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Origin of Rate-Acceleration in Ester Hydrolysis with Metalloprotease Mimics

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Abstract—Mimics of carboxypeptidase A, a prototypical metalloprotease, were synthesized by linking macrocyclicpolyamines to the primary side of β-cyclodextrin followed by complexing with Zn(II). These enzyme mimics exhibit saturation kinetics in hydrolysis of *p*-nitrophenyl acetate (PNPA) and enhance the rate of hydrolysis reaction by almost 300-fold. The effective molarities (EM) of the mimics range from 0.2 to 1.9 M. Origin of the rate acceleration was examined: the reactivity of Zn(II) complexes of [12]aneN_3 [12]aneN_4, and [14]aneN_4 for hydrolyzing PNPA increases with increase in basicity of the zinc bound hydroxides [Zn(II)–OH], yielding a linear Brönsted plot. Free hydroxide fits well on this plot. A similar plot was obtained with the enzyme mimics. The Brönsted relationships indicate that the Zn(II)–OH in the catalytic systems hydrolyzes the ester by direct nucleophilic attack on the ester carbonyl of cyclodextrin-bound but not Zn(II)-coordinated PNPA.

Introduction

Carboxypeptidase (CPA) is a much studied zinc protease which cleaves polypeptide substrates and esters. [1] The essential constituents of the active site of the enzyme are a hydrophobic pocket for substrate recognition and binding, the zinc ion that is coordinated to two imidazole nitrogens of His-69 and His-196 and the carboxylate of Glu-72, and the catalytically essential carboxylate of Glu-270.[1] A water molecule is coordinated to the zinc ion as the fourth ligand. The scissile peptide carbonyl group of the incoming substrate is known to coordinate also to the zinc ion, which results in the activation of the carbonyl carbon for a nucleophilic attack. Much has been learned about CPA through kinetic and mechanistic studies, site-directed mutangenesis, and X-ray crystallography, but details of the chemistry that occurs in the catalytic process is still a matter of debate.

Christianson and Lipscomb proposed that the zinc bound water molecule that is activated by the metal ion and the carboxylate of Glu-270 serves as a nucleophile, attacking at the carbonyl carbon of the scissile peptide bond of substrate to form a tetrahedral transition state that collapses to products (Fig. 1).[2]

Recently, Kimura et al. reported that Zn(II) forms a stable and structurally well defined complex with [12]aneN_3 and a molecule of water.[3] The pK_a value of the zinc bound water molecule was determined to be 7.3 (Fig. 2).[4] This water molecule is thus a strong nucleophile comparable to the Zn(II) bound water molecule of carbonic anhydrase and the complex was thought to be an excellent mimic of the enzyme. Furthermore, Kimura et al. demonstrated that the Zn(II)–[12]aneN_3 complex accelerates the hydrolysis of not only *p*-nitrophenyl acetate (PNPA) but also methyl acetate in neutral aqueous medium.[4]

We thought that [12]aneN_3 can also mimic the three ligands that coordinate to the Zn(II) at the active site of

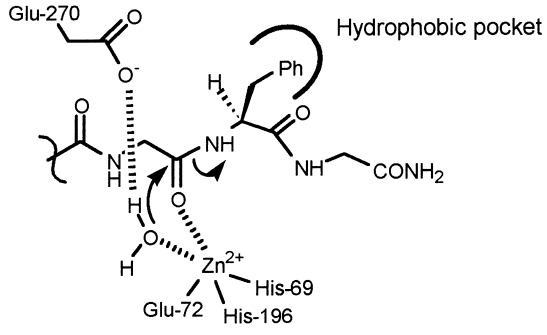

Figure 1. Schematic representation of the general base mechanism proposed for the enzymatic hydrolysis of peptide substrate by carboxypeptidase A.

