After standing overnight at room temperature, the solution was evaporated to dryness. AcOEt was added to the residue and the insoluble part (mainly $Et_3N^+HCl^-$) was filtered off. The AcOEt solution was washed with water, 0.5 mol dm^{-3} aqueous $NaHCO_3$ solution, 0.3 mol dm^{-3} aqueous citric acid solution, and saturated aqueous solution of NaCl. The AcOEt solution was dried over anhydrous Na_2SO_4 and AcOEt was distilled off. To the residual oil, Et_2O was added and the solidified product was recrystallized from EtOH/AcOEt/Et_2O. Yield 7.07 g (72%); mp 69°C.

N-t-Butyloxycarbonyl-D-leucine Succinimide Ester (Boc-D-Leu-OSu) (**IV**). This was synthesized from D-leucine according to the literature[10] which describes the synthesis of Boc-L-Leu-OSu. Yield 72%; mp 111°C [lit.[10] 116°C].

γ-Benzyl-L-glutamyl-L-histidine Methyl Ester Dihydrochloride [*L-Glu(OBzl)-L-His-OMe·2HCl*] (**V**). A solution of **III** (7.07 g, 14.5 mmol) in 4.7 mol dm^{-3} HCl/dioxane (50 cm^3) was allowed to stand for 30 minutes at room temperature, and then the solvent was distilled off. Et_2O (100 cm^3) was added to the residue and the mixture was left overnight at −15°C to solidify the product. The solid obtained was very hygroscopic. Yield 5.96 g (89%).

N-t-Butyloxycarbonyl-D-leucyl-γ-benzyl-L-glutamyl-L-histidine Methyl Ester [*Boc-D-Leu-L-Glu(OBzl)-L-His-OMe*] (**VI**). This was synthesized from **IV** (4.67 g, 12.9 mmol) and **V** (5.96 g, 12.9 mmol) as described for the synthesis of **III**. The product was obtained as an oil. Yield 9.56 g (68%).

N-t-Butyloxycarbonyl-D-leucyl-L-glutamyl-L-histidine Methyl Ester Monoacetate Hydrate (Boc-D-Leu-L-Glu-L-His-OMe·AcOH·H_2O) (**VII**). A solution of **VI** (9.56 g, 8.77 mmol) in 10% AcOH/MeOH (50 cm^3) was hydrogenated with Pd-black as a catalyst. After 12 hours, the solution was filtered and the filtrate was evaporated to dryness. AcOEt was added to the oily residue, and the mixture was kept at −15°C to yield a hygroscopic crystal. It was recrystalized from Me_2CHOH/AcOEt. Yield 4.45 g (86%); mp 112—113°C.

Anal. Calcd for $C_{23}H_{37}N_5O_8 \cdot CH_3COOH \cdot H_2O$: C, 50.92%; H, 7.35%; N, 11.88%. Found: C, 50.33%; H, 7.32%; N, 11.82%.

N-t-Butyloxycarbonyl-D-leucyl-L-glutamyl-L-histidine Hydrazide (Boc-D-Leu-L-Glu-L-His-$NHNH_2$) (**VIII**). A solution of **VII** (1.38 g, 2.34 mmol) and 24% hydrazine hydrate (12 cm^3) in MeOH (30 cm^3) was made to stand at room temperature for 3 days. The solution was then evaporated to dryness, and the residue was washed with Et_2O and solidified. Yield 1.1 g (92%).

D-Leucyl-L-glutamyl-L-histidine Methyl Ester Ditrifluoroacetate (D-Leu-L-Glu-L-His-OMe·$2CF_3CO_2H$) (**IX**). A solution of **VII** (1.21 g, 2.05 mmol) in trifluoroacetic acid (3 cm^3) was stirred for one hour at room temperature, and then evaporated to dryness. The residue was solidified by the addition of Et_2O. Since the product was very hygroscopic, elemental analysis was not performed. Deblocking of *t*-butyloxycarbonyl group was confirmed by infrared spectroscopy. All the Et_2O-insoluble materials were dissolved in $HCONMe_2$ (8 cm^3) and subjected to a condensation reaction with **VIII**.

