Cyclen-Containing Inhibitors of Carboxypeptidase A Synthesized in Search of Target-Selective Artificial Proteases†

Jung Bae Song, Sang Soo Hah, and Junghun Suh*

Department of Chemistry, Seoul National University, Seoul 151-747, Korea
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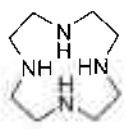
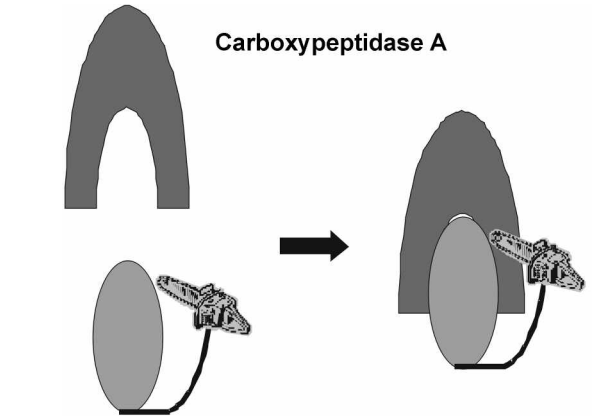
Previously, we reported the first substrate-selective artificial protease by using myoglobin as the substrate.[1-3] Target-selective peptide-cleaving catalysts can be used as drugs if the target is a protein or an oligopeptide related to a disease. For example, a peptide-cleaving catalyst specifically recognizing HIV protease and rapidly cleaving it into two pieces can be used as a new drug for AIDS. Since only a catalytic amount of the drug is needed, the drug dosage and the side effects can be reduced by using the peptide-cleaving catalysts.

The myoglobin-cleaving catalysts reported previously were designed by attaching a catalytic group to a binding site that recognizes the surface of myoglobin.[1,3] The Cu(II) or Co(III) complex of cyclen (Cyc) was used as the catalytic group in view of their catalytic activity in peptide hydrolysis. Thus, connection of a catalytic group to a binding site can be considered as a general method for creation of target-selective peptide-cleaving catalysts. The binding site may recognize either a certain portion on the surface of the target protein as in the case of the myoglobin-cleaving catalyst mentioned above or the active site of the target protein. In view of a vast amount of ligand molecules reported to have high affinity toward many disease-related proteins, the known ligand molecules may be utilized as the binding site of the peptide-cleaving catalysts. In this regard, we undertook synthesis of peptide-cleaving catalysts selective for carboxypeptidase A (CPA) by using CPA inhibitors as the binding site of the catalysts. The idea of designing a CPA-selective peptide-cleaving catalyst based on a known CPA inhibitor is illustrated in Scheme 1.

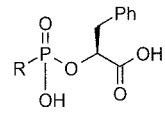


Cyc

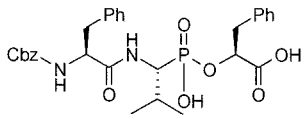
Among many inhibitors of CPA reported in the literature, phosphonate analogues of CPA substrates such as A_1-A_5 are the most potent.[4,5] The X-ray crystallographic study performed on the phosphonate inhibitors disclosed that the L-β-phenyllactic acid (PLA) moiety containing the hydroxyalkylphosphinyl group occupied the active site.[6] Thus, the hydroxyalkylphosphinyl ester (A_0) of PLA can be exploited as binding sites of the CPA-cleaving catalysts. In this study, we synthesized various derivatives of Cyc containing A_0. We speculated that those Cyc derivatives might be converted to effective CPA-cleaving catalysts upon binding of Cu(II) or Co(III) in view of their ability to recognize the active site of CPA. Structures of the Cyc-containing inhibitors synthesized



Scheme 1. Designing CPA-selective peptide-cleaving catalysts based on a known CPA inhibitor.