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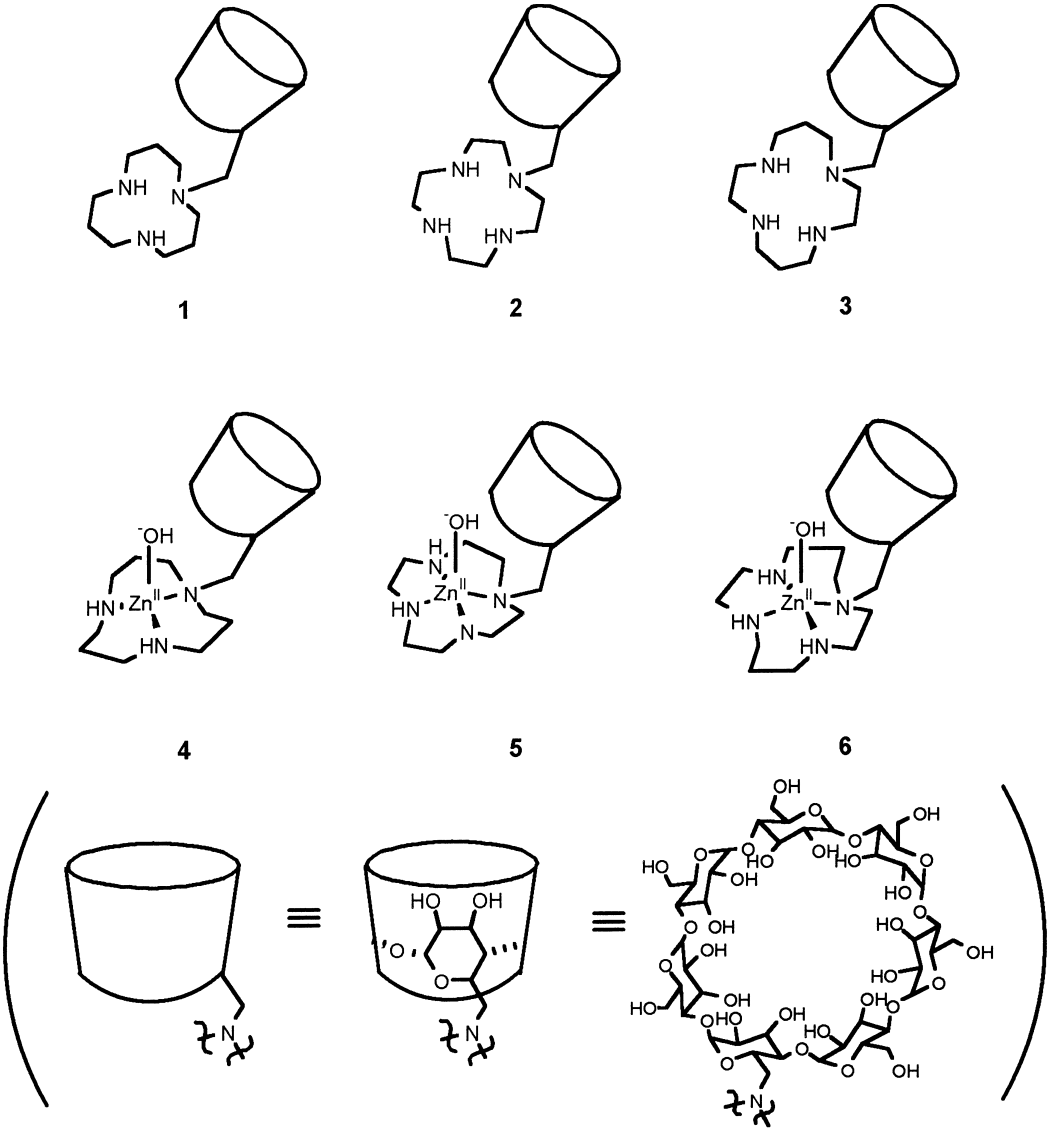
CPA and thus may be useful for the construction of CPA models. However, since CPA bears the P_1' hydrophobic pocket for substrate recognition and binding as discussed above, it was thought to be necessary for the mimics of CPA to have a binding pocket that is structurally complimentary to the substrate in addition to the Zn(II) ligand. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide having the overall shape of a truncated cone.[5] The cone is hydrophobic and forms an inclusion complex with a phenyl ring in water. Thus, β-CD was thought to be a viable mimic of the hydrophobic pocket at the CPA active site. In this paper we describe compounds **4**, **5**,[6] and **6** that are constructed as mimics of CPA by linking a macrocyclicpolyamine to the primary side of β-CD followed by the addition of Zn(II), and their evaluation as CPA models.