N-t-Butyloxycarbonyl-(D-leucyl-L-glutamyl-L-histidyl)$_2$ Methyl Ester Trihydrate [*Boc-(D-Leu-L-Glu-L-His)$_2$-OMe·$3H_2O$*] (**X**). A solution of **VIII** (1.1 g, 2.15 mmol) in $HCONMe_2$ (8 cm^3) was mixed with concentrated hydrochloric acid (1.92 cm^3) and 2 *N* $NaNO_2$ (1.1 cm^3) with stirring at −20°C. After 30 minutes, the solution was cooled to −30°C, and *N*-ethylpiperidine (5.0 cm^3, 36.4 mmol) and a solution of **IX** in $HCONMe_2$ were added. The solution was kept at −5°C for 3 days, and then the solvent was distilled off. To the residue, Me_2CO and EtOH were added and the insoluble *N*-ethylpiperidine hydrochloride was filtered off. The filtrate was evaporated to dryness, and an aqueous solution of the residue was eluted for purification through a Sephadex G-15 column (3.2 × 60 cm). The initial fraction was collected and freeze-dried. Yield 606 mg (31%); mp 138—140°C.

Anal. Calcd for $C_{40}H_{62}N_{10}O_{13} \cdot 3H_2O$: C, 50.84%; H, 7.25%; N, 14.82%. Found: C, 51.11%, H, 7.09%, N, 14.45%.

N-t-Butyloxycarbonyl-(D-leucyl-L-glutamyl-L-histidyl)$_2$ Hydrazide [*Boc-(D-Leu-L-Glu-L-His)$_2$-$NHNH_2$*] (**XI**). In a manner similar to the synthesis of **VIII**, **X** (4.28 mg, 0.45 mmol) was treated with hydrazine hydrate (5 cm^3). The product was solidified by adding Me_2CO. Yield 361 mg (90%).

Cyclo(D-leucyl-L-glutamyl-L-histidyl)$_2$ Dihydro-

chloride 3.5 Hydrate [*cyclo(*D-*Leu*-L-*Glu*-L-*His*)$_2$·2HCl·3.5H$_2$O*] (**XII**). A solution of **XI** (361 mg, 0.405 mmol) in 0.1 mol dm^{-3} HCl/HCOOH was stirred for one hour at room temperature. The solvent was distilled off and the residue was washed with Et_2O. The product was dissolved in $HCONMe_2$ (20 cm^3) and the solution was stirred at −20°C. To the solution, AcOH (1 cm^3), concentrated hydrochloric acid (0.2 cm^3), and 2 mol dm^{-3} aqueous $NaNO_2$ solution (2 cm^3) were added. After 15 minutes, the solution was poured into pyridine (200 cm^3) at −5°C. After being kept at −5°C for 2 days, the solution was evaporated to dryness, and the reaction product was eluted for purification through a column as in the case of **X**. The product was recrystallized from Me_2CO/H_2O. Yield 84 mg (23%); mp 196—200°C. The R_f of the material subjected to TLC in *n*-butanol/acetic acid/water/pyridine (15 : 10 : 12 : 13) was 0.52.

Anal. Calcd for $C_{34}H_{50}N_{10}O_{10}\cdot 2HCl\cdot 3.5H_2O$: C, 45.64%; H, 6.42%; N, 15.65%. Found: C, 45.65%; H, 6.48%; N, 15.79%.

A protected linear tripeptide **VII** was used as the catalyst for a comparison with the cyclic hexapeptide.

As a standard nucleophile, imidazole was used and compared with the peptide catalysts synthesized. Commercial imidazole was recrystallized from benzene.

Substrates

As neutral *p*-nitrophenyl esters of carboxylic acids, $CH_3COOPh(NO_2)$,[1] $CH_3(CH_2)_8COOPh(NO_2)$,[2] and $CH_3(CH_2)_{10}COOPh(NO_2)$[1] were used. Syntheses and purifications of these materials have already been described.

