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Hydrolysis of Active Esters of Aliphatic Carboxylic Acids with Cyclic Dipeptide Catalysts Consisting of L-Histidine and Different Aliphatic α-Amino Acids

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The hydrolyses of a series of *p*-nitrophenyl carboxylates with different acyl chains were investigated at 25 °C, pH 7.8 using cyclic dipeptides[*cyclo*(-L- or D-Xyz-His-) consisting of L-histidine (L-His) and an α-amino acid (Xyz) with different aliphatic side chains] as catalysts. Consequently, *cyclo*(-D-Leu-L-His-) and *cyclo*(-D-Val-L-His-) were found to be specifically effective catalysts for the hydrolysis of *p*-nitrophenyl laurate. These trans (D-L-type) cyclic dipeptides were much more efficient catalysts than their diastereomers, and were more reactive than imidazole, despite the fact that the former are less basic than the latter. The conformation of the cyclic peptides in solution was investigated using proton magnetic resonance spectroscopy and the relationship between conformation and catalytic activity was investigated. Consequently, it was found that the hydrophobic interaction between a catalyst and a substrate and the stereochemical fit for the cooperation of functional groups in the intramolecular nucleophilic catalysis are very important in order to attain a highly efficient catalysis. It was also found that the functional groups should have a certain size and flexibility in order to realize an effective stereochemical fit between functional groups of catalysts and substrates.

The specific catalysis of hydrolytic enzymes has been shown to depend upon the intramolecular cooperation of side-chain functional groups which are confined to a specific spatial conformation by the influence of the secondary structure of a polypeptide chain. In order to develop a catalyst with an enzyme-like character, the use of cyclic peptides seemed to be promising.[1] On the basis of this view, we synthesized cyclic dipeptides consisting of leucine and histidine and investigated the catalysis in the hydrolysis of *p*-nitrophenyl carboxylates. A very high catalytic activity of *cyclo*(D-leucyl-L-histidine)[*cyclo*(-D-Leu-L-His-)] in the hydrolysis of *p*-nitrophenyl laurate [$CH_3(CH_2)_{10}COOC_6H_4NO_2$] at 25 °C, pH 7.9 in 20% dioxane/water at the substrate concentration of 3.0×10^{-5} mol dm^{-3} has been reported by us.[2] *Cyclo*(-D-Leu-L-His-) (pK_a=6.00) is less basic than imidazole (pK_a=7.05) and the former was less efficient than the latter as a catalyst for the hydrolysis of *p*-nitrophenyl esters of short-chain carboxylic acids. However, it was specifically reactive for the hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$, that is, in the above conditions the second-order rate constant k_{cat} was 34—68 dm^3 mol^{-1} min^{-1} which was several tens of times as large as k_{cat}=1.9 dm^3 mol^{-1} min^{-1} by imidazole. It was considered that the hydrophobic interaction between the catlyst and the substrate should have played an important role in the above reactions.

It should be interesting to confirm if the above explanation is commonly applicable to other combinations of catalysts and substrates. This sort of investigation will be effective to explore a substrate-selective catalysis, that is, an efficient catalysis for a specific substrate, by controlling the structure of the cyclic peptide.

Experimental

Cyclic Dipeptides. *N*-Benzyloxycarbonyl α-amino acid or *N*-*t*-butoxycarbonyl α-amino acid and L-histidine methyl ester were condensed by an active ester method or with dicyclohexylcarbodiimide to obtain a linear dipeptide blocked at both terminals. A benzyloxycarbonyl group and *t*-butoxycarbonyl group were removed by catalytic hydrogenation and HCl/dioxane, respectively. The cyclization was carried out by refluxing in methanol. Cyclic dipeptides were recrystallized from water or a water/acetone mixture. It should be emphasized that ion-exchanging resins were not used to purify the intermediates and the products. Syntheses of *cyclo*(-Gly-L-His-), *cyclo*(-L-Leu-L-His-) and *cyclo*(-D-Leu-L-His-) have been reported previously.[2] *Cyclo*(-L-Ala-L-His-), mp 252—254 °C. Calcd for $C_9H_{12}N_4O_2$: C, 51.92; H, 5.81; N, 26.91%. Found: C, 51.70; H, 5.54; N, 26.96%. *Cyclo*(-D-Ala-L-His-), mp 235—236 °C. Calcd for $C_9H_{12}N_4O_2\cdot H_2O$: C, 47.78; H, 6.24; N, 24.76%. Found: C, 47.71; H, 6.10; N, 24.40%. *Cyclo*(-L-Phe-L-His-), mp 263 °C. Calcd for $C_{15}H_{16}N_4O_2$: C, 63.37; H, 5.67; N, 19.17%. Found: C, 63.07; H, 5.60; N, 20.00%. *Cyclo*(-D-Phe-L-His-), mp 255—257 °C. Found: C, 63.17; H, 5.53; N, 19.76%. *Cyclo*(-L-Val-L-His-), mp 220—224 °C. Calcd for $C_{11}H_{16}N_4O_2$: C, 55.92; H, 6.83; N, 23.71%. Found: C, 55.72; H, 6.89; N, 23.02%. *Cyclo*(-D-Val-L-His-), mp 228—230 °C. Found: C, 55.88; H, 6.68; N, 23.46%. *Cyclo*(-DL-Nle-L-His-), mp 207—213 °C. Calcd for $C_{12}H_{18}N_4O_2\cdot H_2O$: C, 53.72; H, 7.51; N, 20.88%. Found: C, 54.41; H, 7.32; N, 20.77%.