Results and Discussion

Compound **1** was synthesized by allowing 6-deoxy-*O*-tosyl-β-CD prepared by the literature method[7] with a modification to react with [12]aneN$_3$ at 100 °C in DMF solution. The crude product, thus obtained, was purified by CM–Sephadex chromatography followed by recrystallization from water/methanol. The structure and the purity of the product were confirmed by ^{1}H NMR, ^{13}C NMR, mass spectroscopy and elemental analysis. In a similar fashion, compounds **2** and **3** were synthesized. Zinc complexes, **4**, **5** and **6** were prepared in situ by adding an equimolar amount of zinc chloride to the pH 7.0 Tris buffer solution containing the respective macrocyclicpolyamine, i.e. **1**, **2** and **3**.

The enzyme mimic (catalyst) is thought to bind the substrate to form a complex that undergoes a chemical reaction to yield products with regeneration of the catalyst (Scheme 1). The breakdown of the complex to the product occurs with the first-order rate constant $k_{cat.}$ The rate constant for the conversion of the substrate into the product in the absence of the catalyst is represented by k_{un}. The observed first order rate constant (k_{obs}) and the dissociation constant (K_m) of the complex may be expressed by eqs (1) and (2), respectively. In



these equations, $[P]$, $[C]_0$ and $[S]_0$ represent the concentration of the product, enzyme mimic and substrate, respectively.

$$d[P]/dt = k_{obs}\{[S]_0 + [C\cdot S]\} = k_{un}[S]_0 + k_{cat}[C\cdot S] \quad (1)$$

$$K_m = \{[C]_0 + [S]0\}/[C\cdot S] \quad (2)$$

Since $[C\cdot S] = [C]_0[S]_0/K_m$, eq (1) may be rewritten to give eq (3) which may be further transformed to eq (4):

$$k_{obs} = (K_m k_{un} + k_{cat}[C]_0)/(K_m + [C]_0) \quad (3)$$

$$(k_{obs} - k_{un})/[C]_0 = -k_{obs}/K_m + k_{cat}/K_m \quad (4)$$

The kinetic studies were carried out at 25 °C in pH 7.0 buffered solution using PNPA as substrate. The hydrolysis of the substrate generates *p*-nitrophenolate which was monitored spectrophotometrically at 400 nm. Under the conditions of a large excess of catalyst over the substrate ($[S]_0 = 1.0 \times 10^{-4}$ M), good pseudo-first-order rate constants (k_{obs}) were obtained. The saturation kinetics were observed for all three enzyme mimics as shown in Figure 3, satisfying the Michaelis–Menten kinetics required as enzyme mimics. We plotted the k_{obs} values for PNPA hydrolysis at varied concentrations of mimic **4** against $(k_{obs} - k_{un})/[\text{CPA mimic}]$ according to eq (4) and obtained k_{cat} and K_m values of 7.72×10^{-4} s^{-1} and 3.32 mM, respectively (Fig. 4) and are collected in Table 1 along with the kinetic parameters obtained with the other two mimics. Highest rate increase was observed with **4** which accelerates the hydrolysis of PNPA by 291-fold. In principle, metal ions can accelerate the rate of hydrolysis of ester by (1) activating the carbonyl group (mechanism A), (2) activating the leaving group (mechanism B), or (3) providing a metal–hydroxide nucleophile (mechanism C).[8] A combination of these activations is also possible for the hydrolysis of a given ester. In order to distinguish between the possible mechanisms that might operate for the present system, we analyzed the reactivities of the Zn(II)–cyclopolyamine complexes for hydrolyzing PNPA. The second order rate constant for the Zn(II)–hydroxide promoted hydrolysis of PNPA increases with increase in the basicity of the Zn(II)–hydroxide to generate a Brönsted plot with $\beta = 0.35$ (Fig. 5) and we found that zinc-free hydroxide fits well on this plot. This observation suggests,

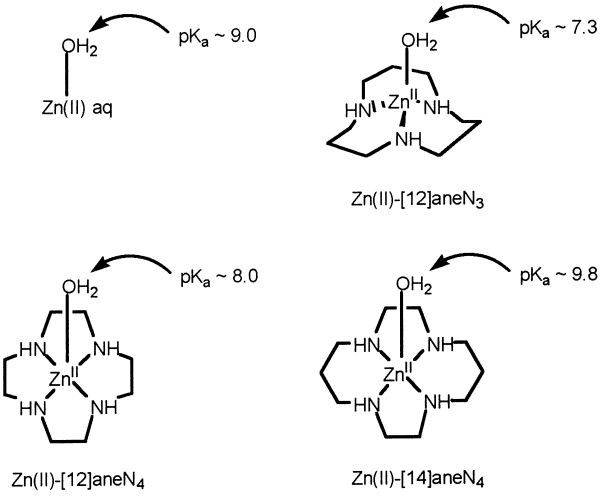
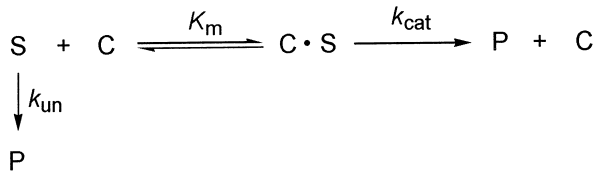


