Macrocyclic Enzyme Model Systems. Catalytic Activity of Cyclic Peptides Involving Hydrophobic Segments[†]

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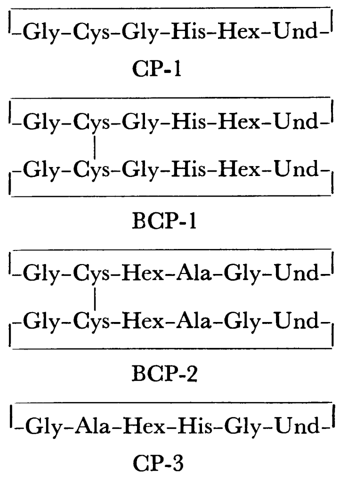
In order to develop an effective hydrolase model, BCP-1 was synthesized and its kinetic behavior investigated. The corresponding linear hexapeptide was prepared by the solid phase method and subjected to cyclization by the azide method. BCP-1 enhanced the hydrolysis of *p*-nitrophenyl carboxylates bearing a long alkyl chain. The pH-rate profile for the hydrolysis of *p*-nitrophenyl hexadecanoate (PNPP) as effected by BCP-1 was not of a bell-shaped type but a sigmoid, from which the kinetic pK_a value was estimated as 12.3. The initial reaction rate was found to level off beyond a certain range of substrate concentration (saturation-type kinetics), the kinetic data being analyzed on the basis of Michaelis-Menten scheme. The large hydrophobic binding ability of BCP-1 toward PNPP (K_m(app) $\simeq 10^{-6}$ M) was attributed to its double-layered bicyclic structure. The Hammett plot for the hydrolysis of substituted phenyl hexadecanoates gave a satisfactory linear correlation. Consequently, the acyl transfer from the bound substrate to the imidazole anion of the histidyl residue of BCP-1 was referred to the rate-determining step. The large negative entropy change for the acyl transfer step seems to indicate that the Michaelis complex does not have a conformation favorable for such acyl transfer process. The catalytic effectiveness (k_{cat}/K_m(app)) for the BCP-1 catalysis in PNPP hydrolysis is comparable to that for enzymatic hydrolysis.

Catalytic functions of macrocyclic compounds such as cycloamyloses,[1)] cyclophanes,[2)] and cyclic peptides[3)] have been investigated in order to explore the possibility of developing novel and efficient enzyme models. Cyclic peptides would simulate enzyme functions in a more favorable manner among these model systems since all enzymes are polypeptides. A few cyclic peptides so far synthesized were constructed only with α-amino acids, their hydrolase-like functions being much lower than those of real enzymes. The low efficiency may be primarily attributed to the network formation in their interior cavity by intramolecular hydrogen bonding, so that the hydrophobic binding ability can not be exercised. Such extensive intramolecular hydrogen bonding has been observed for cyclic hexapeptides by means of NMR[4)] and CD[5)] measurements.

In the present study, we designed cyclic peptides which bear long-chain alkyl segments in their skeleton to reduce the extent of intramolecular hydrogen bonding interaction, providing an efficient hydrophobic cavity for binding substrates. CP-1 was designed as a papain model with the aid of a space-filling molecular model (CPK model) so that the cyclic peptide has both an imidazole group of L-His and a thiol group of L-Cys at the juxtaposition within hydrogen bonding distance. In spite of this interesting aspect, CP-1 was not obtained but its dimeric form with a disulfide bond (BCP-1) was isolated. BCP-1 has only imidazole moieties as active functional groups and is considered to exist as a double-layered structure in aqueous media due to strong hydrophobic interaction between the two cyclic skeletons. We have investigated the esterase-like activity of BCP-1 in the ester hydrolysis for which the model compound would show a profound binding ability toward the substrates. In order to clarify the catalytic mechanism involved in the ester hydrolysis effected by BCP-1, other related compounds BCP-2 and CP-3 were also prepared and their kinetic effects studied.

Chart 1:



(Hex, 6-aminohexanoyl; Und, ω-aminoundecanoyl)

CP-1, *cyclo*-glycyl-L-cysteinylglycyl-L-histidyl-6-aminohexanoyl-ω-aminoundecanoyl; BCP-1, *S,S'*-bis(*cyclo*-glycyl-L-hemicystylglycyl-L-histidyl-6-aminohexanoyl-ω-aminoundecanoyl); BCP-2, *S,S'*-bis(*cyclo*-glycyl-L-hemicystyl-6-aminohexanoyl-L-alanylglycyl-ω-aminoundecanoyl); CP-3, *cyclo*-glycyl-L-alanyl-6-aminohexanoyl-L-histidylglycyl-ω-aminoundecanoyl.

Experimental

The synthetic procedures for the protected CP-1 (PCP-1) and BCP-1 are outlined in Scheme. 1. The linear hexapeptide hydrazide (**4**) was prepared by the solid phase method[6)] and subjected to cyclization by the azide method.

Chloromethylated Styrene-2%Divinylbenzene Copolymer[7)] *(**1**).* Copolystyrene-2%divinylbenzene (Protein Research Foundation, 100—200 mesh; 48 g) was made to swell in chloroform (300 ml) by stirring at room temperature for 2.5 h and was then cooled down to 0 °C. A solution of anhydrous stannic chloride (7.5 ml) in chloromethyl methyl ether (50 ml) was added to this mixture at 0 °C in 30 min, and stirred for 1 h at the same temperature. The mixture was filtered and the solid recovered was washed with dioxane-water (3 : 1 by volume, 1 litre) and subsequently with dioxane-3M HCl

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[†] Contribution No. 483 from this Department.