To synthesize *cyclo*(-D-Pip-L-His-) a racemic pipecolic acid-(2-piperidinecarboxylic acid) was optically resolved to obtain (*R*)-pipecolic acid(D-Pip). (+)-Tartaric acid was added to the racemic pipecolic acid to form a (*R*)-pipecolic acid — (+)-tartaric acid complex. To the complex, lead (IV) acetate was added to remove (+)-tartaric acid and to obtain (*R*)-pipecolic acid.[3] (*R*)-Pipecolic acid — (+)-tartaric acid complex, mp 182—185 °C (lit,[3] 182 °C), $[\alpha]_D$ 19.9 (H_2O) (lit,[3] 20.0). (*R*)-pipecolic acid, $[\alpha]_D$ 26.6 (H_2O)(lit,[4] 26±1). (*R*)-*N*-(Benzyloxycarbonyl)pipecolic acid was condensed with methyl L-histidinate using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline to obtain a linear dipeptide blocked at both terminals. The subsequent procedure leading to *cyclo*-(-D-Pip-L-His-) was the same as the above. (*R*)-*N*-(Benzyloxycarbonyl)pipecolic acid, mp 110—113 °C (lit,[4] 112—113 °C); $[\alpha]_D$ 60.0 (AcOH) (lit,[5] 57.2±1). *Cyclo*(-D-Pip-L-His-), mp 185—195 °C. Calcd for $C_{12}H_{16}N_4O_2$: C, 58.05; H, 6.50; N, 22.57%. Found: C, 58.21; H, 6.57; N, 22.34%.

Methyl *N*-methoxycarbonyl-L- or D-leucyl-L-histidinate

(MeOCO-L or D-Leu-L-His-OMe) was used as a reference catalyst for the hydrolysis. They were synthesized in the same way as reported previously.[2]

Substrate. Vaious carboxylic acids were condensed with *p*-nitrophenol using dicyclohexylcarbodiimide, and the active esters were purified by recrystallization from ethanol. All esters were proven to be pure by an elemental analysis. Syntheses and purifications of *p*-nitrophenyl acetate [$CH_3COOC_6H_4NO_2$], 3-phenylpropionate [$Ph(CH_2)_2COOC_6H_4NO_2$] and laurate [$CH_3(CH_2)_{10}COOC_6H_4NO_2$] have been reported previously.[2] *p*-Nitrophenyl 5-phenylvalerate[$Ph(CH_2)_4COOC_6H_4NO_2$], mp 64.5—65.0°C. Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68%. Found: C, 68.07; H, 5.80; N, 4.90%. *p*-Nitrophenyl decanoate [$CH_3(CH_2)_8COOC_6H_4NO_2$], mp 32.5—33°C. Calcd for $C_{16}H_{23}NO_4$: C, 65.51; H, 7.90; N, 4.77%. Found C, 65.75; H, 7.91; N, 4.74%. *p*-Nitrophenyl cyclohexanecarboxylate [$c\text{-}C_6H_{11}COOC_6H_4NO_2$], mp 50°C. Calcd for $C_{13}H_{15}NO_4$: C, 62.64; H, 6.07, N, 5.62%. Found: C, 62.91; H, 6.11; N, 5.56%. *p*-Nitrophenyl pivalate-[$Me_3CCOOC_6H_4NO_2$], mp 98—99°C (lit,[5] 94—95°C).

Hydrolytic Reaction. Hydrolyses were carried out at 25°C, pH 7.8 (KH_2PO_4-NaOH buffer) in 20% dioxane/water mixture. The catalyst concentration with respect to the imidazolyl group was in the range 1.65—11.2×10^{-4} mol dm^{-3}. It was lowered to 5.49×10^{-5} mol dm^{-3} when the effect of the dilution of the catalyst was examined. The substrate concentration was 3.0×10^{-5} mol dm^{-3}. However, the concentration of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ and $CH_3(CH_2)_8COOC_6H_4NO_2$ was 6.0×10^{-6} mol dm^{-3} or 2.0×10^{-6} mol dm^{-3} to avoid association. A mixture of the buffer solution and the aqueous catalyst solution was kept at a constant temperature in an ultraviolet cell and a dioxane solution of substrate, which was kept at the same temperature, was added to start the hydrolytic reaction. By this procedure, an excellent reproducibility of the reaction was obtained even when a slight turbidity was observed in the hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$. To determine the reaction rate, a JASCO UVIDEC-1-type spectrophotomer was employed. The concentration of the *p*-nitrophenolate ion liberated in the reaction was determined by the absorbance at 400 nm, and the rate of hydrolysis was calculated. Optical cells having optical path lengths of 1 cm and 5 cm were used when the substrate concentration was 3.0×10^{-5} mol dm^{-3} and 6.0×10^{-6} mol dm^{-3} or 2.0×10^{-6} mol dm^{-3}, respectively. A second-order rate constant k_{cat} was calcutated from the pseudo-first-order rate constant k_1, the rate constant for the spontaneous hydrolysis k_w, and the effective imidazolyl concentration under reaction conditions. With all cyclic dipeptide catalysts the side-chain imidazolyl group of L-histidyl residue is in its neutral form at pH 7.8, so that the effective concentration is the same as the total concentration. On the other hand, with imidazole as a catalyst the fraction of catalytically active imidazole was 0.85 at pH 7.8. The second-order kinetics has been described previously.[2] The catalytic activities were compared in terms of k_{cat}.

Conformational Analysis. The side-chain conformation of cyclic peptides in solution was investigated by 220 MHz and 100 MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Varian HR-220 and HA-100 spectrometers were used. Dimethyl-d_6-sulfoxide(Me_2SO-d_6) and D_2O (NaOD-KD_2PO_4 buffer, pH 7.8) were used as solvent. Tetramethylsilane (Me_4Si) was used as an internal standard for the Me_2SO-d_6 solution. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate[$Me_3Si(CH_2)_3SO_3Na$] and Me_4Si were used as an internal standard and an external standard for the D_2O solution, respectively.