Figure 2. pK_a values of the water molecule bound to Zn(II)-macrocyclicpolyamide.[3]



Scheme 1.

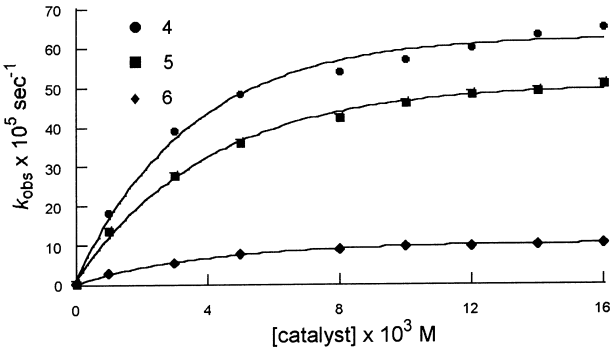


Figure 3. Plots of k_{obs} versus concentrations of enzyme mimic (**4**, **5** and **6**) in the hydrolysis of PNPA.

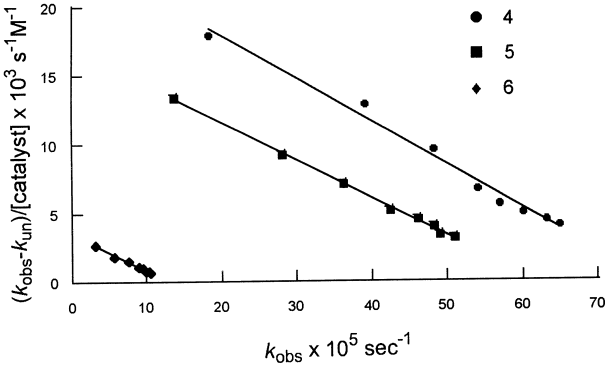


Figure 4. Plots of $(k_{obs}–k_{un})/[\text{CPA mimic}]_0$ versus k_{obs} for the hydrolysis of PNPA by **4**, **5** and **6**. In the plot the slope of the straight line represents $-1/K_m$ and the *y*-intercept corresponds to k_{cat}/K_m, the second order rate constant.

Table 1. Kinetic constants for the hydrolysis of PNPA at pH 7.0 (Tris buffer) and 25 °C in the presence of the enzyme mimic (catalyst)

Catalyst	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/k_{un} [a]	EM (M)
Zn(II)–[12]aneN$_3$			3.6×10^{-3}		
Zn(II)–[12]aneN$_4$			1.8×10^{-3}		
Zn(II)–[14]aneN$_4$			0.75×10^{-3}		
4	7.72×10^{-4}	3.32	233×10^{-3}	291	0.21
5	6.21×10^{-4}	3.64	171×10^{-3}	234	0.35
6	1.28×10^{-4}	3.70	34.6×10^{-3}	48	0.17

[a]The value of k_{un} for the hydrolysis of PNPA was reported to be 2.65×10^{-6} s^{-1} (Breslow, R.; Nesnas, N. *Tetrahedron Lett*. **1999**, *40*, 3335).

strongly, that the mechanism for the hydrolysis reactions involves direct nucleophilic attack of the metal-hydroxide on the uncoordinated ester (mechanism C). The pK_a value of the Zn(II) bound water molecules in **4**, **5** and **6** were determined to be lower compared with the p*K*a of the water molecule bound to the corresponding Zn(II)–cyclicpolyamines, i.e. Zn(II)–[12]aneN_3, Zn(II)–[12]aneN_4 and Zn(II)–[14]ane N_4, respectively by about 0.05 unit. Figure 5 also shows the Brönsted plot ($\beta = 0.42$) constructed using the pK_a values obtained with the enzyme mimics. It can be seen that the Co(III)-cyclen attached β-CD that was reported by Akkaya and Czarnik[9] also fits reasonably well on the Brönsted plot. Hence, we hasten to propose that the direct nucleophilic attack of the Zn(II)-hydroxide on cyclodextrin-bound but not the Zn(II)-coordinated ester is also in operation for the enzyme mimic promoted hydrolysis of PNPA. Contrary to expectation, the carbonyl group of PNPA in the present models does not appear to be activated by the Zn(II). The present results are in accord with the mechanism that the zinc bound water molecule at the active site of CPA is sufficiently nucleophilic and thus attacks directly on the scissile bond of the substrate.[2,10,11] It is worth noting that by being the substrate inserted in the hydrophobic pocket of the β-CD the rate of ester hydrolysis is enhanced by about two orders of magnitude (Fig. 5).