Boc-Und-OCH_2-P (**2**; 4.0 g, 3.2 mmol)

1. CF_3CO_2H/CH_2Cl_2 (2 : 3)
2. $(C_2H_5)_3N/CH_2Cl_2$ (1 : 10)
3. Boc-Hex-OH (2.22 g, 9.6 mmol), DCC/CH_2Cl_2

↓

Boc-Hex-Und-OCH_2P

1. CF_3CO_2H/CH_2Cl_2 (2 : 3)
2. $(C_2H_5)_3N$/DMF (1 : 10)
3. Boc-His(Bzl)-OH (3.31 g, 9.6 mmol), DCC/DMF

↓

Boc-His(Bzl)-Hex-Und-OCH_2-P

1. 1 M-HCl (in CH_3CO_2H)
2. $(C_2H_5)_3N/CH_2Cl_2$ (1 : 10)
3. Boc-Gly-OH (1.68 g, 9.6 mmol)/[CH_2Cl_2/DMF], DCC/CH_2Cl_2

↓

Boc-Gly-His(Bzl)-Hex-Und-OCH_2-P

1. 1 M-HCl (in CH_3CO_2H)
2. $(C_2H_5)_3N/CH_2Cl_2$ (1 : 10)
3. Boc-Cys(Bzl)-OH (2.99 g, 9.6 mmol), DCC/CH_2Cl_2

↓

Boc-Cys(Bzl)-Gly-His(Bzl)-Hex-Und-OCH_2-P

1. 1 M-HCl (in CH_3CO_2H)
2. $(C_2H_5)_3N/CH_2Cl_2$ (1 : 10)
3. Boc-Gly-OH (1.68 g, 9.6 mmol)/[CH_2Cl_2/DMF], DCC/CH_2Cl_2

↓

Boc-Gly-Cys(Bzl)-Gly-His(Bzl)-Hex-Und-OCH_2-P (**3**)

↓ NH_2NH_2/DMF

Boc-Gly-Cys(Bzl)-Gly-His(Bzl)-Hex-Und-$NHNH_2$ (**4**)

1. 2 M-HCl (in CH_3OH)
2. 2 M-HCl (in THF), $(CH_3)_2CHCH_2CH_2NO_2$/DMF
3. $(C_2H_5)_3N$/DMF

↓

cyclo-Gly-Cys(Bzl)-Gly-His(Bzl)-Hex-Und (**5**, PCP-1)

↓ Na/liq. NH_3

cyclo-Gly-Cys-Gly-His-Hex-Und
| (BCP-1)
cyclo-Gly-Cys-Gly-His-Hex-Uud

Scheme 1. Synthetic procedures for BCP-1: Hex, 6-aminohexanoyl: Und, ω-aminoundecanoyl; Boc, *t*-butoxycarbonyl; Bzl, benzyl; P, styrene-2%divinylbenzene copolymer.

(3 : 1 by volume, 1 litre). The cream-colored beads were washed further with water first and then with aqueous dioxane, the dioxane content of which was increased gradually up to 100%. After the beads had been washed with pure dioxane, they were washed with dioxane-methanol with gradual increase in methanol content and finally with pure methanol. No abrupt change in solvent composition was made during the course of washing. The product was dried *in vacuo* at 100 °C: yield 53.2 g; 1.98 mmol Cl/g.

N-t-*Butoxycarbonyl-ω-aminoundecanoyl Resin* (**2**). A solution of *N-t*-butoxycarbonyl-ω-aminoundecanoic acid[8] (8.94 g, 29.7 mmol) and triethylamine (3.3 ml, 24.0 mmol) in 40 ml of dry ethanol was mixed with **1** (100—200 mesh, 10.0 g). The mixture was stirred under reflux for 144.5 h and filtered to recover the resin, which was subsequently washed thoroughly with ethanol, hot ethanol, aqueous ethanol, water, aqueous methanol, and methanol, and then dried *in vacuo*; the content of ω-aminoundecanoic acid by nitrogen analysis, 0.80 mmol/g (conversion 40.4%).

N-t-*Butoxycarbonylglycyl*-S-*benzyl*-L-*cysteinylglycyl*-N^{im}-*benzyl*-L-*histidyl-6-aminohexanoyl-ω-aminoundecanoyl Resin* (**3**). A portion of **2** (4.0 g, 3.2 mmol) was coupled first with *N-t*-butoxycarbonyl-6-aminohexanoic acid[8] (Table 1) with a Shimadzu peptide synthesizer APS-80. The subsequent coupling reactions were carried out in a similar manner; modifications with regard to solvents and reagents are shown in Scheme 1. Additional washing with a solvent for the corresponding coupling reaction was carried out for the subsequent coupling steps between treatment Nos. 10 and 11 in Table 1. *N-t*-Butoxycarbonyl-N^{im}-benzyl-L-histidine[9] and *N-t*-butoxycarbonyl-*S*-benzyl-L-cysteine[10] were prepared according to the procedures reported.

N-t-*Butoxycarbonylglycyl*-S-*benzyl*-L-*cysteinylglycyl*-N^{im}-*benzyl*-L-*histidyl-6-aminohexanoyl-ω-aminoundecanoic Acid Hydrazide* (**4**). To a suspension of protected linear hexapeptide resin **3** (4.85 g) in *N,N*-dimethylformamide (30 ml) was added freshly prepared anhydrous hydrazine (bp 113.5 °C; 5.0 ml, 157 mmol). The mixture was stirred at room temperature for 48 h, and filtered to separate the resin which was subsequently washed with *N,N*-dimethylformamide (100 ml). The filtrate and washings were combined and evaporated *in vacuo*, and the residue was treated with water (200 ml) in order to isolate the insoluble product (610 mg). The crude material was purified on a column (2.5 × 81.0 cm) of Sephadex LH-20 with methanol as an eluent: yield 560 mg (18.2% based on *N-t*-butoxycarbonyl-ω-aminoundecanoyl resin); ninhydrin test, positive; hydrazide test, positive; R_f with 1-butanol-water-acetic acid (4:2:1), 0.89; mp 144—147 °C. IR (KBr disc): 3280 (NH str.); 2910 and 2840 (CH str.); 1635 (C=O str.); 770, 730, and 700 cm^{-1} (CH bend. of benzene). NMR (DMSO-d_6, TMS): δ 1.23 (22H, s, $-NHCH_2(\underline{CH_2})_{n-2}CH_2CO-$), 1.38 (9H, s, $(\underline{CH_3})_3CO-$), 2.02 (4H, broad, $-NHCH_2(CH_2)_{n-2}\underline{CH_2}CO-$), 2.85 (2H, broad,