As *p*-nitrophenyl ester hydrochlorides of α- and ω-aminocarboxylic acid, which carry a positive charge at the end of the acyl chain, Gly-$OPh(NO_2)\cdot HCl$ and $Cl^-H_3N^+(CH_2)_{11}COOPh(NO_2)$ were used. Syntheses and purifications of these materials have been reported.[3]

As *p*-nitrophenyl ester hydrochlorides of chiral α-amino acid, D- and L-Val-$OPh(NO_2)\cdot HCl$ and D- and L-Leu-$OPh(NO_2)\cdot HCl$ were used. Syntheses and purifications of these materials have been reported.[3]

Hydrolytic Reaction

A solution of neutral *p*-nitrophenyl ester of carboxylic acid in dioxane was added to a mixture of an aqueous solution of catalyst and a buffer solution to start the hydrolysis.

A stock solution of $Cl^-H_3N^+(CH_2)_{11}COOPh(NO_2)$ in 0.01 mol dm^{-3} hydrochloric acid containing a small amount of MeOH was mixed with an aqueous solution of catalyst and a buffer solution to start the hydrolysis.

A stock solution of α-amino acid *p*-nitrophenyl ester in 0.01 mol dm^{-3} hydrochloric acid was mixed with an aqueous solution of catalyst and a buffer solution to start the hydrolysis.

The concentration of phenolate ion liberated during the hydrolytic reaction was determined by the optical density of 400 nm absorption. This determination was carried out using a JASCO UVIDEC-1 spectrophotometer. Optical cells having path lengths of 5 cm and 1 cm were used, respectively, when the substrate concentrations were 6.0×10^{-6} mol dm^{-3} and higher than 3.0×10^{-5} mol dm^{-3}. The second-order rate constant k_{cat} was determined as reported previously.[1]

Determination of pK_a of Catalyst

270 MHz ^{1}H NMR spectra of D_2O solution of cyclo(D-Leu-L-Glu-L-His)$_2$ were measured at 23°C with Bruker WH270 spectrometer using $Me_3Si(CH_2)_3SO_3Na$ as an internal standard. pK_a of the Glu-COOH group was determined from the chemical shift change in the Glu-C$^\gamma$H$_2$ signal with pD. pK_a of the His-imidazolyl-group was determined from the chemical shift change in the imidazolyl-C^{2}H signal with pD.

RESULTS AND DISCUSSION

Determination of the pK_a of Cyclic Hexapeptide

From the pD dependence of the 270 MHz ^{1}H NMR spectrum of a D_2O solution of cyclo(D-Leu-L-Glu-L-His)$_2$, pK_a of Glu-COOH was determined to be 4.30. pK_a of the γ-COOH group of glutamic acid is-4.07 and that of Glu-COOH in cyclo(L-Glu-L-His) has been reported to be 4.4.[5] These values are not particularly different from each other and therefore indicate the absence of specific interactions of carboxyl groups in cyclo(D-Leu-L-Glu-L-His)$_2$.

As will be reported in the next paper,[11] cyclo(D-Leu-L-Glu-L-His)$_2$ takes several conformations in D_2O and four signals of imidazolyl-C^{2}H appear. From the pD dependence of each signal, the pK_a

values of the His-imidazoyl groups involved in major conformations were determined to be in the range of 6.5—6.85 and those involved in minor conformations in the range of 7.3—7.5. Allowing for pK_a of the His-imidazolyl group in cyclo(L-Glu-L-His) being 6.6,[5] those for minor conformations are much higher than usual. In the minor conformations, there may possibly be some side chain interactions or conformational change at about pD 6.5 causing an increase in the pK_a of the His-imidazoyl group.

The CD spectra of aqueous solution of cyclo(D-Leu-L-Glu-L-His)$_2$ were measured at pH 4.0, 7.0, and 10.0, but no serious change in the spectrum was observed. This indicates that no change in the major conformation took place during the pH titration.