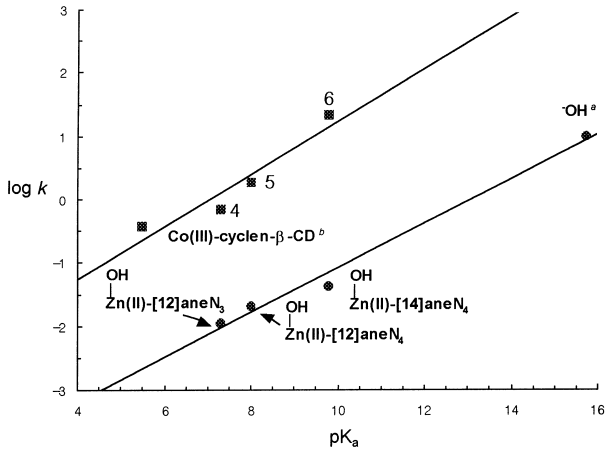
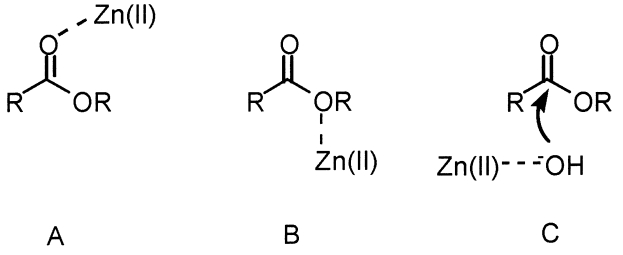


Figure 5. Brönsted plots for the hydrolysis of PNPA by enzyme mimics and Zn(II)–cyclicpolyamine complexes. The second order rate constants are corrected for Zn(II)–OH concentration. [a] From ref 9. [b] Jencks, W.P.; Gilchrist, M. *J. Am. Chem. Soc.* **1968**, *90*, 2622.



The mechanistic pathway for the hydrolysis of PNPA by the mimics at neutral pH may be represented by Figure 6. Firstly, the phenyl ring of the substrate fits in the hydrophobic cone of β-CD to form a complex. A nucleophilic attack by the Zn(II)-hydroxide on the ester carbonyl carbon would then ensue to generate a tetrahedral transition state which collapses to the products. Since we failed to observe catalytic turnover with the present mimics, it appears that the *p*-nitrophenolate that is formed remains mostly in the cone of β-CD. Analogous reaction paths are also envisioned for the PNPA hydrolysis in the presence of **5** or **6**.