Results and Discussion

The Hydrolysis of p-*Nitrophenyl Laurate with* Cyclo(-L-*or* D-*Leucyl*-L-*Histidyl).* In a series of experiments to confirm the previous experimental results, we could not reproduce the rapid hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ by *cyclo*(-D-Leu-L-His-) under the same conditions. A trace amount of some long-chain alkylamine, which was contaminated into the *cyclo*(-D-Leu-L-His-) synthesized in the previous way, might have catalyzed the apparently fast hydrolysis. It has been reported that the hydrolyses of *p*-nitrophenyl esters of long-chain carboxylic acids are greatly accelerated by long-chain alkylamine.[6,7] Since a trace amount of long-chain alkylamine may be concentrated in a cyclic peptide during the purification with anion-exhange resin Amberlite IRA-400 which was used in the previous experiments, cyclic peptide catalysts were resynthesized without using ion-exchangers in the present investigation. The hydrolytic experiments of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ with a pure *cyclo*(-D-Leu-L-His-) and related peptide catalysts were carried out nearly the same conditions as before, and k_{cat} values are shown in Table 1 together with other data. It is remarkable that the k_{cat} value, by pure *cyclo*(-D-Leu-L-His-), is much smaller than that reported previously. We must conclude that k_{cat}=34—68 dm^3 mol^{-1} min^{-1} reported in a previous paper is much larger than the real value. The origin of the error is perhaps some hydrophobic amine contaminated from an ion-exchanger during the synthesis of *cyclo*(-D-Leu-L-His-).

Table 1. Second-order rate constants(k_{cat}, dm^3 mol^{-1} min^{-1}) for the hydrolysis of *p*-nitrophenyl laurate[a]

Catalyst	pK_a	$[S]_0^{b)}$=6.0×10^{-6} mol dm^{-3}	$[S]_0^{b)}$=3.0×10^{-5} mol dm^{-3}	
			Present experiment	Previous experiment
None (k_w, min^{-1})	—	0.24×10^{-3}	0.07×10^{-3}	0.20×10^{-3}
Imidazole	7.05	1.6	3.8	1.9
MeOCO-L-Leu-L-His-OMe	6.60	1.3	0.73	≈0
MeOCO-D-Leu-L-His-OMe	6.60	2.7	0.77	0.18
Cyclo(-L-Leu-L-His-)	6.25	0.05	0.08	0.25
Cyclo(-D-Leu-L-His-)	6.00	2.6	1.1	34—68

a) 25°C, pH 7.8, 20% dioxane/water. b) Initial substrate concentration.

However, it was not possible to isolate and identify the hydrophobic amine.

In previous experiments in which the substrate concentration was 3.0×10^{-5} mol dm^{-3}, sometimes the solution was trubid and precipitation occurred during the reaction. In these cases, the first-order plot with respect to the concentration of the reaction product was not linear. In view of several papers,[8,9] $CH_3(CH_2)_{10}COOC_6H_4NO_2$ tends to associate in a 20% dioxane/water solution at a concentration of 3.0×10^{-5} mol dm^{-3} and the association affects the k_{cat} value. A similar situation will be met with $CH_3(CH_2)_8COOC_6H_4NO_2$, too. Therefore, the hydrolyses of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ with pure cyclic dipeptides containing L-histidine were carried out at substrate concentrations of 6.0×10^{-6} mol dm^{-3} and 2.0×10^{-6} mol dm^{-3}, which seemed to be low enough to avoid association. Under these conditions the reaction solution was nearly transparent. In particular, at the substrate concentration of 2×10^{-6} mol dm^{-3}, the reaction solution was perfectly transparent, and a first-order plot on the basis of the absorption due to dissociated *p*-nitrophenolate ion was linear except for the very early stage of the hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$. The k_{cat} values were determined from the linear part of first-order plot and are summarized in Table 2. In Table 2 the effect of dilution of catalyst on k_{cat} is also shown.

In Table 2, the catalyst dilution does not induce a definite change in k_{cat}. Therefore, the catalysts should be completely soluble under the conditions employed in the present investigation. An inspection of the experimental data shown in Tables 1 and 2 shows that a substrate dilution from 3.0×10^{-5} mol dm^{-3} to 6.0×10^{-6} mol dm^{-3} increased k_{cat} for $CH_3(CH_2)_{10}COOC_6H_4NO_2$ with peptide catalysts, that the reproducibility of the hydrolyses of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ and $CH_3(CH_2)_8COOC_6H_4NO_2$ at a concentration 6.0×10^{-6} mol dm^{-3} is excellent, and that the further dilution of both substrates from 6.0×10^{-6} mol dm^{-3} to 2.0×10^{-6} mol dm^{-3} increased the k_{cat} values again. Since the aggregation of these hydrophobic substrates in 20% dioxane/water at a concentration of 2.0×10^{-6} mol dm^{-3} is suppressed to the minimum extent as described above, the k_{cat} values obtained at 2.0×10^{-6} mol dm^{-3} are regarded as the true values. It is remarkable that the cyclic peptides, consisting of D-leucyl or D-valyl and L-histidyl residues, have a high catalytic activity toward $CH_3(CH_2)_{10}COOC_6H_4NO_2$ under diluted conditions as compared to imidazole. It should be a marked result because these cyclic peptide catalysts are less basic than imidazole. The binding of hydrophobic substrates by a hydrophobic side chain of leucyl or valyl residue must have increased the reaction rate. In fact, in a mixed solvent containing more dioxane, the k_{cat} of *cyclo*(-D-Leu-L-His-) and *cyclo*(-D-Val-L-His-) was much smaller than that of imidazole as shown in Table 3.