Hydrolysis of Neutral p-Nitrophenyl Esters of Carboxylic Acids

Since hydrophobic Leu and nucleophilic His residues take a D-L sequence in cyclo(D-Leu-L-Glu-L-His)$_2$, the cyclic hexapeptide should be an efficient catalyst for the hydrolysis of hydrophobic carboxylic acid esters.[1,2] Hydrolyses of a series of aliphatic carboxylic acid *p*-nitrophenyl esters, $CH_3(CH_2)_{n-2}COOPh(NO_2)$ ($n=2$, 10, 12), were carried out in a 20% dioxane/H_2O mixture, and the results are shown in Table I. To avoid any possible association of $CH_3(CH_2)_8COOPh(NO_2)$ and $CH_3(CH_2)_{10}COOPh(NO_2)$ in this solvent,[2] the initial substrate concentration was kept as low as 6.0×10^{-6} mol dm^{-3}. In the hydrolyses with imidazole as the catalyst, with increasing n from 2 to 10 and finally to 12, the steric hindrance in the nucleophilic catalysis increased and consequently k_{cat} decreased. However, in the hydrolyses with the linear tripeptide as the catalyst, with increasing chain length of substrate, k_{cat} increased slightly. This slight acceleration is due to an intramolecular cooperative catalysis in which a nucleophilic attack by the His-imidazolyl group occurs on long-chain substrates having $n=10$ and 12 and bound with a Leu residue of the linear tripeptide by hydrophobic interaction. In hydrolysis with the cyclic hexapeptide, the reaction rates of substrates having $n=10$ and 12 increased very much. For example, in the hydrolysis of $CH_3(CH_2)_{10}COOPh(NO_2)$ k_{cat} with the cyclic hexapeptide was about 20 times as large as k_{cat} with imidazole. The cyclic hexapeptide should be more hydrophobic and bind to the substrate more strongly than the linear tripeptide.

Table I. Second-order rate constant k_{cat} (dm^3 mol^{-1} min^{-1}) for the hydrolysis of $CH_3(CH_2)_{n-2}COOPh(NO_2)$[a]

Catalyst	$n=2$[b]	$n=10$[c]	$n=12$[c]
None ($k_w\times10^3$, min^{-1})	0.92	0.89	0.24
Imidazole	28	15	1.8
Boc-D-Leu-L-Glu-L-His-OMe	6.2	7.9	9.5
Cyclo(D-Leu-L-Glu-L-His)$_2$	5.8	46	34

[a] In 20% dioxane/H_2O mixture at 25°C and pH 7.8 (KH_2PO_4/NaOH buffer).
[b] In 3% dioxane/H_2O mixture, $[Substrate]_0=3.0\times10^{-5}$ mol dm^{-3}.
[c] $[Substrate]_0=6.0\times10^{-6}$ mol dm^{-3}.

To investigate the contribution of hydrophobic interaction to the hydrolysis of $CH_3(CH_2)_{10}COOPh(NO_2)$, hydrolytic reactions were carried out in media of various water/organic solvent compositions. Under the conditions of the initial substrate concentration being 3.0×10^{-5} mol dm^{-3}, pH 7.8, and the temperature 25°C, k_{cat} values by the cyclic hexapeptide and linear tripeptide were nearly zero either in a 33.3% EtOH/16.7% dioxane/H_2O mixture or in a 50% dioxane/H_2O mixture. It is difficult to explain the change in reactivity in terms of solvent-induced conformational change in the cyclic hexapeptide, since the main-chain conformation of the cyclic hexapeptide without *N*-substituted α-amino acid residues should hardly be affected by solvent. Therefore, the above findings indicate that the cyclic hexapeptide is an efficient catalyst only when powerful hydrophobic interaction occurs between the substrate and catalyst in water-rich solvents.