TABLE 1. OPERATIONAL SEQUENCE FOR COUPLING REACTION OF Boc-Hex-OH WITH **2** PROGRAMMED ON A SHIMADZU PEPTIDE SYNTHESIZER (ASP-80)

Treatment No.	Reagent	Shaking period	Repeating frequency	Each amount
1	CH_2Cl_2	4 min	3	40 ml
2	CF_3CO_2H-CH_2Cl_2 (2 : 3 v/v)	60 min	1	40 ml
3	CH_2Cl_2	4 min	3	40 ml
4	C_2H_5OH	4 min	3	40 ml
5	CH_2Cl_2	4 min	3	40 ml
6	$(C_2H_5)_3N$-CH_2Cl_2 (1 : 10 v/v)	10 min	1	50.4 ml
7	CH_2Cl_2	4 min	5	40 ml
8	Charge: Boc-Hex-OH/CH_2Cl_2	10 min	1	2.22 g/29 ml
9	Charge: DCC/CH_2Cl_2	19 h	1	1.98 g/20.5 ml
10	CH_2Cl_2	4 min	4	40 ml
11	C_2H_5OH	20 min	2	40 ml

$-NHCH(C\underline{H}_2SCH_2Ph)CO-$), 3.00 (4H, broad, $-NHC\underline{H}_2(CH_2)_{n-2}CH_2CO-$), 3.50 (4H, s, $-NHC\underline{H}_2CO-$), 3.68 (2H, broad, $-NHCH(C\underline{H}_2ImCH_2Ph)CO-$), 3.75 (2H, s, $-SC\underline{H}_2Ph$), 4.50 (2H, broad, $-C\underline{H}-$), 5.12 (2H, s, $N^{Im}-C\underline{H}_2Ph$), 6.85 (1H, s, imidazolyl H), 7.30 (10 H, s, phenyl H's), 7.62 (1H, s, imidazolyl H), 7.83—8.60 (6H, broad, $-N\underline{H}-$), and 9.00 (2H, broad, $-NHN\underline{H}_2$). Found: C, 60.83; H, 7.76; N, 14.20%. Calcd for $C_{49}H_{74}N_{10}O_8S$: C, 61.10; H, 7.74; N, 14.54%.

cyclo-*Glycyl-*S*-benzyl-*L*-cysteinylglycyl-*N^{Im}*-benzyl-*L*-histidyl-6-aminohexanoyl-ω-aminoundecanoyl (**5**, PCP-1).* Linear hexapeptide hydrazide **4** (884 mg, 0.98 mmol) was dissolved in freshly prepared 2M hydrogen chloride in dry methanol (60 ml), and the mixture was stirred at room temperature for 3 h and then evaporated *in vacuo*. The residue was triturated in anhydrous ether, washed with the same solvent, and dried *in vacuo* at 40 °C to give pale yellow crystals. To the product dissolved in dry *N,N*-dimethylformamide (70 ml) was added freshly prepared 1.85 M hydrogen chloride in dry tetrahydrofuran (35 ml) and isopentyl nitrite (0.5 ml, 3.69 mmol) at −30 °C. After the reaction mixture being allowed to stand at −30 °C for 90 min, the absence of residual hydrazide in it was confirmed. A mixture of 0.3 M iron(III) chloride in 0.1 M acetic acid and 0.2 M potassium hexacyanoferrate(III) in 0.1 M acetic acid was used for detection. The mixture was diluted with dry *N,N*-dimethylformamide (540 ml) at −60 °C, followed by addition of dry triethylamine (21.7 ml, 155.4 mmol). The reaction mixture was allowed to stand at −10 °C for 48 h with stirring, and evaporated *in vacuo*. The residue was treated with water (200 ml) in order to recover the insoluble product. The crude product was purified by chromatography on columns of Amberlite IR-45 (1.5×27 cm) and Amberlite IRC-50 (1.5×27 cm) with dichloromethane–methanol–*N,N*-dimethylformamide–water (32:17:5:6 by volume), and then on a column of Sephadex LH-20 (2.5×81.0 cm) with methanol to give a white solid: yield 165.2 mg; mp(dec) 207—210 °C; ninhydrin test, negative; hydrazide test, negative. IR (KBr disc): 3300 (NH str.); 2920 and 2860 (CH str.); 1640 (C=O str.); and 770, 730, and 700 cm^{-1} (CH bend. of benzene). NMR (DMSO-d_6, TMS): δ 1.25 (22H, s, $-NHCH_2(C\underline{H}_2)_{n-2}CH_2CO-$), 2.08 (4H, broad, $-NHCH_2(CH_2)_{n-2}C\underline{H}_2CO-$), 2.85 (2H, broad, $-NHCH(C\underline{H}_2SCH_2Ph)CO-$), 3.07 (4H, broad, $-NHC\underline{H}_2(CH_2)_{n-2}CH_2CO-$), 3.42 (4H, s, $-NHC\underline{H}_2CO-$), 3.65(2H, broad, $-NHCH(C\underline{H}_2ImCH_2Ph)CO-$), 3.80 (2H, s, $-SC\underline{H}_2Ph$), 4.50 (2H, broad, $-CH-$), 5.22 (2H, s, $N^{Im}-C\underline{H}_2Ph$), 7.00 (1H, s, imidazolyl H), 7.38 (10H, s, phenyl H's), 7.85 (1H, s, imidazolyl H), and 7.96—8.58 (7H, broad, $-N\underline{H}-$). Mol wt: osmometry (methanol), 830; calcd for $C_{44}H_{62}N_8O_6S$, 831.1. Found: C, 62.16; H, 7.61; N, 12.82%. Calcd for $C_{44}H_{62}N_8O_6S \cdot H_2O$: C, 62.24; H, 7.63; N, 13.20%.

