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IRREVERSIBLE INHIBITION OF ZINC-CONTAINING PROTEASE BY OXAZOLIDINONE DERIVATIVES. NOVEL INACTIVATION CHEMISTRY

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Abstract: α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic acid (BOOA) having (α*R*,4*S*) and (α*S*,4*R*) configurations were designed and synthesized as a novel type of mechanism-based inactivators for carboxypeptidase A (CPA), and kinetic analysis demonstrated that they indeed inhibit the enzyme in a time-dependent manner with the second order inhibitory rate constants (k_{inact}/K_I) of 1.52 and 1.39 $M^{-1} s^{-1}$ for (α*S*,4*R*)-BOOA and (α*R*,4*S*)-BOOA, respectively. © 1998 Elsevier Science Ltd. All rights reserved.

Carboxypeptidase A (CPA, EC 3. 4. 17. 1) is a much studied zinc containing protease which cleaves off the C-terminal amino acid residue having a hydrophobic side chain from peptide substrate.[1] As a prototypical enzyme for a large family of zinc proteases, this enzyme has been served as a model enzyme in the development of enzyme inhibitor design principles.[2,3,4] The design strategies developed using CPA as a target enzyme[5,6] are highly valuable since they can be utilized for the design of inhibitors of other zinc proteases of medicinal interest such as angiotensin converting enzyme,[2] enkephalinase[3] and collagenases.[4] We wish to report herein a novel strategy employed in the design of mechanism-based inactivators for CPA exploiting an oxazolidinone ring as a latent inactivating functionality.

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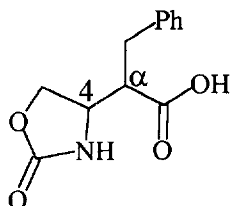


BOOA

Expectedly, the designed inhibitor of α-benzyl-2-oxo-1,3-oxazolidine-4-acetic acid (**BOOA**) binds to the active site of CPA with its carboxylate group forming hydrogen bonds with the guanidinium moiety of Arg-145 and the phenyl group fitting into the hydrophobic S_1' pocket (Figure 1).[7] This binding mode would bring the urethane carboxamide of the oxazolidinone moiety to rest at the close proximity to the zinc ion to form a bidentate coordinative bond, whereby the zinc-bound water molecule is displaced. The nucleophilic attack at the activated carbonyl carbon of the enzyme-bound **BOOA** by the carboxylate of Glu-270 may then occur,[8] and subsequent intramolecular rearrangements would effect a covalent modification of the Glu-270 carboxylate group, impairing the catalytic power of CPA permanently (path *a*). The attack of the carboxylate at the C_5 of the oxazolidinone ring to generate **2** directly (path *b*) is also a possibility. The design rationale is depicted in Figure 1.

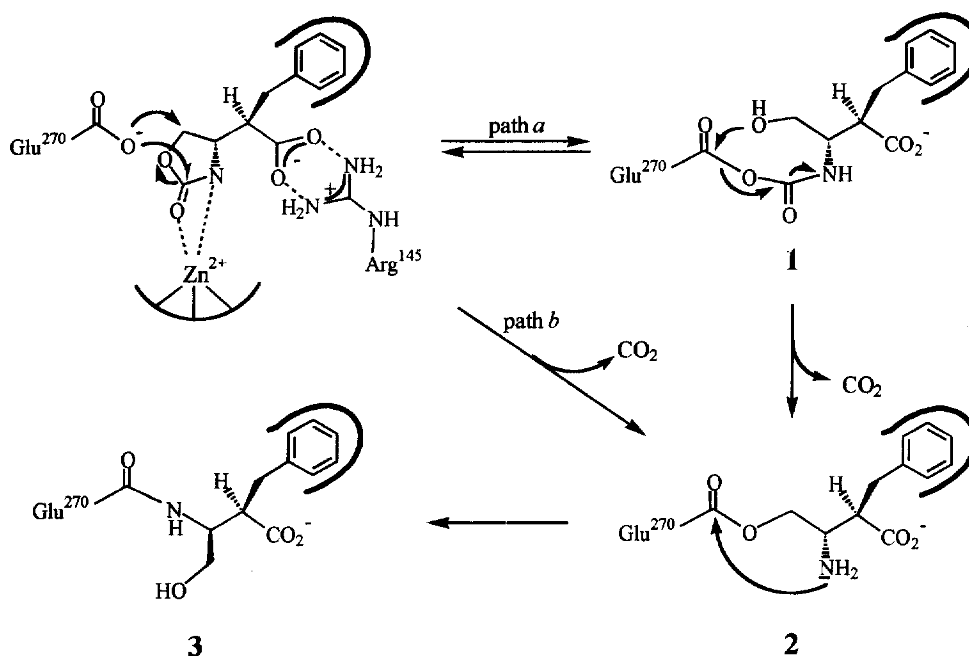
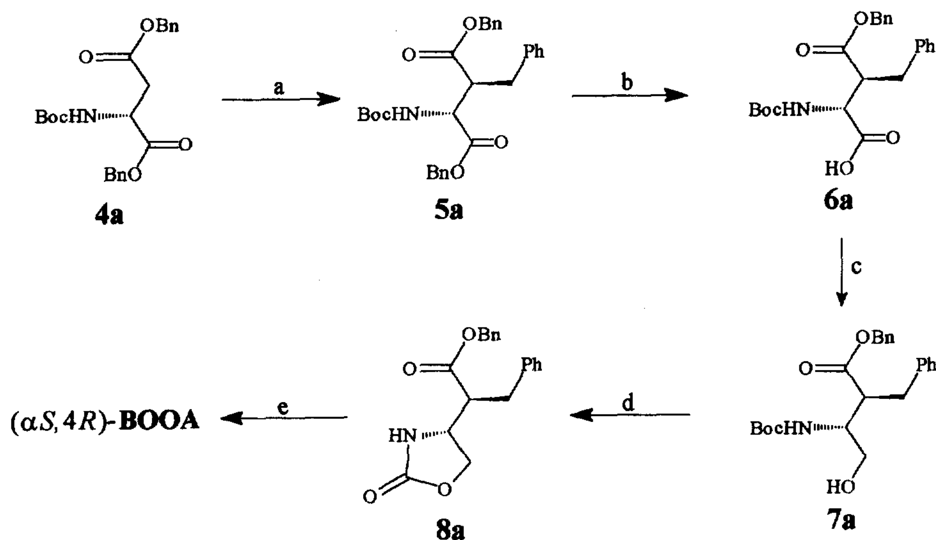