Since two Glu residues and two His residues are present in the cyclic hexapeptide, the latter could be a multifunctional catalyst by intramolecular cooperation of these functional groups. Hydrolyses of $CH_3COOPh(NO_2)$ with the cyclic hexapeptide were carried out in a 20% dioxane/H_2O mixture and the pH dependence of k_{cat} was examined in a pH range from 4 to 8. In actuality, the reactions were carried out at pH 4.03 (citrate/HCl/NaOH buffer), pH 4.97 and 6.01 (citrate/NaOH buffer), and pH 6.95 and 7.81 (KH_2PO_4/NaOH buffer). The results are shown in Figure 2, in which the filled circles represent the observed values and the real line the

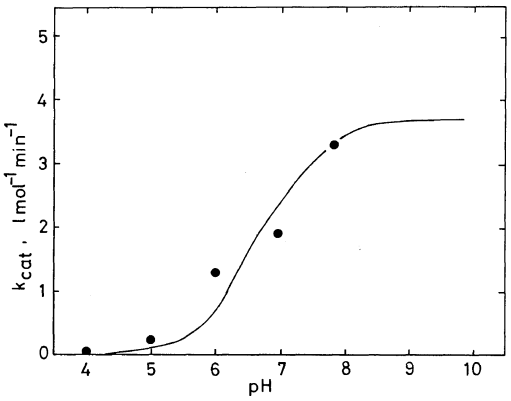

Figure 2. k_{cat} *versus* pH plot in the hydrolysis of *p*-nitrophenyl acetate with cyclo(D-Leu-L-Glu-L-His)$_2$ in 20% dioxane/H_2O mixture at 25°C: ●, observed value; ——, calculated value (see the text for details).

Table II. Second-order rate constant k_{cat} for the hydrolysis of $Cl^-H_3N^+(CH_2)_nCOOPh(NO_2)$

Catalyst	$n=1$[a]	$n=11$[b]
	$dm^3\,mol^{-1}\,s^{-1}$	$dm^3\,mol^{-1}\,min^{-1}$
Imidazole	3	13
Boc-D-Leu-L-Glu-L-His-OMe	0.55	4.7
Cyclo(D-Leu-L-Glu-L-His)$_2$	0.49	5.1

[a] In aqueous solution at 25°C and pH 6.95 (KH_2PO_4/NaOH buffer), $[Substrate]_0=3.0\times10^{-5}$ mol dm^{-3}, $k_w=5.60\times10^{-3}\,s^{-1}$.
[b] In 1% MeOH/H_2O mixture at 25°C and pH 7.8 (KH_2PO_4/NaOH buffer), $[Substrate]_0=3.0\times10^{-5}$ mol dm^{-3}, $k_w=0.98\times10^{-3}\,min^{-1}$.

calculated values. The calculation was made with a standard k_{cat} value of 3.30 $dm^3\,mol^{-1}\,min^{-1}$ at pH 7.81 going on the assumption that k_{cat} is linearly proportional to the concentration of neutral imidazolyl functions. The agreement between the observed and calculated values in Figure 2 indicates the single catalysis of the imidazolyl function and the absence of an intramolecular interaction of the imidazolyl group with another imidazolyl group or carboxyl groups.

Hydrolysis of Carboxylic Acid p-Nitrophenyl Esters Having a Positive Charge at the End of the Acyl Chain

Since anionic Glu residues and nucleophilic His residues take an L-L sequence in cyclo(D-Leu-L-Glu-L-His)$_2$, the cyclic hexapeptide should be an efficient catalyst for the hydrolysis of cationic carboxylic acid esters.[5] α- and ω-Aminocarboxylic acid active ester hydrochlorides having a positive charge at the end of acyl chain, $Cl^-H_3N^+(CH_2)_nCOOPh(NO_2)$ ($n=1$ and 11), were hydrolyzed and the results are shown in Table II.

In the hydrolysis of Gly-$OPh(NO_2)\cdot HCl$ or $Cl^-H_3N^+(CH_2)_{11}COOPh(NO_2)$, neither the linear tripeptide nor cyclic hexapeptide exceeded the catalytic activity of imidazole. In water-rich media, the cyclic hexapeptide seems to fail in binding with the substrate by electrostatic interactions. Furthermore, hydrophobic interactions between $Cl^-H_3N^+(CH_2)_{11}COOPh(NO_2)$ and the cyclic hexapeptide may have been weakened by a hydration shell about the charged substrate.[3]