A_0



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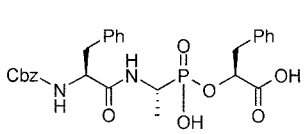

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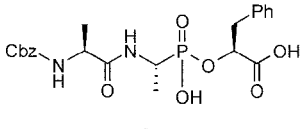

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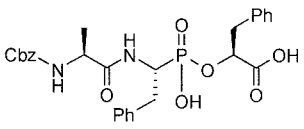

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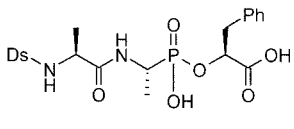

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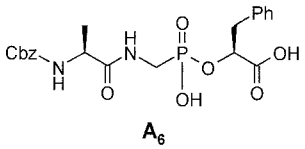


A_6

†This paper is dedicated to Professor Yong Hae Kim for his distinguished achievements in chemistry.

*Corresponding Author. e-mail: jhsuh@snu.ac.kr

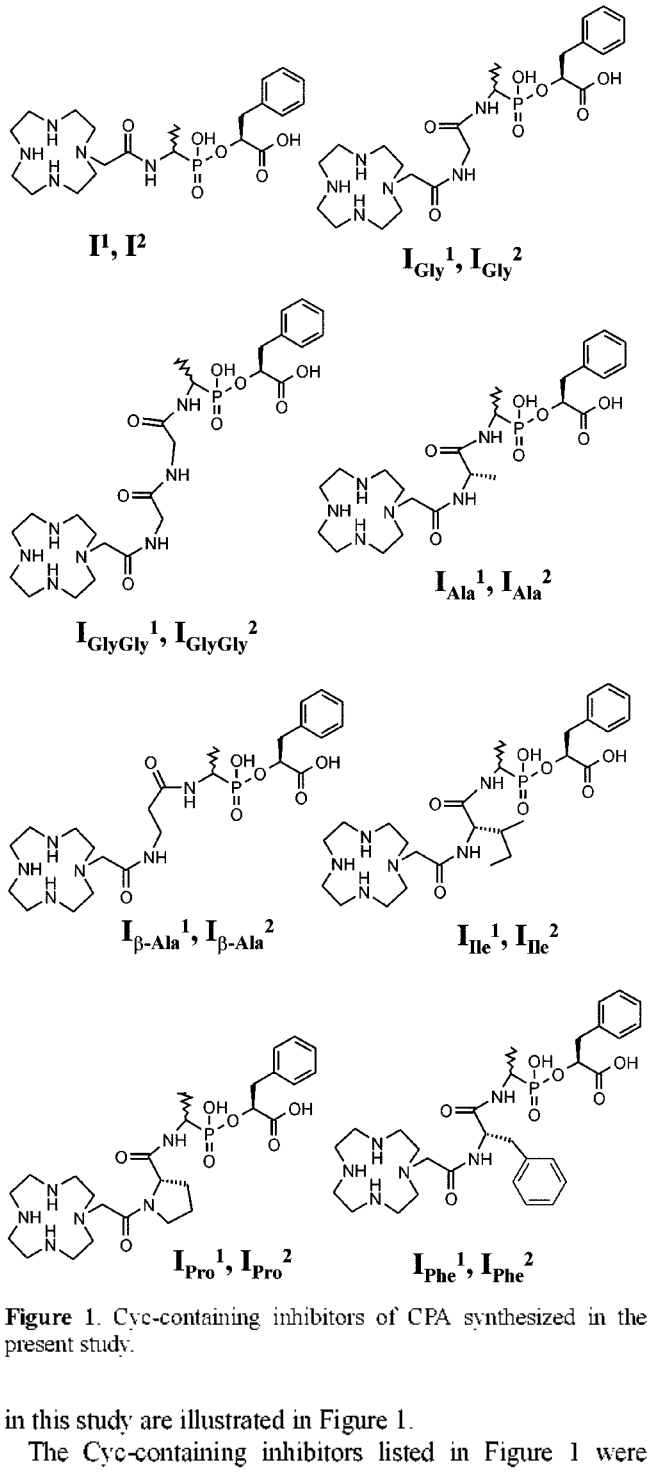


Figure 1. Cyc-containing inhibitors of CPA synthesized in the present study.

in this study are illustrated in Figure 1.