Most interestingly, the catalysis by *cyclo*(-D-Leu-L-His-) or *cyclo*(-D-Val-L-His-) was much stronger than that by *cyclo*(-L-Leu-L-His-) or *cyclo*(-L-Val-L-His-), although both enantiomorphic pairs of catalysts are considered to be equally hydrophobic. The different activities of enantiomorphic catalysts may have been caused by different orientations of side chains in these cyclic dipeptides. In *cyclo*(-L-Leu-L-His-) or *cyclo*(-L-Val-L-His-), the side chains exist at the opposite side of the cyclic dipeptide plane.[10] To realize the hydrophobic binding of a substrate in an aqueous solution and subsequent intramolecular nucleophilic catalysis, *cyclo*(-D-Leu-L-His-) and *cyclo*(-D-Val-L-His-)

TABLE 2. EFFECTS OF DILUTION OF CATALYST AND SUBSTRATE ON THE SECOND-ORDER RATE CONSTANT (k_{cat}, dm^3 mol^{-1} min^{-1}) FOR THE HYDROLYSIS OF HYDROPHOBIC ESTERS WITH HYDROPHOBIC CYCLIC DIPEPTIDE CATALYSTS

Effect of Catalyst Dilution[a]

Catalyst	Low[b]	Intermediate[c]	High[d]
None (k_w, min^{-1})	2.6×10^{-4}	3.4×10^{-4}	3.3×10^{-4}
Imidazole	1.8	1.4	0.31
Cyclo(-D-Val-L-His-)	2.6	2.7	1.8
Cyclo(-D-Leu-L-His-)	2.5	3.2	2.4

a) 25 °C, pH 7.8, 20% dioxane/water, $[CH_3(CH_2)_{10}COOC_6H_4NO_2]_0=6.0\times10^{-6}$ mol dm^{-3}. b) [Catalyst]=$(5.49—5.87)\times10^{-5}$ mol dm^{-3}. c) [Catalyst]=$(1.64—1.76)\times10^{-4}$ mol dm^{-3}. d) [Catalyst]=$(4.94—5.28)\times10^{-4}$ mol dm^{-3}.

Effect of Substrate Dilution[e]

Catalyst	$CH_3(CH_2)_8COOC_6H_4NO_2$		$CH_3(CH_2)_{10}COOC_6H_4NO_2$		
	6.0×10^{-6} mol dm^{-3}	2.0×10^{-6} mol dm^{-3}	6.0×10^{-6} mol dm^{-3}		2.0×10^{-6} mol dm^{-3}
None (k_w, min^{-1})	0.86×10^{-3}, 0.38×10^{-3}	0.47×10^{-3}, 0.59×10^{-3}	0.21×10^{-3}, 0.34×10^{-3}		0.22×10^{-3}
Imidazole	7.4, 7.1	9.7	1.6	1.4	4.9
Cyclo(-L-Val-L-His-)	1.1	1.2		0.15	0.11
Cyclo(-D-Val-L-His-)	9.9	3.5		2.7	16
Cyclo(-L-Leu-L-His-)	1.4	1.2	0.50	0.68	0.15
Cyclo(-D-Leu-L-His-)	1.6	6.3	2.6	3.2	16

e) 25 °C, pH 7.8, 20% dioxane/water, [Catalyst]=$(1.65—1.80)\times10^{-4}$ mol dm^{-3}.

Table 3. Second-order rate constants (k_{cat}, $dm^3 mol^{-1} min^{-1}$) for the hydrolysis of p-nitrophenyl laurate[a]

Catalyat	20% dioxane/water		50% dioxane/water	
None (k_w, min^{-1})	0.21×10^{-3}	0.22×10^{-3}[b]	2.6×10^{-3}	0.21×10^{-3}[b]
Imidazole	1.6	4.9[b]	2.3	1.9[b]
Cyclo(-D-Val-L-His-)	—	16[b]	—	0.67[b]
Cyclo(-D-Leu-L-His-)	2.6	—	0.5	—

a) 25°C, pH 7.8, $[CH_3(CH_2)_{10}COOC_6H_4NO_2]_0=6.0\times10^{-6}$ mol dm^{-3}. b) $[CH_3(CH_2)_{10}COOC_6H_4NO_2]_0=2.0\times10^{-6}$ mol dm^{-3}.

Table 4. Second-order rate constants(k_{cat}, $dm^3 mol^{-1} min^{-1}$) for the hydrolysis of *p*-nitrophenyl acetate and *p*-nitrophenyl decanoate by various cyclic dipeptide catalysts *Cyclo*(-Xyz-L-His-)[a]

Xyz residue	Gly	L-Als	D-Ala	L-Phe	D-Phe	L-Val	D-Val	L-Leu	D-Leu	Imidazole
pK_a	6.20	6.25	6.20	6.35	6.35	6.35	6.40	6.25	6.00	7.05
$CH_3COOC_6H_4NO_2$	1.5	1.9	2.2	1.9	3.2	1.9	3.5	1.6	3.2	17.5
$CH_3(CH_2)_8COOC_6H_4NO_2$	—	—	—	0.4[b]	1.9[b]	1.2	3.5	1.2	6.3	9.7

a) 25°C, pH 7.8, 20% dioxane/water, $[S]_0=3.0\times10^{-5}$ mol dm^{-3} for $CH_3COOC_6H_4NO_2$, 2.0×10^{-6} mol dm^{-3} for $CH_3(CH_2)_8COOC_6H_4NO_2$. b) $[S]_0=3.0\times10^{-5}$ mol dm^{-3}. c) $[S]_0=6.0\times10^{-6}$ mol dm^{-3}.