Hydrolysis of α-Amino Acid p-Nitrophenyl Ester Hydrochlorides

Since active ester hydrochlorides of valine and leucine are hydrophobic and cationically charged, they are expected to be hydrolyzed efficiently with the cyclic hexapeptide which is hydrophobic and anionically charged. Furthermore, an enantiomer-selective catalysis ought to occur, provided that a stereochemical fit of the cyclic hexapeptide with a specific optical enantiomer is achieved. With this in mind, the hydrolyses of Val-$OPh(NO_2)\cdot HCl$ and Leu-$OPh(NO_2)\cdot HCl$ were investigated. The experimental results obtained in aqueous solution at pH 6.95 (phosphate buffer) are shown in Table III. Under these conditions, neither the linear tripeptide nor cyclic hexapeptide exceeded the catalytic activity of imidazole, and enantiomer-selective hydrolysis did not take place.

Similar reactions were carried out in aqueous solution at pH 6.01 (citrate buffer) with the addition of $Cu(ClO_4)_2$. The experimental results are shown in Table IV. Under the present conditions the apparent catalytic activity of the tripeptide slightly increased and was more than that of imidazole. The reason for the increased reactivity of the linear tripeptide with the addition of $Cu(ClO_4)_2$ should be sought. However, no definite comments in regard to this can be made since the k_{cat} values shown in

Table III. Second-order rate constant k_{cat} ($dm^3\,mol^{-1}\,s^{-1}$) for the hydrolysis of α-amino acid *p*-nitrophenyl ester hydrochloride[a]

Catalyst	Val-OPh(NO_2)·HCl		Leu-OPh(NO_2)·HCl	
	L	D	L	D
None ($k_w \times 10^3$, s^{-1})	3.26	3.31	4.46	4.70
Imidazole	1.2	1.2	2.1	2.0
Boc-D-Leu-L-Glu-L-His-OMe	0.63	0.76	0.93	0.82
Cyclo(D-Leu-L-Glu-L-His)$_2$	0.13	0.26	0.40	0.36

[a] In aqueous solution at 25°C and pH 6.95 (KH_2PO_4/NaOH buffer), $[Substrate]_0 = 6.0 \times 10^{-5}$ mol dm^{-3}.

Table IV. Second-order rate constant k_{cat} ($dm^3\,mol^{-1}\,s^{-1}$) for the hydrolysis of α-amino acid *p*-nitrophenyl ester hydrochloride[a]

Catalyst	Val-OPh(NO_2)·HCl		Leu-OPh(NO_2)·HCl	
	L	D	L	D
None ($k_w \times 10^3$, s^{-1})	0.983	1.07	2.84	2.79
Imidazole	0.8	0.5	1.1	1.1
Boc-D-Leu-L-Glu-L-His-OMe	2.0	1.4	2.3	2.0
Cyclo(D-Leu-L-Glu-L-His)$_2$	0.3	0.3	0.8	0.3

[a] In aqueous solution at 25°C and pH 6.01 (citrate buffer), $[Substrate]_0 = 6.0 \times 10^{-5}$ mol dm^{-3}, $[Cu(ClO_4)_2] = 7.0 \times 10^{-4}$ mol dm^{-3}.

Table V. Spontaneous rate constant k_w', pseudo-first-order rate constant k_1, and second-order rate constant k_{cat} for the hydrolysis of α-amino acid *p*-nitrophenyl ester hydrochloride[a]

	Val-OPh(NO_2)·HCl		Leu-OPh(NO_2)·HCl	
	L	D	L	D
$k_w' \times 10^3$ (s^{-1})[b]	1.27	1.51	1.66	1.70
$k_1 \times 10^3$ (s^{-1})[c]	6.31	5.80	27.9	24.4
k_{cat} ($dm^3\,mol^{-1}\,s^{-1}$)[c]	11.6	9.9	60.6	52.4

[a] In aqueous solution at 25°C and pH 6.04 (KH_2PO_4/NaOH buffer), $[Substrate]_0 = 6.0 \times 10^{-5}$ mol dm^{-3}.
[b] $[Cu(ClO_4)_2] = 0$, $[Cyclo(D\text{-}Leu\text{-}L\text{-}Glu\text{-}L\text{-}His)_2] = 0$.
[c] $[Cu(ClO_4)_2] = 7.8 \times 10^{-4}$ mol dm^{-3}, $[Cyclo(D\text{-}Leu\text{-}L\text{-}Glu\text{-}L\text{-}His)_2] = 4.33 \times 10^{-4}$ mol dm^{-3}.