The Cyc-containing inhibitors listed in Figure 1 were prepared according to the synthetic route summarized in Scheme 2. Since the enantiomeric mixture of **1** was used in the synthesis, diastereomeric mixtures were obtained for the Cyc-containing inhibitors. The diastereomeric mixture was separated by HPLC as described in the Experimental Section and the isomer with the shorter elution time was denoted by superscript 1 in the nomenclature and the one with the longer elution time by superscript 2. As will be shown later, the isomers with longer elution times were more potent inhibitors for CPA compared with the respective isomers with shorter elution times. Since it is well established that L-configuration at the chiral carbon has much greater affinity toward CPA.[4,5] L-configuration was assigned to the stereoisomers with longer elution times. Concentrations of the Cyc-containing inhibitors separated by HPLC were determined by spectral titration using $CuCl_2$ by following the formation of Cu(II) complex of Cyc.

Inhibition constants (K_i) for the Cyc-containing inhibitors in the action of CPA were measured without insertion of Cu(II) or Co(III) ion into the Cyc moieties. The kinetic data were collected by using *p*-chlorocinnamoyl PLA (ClCPL)[7,8] as the substrate by following the disappearance of the substrate (S) spectrophotometrically at 315 nm. As reported previously,[8] the CPA-catalyzed hydrolysis of esters like ClCPL is complicated by product inhibition by PLA and the plots of ln [S] against time deviate from the pseudo-first-order kinetic behavior. Even for the enzymatic reactions competitively inhibited by the product, the initial rate is expressed as eq. (1) under the conditions of $S_o >> E_o$. From the dependence of the initial rate constant, k_{in} (= v_o/S_o), on the initially added substrate concentration (S_o). k_{cat} and K_m can be estimated.

