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INACTIVATION OF A PROTOTYPIC ZINC-CONTAINING PROTEASE WITH (*S*)-2-BENZYL-2-(3-OXO-2-ISOXAZOLIDINYL)ACETIC ACID

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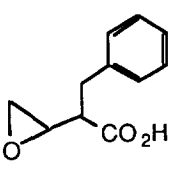
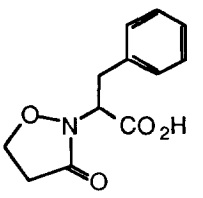
Abstract: (S)-2-Benzyl-2-(3-oxo-2-isoxazolidinyl)acetic acid was designed, synthesized, and kinetically proven to be a mechanism-based inactivator for carboxypeptidase A, a representative zinc-containing protease.

Zinc containing proteases constitute an important class of proteolytic enzymes and thus have received much attention. They play a vital role in numerous physiological processes.[1] Carboxypeptidase A (CPA)[2] which catalyzes the cleavage of C-terminal amino acid residue having a hydrophobic side chain from polypeptide substrates serves as a leading representative for these enzymes. We have been interested in developing design strategies for inhibitors of the zinc containing enzymes using CPA as a model.[3] In this communication we wish to report that (*S*)-2-benzyl-2-(3-oxo-2-isoxazolidinyl)acetic acid (BIAA) is a newly designed mechanism-based inactivator for CPA.



BEBA



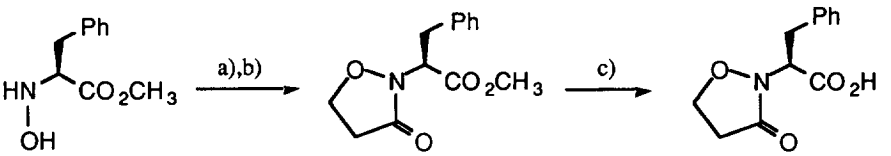
BIAA

2-Benzyl-3,4-epoxybutanoic acid (BEBA) has been reported to be a fast acting and extremely efficient irreversible inhibitor for CPA.[3c] Upon binding of this inhibitor to the enzyme, its oxirane ring is thought to be activated for an electrophilic reaction by the active site zinc ion, a Lewis acid. The carboxylate of Glu-

270 attacks the activated oxirane moiety at the 4-position to form a covalent linkage with a concurrent opening of the epoxide ring. Thus, the inhibitor becomes permanently tethered to the enzyme. However, oxiranes are in general unstable especially under acidic conditions, therefore it was thought to be prudent to replace the oxirane with a moiety which can survive under acidic conditions but still can be activated by the zinc ion thus to undergo the electrophilic reaction with the Glu-270 carboxylate.

We envisioned that 3-isoxazolidinone may be a viable candidate for the replacement of the oxirane moiety in BEBA. As being activated by the electronegative oxygen next to the lactam nitrogen, the heterocycle ring may be considered as a β-lactam equivalent in chemical reactivity. In fact, 4-amino-3-isoxazolidinone (D-cycloserine)[4] and its derivative[5], lactivicin are known to inhibit D-alanyl-D-alanine transpeptidase in a manner similar to β-lactam antibiotics inhibit the enzyme, that is, they acylate irreversibly the serine hydroxyl at the active site of the transpeptidase. A substrate analog having the isoxazolidinone moiety such as BIAA was thus conceived as a potential inactivator for CPA. Like BEBA, the prototypic inhibitor, the carboxylate of BIAA would interact with the guanidinium moiety of the active site Arg-145, the aromatic ring is anchored in the primary recognition pocket of the enzyme, and the carbonyl oxygen of the heterocycle coordinates to the active site zinc ion. As being a substrate analog, the *S*-configuration, the stereochemistry of substrates was maintained.

The designed inhibitor was synthesized starting with *N*-hydroxy-L-phenyalanine methyl ester[6] following the sequences of reactions indicated in Scheme 1.[7] Hydrolysis of the ester to BIAA was effected by α-chymotrypsin at pH 7.8 in order to minimize a possible racemization at the α-position upon chemical treatment.