Table IV have not been corrected for effective concentrations of imidazolyl functions. The cyclic hexapeptide was inferior to imidazole under the present conditions. Again, an enantiomer-selective hydrolysis of Val-OPh(NO_2)·HCl or Leu-OPh(NO_2)·HCl was not possible with either of the peptide catalysts.

Added copper ions may interact more strongly with citrate ions as a buffer reagent than the cyclic hexapeptide. Therefore, a weakly interacting phosphate buffer was used in the hydrolysis in the presence of copper ions. Hydrolytic reactions were carried out in aquous solution at pH 6.04 (KH_2PO_4/NaOH buffer) with the addition of $Cu(ClO_4)_2$. The experimental results are shown in Table V. k_w in the presence of $Cu(ClO_4)_2$ (7.85×10^{-4} mol dm^{-3}) and

the absence of cyclic hexapeptide was necessary to determine k_{cat}. However, at pH 6.04 in a phosphate-buffered solution, $Cu(OH)_2$ was precipitated without the cyclic hexapeptide and consequently determination of k_w was impossible. We could determine only k_w', the rate constant for the spontaneous hydrolysis under the absence of copper ions. However, under our conditions the catalysis by copper ions seems not to occur, since the spontaneous hydrolysis of α-amino acid ester (k_w in Table III) was not much different from that in the presence of Cu^{2+} (k_w in Table IV), allowing for pH difference. Therefore, apparent k_{cat} values were calculted from k_1 and k_w' instead of k_w. The comparison of the apparent k_{cat} with those in Tables III and IV shows a remarkable increase (up to 150-fold increase) of k_{cat} as a result of the addition of Cu^{2+} to the phosphate-buffered solution containing the cyclic hexapeptide. In Tables III and IV, it is also seen that Leu-OPh(NO_2)·HCl is at most twice as reactive as Val-OPh(NO_2)·HCl with any hydrolytic catalyst. However, it is shown in Table V that Leu-OPh(NO_2)·HCl is almost five times as reactive as Val-OPh(NO_2)·HCl with the cyclic hexapeptide plus copper ion. These two points indicate that the hydrophobic interaction between the cyclic hexapeptide and the substrate is much more important when copper ions are present than when copper ions are absent. This may be closely related to the conformational change in cyclic hexapeptide induced by copper ions which will be dealt with in the next paper.[11]

In Table V, the apparent k_{cat} values of L-Val-OPh(NO_2)·HCl and L-Leu-OPh(NO_2)·HCl are slightly larger than those of the D-enantiomers. Since the catalytic hydrolysis was fast enough to determine the k_1 values accurately, we believe that the difference in the k_{cat} values of the L- and D-enantiomers is definitely more than the experimental error. With reference to the L-enantiomer selectivity of the cyclic hexapeptide in the presence of copper ions, catalytic hydrolysis by the linear tripeptide under similar conditions should be investigated. However, reproducible rate constants were not obtained in the pH range 6.0—7.4. In the pH range 6.0—7.0, the precipitation of $Cu(OH)_2$ seriously affected the determination of the reaction rate. In a pH range above 7, the precipitation of $Cu(OH)_2$ was less marked but hydrolysis was too fast to be determined accurately by the present method. The order of mixing the buffer solution, the metal salt and the tripeptide has been variously changed, but with no improvement.