$$v_o = (-d[S]/dt)_{t=o} = k_{in}S_o = k_{cat}E_oS_o/(K_m + S_o) \quad (1)$$

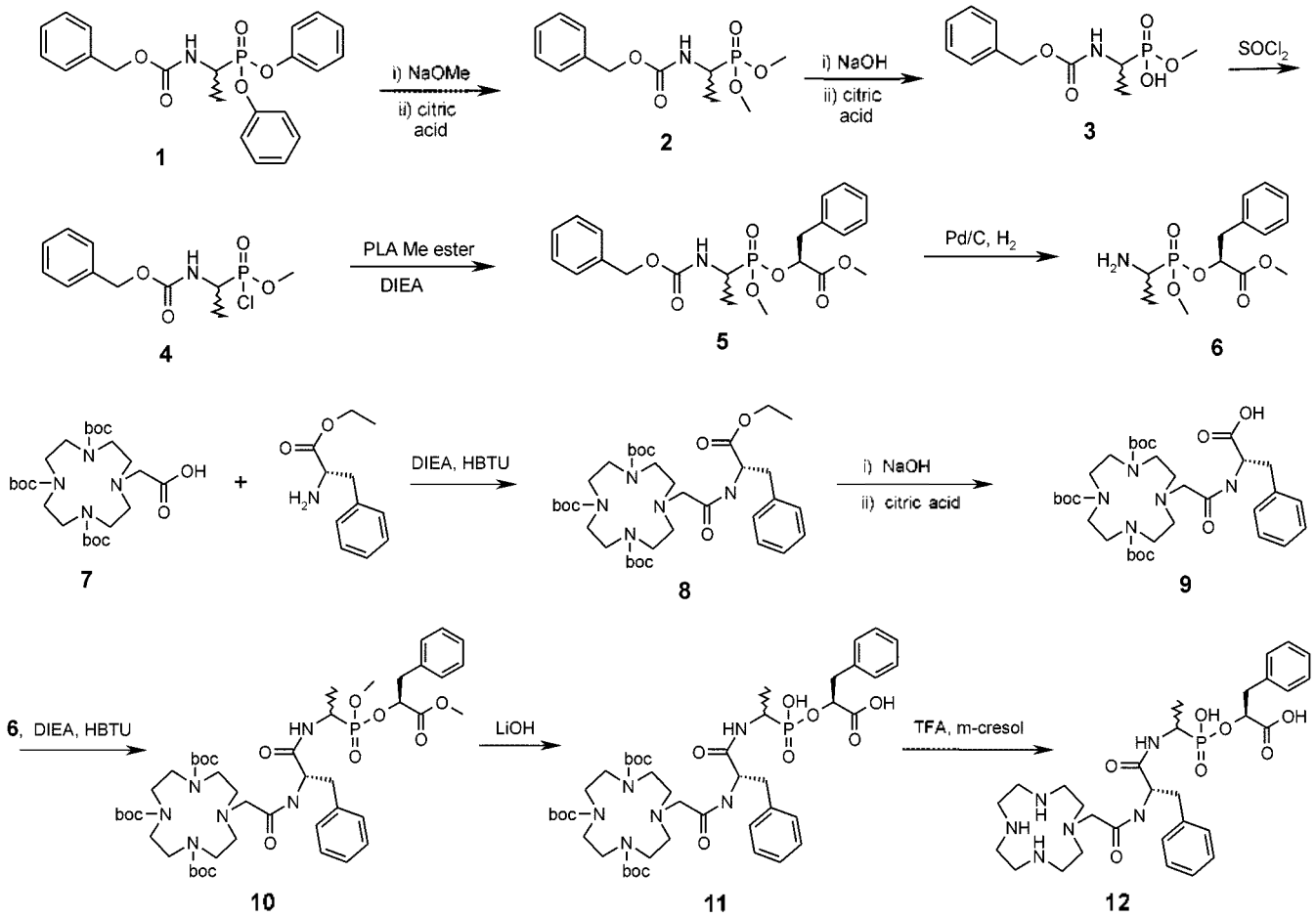
We estimated k_{in} by fitting the absorbance change observed during the CPA-catalyzed hydrolysis of ClCPL according to eq. (2). Here, A_i and A_p represents the absorbance reading for the reaction mixture measured at time t_i and the absorbance reading of the product solution measured after completion of the reaction, respectively. The term ln ($A_i - A_p$) is related to ln [S]$_i$ and the term at_i^2 reflects the degree of deviation from the pseudo-first-order kinetic behavior. By least-square analysis using a computer program (Sigma Plot), the best parameter values were estimated for a, b, and c. Since b is the slope of the tangent line of ln ($A_i - A_p$) drawn at the initial reaction time, b is taken as k_{in}. The k_{in} values measured at various S_o values were fitted to eq. (1) by the Sigma Plot program: k_{cat} of 109 ± 5 s^{-1} and K_m = 82.0 ± 10.2 μM (k_{cat}/K_m = 1.33 × 10^6 $s^{-1}M^{-1}$) were obtained which may be compared with the literature values[8] of 144 s^{-1} and 136 μM (k_{cat}/K_m = 1.06 × 10^6 $s^{-1}M^{-1}$), respectively, measured with a different isozyme of CPA.

$$-\ln (A_i - A_p) = at_i^2 + bt_i + c \quad (2)$$

In the presence of a competitive inhibitor with initial concentration of I_o, the initial rate is expressed as eq. (3) under the conditions of $S_o >> E_o$ and $I_o >> E_o$. By fixing S_o and E_o at constant values, k_{in} values were measured at various I_o values. By analyzing the dependence of k_{in} on I_o, K_i values were estimated. For correct estimation of K_i, I_o should be neither too large nor too small compared with K_i.

$$k_{in} = k_{cat}E_o/(K_m + I_oK_m/K_i - S_o) \quad (3)$$

Equilibrium between CPA and the inhibitors can be reached within less than a few minutes when I_o is sufficiently high. In the case of very potent inhibitors, the initial equilibrium mixtures obtained by mixing CPA and the inhibitor was further diluted in order to lower I_o to a value



Scheme 2. A typical synthetic route for the Cyc-containing inhibitors of CPA.