Table 5. Second-order rate constants(k_{cat}, $dm^3 mol^{-1} min^{-1}$) for hydrolysis of *p*-nitrophenyl esters of aliphatic carboxylic acids with different acyl chain lenghts by hydroophobic cyclic dipeptide catalysts[a]

Catalyst	$CH_3COOC_6H_4NO_2$	$Ph(CH_2)_2COOC_6H_4NO_2$	$Ph(CH_2)_4COOC_6H_4NO_2$	$CH_3(CH_2)_8COOC_6H_4NO_2$	$CH_3(CH_2)_{10}COOC_6H_4NO_2$
Cyclo(-Gly-L-His-)	1.5	1.7	3.5	—	1.1
Cyclo(-L-Phe-L-His-)	1.9	1.9	1.2	0.4	—
Cyclo(-D-Phe-L-His-)	3.2	3.4	2.5	1.9[b]	0.8[b]
Cyclo(-L-Val-L-His-)	1.9	1.6	—	1.2[c]	0.11[c]
Cyclo(-D-Val-L-His-)	3.5	3.7	2.4	3.5[c]	16[c]
Cyclo(-L-Leu-L-His-)	1.6	1.5	—	1.2[c]	0.15[c]
Cyclo(-D-Leu-L-His-)	3.2	4.1	2.1	6.3[c]	16[c]
Cyclo(-DL-Nle-L-His-)	3.7	4.2	3.4	—	—
Imidazole	17.5	14.0	8.9	9.7[c]	4.9[c]

a) 25°C, pH 7.8, 20% dioxane/water, $[S]_0=3.0\times10^{-5}$ mol dm^{-3}. b) $[S]_0=6.0\times10^{-6}$ mol dm^{-3}. c) $[S]_0=2.0\times10^{-6}$ mol dm^{-3}.

must have a suitable orientation of the functional groups and a stereochemical fit with a substrate.

Hydrolysis Catalyzed by Cyclic Dipeptides Carring an Alkyl Side Chain of Different Chain Lengths. The hydrophobicity of cyclic dipeptide catalysts can be controlled by the kind of α-amino acid (Xyz) which is coupled with L-histidine in the cyclic dipeptide. Thus, we can investigate the effect of the nature of cyclic dipeptide catalysts on the hydrolyses of a highly hydrophobic $CH_3(CH_2)_8COOC_6H_4NO_2$ and a less hydrophobic $CH_3COOC_6H_4NO_2$. The experimental results are summarized in Table 4.

The basicities of the side-chain imidazolyl groups of cyclic dipeptides are lower by *ca.* 0.7 p*K* unit than that of imidazole. The basicities are not seriously affected by the different natures and configurations of the α-amino acid residues which are coupled with L-histidine in the cyclic dipeptides.

For the hydrolysis of $CH_3COOC_6H_4NO_2$, which has the shortest acyl chain among the substrates investigated, imidazole was the most efficient catalyst and none of the cyclic dipeptide catalysts used were found to be more efficient than imidazole. No particular interaction, such as a hydrophobic interaction, seems to operate between $CH_3COOC_6H_4NO_2$ and the cyclic dipeptides. As a consequence, the cyclic dipeptide catalysts, which are less basic and are accompanied by a higher steric hindrance in the nucleophilic reaction, are less efficient than imidazole. It should be pointed out that with all diastereomeric catalysts a D-L-type dipeptide is somewhat more(up to twice) reactive than an L-L-type cyclic dipeptide. As will be described subsequently, this difference must be due to a less steric hindrance in the reaction of an imidazolyl group of the D-L-type cyclic dipeptide.

On the other hand, in the hydrolysis of $CH_3(CH_2)_8COOC_6H_4NO_2$, which has a long acyl chain, cyclic dipeptide catalysts carrying a bulky side chain were found to be relatively reactive. More interestingly, under these conditions *cyclo*(-D-Xyz-L-His-) s were 3—5 times more reactive than their diastereomers. This reactivity difference should be ascribed to a favorable orientation of the side chains of *cyclo*(-D-Xyz-L-His-) in the intramolecular catalysis toward a bound substrate.

Next, the hydrolytic reactions of *p*-nitrophenyl carboxylates having different lengths of an acyl chain with some hydrophobic cyclic dipeptide catalysts were investigated. The experimental results are summarized in Table 5.

The second-order rate constants k_{cat} for the hydrol-

yses with imidazole as a catalyst decreased on going from $CH_3COOC_6H_4NO_2$ to $CH_3(CH_2)_{10}COOC_6H_4NO_2$. The same trend was also observed in a hydrolyses catalyzed by L-L-type cyclic dipeptide catalysts. These experimental findings indicate an increasing steric hindrance against a nucleophilic attack by imidazole or an imidazolyl group as the length of the acyl chain of the substrates increases.

On the other hand, the k_{cat} values for the hydrolyses catalyzed by D-L-type cyclic dipeptides did not decrease markedly as the acyl chain length of the substrates increased. Interestingly, in the hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$, *cyclo*(-D-Val-L-His-) and *cyclo*(-D-Leu-L-His-) were more reactive than imidazole. In these experimental results, the important contribution of the hydrophobic binding of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ by the hydrophobic cyclic dipeptides is again indicated.

The reactivity difference between the *cyclo*(-D-Xyz-L-His-)s, where Xyz is Leu, Val, and Phe, and their diastereomers is much larger in the hydrolysis of $CH_3(CH_2)_{10}COOC_6H_4NO_2$ than in the hydrolysis of $CH_3(CH_2)_8COOC_6H_4NO_2$, which is in turn larger than those in the hydrolyses of $CH_3COOC_6H_4NO_2$, $Ph(CH_2)_2COOC_6H_4NO_2$, and $Ph(CH_2)_4COOC_6H_4NO_2$. In other words, the more important the hydrophobic interaction between substrate and cyclic dipeptide, the larger the reactivity difference between the diastereomers of the cyclic dipeptides. *Cyclo*(-D-Xyz-L-His-) is intrinsically more reactive in a nucleophilic reaction toward $CH_3COOC_6H_4NO_2$, $Ph(CH_2)_2COOC_6$-H_4NO_2, and $Ph(CH_2)_4COOC_6H_4NO_2$ because of less steric hindrance than *cyclo*(-L-Xyz-L-His-). In addition to this, the conformation of *cyclo*(-D-Xyz-L-His-) is far more favorable in the intramolecular reaction toward a bound substrate than that of *cyclo*(-L-Xyz-L-His-).