In the presence of cyclo(D-Leu-L-Glu-L-His)$_2$ $Cu(OH)_2$ did not precipitate from the phosphate-buffered solution, so copper ions must have complexed with the cyclic hexapeptide. The interaction of cyclo(D-Leu-L-Glu-L-His)$_2$ with copper ions in a phosphate-buffered solution has been verified by circular dichroism spectroscopy and will be described in the next paper.[11] The precipitation of $Cu(OH)_2$ in a phosphate-buffered solution containing Boc-D-Leu-L-Glu-L-His-OMe indicates that no interaction occurred. In fact, the circular dichroism spectra of the linear tripeptide in phosphate-buffered solution at pH 6.0, 7.0 and 7.8 did not change at all by the addition of $Cu(ClO_4)_2$. Therefore, the enormous rate increase and slight selectivity of L-enantiomers in the hydrolysis of Val-OPh(NO_2)·HCl and Leu-OPh(NO_2)·HCl must have come about through the cyclo(D-Leu-L-Glu-L-His)$_2$–Cu^{2+} complex.

Effects of Metal Ions

A remarkable increase in reaction rate by the addition of metal ions has been observed in the hydrolysis of 8-acetoxyquinoline-2-carboxylic acid[12] and in the aminolysis of penicillin.[13] In these examples, a metal ion coordinates to the substrate and activates it for reaction. However, under our conditions, we found no evidence for the interaction of α-amino acid ester hydrochloride with metal ions.

As stated in the previous section, a copper ion is considered to be bound to the cyclic hexapeptide in phosphate-buffered solution. Coordination of metal ions to a multifunctional catalyst and activation of nearby catalytic groups have been reported.[14-16] Under these conditions, orientation of the substrate seems necessary for acceleration of the reaction. However, no experimental evidences for alignment of functional groups, metal ion, and substrate are presented in these examples. In our cyclic hexapeptide, the two carboxyl groups must be ligand groups bound to a copper ion.

The present cyclic hexapeptide was an efficient catalyst for the hydrolysis of *p*-nitrophenyl esters of neutral, long-chain carboxylic acid, but not very efficient for that of cationically charged, long-chain carboxylic acid. A similar trend was observed with cyclic dipeptide catalysts.[3] Overberger and

Podsiadley[17] reported that copolymers of 5(6)-vinylbenzimidazole and acrylic acid accelerated the solvolysis of cationically charged, long-chain carboxylic acid esters because of the contribution of apolar interactions. It was therefore considered[3] that a hydrophobic domain should be formed about the catalytic site to ensure the occurrence of hydrophobic interactions between a cationically charged substrate and catalyst. The highly efficient catalysis by the cyclo(D-Leu-L-Glu-L-His)$_2$–Cu^{2+} complex in the hydrolysis of cationically charged α-amino acid ester hydrochlorides should, therefore, indicate the formation of a hydrophobic domain by coordination of copper ion. Further investigation is necessary to confirm this consideration, whether the cyclic hexapeptide is an efficient catalyst for $CH_3(CH_2)_{10}COOPh(NO_2)$, $CH_3(CH_2)_8COOPh(NO_2)$, and $Cl^-H_3N^+(CH_2)_{11}$-$COOPh(NO_2)$ in the presence of copper ions.

The cyclo(D-Leu-L-Glu-L-His)$_2$–Cu^{2+} complex may be the first example showing that a cyclic peptide is an enantiomer-selective catalyst of ester hydrolysis, although the difference in the reaction rates of the L- and D-enantiomers was not very large. The enantiomer-selective hydrolysis of phenylalanine esters with poly(L-lysine)–Cu^{2+} complex has been reported by Hatano, *et al.*[18,19] In these examples, the copper ion is a catalyst and the polypeptide only a chiral ligand. Under our conditions, catalysis by copper ions does not seem to occur as described above.

A hydrophobic α-amino acid ester hydrochloride should be bound to a hydrophobic domain of cyclo(D-Leu-L-Glu-L-His)$_2$–Cu^{2+} complex and subjected to intramolecular catalysis by His-imidazolyl groups. Either the substrate binding or the intramolecular catalysis or both should be enantiomer-selective. Since for cyclic peptides conformation can be made clear by sepectroscopy, investigation of the stereochemistry of cyclo(D-Leu-L-Glu-L-His)$_2$–Cu^{2+} complex may provide a clue in elucidating the mechanism of enantiomer-selective hydrolysis.[11]

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