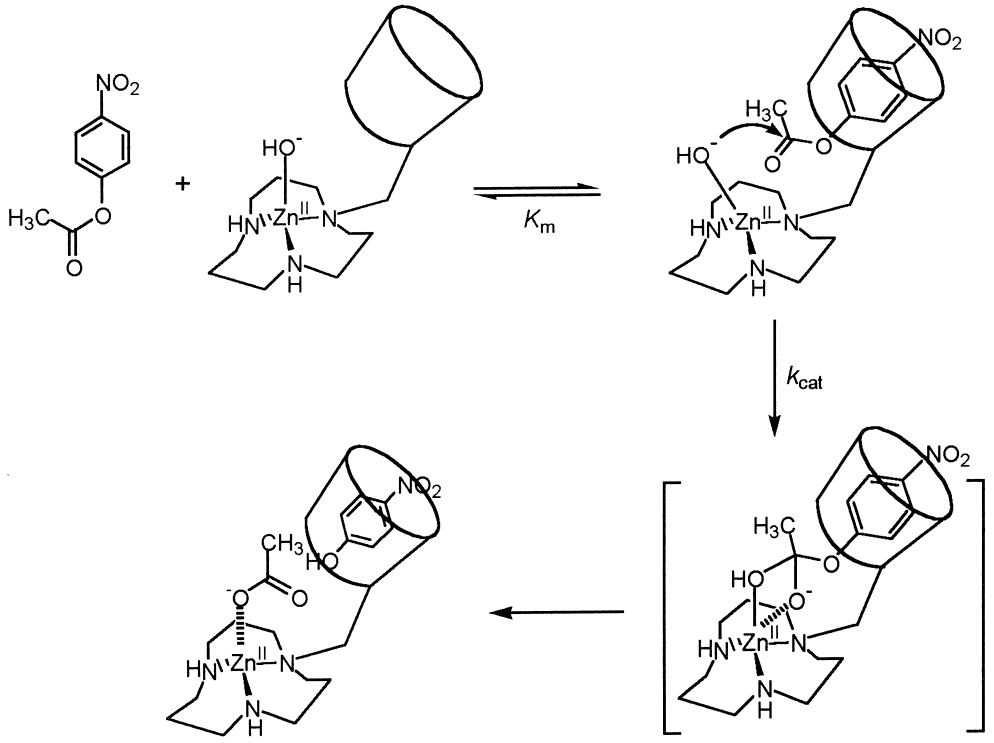


Figure 6. Schematic representation of the PNPA hydrolysis catalyzed by **4**.

It is well known that catalytic groups can provide much greater rate-acceleration in intramolecular reactions than in intermolecular reactions. The effective molarity (EM) defined as the ratio of the rate constant for the intramolecular reaction to that of the corresponding intermolecular reaction is a parameter that reflects the effectiveness of a catalytic system.[12] Effective molarities in the range of 10^5–10^8 M are common for intramolcular nucleophilic catalytic reactions. We were interested in finding out what the effective molarities would be of the enzyme mimics for hydrolyzing PNPA. The EM values of 0.21, 0.35 and 0.17 M are found for **4**, **5** and **6**, respectively (Table 1). Although the effective molarities are large, they are not as enormous as in a carefully designed covalent system. This is not surprising in view of the loose association between the substrate and the enzyme mimics and the flexible linker between the metal complex and β-CD.

Conclusion

We have synthesized mimics of zinc proteases by bridging Zn(II) complexes of macrocyclicpolyamines to β-CD. These enzyme mimics exhibit saturation kinetics in hydrolysis of PNPA as enzymes do, and promote the hydrolysis reaction by almost 300-fold. The effective molarities of these systems range from 0.17 to 0.35 M. From the analysis of the Brönsted plots obtained with the mimics and the Zn(II)-macrocyclicpolyamines for the PNPA hydrolysis, it was concluded that the Zn(II)-bound water molecule in the mimics attacks on the ester carbonyl of the PNPA that is CD-bound but not Zn(II)-coordinated.

Experimental

Melting points (MP) were determined on a Thomas–Hoover capillary MP apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker FT-NMR spectrometer (300 or 500 MHz) and chemical shifts are expressed in ppm relative to tetramethylsilane. Samples were dissolved in a mixture of deuterium oxide and deuteriomethylsulfoxide. Low resolution mass spectra were obtained with a KRATOS MS 25 RFA spectrometer. Kinetic study was carried out using a Perkin–Elmer HP 8453 UV–vis spectrometer. Elemental analyses were performed at POSTECH (CBM), Pohang, South Korea. Purification of cyclodextrin derivatives were performed by CM-25 Sephadex chromatography. All chemicals were of reagent grade obtained from Aldrich Chemical Co. *N,N*-Dimethylformamide was distilled over magnesium sulfate and stored under nitrogen.

Mono-6-deoxy-6-(*p*-toluenesulfonyl)-β-cyclodextrin (β-CD-6-OTs). A solution of *p*-toluenesulfonyl chloride (1.5 g, 7.9 mmol) in anhydrous pyridine (10 mL) was added with stirring to a solution of β-CD (5.0 g, 4.4 mmol, dried under vacuum at 100 °C for 12 h) dissolved in anhydrous pyridine (50 mL). After 5 h at 4 °C, pyridine was removed under reduced pressure at 40 °C and a light yellowish syrup was obtained. Upon the addition of acetone (300 mL) to the residue a colorless solid precipitated which was collected by suction filtration and washed with acetone. Recrystallization of the crude product from water yielded an analytically pure sample (1.8 g, 30% yield) as a white crystal: mp 168–170 °C (dec.); ^{1}H NMR (DMSO-d_6) δ 2.43 (3H, s), 3.33–3.57 (42H, m), 4.32 (6H, s), 4.84 (7H, s), 5.67 (14H, s), 7.43 (2H, d), 7.76 (2H, d); ^{13}C NMR (DMSO-d_6) δ 21.80, 59.76, 71.90, 72.88, 81.36, 101.73, 127.35, 129.66, 132.47, 144.56.