Conformational Analysis of Side-Chain Group of Cyclic Peptide by Nuclear Magnetic Resonance Spectroscopy. An attempt was made to elucidate the hydrolytic activity — conformation relationship of the cyclic peptide catalysts through an analysis of the side-chain conformation of the cyclic peptides by NMR spectroscopy.

^1H NMR spectra of *cyclo*(-Xyz-L-His-) in Me_2SO-d_6 or D_2O solution were obtained, and the chemical shifts (δ^α, δ^β) of H^α and H^β of the two α-amino acid residues and the coupling constants($J^{\alpha\beta}$) between them were determined. These values are summarized in Table 6.

The $J^{\alpha\beta}$ values were substituted into a Karplus-type equation, by which the proportions of conformers existing in three conformational energy minima were calculated with reference to the internal rotation around the C^α–C^β bond. On that basis, the proportion F, which represents the folded conformation with the side chain stacking over the plane of cyclic dipeptide, and the proportion U, which represents the unfolded conformation with the side chain protruding outside the plane of cyclic peptide, were calculated and are shown in Table 7. In the same table, those values that have been determined by NMR spectroscopy with *cyclo*(-L- or D-Leu-L-His-) and *cyclo*(-Gly-L-His-) are also shown.[10]

The backbone conformation of these cyclic dipeptides was not investigated in detail. However, it may be considered, on the basis of the previous conclusion obtained with *cyclo*(-L- or D-Leu-L-His-),[10] that L-L-type cyclic dipeptides take a flagpole-boat conformation and that D-L-type cyclic dipeptides assume a nearly planar conformation. In all the cyclic dipeptides investigated, the fraction with a folded conformation of the side chain of L-histidyl residue was more than 1/3, the statistical value. This tendency is particularly marked for D_2O. The folded conformation is stabilized by an aromatic — amide interaction between an imidazolyl group and the backbone of the cyclic di-

TABLE 6. CHEMICAL SHIFT (δ, ppm) AND COUPLING CONSTANT (J, Hz) OF H^α AND H^β OF *Cyclo*(-Xyz-L-His-) IN ^1H NMR SPECTRUM[a]

Xyz residue	Solvent	L-His residue			Xyz residue		
		δ^α	δ^β	$J^{\alpha\beta}$	δ^α	δ^β	$J^{\alpha\beta}$
L-Ala	Me_2SO-d_6	4.09	2.87	5.0	3.84	0.99	7.0
			3.04	6.0			
D-Ala	Me_2SO-d_6	4.02	2.93	5.0	3.44	1.19	6.3
L-Phe	Me_2SO-d_6	4.16	2.86	5.0	3.89	1.68	4.0
						2.61	9.0
D-Phe	Me_2SO-d_6	3.24	≈2.84	—[b]	3.87	2.87	4.2
						3.10	5.3
L-Val	Me_2SO-d_6	4.08	2.79	4.1	3.67	2.04	3.5
			3.08	7.6			
D-Val	Me_2SO-d_6	4.05	2.83	4.8	3.39	2.11	4.0
			3.06	6.8			
L-Val	D_2O	4.88	3.52	5.2	4.35	2.33	4.2
			3.72	4.5			
D-Val	D_2O	4.87	3.52	4.1	3.92	2.67	3.0
			3.71	4.4			

a) The standard substance is Me_4Si in Me_2SO-d_6 and capillary Me_4Si in D_2O. b) Overlapping with H^β signal obstacled the determination of the coupling constant.

Table 7. Side-chain orientation of *Cyclo*(-Xyz-L-His-) in solution as determined by ^1H NMR spectroscopy[a]

Xyz residue	Solvent	L-His residue		Xyz residue	
		F(%)	*U*(%)	*F*(%)	*U*(%)
Gly	Me_2SO-d_6	56	44	—[d]	—[d]
L-Ala	Me_2SO-d_6	47	53	—[d]	—[d]
D-Ala	Me_2SO-d_6	56	44	—[d]	—[d]
L-Phe	Me_2SO-d_6	56	44	29	71
D-Phe	Me_2SO-d_6	—[c]	—[c]	61	39
L-Val	Me_2SO-d_6	41	59	—[e]	—[e]
D-Val	Me_2SO-d_6	42	58	—[e]	—[e]
L-Leu	Me_2SO-d_6	48	52	29	71
D-Leu	Me_2SO-d_6	38	62	38	62
Gly[b]	D_2O	57	42	—[d]	—[d]
L-Val	D_2O	59	41	—[c]	—[c]
D-Val	D_2O	68	32	—[c]	—[c]
L-Leu	D_2O	70	30	22	78
D-Leu	D_2O	60	40	50	50

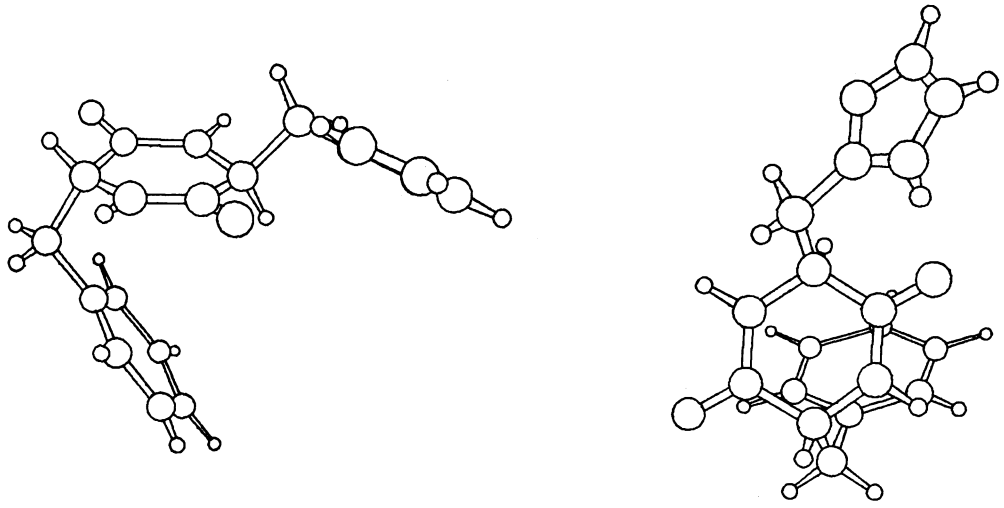
a) The standard substance is Me_4Si in Me_2SO-d_6 and capillary Me_4Si in D_2O. b) The standard substance is $Me_3Si(CH_2)_3SO_3Na$. c) Coupling constant was not determined (see Table 3). d) No C^γ atom is present. e) Two methyl groups are present at the γ position.