S,S′*-Bis*(cyclo*-glycyl-*L*-hemicystylglycyl-*L*-histidyl-6-aminohexanoyl-ω-aminoundecanoyl) (BCP-1).* To a solution of the protected cyclic peptide (**5**) (100 mg, 0.12 mmol) in liquid ammonia (100 ml, distilled in the presence of sodium metal) was added a small amount of sodium metal with stirring until the blue color of the solution remained for at least 2 min. In order to quench the excess sodium metal, a small amount of ammonium chloride was added to the solution which was subsequently evaporated to dryness *in vacuo* at room temperature. The residue was then treated with water (100 ml). The insoluble product was recovered by filtration, washed thoroughly with water, and dried *in vacuo* to give a white solid: yield 52.5 mg (67%); ninhydrin test, negative; Pauly test, positive; nitroprusside test, negative; Ellman test, negative. IR (KBr disc): 3300 (NH str.); 2920 and 2860 (CH str.); 1645 cm^{-1} (C=O str.). NMR (DMSO-d_6, TMS): δ 1.23 (22H, s, $-NHCH_2(C\underline{H}_2)_{n-2}CH_2CO-$), 2.10 (4H, broad, $-NHCH_2(CH_2)_{n-2}C\underline{H}_2CO-$), 2.80 (2H, broad, $-NHCH(C\underline{H}_2S-)CO-$), 3.13 (4H, s, $-NHC\underline{H}_2(CH_2)_{n-2}CH_2CO-$), 3.38 (4H, s, $-NHC\underline{H}_2CO-$), 3.65 (2H, s, $-NHCH(C\underline{H}_2Im)CO-$), 4.46 (2H, broad, $-C\underline{H}-$), 6.80 (1H, s, imidazolyl H), and 7.54 (1H, s, imidazolyl H). Amino acid analysis: Hex, 0.9 (1); His, 1.0 (1); Gly, 1.9 (2); Cys, 0.8 (1). The product showed very limited solubility in water and in most organic solvents; molecular weight determination by vapor pressure osmometry was not possible. Found: C, 54.21; H, 7.77; N, 16.09%. Calcd for $C_{60}H_{98}N_{14}O_{12}S_2 \cdot 2H_2O$: C, 53.87; H, 7.84; N, 16.75%.

Other Cyclic Peptides. *cyclo*-Glycyl-L-cysteinyl-6-aminohexanoyl-L-alanylglycyl-ω-aminoundecanoyl (CP-2) and CP-3 were prepared in a manner similar to that described for BCP-1. Elimination of the protecting group in the last step of the CP-2 synthesis was performed with anhydrous hydrofluoric acid.

CP-2: white powder; ninhydrin negative, nitroprusside positive, and Ellman positive at pH 8. IR (KBr disc): 3295 (N$\underline{H}$ str.); 2920 and 2850 (CH str.); 1625 and 1540 cm^{-1} (C=O str.). NMR (DMSO-d_6, TMS): δ 1.22 (22H, s, $-NHCH_2(C\underline{H}_2)_{n-2}CH_2CO-$), 2.15 (4H, broad, $-NHCH_2(CH_2)_{n-2}C\underline{H}_2CO-$), 2.77 (2H, broad, $-C\underline{H}_2SH$), 3.04 (4H broad, $-NHC\underline{H}_2(CH_2)_{n-2}CH_2CO-$), 3.63 (4H, s, $-NHC\underline{H}_2CO-$), and 4.58 (2H, broad, $-C\underline{H}-$). Amino acid analysis: Gly, 2.2 (2); Ala, 1.1 (1); Cys, 1.0 (1); Hex, 1.1 (1). BCP-2 was prepared by air-oxidation of CP-2 before use until nitroprusside test turned out to be completely negative.

CP-3: white powder; ninhydrin negative, and Pauly positive; R_f with 1-butanol–water–acetic acid (4 : 2 : 1 by volume), 0.53. IR (KBr disc): 3280 (NH str.); 2920 and 2850 (CH str.); 1640 and 1550 cm^{-1} (C=O str.). NMR (methanol-d_4, TMS): δ 1.32 (22H, s, $-NHCH_2(C\underline{H}_2)_{n-2}CH_2CO-$), 2.26 (4H, broad, $-NHCH_2(CH_2)_{n-2}C\underline{H}_2CO-$), 3.79 (4H, s, $-NHC\underline{H}_2CO-$), 4.26 (2H, broad, $-C\underline{H}-$), 6.91 (1H, s, imidazolyl H), and 7.67 (1H, s, imidazolyl H). Amino acid analysis: Gly, 2.2 (2); Ala, 1.1 (1); His, 1.0 (1); Hex, 0.7 (1). Found: C, 56.79; H, 8.22; N, 17.02%; M$^+$, 619. Calcd for $C_{30}H_{50}N_8O_6 \cdot H_2O$: C,56.58; H, 8.23; N, 17.59%; mol wt ($C_{30}H_{50}N_8O_6$), 618.81.

Substrates. Substituted phenyl esters were prepared by the general method[11] from acid chlorides and substituted phenols in the presence of a trace amount of magnesium and confirmed by IR and NMR spectroscopy. Analytical and physical data for the esters are given in Table 2.