Scheme 1. *Reagents and conditions*: (a) TBSCl, imidazole, DMF; (b) 3-bromopropionyl-chloride, pyridine (1equiv), THF; (c) α - chymotrypsin, pH 7.8 phosphate buffer (0.1 M)

Addition of BIAA to a solution of CPA leads to irreversible inhibition of the enzyme. The inactivation was followed spectrophotometrically at 320 nm using *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (Cl-CPL)[8] as the substrate following the competitive substrate assay method[3c] at pH 7.5, 0.05M Tris buffer (0.5M NaCl)and at 25 ^{0}C. Semilogarithmic plot of the percent remaining activity *vs* time gives straight lines (Figure

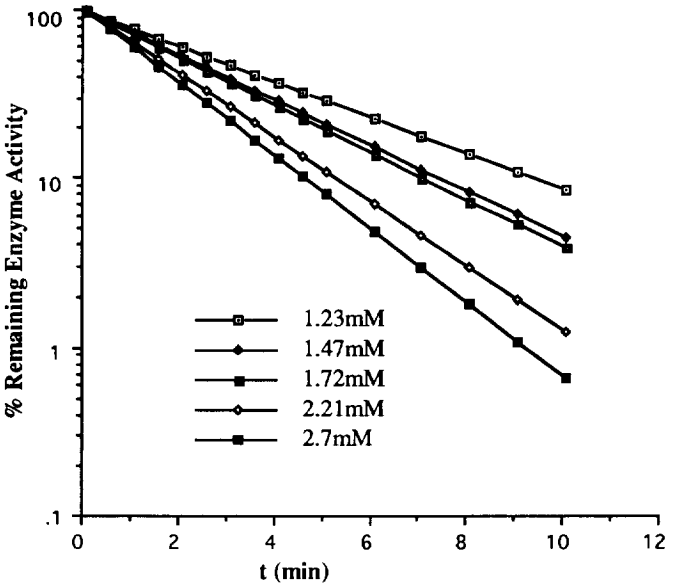


Figure 1. Semilogarithmic plot of percent remaining activity *vs* time give straight lines with a slope of $-k_{obs}$ for each inhibitor concentration.

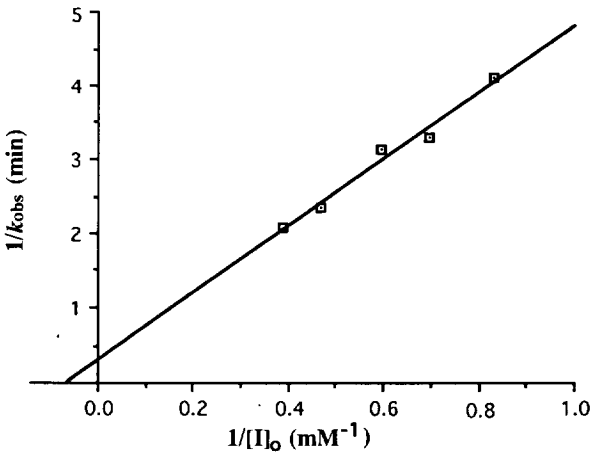


Figure 2. Double reciprocal plot of k_{obs} *vs* $[I]_o$ gives a straight line whose y-intercept corresponds $1/k_{inact}$.

Equation 1

$$\frac{1}{k_{obs}} = \frac{K_i}{k_{inact}}\left(1 + \frac{[S]_o}{K_m}\right)\frac{1}{[I]_o} + \frac{1}{k_{inact}}$$