peptide. This interaction seemed to be more important in D_2O than in Me_2SO.[11,12] The high-field shift of the H^α signal of a D-Xyz residue of *cyclo*(-D-Xyz-L-His-), as compared with that of L-Xyz residue of *cyclo*-(-L-Xyz-L-His-) (see Table 6), might be due to a magnetic-shielding effect of the imidazolyl group. This indicates a molecular conformation in which H^α of D-Xyz residue protrudes over the plane of the imidazolyl group in the folded conformation of the side chain of the L-histidyl residue. Similarly, in Table 6, the H^β signal of the L-Xyz residue in *cyclo*(-L-Xyz-L-His-) is shifted to a higher magnetic field than the H^β signal of the D-Xyz residue in *cyclo*(-D-Xyz-L-His-). When two H^β atoms are concerned, they are nonequivalent and one of them is more strongly shifted to a high field than the other. These phenomena might have been caused by a magnetic-shielding effect of the imidazolyl group, and again indicate a folded conformation of the side chain of L-histidyl residue in which the H^β of L-Xyz residue protrudes over the plane of imidazolyl group.

These experimental NMR spectroscopy results indicate that in *cyclo*(-D-Xyz-L-His-) two side chains exist on different sides of the almost planar backbone plane of the cyclic dipeptide, and that in *cyclo*(-L-Xyz-L-His-) two side chains exist on the same side of the boat-type plane of the cyclic dipeptide. This conclusion means that the molecular conformation[10] proposed previously for *cyclo*(-L- or D-Leu-L-His-) is commonly available in other cyclic dipeptides. It is, therefore, conceivable to say that a conformation in which an isobutyl side chain of the D-Leu residue exists on the other side of an imidazolylmethyl side chain of the L-His residue with respect to the cyclic dipeptide backbone enables a stereochemical fit in binding a long-chain $CH_3(CH_2)_{10}COOC_6H_4NO_2$ by hydrophobic interaction with the isobutyl group and in the intramolecular nucleophilic attack by the imidazolylmethyl group.

There are two γ-methyl groups in *cyclo*(-L- or D-Val-L-His-), so that a Karplus-type equation is not applicable to this cyclic dipeptide. Therefore, the internal rotation around the C^α-C^β bond cannot be investigated on the basis of the coupling constant. In this cyclic peptide, however, one or two methyl groups are present in an unfolded position in any rotational state. Therefore, with *cyclo*(-D-Val-L-His-) a conformation becomes available in which one or two methyl groups situate fairly close to the backbone of the cyclic dipeptide and on the other side of the imidazolylmethyl group. This sort of arrangement of the functional groups may provide a favorable stereochemical fit with $CH_3(CH_2)_{10}COOC_6H_4NO_2$ for a concerted hydrophobic binding — intramolecular nucleophilic catalysis.

Cyclo(-D-Phe-L-His-) was found not to be so effective for the hydrolysis of *p*-nitrophenyl esters of long-chain carboxylic acids, although the side chain benzyl group of the Phe residue may well be hydrophobic comparable to the isobutyl side chain of the Leu residue and the isopropyl side chain of the Val residue. The reason for the low catalytic activity may exist in the peculiar orientation of the two side chains of *cyclo*(-D-Phe-L-His-). It is desirable to know the conformation of *cyclo*(-D-Phe-L-His-) in an aqueous solution, because the hydrolysis was carried out in a 20% dioxane/water mixture. Unfortunately, for the limited solubility of *cyclo*(-D-Phe-L-His-) in water, only the conformation in Me_2SO was investigated. As shown in Table 6, the NMR chemical shift of the H^α of the Phe residue in *cyclo*(-D-Phe-L-His-) is nearly the same as that in *cyclo*(-L-Phe-L-His-). In other words, the H^α of the Phe residue in *cyclo*(-D-Phe-L-His-) is free from the magnetic-shielding effect of the imidazolyl group of the His residue, which is a significant exception in the *cyclo*(-D-Xyz-L-His-) series. This indicates that the extent of the folded orientation of the imidazolylmethyl side chain of His residue in *cyclo*(-D-Phe-L-



(a) (b)

Fig. 1. Molecular conformation of *cyclo*(-D-Phe-L-His-) in Me_2SO-d_6 simulated by computer. Internal rotation angles: amide bond, planar cis; ϕ, −9.3°; ψ, 9.3°: His-χ^1, gauche: Phe-χ^2, 90°. Bond lengths and bond angles were usual values.
a, side view, b, top view.

His-) is less than that of others. As shown in Table 7, the folded conformation of the side-chain benzyl group of *cyclo*(-D-Phe-L-His-) amounts to nearly 60% in Me_2SO. The contribution of the folded conformation will be more important in an aqueous solution than in Me_2SO. Since the amide — aromatic interaction of the cyclic peptide backbone is stronger with a phenyl group than with an imidazolyl group,[11,12] *cyclo*(-D-Phe-L-His-) should have been forced to take the conformation depicted in Fig. 1 in an aqueous solution. Evidence supporting the conformation of Fig. 1 is collected in Table 6. For example, the NMR signal of the H^{α} of His residue in *cyclo*(-D-Phe-L-His-) shifts extensively to the upfield. This means that the benzyl group of the Phe residue situates very near to the H^{α} of the His residue. This type of arrangement of functional groups should lead to a poor stereochemical fit for the hydrophobic binding of *p*-nitrophenyl esters of long-chain carboxylic acids and the intramolecular nucleophilic attack.