comparable to K_i with maintaining the condition of $I_o >> E_o$. To reach the new equilibrium, *EI* complex should dissociate to *E* and *I* which can take long when K_i is very low.[4,5] For the potent inhibitors examined in this study, the new equilibrium was attained within 1-2 hours as checked by assay with ClCPL. Dissociation of *EI* during readjustment of the equilibrium states demonstrates the reversible nature of the *EI* formation in the case of the Cyc-containing inhibitors. This excludes the possibility of Zn(II) abstraction from CPA to the Cyc moiety in the *EI* complex. In the X-ray crystallographic structures of CPA complexes formed with the A_1, A_3, and related inhibitors, the two oxygen atoms attached to the phosphorus atom of the inhibitor is bound by the Zn(II) ion of CPA.[6] If a similar binding mode is operative for the Cyc-containing inhibitors, it is not possible to transfer the Zn(II) bound by the phosphonyl oxygen to the Cyc moiety in the *EI* complex.

The K_i values for the Cyc-containing inhibitors of CPA investigated in this study are summarized in Table 1, together with those for A_1-A_6 reported in the literature. The most potent inhibitor synthesized in this study is I_{Pro}^2. Inhibitors A_2, A_3, and A_5 as well as the Cyc-containing inhibitors contain phosphonic acid analogue of L-Ala. I_{Ala}^2 is an analogue of A_3 and A_5 which differ only in the acyl group attached to the *N*-termini. Similarly, I_{Phe}^2 is an analogue of A_2. Although I_{Phe}^2 is a considerably weaker inhibitor of CPA compared with A_2, I_{Ala}^2 is as potent as A_3 or A_5. This suggests that the effect of the Cyc-acetyl group on CPA inhibition is similar to those of Cbz or dansyl groups, despite the unique structure of Cyc. Cyc is present as a protonated form at neutral pHs and contains several sites capable of hydrogen-bond formation with CPA. These structural features apparently did not affect the binding of the Cyc-containing inhibitors by CPA.

To test whether the Cu(II) or Co(III) complexes of the Cyc-containing inhibitors act as CPA-cleaving catalysts, the Cu(II) or Co(III) complex of I_{pro}^2 or I_{Phe}^2 was mixed with CPA under the conditions of $E_o > I_o$. The activity of CPA was inhibited to similar extents by the metal-bound or metal-free Cyc-containing inhibitors, indicating that the metal complexes are effectively bound by CPA. In order to obtain positive evidence for cleavage of CPA by the Cu(II)Cyc- or Co(III)Cyc-containing inhibitors, two kinds of experiments were performed. First, whether the degree of inhibition of CPA activity by the added inhibitor exceeds the amounts of the inhibitor was checked to examine the catalytic turnover of the CPA cleavage. Second, formation of new protein fragments upon incubation of CPA with the Cu(II)Cyc or

Table 1. Values of K_i for various CPA inhibitors[a,b]

Inhibitor	K_i (pM)	Inhibitor	K_i (pM)
I^1	No inhibition	I^2	900 ± 90
$I_{Gly}{}^1$	4200 ± 1000	$I_{Gly}{}^2$	82 ± 5
$I_{GlyGly}{}^1$	3200 ± 900	$I_{GlyGly}{}^2$	26 ± 3
$I_{Ala}{}^1$	90 ± 22	$I_{Ala}{}^2$	8.4 ± 0.2
$I_{\beta\text{-}Ala}{}^1$	900 ± 90	$I_{\beta\text{-}Ala}{}^2$	180 ± 30
$I_{Ile}{}^1$	260 ± 80	$I_{Ile}{}^2$	15 ± 2
$I_{Pro}{}^1$	77 ± 28	$I_{Pro}{}^2$	4.8 ± 0.4
$I_{Phe}{}^1$	2100 ± 400	$I_{Phe}{}^2$	59 ± 27
A_1	0.02	A_2	1
A_3	3	A_4	4
A_5	20	A_6	710

[a]25 °C, pH 7.5, 0.5 M NaCl, 0.05 M Tris or Hepes. [b]Data for A_1-A_6 were taken from the literature.[4,5]

Co(III)Cyc derivatives was examined by MALDI-TOF MS. Several experiments were performed under various conditions, but no positive evidence was obtained for the cleavage of CPA by the inhibitors.