1), suggesting that the inhibition occurs in an irreversible fashion. Kinetic parameters, K_I and k_{inact} for the inactivation were estimated to be 4.35 mM and 3.85 min^{-1}, respectively, from the double reciprocal plot of $[I]_o$ *vs* k_{obs} (Figure 2) and equation 1. In the Figure and equation, k_{obs} represents peudo first-order rate constant obtained from the inhibition kinetic experiment (obtainable directly from the computer-assisted UV spectrophotometer), $[S]_o$ the initial concentration of the substrate, and K_m the Michaelis-Menten constant (136 μM). The inactivation potency of BIAA expressed in k_{inact}/K_I was calculated to be 14.2 $M^{-1} s^{-1}$. No return of the enzymic activity was observed upon dialysis of the inactivated enzyme. The reversible inhibition constant (K_I) which reflects the affinity of the enzyme for the inhibitor was calculated from the Dixon plot[9] (not shown) of initial velocity to furnish 1.19 mM, and the partition ratio which is an indication of the efficiency of the inactivator was obtained by the titration method[10] to provide 738 (Figure 3).

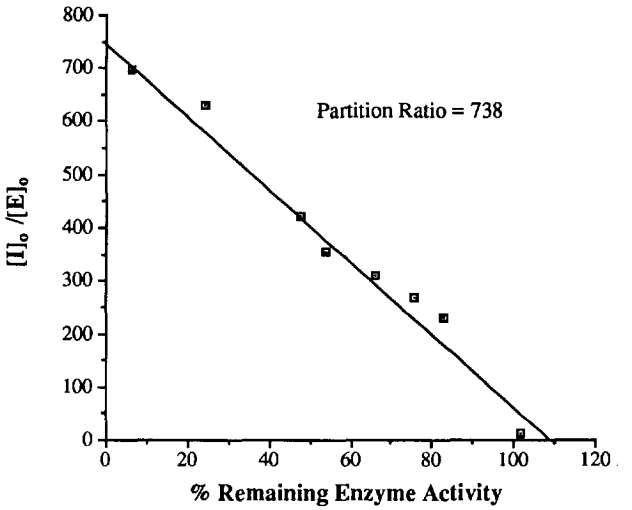
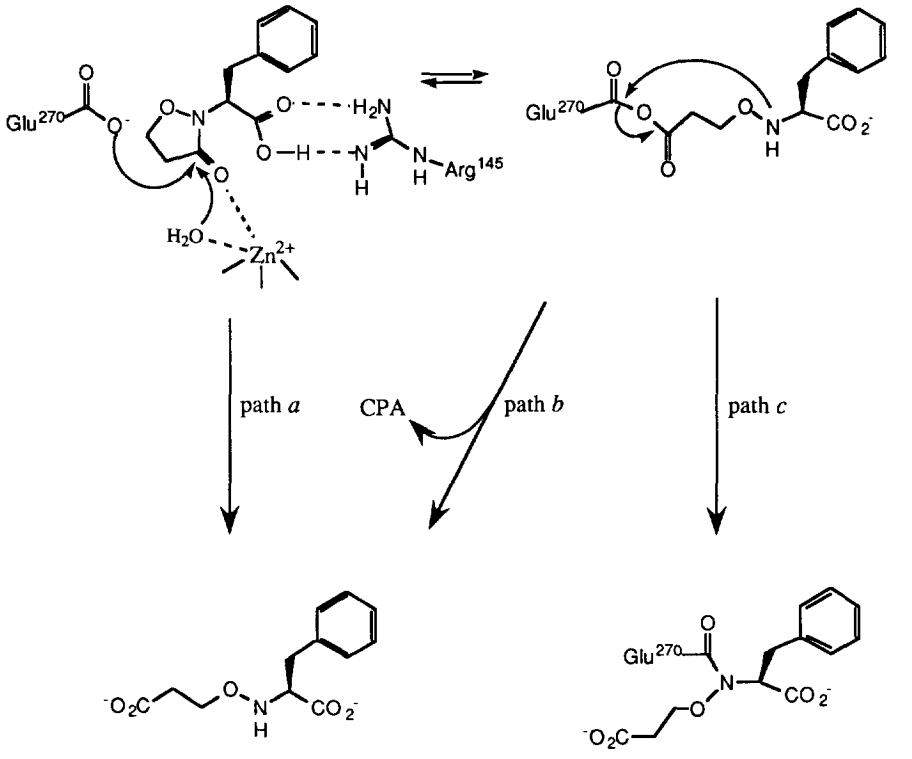


Figure 3. Determination of partition ratio. $[I]_o/[E]_o$ is plotted against the remaining activity after dialysis of the incubated enzyme and inhibitor mixture for 36 h.