Kinetic Measurements. Rates of phenol liberation from a substituted phenyl ester were measured either with a Union Giken high sensitive spectrophotometer SM-401 or with a Hitachi 124 spectrophotometer at the following wavelengths: *p*-cyanophenyl hexadecanoate, 275 nm; *p*-acetylphenyl hexadecanoate, 330 nm; *m*-nitrophenyl and *p*-nitrophenyl hexadecanoate, and other *p*-nitrophenyl esters, 400 nm. Each run was initiated by adding a dioxane solution(30 μl) of a substrate ester to a mixture of a reaction medium (3.0 ml) and a *N,N*-dimethylformamide solution (30 μl) of a catalyst, which was pre-equilibrated at an appropriate temperature in a thermostatted cell set in the spectrophotometer. The reaction temperature was maintained constant within an accuracy of ±0.1 °C. The reaction medium was prepared as follows: 10.0 ml of 1.0 M aqueous potassium chloride, 10.0 ml of an appropriate aqueous buffer, and 10.0 ml of dry dioxane were placed in a 100-ml volumetric flask; and subsequently the flask was filled with deionized and distilled water. Aqueous buffer solutions used are as follows:

1/10 M potassium dihydrogenphosphate-1/20 M sodium borate for pH 6—9, 1/20 M sodium borate-1/20 M sodium carbonate for pH 10—11, and 1/10 M sodium hydroxide —1/10 M sodium hydrogenphosphate for pH 11—12. pH Measurements were carried out with a TOA HM-9A pH meter equipped with a TOA GC-125 combined electrode after calibration with a combination of appropriate aqueous standard buffers.

Results and Discussion

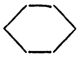
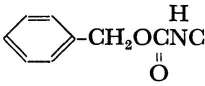
Catalytic Effects and Substrate Specificity. The catalytic efficiency of BCP-1 in the hydrolysis of *p*-nitrophenyl esters of some carboxylic acids has been investigated by the kinetic method in 10.0%(v/v) dioxane–1.0%(v/v) *N*,*N*-dimethylformamide–water at μ 0.10 (KCl). Apparent pseudo-first-order rate constants were obtained from the initial stage of the reaction by measuring the amount of liberated *p*-nitrophenol. The first-order kinetics was found to hold at least up to 65% conversion of the substrate under the conditions [S]>[E]; S and E stand for substrate and catalyst species, respectively. The kinetic data are summarized in Table 3. The result shows BCP-1 only catalyzes the hydrolysis of *p*-nitrophenyl esters bearing a long alkyl chain such as *p*-nitrophenyl hexadecanoate (PNPP) and dodecanoate (PNPL). In order to observe the catalytic effect by BCP-1, the substrate is required to involve a bulky group of suf-

Table 2. Analytical and physical data for substrates

Substrate[a]	Formula	Found (%) C	Found (%) H	Found (%) N	Calcd (%) C	Calcd (%) H	Calcd (%) N	Mp/Bp
PNPP	$C_{22}H_{35}NO_4$	70.06	9.08	3.90	69.99	9.34	3.71	62—64 °C
MNPP	$C_{22}H_{35}NO_4$	70.07	9.41	3.63	69.99	9.34	3.71	61—62 °C
PAPP	$C_{24}H_{38}O_3$	76.84	10.84		76.96	10.23		62—63 °C
PCPP	$C_{23}H_{35}NO_2$	77.21	9.94	3.84	77.26	9.87	3.92	75—76 °C
PNPCHC	$C_{13}H_{15}NO_4$	62.75	6.16	5.40	62.64	6.06	5.61	154—174 °C/0.7 mmHg
PNPG	$C_{16}H_{14}N_2O_6$	58.17	4.29	8.53	58.18	4.27	8.48	130—132 °C

a) Abbreviations: PNPP, *p*-nitrophenyl hexadecanoate; MNPP, *m*-nitrophenyl hexadecanoate; PAPP, *p*-acetylphenyl hexadecanoate; PCPP, *p*-cyanophenyl hexadecanoate; PNPCHC, *p*-nitrophenyl cyclohexanecarboxylate; PNPG, *p*-nitrophenyl *N*-benzyloxycarbonylglycinate.

Table 3. Apparent first-order rate constants for the BCP-1 catalyzed hydrolysis of *p*-nitrophenyl carboxylates at 40.0±0.1 °C and μ 0.10 (KCl) in 10.0%(v/v) dioxane–1.0%(v/v) *N*,*N*-dimethylformamide–water[a]

Substrate, R[b]	$k_{\text{hyd}}\times 10^5$ (s^{-1})	$k_{\text{obsd}}\times 10^5$ (s^{-1})	$k_c\times 10^5$ [c] (s^{-1})	pH
$CH_3(CH_2)_{14}$-	2.78	4.77	1.99	11.45
$CH_3(CH_2)_{10}$-	30.7	34.5	3.80	10.91
$CH_3(CH_2)_8$-	300	300	—	10.91
CH_3-	1625	1625	—	10.91
	400	400	—	10.91
	1385	1385	—	9.72

a) Initial concentrations: substrates, 0.984—0.989×10^{-5} M; BCP-1, 0.49×10^{-6} M. b) R for p-NO_2-C_6H_4-OC(O)R. c) $k_c=k_{\text{obsd}}-k_{\text{hyd}}$; k_{obsd}, observed first-order rate constants; k_{hyd}, hydrolysis rate constant in the absence of the catalyst.

Table 4. Apparent first- and second-order rate constants for the catalyzed hydrolysis of *p*-nitrophenyl hexadecanoate at 40.0±0.1 °C and μ 0.10 (KCl) in 10.0%(v/v) dioxane–1.0%(v/v) *N*,*N*-dimethylformamide–water[a]

Catalyst species[b] (E)	$k_{\text{obsd}}\times 10^4$ (s^{-1})	$k_c\times 10^4$ [c] (s^{-1})	k_c/[E] (s^{-1} M^{-1})	pH
BCP-1 (0.49×10^{-6} M)	3.13	1.03	210	12.30
CP-3 (7.63×10^{-6} M)[d]	2.10	0	—	12.28
PCP-1 (2.55×10^{-5} M)	1.39	inhibition	—	12.30
BCP-2 (0.51×10^{-5} M)	1.12	inhibition	—	12.26
Imidazole (0.987×10^{-2} M)	2.60	0.50	5.07×10^{-3}	12.30
OH$^-$ [e]	2.10	—	1.05×10^{-2}	12.30

a) Initial concentration of PNPP, 0.985×10^{-5} M. b) The concentration of each catalyst (E) is indicated in the parentheses right after its name. c) $k_c=k_{\text{obsd}}-k_{\text{hyd}}$. d) The maximum attainable concentration under the present conditions. e) Alkaline hydrolysis.