The Cu(II) or Co(III) complex of $I_{pro}{}^2$ or $I_{Phe}{}^2$ contains an effective binding site for CPA as well as the peptide-cleaving catalytic center. Although the Cu(II) or Co(III) complex of $I_{pro}{}^2$ or $I_{Phe}{}^2$ occupies the active site of CPA, it failed to cleave the peptide backbone of CPA. It appears that the Cu(II)Cyc or Co(III)Cyc moiety was not positioned in a productive location close to a peptide group in the complex formed with CPA. Results of the present study suggest that the structure of the linker (Scheme 1) connecting the catalytic group and the binding site must be optimized in order to design effective target-selective peptide-cleaving catalysts exploiting known ligands of the target protein.

Experimental Section

ClCPL was prepared as reported in the literature.[7,8] For synthesis of Cyc-containing inhibitors, **1** and **7** were prepared according to the reported methods.[3,9] MALDI-TOF MS data for the inhibitors listed in Figure 1 are: *m/z* 486.68 $(M+H)^+$ for I^1/I^2 ($C_{21}H_{36}N_5O_6P$ calcd. 486.52); *m/z* 543.62 $(M+H)^+$ for $I_{Gly}{}^1/I_{Gly}{}^2$ ($C_{23}H_{39}N_6O_7P$ calcd. 543.58); *m/z* 600.70 $(M+H)^+$ for $I_{GlyGly}{}^1/I_{GlyGly}{}^2$ ($C_{25}H_{42}N_7O_8P$ calcd. 600.63); *m/z* 557.51 $(M+H)^-$ for $I_{Ala}{}^1/I_{Ala}{}^2$ ($C_{24}H_{41}N_6O_7P$ calcd. 557.60); *m/z* 557.52 $(M+H)^-$ for $I_{\beta\text{-}Ala}{}^1/I_{\beta\text{-}Ala}{}^2$ ($C_{24}H_{41}N_6O_7P$ calcd. 557.60); *m/z* 599.61 $(M+H)^+$ for $I_{Ile}{}^1/I_{Ile}{}^2$ ($C_{27}H_{47}N_6O_7P$ calcd. 599.69); *m/z* 583.69 $(M+H)^+$ for $I_{Pro}{}^1/I_{Pro}{}^2$ ($C_{26}H_{43}N_6O_7P$ calcd. 583.64); *m/z* 633.63 $(M+H)^-$ for $I_{Phe}{}^1/I_{Phe}{}^2$ ($C_{30}H_{45}N_6O_7P$ calcd. 633.70). For separation of the diastereomeric mixtures of the Cyc-containing inhibitors, a 100 series HPLC system (Agilent Technology) was used with the variable-wavelength UV detector set at 260 nm. A 250 × 4.6 mm I.D. reversed-phase Hypersil BDS C_{18} column (Hypersil) was used. A gradient separation was performed with solvents A and B (A, 0.05 N triethylammonium bicarbonate in H_2O pH 7.4; B, 0.05 N triethylammonium bicarbonate in 47% aqueous methanol, pH 7.4): 0-10 min with 10% B; 10-30 min with 10% to 100% B; 30-35 min with 100% to 10% B. The Cu(II) complexes of the Cyc-containing inhibitors were generated by adding 0.8 equiv of $CuCl_2$ to the solution of the respective inhibitors. The Co(III) complexes of the Cyc-containing inhibitors were obtained as described previously.[3] CPA (Cox form) was purchased from Sigma and was used after washing the enzyme crystals with water at 4 °C. To prevent deactivation of CPA at < 1 μM, bovine serum albumin (0.1 mg/mL) and $ZnCl_2$ (1 μM) was added.[4,5]

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