The enzyme inactivation results presented in this study may be rationalized by invoking reaction path depicted in Scheme 2.[11] BIAA binds the enzyme at the active site to form a Michaelis-Menten complex. The isoxazolidinone ring the bound BIAA is activated by the coordination of its carbonyl oxygen to the zinc ion and undergoes a chemical reaction at the carbonyl carbon with the carboxylate of Glu-270, generating an anhydride intermediate. The reactive intermediate thus formed may undergo a further reaction of an intramolecular rearrangement, whereby the carboxylate becomes tethered to the inhibitor in the form of a carboxamide to impaire the enzymic activity permanently (path *c*). However, the possibility (path *a*) that the zinc bound water molecule may also function as a nucleophile, attacking the isoxazolidinone ring of the bound BIAA would not be eliminated. This reaction path is analogous to a proposed mechanism for the

catalytic hydrolytic reaction of CPA.[2b] Furthermore, the anhydride intermediate formed in the inactivation process may be hydrolyzed by water with regeneration of the enzyme (path b). The high partition ratio obtained in the inactivation with BIAA is then envisaged.



Scheme 2. A proposed pathway for the irreversible inhibition of CPA by BIAA.

Acknowledgment: We thank the Korea Science and Engineering Foundation for the financial support of this work, and S. J. Chung for the suggestion of an alternate pathway for the inactivation of CPA by BIAA.

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7. All new compounds were fully characterized. For **BIAA**: mp 180-182°C; $[\alpha]_D$ = -6.4°(c 1.02, DMSO); IR(thin film) 3300-3500, 1700, 1650 cm^{-1}; ^1H NMR(Methanol-d4, 300 MHz) δ 2.60(m, 1H), 2.71(m, 1H), 3.12(dd, 1H), 3.30(dd, 1H), 4.21(q, 1H), 4.36(q, 1H), 4.91(dd, 1H), 7.24-7.27(m, 5H); ^{13}C NMR(Methanol-d4, 75 MHz) 33, 35, 60, 68, 128-140, 172, 174; EI MS 235(M^+); Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.96. Found: C, 61.04; H, 5.62; N, 5.72.
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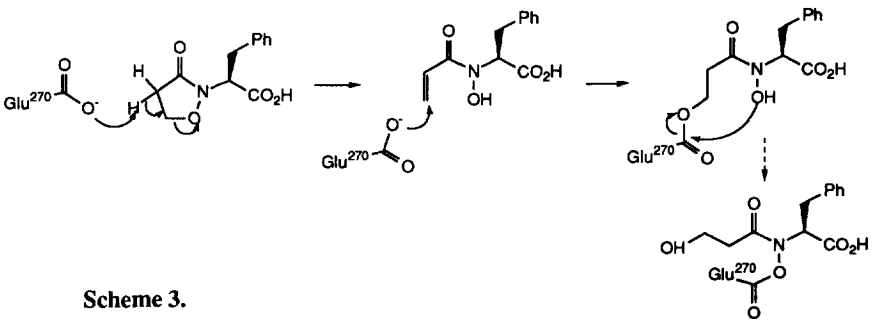
11. An alternative pathway for the inactivation of CPA by BIAA is possible: The carboxylate of Glu-270 functions as a base abstracting an acidic proton next to the carbonyl[12] of the isoxazolidinone ring in the bound BIAA to generate an enone which in turn captures the carboxylate as shown in Scheme 3.



Scheme 3.

12. (a) Mobashery, S.; Ghosh, S. S.; Tamaura, S. Y.; Kaiser, E. T. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 578 - 582. (b) Ghosh, S. S.; Wu, Y.-Q.; Mobashery, S. *J. Biol. Chem.* **1991**, *266*, 8759 - 8764.

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