Effect of the Nature of Hydrophobic Group on the Intramolecular Cooperation of a Hydrophobic Binding Group and a Nucleophilic Catalytic Group. It has been shown that the orientation of functional groups in the side chains of a cyclic dipeptide consisting of a hydrophobic D-amino acid and L-histidine is suitable to their intramolecular cooperation toward the hydrolysis of hydrophobic nitrophenyl carboxylates.[2] It was also shown above that subtle change in the structure of a hydrophobic side chain delicately influences the intramolecular cooperation, as in *cyclo*(-D-Phe-L-His-). To elucidate this point and to gain more information regarding the design of an efficient catalyst, the effect of the structure of the hydrophobic group in both catalyst and substrate on the reactivity was investigated.

First of all, $Me_3CCOOC_6H_4NO_2$ and *c*-$C_6H_{11}COOC_6H_4NO_2$ were chosen as the *p*-nitrophenyl carboxylates to be tested. They have a hydrophobic acyl chain which is different in structure from those investigated so far. These esters were hydrolyaed with a series of *cyclo*(-L- or D-Xyz-L-His-) and the experimental results are shown in Table 8.

Either $Me_3CCOOC_6H_4NO_2$ or *c*-$C_6H_{11}COOC_6H_4NO_2$ carries a highly hydrophobic acyl chain. However, both were not hydrolyzed efficiently with *cyclo*(-D-Xyz-L-His-) bearing a hydrophobic side chain. According to the examination regarding the molecular model, the acry group of $Me_3CCOOC_6H_4NO_2$ is spheric. Therefore, the arrangement of the acyl group and the ester group of a substrate does not fit with the arrangement of the functional groups of *cyclo*(-D-Xyz-L-His-). Similary, the cyclohexane ring of *c*-$C_6H_{11}COOC_6H_4NO_2$ assumes a rigid conformation, which makes a stereochemical fit between functional groups of the substrate and the catalyst difficult. This situation must have led to the sluggish reaction.

Next, *cyclo*(-D-Pip-L-His-) was synthesized as a cata-

Table 8. Second-order rate constants(k_{cat}, $dm^3 mol^{-1} min^{-1}$) for the hydrolysis of *p*-nitrophenyl pivalate and *p*-nitrophenyl cyclohexanecarboxylate by hydrophobic cyclic dipeptide catalysts *Cyclo*(-Xyz-L-His-)[a]

Xyz residue	Gly	L-Phe	D-Phe	L-Val	D-Val	L-Leu	D-Leu	DL-Nle	Imidazole
$Me_3CCOOC_6H_4NO_2$	0.2	0.02	0.04	—	0.03	0.04	0.10	0.06	0.2
c-$C_6H_{11}COOC_6H_4NO_2$	1.0	0.8	2.0	1.1	2.1	0.7	1.9	—	8.3

a) 25 °C, pH 7.8, 20% dioxane/water, $[S]_0 = 3.0 \times 10^{-5}$ mol dm^{-3}.

Table 9. Second-order rate constants(k_{cat}, $dm^3 mol^{-1} min^{-1}$) for the hydrolysis of *p*-nitrophenyl esters of several aliphatic carboxylic acids by *Cyclo*(-D-Pip-L-His-)[a]

Substrate	$CH_3COOC_6H_4NO_2$	$Ph(CH_2)_2COOC_6H_4NO_2$	$CH_3(CH_2)_8COOC_6H_4NO_2$	$c\text{-}C_6H_{11}COOC_6H_4NO_2$
Cyclo(-D-Pip-L-His-)	0.9	1.3	0.5	1.8
Imidazole	17.5	14.0	7.4[b]	8.3

a) 25°C, pH 7.8, 20% dioxane/water, $[S]_0=3.0\times10^{-5}$ mol dm^{-3}. b) $[S]_0=6.0\times10^{-6}$ mol dm^{-3}.

lyst having a rigid hydrophobic functional group, and was used in the hydrolyses of *p*-nitrophenyl carboxylates. The experimental results are shown in Table 9.

In the hydrolysis catalyzed by *cyclo*(-D-Pip-L-His-), an efficient catalysis, as has been observed with other hydrophobic cyclic dipeptides [*cyclo*(-D-Xyz-L-His-)], was never observed. According to Bláha *et al.*,[13,14] the backbone of the cyclic dipeptide containing pipecolic acid, *cyclo*(-D-Pip-Gly-), *cyclo*(-D-Pip-L-Leu-), and *cyclo*(-D-Pip-L-Phe), is nearly planar. The piperidine ring of the Pip residue is not so strained as to seriously alter the backbone conformation of the cyclic dipeptide. As estimated from $J^{\alpha\beta}$ of the NMR spectrum of *cyclo*(-D-Pip-L-His-) in Me_2SO-d_6, 70% of the imidazolylmethyl groups take the folded form (*F*) and the rest (30%) the unfolded form (*U*). Therefore, *cyclo*(-D-Pip-L-His-) takes nearly the same conformation of functional groups as other hydrophobic *cyclo*(-D-Xyz-L-His-). The unexpectedly low catalytic activity of *cyclo*(-D-Pip-L-His-) should therefore be due to the insufficient flexibility of the peperidine ring of the Pip residue to inhibit a satisfactory stereochemical fit of functional groups between catalyst and substate.

The above experimental results indicate that cylic dipeptides consisting of a hydrophobic D-amino acid and an L-histidine could be an efficient catalyst for the hydrolysis of hydrophobic *p*-nitrophenyl carboxylates, that the efficient catalysis is based upon a satisfactory stereochemical fit of functional groups between catalyst and substrate, and that the functional group should bear a certain size and a moderate flexibility in order to attain the sterochemical fit.

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