ficient hydrophobicity as observed for the paracyclophane catalysis.[2] The rate constants for the hydrolysis of PNPP in the presence of various synthetic catalysts are summarized in Table 4. Among the synthetic cyclic peptides employed, only BCP-1 showed a significant catalytic activity toward PNPP. On the other hand, PCP-1 and BCP-2 inhibited the alkaline hydrolysis of PNPP. This indicates that both cyclic compounds can incorporate PNPP into their cyclic skeletons, preventing the hydroxide attack on the bound substrate. It seems that hydrophobicity provided by the single layered structure of CP-3 in aqueous media is not sufficient to incorporate PNPP. The apparent second-order rate constant ($k_c/[E]$) for the hydrolysis catalyzed by BCP-1 is larger than that for imidazole catalysis by 4.2×10^4 fold. The high catalytic efficiency of BCP-1 is attributed to the binding capability of its hydrophobic cavity toward the substrate of hydrophobic character.

pH-Dependence of Kinetic Behavior. The pH-rate profile is not the bell-shaped type observed in enzymatic reactions,[12] but a sigmoid as shown in Fig. 1. This indicates that BCP-1 has a functional group or groups which can be activated by deprotonation above pH 12. The active functional group is referred to the anionic imidazole group of the histidyl residue: pK_a (graphically estimated), 12.3. Relative to the corresponding pK_a value for imidazole (14.5 at 25 °C and μ 0.5 in aqueous medium),[13] the pK_a value obtained for BCP-1 is somewhat lower. The intramolecular hydrogen bonding between two imidazole groups in the single molecule may act to reduce the pK_a value since these two imidazole groups in BCP-1 can be placed at the juxtaposition within their hydrogen bonding distance according to the CPK model. The access of these groups would be enhanced by the intramolecular hydrophobic interaction between the two cyclic skeletons. Our attempt to determine the pK_a of the imidazole group of L-His residue of BCP-1 by spectrophotometric or potentiometric titration was not successful due to its limited solubility. Overberger *et al.* observed that the solvolysis rate of *p*-nitrophenyl acetate is enhanced by homopolymers of 4-vinylimidazole and 5-vinylbenzimidazole. They attributed the rate enhancement to the reduction of pK_a of the polymers brought about by internal hydrogen bonding interaction.[14]

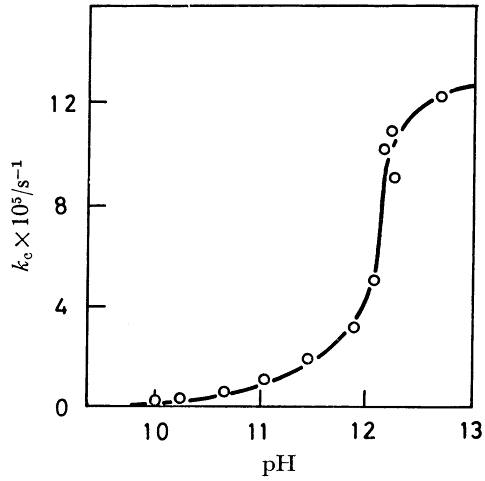


Fig. 1. pH-Rate profile for the BCP-1 catalyzed hydrolysis of *p*-nitrophenyl hexadecanoate (PNPP) in 10.0% (v/v) dioxane–1.0% (v/v) *N,N*-dimethylformamide–water at 40.0±0.1 °C and μ 0.10 (KCl); $k_c=k_{obsd}-k_{hyd}$. Initial concentrations: PNPP, 0.985×10^{-5} M; BCP-1, 0.49×10^{-6} M.

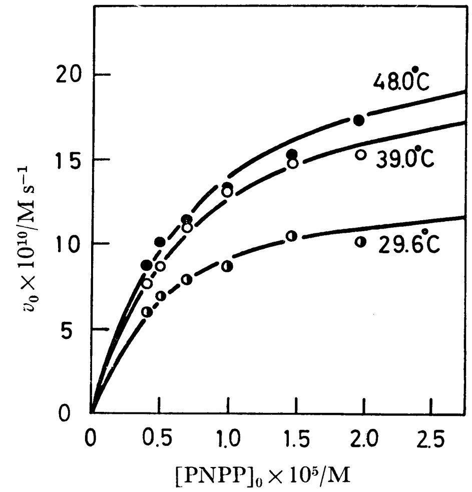


Fig. 2. Saturation-type kinetics for the hydrolysis of *p*-nitrophenyl hexadecanoate as catalyzed by BCP-1 in 10.0% (v/v) dioxane–1.0% (v/v) *N,N*-dimethylformamide–water at pH 12.35 and μ 0.10 (KCl): initial concentration of BCP-1, 0.49×10^{-6} M; v_0, initial reaction velocity; $[PNPP]_0$, initial concentration of PNPP.

Michaelis-Menten Analysis. The initial hydrolysis rate (v_0) as catalyzed by BCP-1 was determined at various concentrations of PNPP. The initial rate was found to level off beyond a certain range of substrate concentration (saturation-type kinetics) as seen in Fig. 2. The kinetic data were analyzed in terms of the Michaelis-Menten kinetics based on the overall reaction pathway given by the following:

$$\begin{array}{ccccccc} E+S & \overset{K_S}{\rightleftharpoons} & (ES) & \xrightarrow{k_2} & (ES)' & \xrightarrow{k_3} & E+P_2 \\ \Big\downarrow k_{hyd} & & & & + & & \\ P_1+P_2 & & & & P_1 & & \end{array} \qquad (1)$$

where P_1 and P_2 stand for the alcohol and the acid moiety, respectively, formed in the hydrolysis of ester substrate S; (ES) and (ES)′ are the Michaelis-type complex and the acylated catalyst, respectively; k_2 and k_3 refer to the rate constants for acylation and deacylation steps performed on the catalyst, respectively. For the three-step kinetic model, apparent Michaelis constant K_m(app) and catalytic coefficient k_{cat} are defined by Eqs. 2 and 3, respectively, on the basis of the steady-state approximation, where $K_s=[E][S]/[ES]$.