Mono-6-deoxy-6-(1,5,9-triazacyclododecanyl)-β-cyclodextrin (1). Dried β-CD-6-OTs, (0.65 g, 0.5 mmol) was dissolved in DMF (20 mL) and 1,5,9-triazacyclododecane (0.43 g, 2.5 mmol) was added to this solution. The resulting mixture was heated at 100 °C for 5 h under N_2 atmosphere. After completion of the reaction, the mixture was evaporated to dryness in vacuo at 40 °C and acetone (100 mL) was added to the residue. The solid thus formed was collected by suction filtration and washed with acetone. The residue was purified by CM-25 Sephadex chromatography eluting with water followed by 0.1 M NH_4OH to give an analytically pure sample (0.46 g, 72% yield) as a white solid mp 232 °C (dec.); ^{1}H NMR (D_2O) δ 2.08–2.18 (6H, m), 3.27–3.34 (12H, m), 3.43–4.20 (42H, m), 5.06 (7H, s); ^{13}C NMR (D_2O) δ 27.48, 47.93, 59.76, 71.90, 72.88, 81.36, 101.73; Fab-MS (*m*/*z*) 1288 (M + 1); anal. calcd for $C_{51}H_{89}N_3O_{34}\cdot 4.5H_2O$: C, 44.73; H, 7.21; N, 3.07. Found: C, 44.41; H, 6.98; N, 3.12.

Mono-6-deoxy-6-(1,4,7,10-tetraazacyclododecanyl)-β-cyclodextrin (2). This compound was synthesized in 64% yield by the same procedure as described above mp 246 °C (dec.); ^{1}H NMR (D_2O) δ 2.73–2.78 (16H, m), 3.45–4.18 (42H, m), 5.07 (7H, s); ^{13}C NMR (D_2O) δ 46.03, 59.66, 71.88, 73.08, 81.38, 101.79; Fab-MS (*m*/*z*) 1289 (M + 1); anal. calcd. for $C_{50}H_{88}N_4O_{34}\cdot 6.5H_2O$: C, 42.70; H, 7.24; N, 3.98. Found: C, 42.59; H, 7.06; N, 3.99.

Mono-6-deoxy-6-(1,4,8,11-tetraazacyclotetradecayl)-β-cyclodextrin (3). This compound was synthesized in 52% yield by the same procedure as described above: mp 254 °C (dec.); ^{1}H NMR (D_2O) δ 1.78–1.84 (4H, m), 2.75–2.80 (8H, m), 2.83–2.90 (8H, m), 3.45–4.20 (42H, m), 5.09 (7H, s); ^{13}C NMR (D_2O) δ 29.85, 40.63, 42.82, 60.06, 70.98, 73.08, 81.86, 101.51; Fab-MS (*m*/*z*) 1317 (M + 1); anal. calcd. for $C_{52}H_{92}N_4O_{34}\cdot 6H_2O$: C, 43.00; H, 7.22; N, 3.86. Found : C, 42.87; H, 7.07; N, 3.92.

Kinetics

Hydrolysis rate of *p*-nitrophenyl acetate in aqueous solution in the presence of an enzymic mimic was measured by following the increase in the 400 nm absorption using a computer-linked UV spectrometer. The reaction solution was maintained at 25 °C using a buffered solution containing 0.05 M Tris buffer (pH 7.0), the ionic strength of which was adjusted to 0.10 with sodium chloride. The typical procedure was as follows: the enzyme mimic, zinc chloride (both final concentrations of 1.0–10.0 mM) and *p*-nitrophenyl acetate (the final concentration of 0.1 mM) in 5% acetonitrile

solution were mixed and the absorption at 400 nm was recorded as a function of time. The observed rate constants (k_{obs}) were obtained directly from the computer interfaced UV spectrometer. All experiments were run in duplicate and the tabulated data represent the average of these experiments.

Acknowledgements

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