$$K_m(\mathrm{app})=\frac{k_3}{k_2+k_3}K_S \qquad (2)$$

$$k_{cat}=\frac{k_2k_3}{k_2+k_3} \qquad (3)$$

The rate of *p*-nitrophenolate (P_1) liberation was found to follow the apparent first-order kinetics for at least 65% conversion of PNPP when [S]>[E], no detectable induction period being observed for all runs of the present study. The observations are consistent with a reaction mechanism which involves complex formation between the substrate and the catalyst followed by the rate-determining acyl-transfer step (k_2) to form acylated catalyst (ES)′. The catalyst species was regenerated through the final fast deacylation step (k_3); so-called turnover behavior was observed. In order to confirm that the acylation step is rate determining, the kinetic data obtained under [S]>[E] for the hydrolysis of substituted phenyl esters of hexadecanoic acid were treated in terms of Hammett equation. A good linear correlation (Fig. 3) indicates that the acylation step is rate determining: Eqs. 2 and 3 can be simplified as $K_m(\mathrm{app})=K_s$ and $k_{cat}=k_2$, respectively. The evaluated ρ-value (3.5) is quite large for the ordinary nucleophilic displacement reaction at the substituted phenyl ester bond. This high sensitivity may result from a tight interaction of the ester bond with the anionic nucleophile in the desolvated cage. It seems that the reaction center is located in the hydrophobic cavity of BCP-1. The Michaelis-Menten parameters obtained by the Lineweaver-Burk plots are given in Table 5.

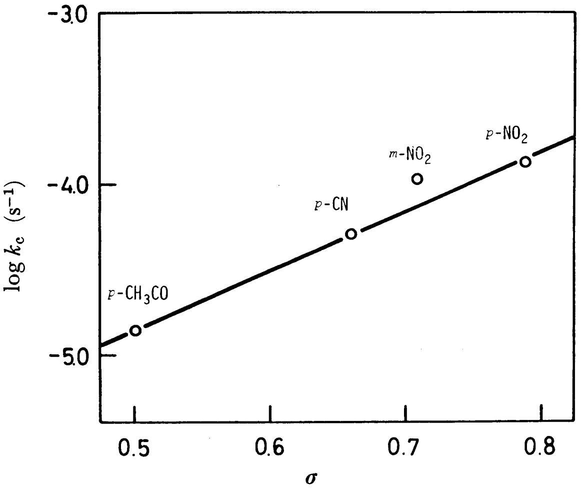


Fig. 3. Correlation of apparent catalytic rate constant k_c with Hammett σ. All runs were carried out in 10.0% (v/v) dioxane-1.0% (v/v) *N,N*-dimethylformamide-water at pH 12.30, 40.0±0.1 °C, and μ 0.10 (KCl). Initial concentrations: BCP-1, 0.49×10^{-6} M; PNPP, 0.985×10^{-5} M; MNPP, 0.984×10^{-5} M; PCPP, 0.984×10^{-5} M; PAPP, 0.981×10^{-5} M.

Nakajima and Okawa investigated the catalytic behavior of *cyclo*(His–Gly–Cys–D-Phe–Gly)$_2$ in the hydrolysis of *p*-nitrophenyl acetate:[3e] $k_{cat}=2.8\times10^{-3}$ s^{-1}, $K_m(\mathrm{app})=2.7\times10^{-3}$ M, $k_{cat}/K_m(\mathrm{app})=1.04$ M^{-1} s^{-1} at pH 7.73 and 24.0 °C. The k_{cat} value for their cyclic peptide is comparable to that for BCP-1 of the present study. However, the $k_{cat}/K_m(\mathrm{app})$ value of the cyclic decapeptide is much smaller than that of BCP-1 by an order of 5×10^2, most likely due to the lower hydrophobic effect of the former. The importance of the hydrophobic effect for incorporation of the bulky substrate was further confirmed by a solvent effect study on the BCP-1 catalyzed hydrolysis. The catalytic ability of BCP-1 completely disappeared when the content of dioxane was raised to 20% (v/v) in reaction media.

The thermodynamic parameters for the substrate-binding process (Table 6) indicate that the formation of a substrate-catalyst complex is primarily controlled by the entropy effect for which the hydrophobic interaction between BCP-1 and PNPP is responsible. In reference to the activation parameters for both BCP-1 catalyzed and alkaline hydrolyses of PNPP, the rate enhancement by BCP-1 (k_{cat}) over the corresponding alkaline hydrolysis (k_{hyd}) is attributed to the enthalpy effect. The unfavorable entropy change is more than compensated by the enthalpy change. The unfavorable entropy effect in the pseudo-intramolecular acyl-transfer was unexpected and might be due to particular steric conformation in the Michaelis-type complex not favorable for the subsequent acylation reaction.

Effect of Mercury(II) Ion. An appropriate metal ion is expected to show a unique effect on the reactivity of BCP-1 since the catalyst has several donor atoms. The mercuric ion gave no effect in the hydro-

TABLE 6. THERMODYNAMIC PARAMETERS FOR THE HYDROLYSIS OF *p*-NITROPHENYL HEXADECANOATE AS CATALYZED BY BCP-1[a)]

Quantity	ΔG_{312K} (kcal/mol)	ΔH (kcal/mol)	ΔS (e. u.)
$K_m(\mathrm{app})^{b)}$	7.33	0±2	−23.5±0.1
$k_{cat}{}^{c)}$	21.6	5.32	−52.7
$k_{hyd}{}^{c)}$	23.5	12.9	−33.9

a) Based on the data given in Table 5. b) $\Delta G^0=-RT\ln K_m(\mathrm{app})=\Delta H^0-T\Delta S^0$. c) $\Delta G^{*}=RT\ln(\kappa T/\mathrm{h}k_r)=\Delta H^{*}-T\Delta S^{*}$; κ, Boltzmann's constant; h, Plank's constant; k_r, rate constant (k_{cat} or k_{hyd}).

TABLE 5. KINETIC PARAMETERS FOR THE BCP-1 CATALYZED HYDROLYSIS OF *p*-NITROPHENYL HEXADECANOATE AT pH 12.32 AND μ 0.10 (KCl)[a)]

Temp (°C)	$v_{max}{}^{b)}\times10^9$ (M s^{-1})	$K_m(\mathrm{app})^{b)}\times10^6$ (M)	$k_{cat}{}^{c)}\times10^3$ (s^{-1})	$[k_{cat}/K_m(\mathrm{app})]\times10^{-2}$ (M^{-1} s^{-1})
29.6	1.39	5.20	2.84	5.46
39.0	2.20	7.39	4.50	6.08
48.0	2.38	7.01	4.86	6.94

a) In 10.0% (v/v) dioxane-1.0% (v/v) *N,N*-dimethylformamide-water with the initial BCP-1 concentration ($[E]_0$) of 0.49×10^{-6} M. b) Kinetic parameters, v_{max} and $K_m(\mathrm{app})$, were evaluated from the initial rates measured at various substrate concentrations ($0.394-1.97\times10^{-5}$ M). c) $k_{cat}=v_{max}/[E]_0$.

TABLE 7. FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF *p*-NITROPHENYL HEXADECANOATE IN THE PRESENCE AND ABSENCE OF MERCURY(II) ION[a)]

$HgCl_2$ (M)	k_{hyd}[b)] $\times 10^3$ (s^{-1})	k_{obsd}[c)] $\times 10^3$ (s^{-1})
None	2.50	3.65
0.988×10^{-6}	2.50	1.96
0.988×10^{-5}	2.50	1.83

a) In 10.0% (v/v) dioxane-1.0% (v/v) *N,N*-dimethylformamide-water; pH, 12.45; μ, 0.10 (KCl); temp, 40.0±0.1 °C. b) Apparent first-order rate constant for the alkaline hydrolysis of PNPP. c) Apparent first-order rate constant for the hydrolysis of PNPP in the presence of BCP-1; initial concentration of BCP-1, 0.49×10^{-6} M.

lysis of PNPP without BCP-1, but considerably retarded the reaction with cooperation of the cyclic peptide (Table 7). The reaction rate was reduced below the catalyst-absence level (k_{hyd}). The result suggests that BCP-1 deactivated by the coordination of the mercuric ion at its imidazole group still holds a significant ability to bind the substrate.

Substrate-Binding Ability of BCP-1 and PCP-1. PCP-1 retarded the alkaline hydrolysis of PNPP by incorporating the substrate into its cavity in line with the kinetic pathway expressed by the following:

$$\begin{tikzcd} S + C \arrow[r, shift left, "K_b"] \arrow[d, "k_{hyd}"] & (SC) \arrow[l, shift left] \arrow[r, "k_{in}"] & P \\ P & & \end{tikzcd} \qquad (4)$$

where cyclic peptide C and substrate S yield association complex (SC) with binding constant K_b, and the free and bound substrates undergo hydrolysis with rate constants k_{hyd} and k_{in}, respectively. The observed pseudo-first-order rate constant (k_{obsd}) is given by

$$k_{obsd} = \frac{k_{hyd} + k_{in}K_b[C]_0}{1 + K_b[C]_0}, \qquad (5)$$

where $[C]_0$ stands for the initial concentration of PCP-1. Equation 5 can be rearranged to give

$$\frac{1}{k_{hyd} - k_{obsd}} = \frac{1}{k_{hyd} - k_{in}} + \frac{1}{(k_{hyd} - k_{in})K_b[C]_0}. \qquad (6)$$

A good linear relationship has been obtained by plotting $1/(k_{hyd} - k_{obsd})$ against $1/[C]_0$. The values of k_{in} and K_b obtained are $1.68 \times 10^{-4}\ s^{-1}$ and $8.47 \times 10^4\ M^{-1}$, respectively, at pH 12.3, 40.0±0.1 °C, and μ 0.10. The hydrolysis constant for the bound PNPP is smaller than that for the free ($k_{hyd} = 2.91 \times 10^{-4}\ s^{-1}$) by 1.7-fold. The binding constant for the complex of PCP-1 and PNPP is smaller than that for the complex of BCP-1 and PNPP by 1.6-fold. BCP-2 also retarded the alkaline hydrolysis of PNPP to a similar extent to that observed for PCP-1 (Table 4). CP-3 did not give out any meaningful effect on the alkaline hydrolysis of PNPP. The result indicates that the hydrophobic effect provided by the CP-3 cavity is not enough to incorporate PNPP.

Conclusion. Since CP-3 has no catalytic activity in the hydrolysis of PNPP, depth of the single-layered cyclic peptide does not seem to provide sufficient hydrophobic effect to incorporate a hydrophobic substrate. On the other hand, BCP-1, being a double-layered bicyclic species, provides a strong hydrophobic cavity. The efficient hydrophobic nature of the double-layered cyclic peptide was further substantiated by investigation on the substrate-binding property of BCP-2. Although we were not successful in developing an efficient papain model, the thiol group of L-Cys residue not being left active upon disulfide-bond formation during the course of CP-1 synthesis, the apparent second-order rate constant (k_{cat}/K_m(app)) for the PNPP hydrolysis effected by BCP-1 is comparable to those for the papain-catalyzed hydrolysis of esters and amides.[15)] The overall rate enhancement by BCP-1 is apparently provided by a much stronger hydrophobic binding ability than that of papain and chymotrypsin toward the present particular substrate. In order to develop a more effective hydrolase model, the steric control needs to be exercised toward a bound substrate, so that the unfavorable entropy effect observed in the acylation step of the present study would be markedly improved.

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