Figure 1. Postulated reaction path employed in the design of (α*S*,4*R*)-**BOOA** as a mechanism-based inactivator for CPA.

Of four possible stereoisomers of **BOOA**, two isomers having configurations of (α*S*,4*R*) and (α*R*,4*S*) were synthesized for the present study. They were chosen on the basis of the inhibitory stereospecificity reported for 2-benzyl-3,4-epoxybutanoic acid (BEBA) in its inactivation of CPA:[6] Stereisomers having (2*S*,3*R*)– and (2*R*,3*S*)–configurations are potent inactivators for the enzyme, but the other two stereisomers do not exhibit irreversible inhibitory activity.[9] Synthetic pathway for (α*S*,4*R*)-**BOOA** is shown in Scheme 2.[10] In an analogous fashion, (α*R*,4*S*)–**BOOA**[11] was synthesized starting with L–aspartic acid. The cyclization of the *N*-Boc amino alcohol derivative to form the oxazolidinone has precedence: Curran *et al.* reported recently that treatment of *N*-Boc,*O*-tosyl derivatives of aminoethanols with base affords oxazolidinones in good yields. [12]



Scheme 1. *Reagents, conditions, and (yields):* (a) LHMDS (2 eq), BnBr, -78 ℃, (46%); (b) 1N NaOH, dioxane-water (5:1), rt, 24 h, (90%); (c) isobutyl chloroformate, 1-ethylmorpholine, DME, -10 ℃, 5 min, and then $NaBH_4$, - 10 ℃, 5 min; (d) TsCl, pyridine, rt, 7 d, (two step yield: 33%); (e) H_2/Pd-C, (80%).

Inhibitory activity of the above oxazolidinone derivatives towards CPA was assayed as reported previously. [6d] The rate of CPA-catalyzed hydrolysis of hippuryl-L-Phe (substrate) in the presence of various concentrations of the inhibitors was followed by the change of absorption intensity at 254 nm.

First-order inactivation rate constants (k_{obs}) were obtained directly from the computer-assisted *UV* spectrometer. Both compounds inhibited CPA in a time-dependent and saturable manner as shown in

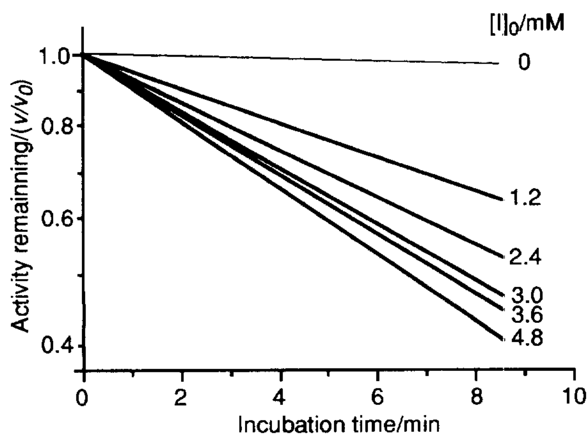


Figure 2. Time-dependent loss of CPA enzymic activity by (αS,4*R*)-**BOOA**.

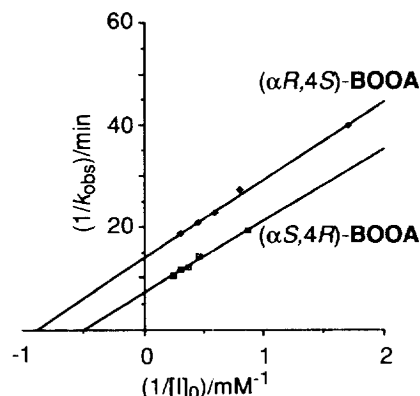


Figure 3. Double reciprocal plotting of k_{obs} *vs* $[I]_0$ gives a straight line whose *y* intercept corresponds to $1/k_{inact}$.

Figures 2 and 3, which suggest that the inhibitions occur irreversibly.[13] The irreversibility of the inhibition was ascertained by dialysis experiments in which exhaustive dialysis of the inactivated enzyme against the buffered reaction medium for 2 days failed to regenerate the enzymic activity. Kinetic constants, K_I, and k_{inact} were calculated from double reciprocal plots[14] of k_{obs} *vs* $[I]_o$ (Figure 3). The inhibitory kinetic parameters thus obtained are listed in Table 1 along with values of k_{inact}/K_I (inactivation potency) which reflects a measure of effectiveness of an enzyme inhibitor. Protection from inactivation was observed for both compounds in the presence of benzylsuccinic acid, a potent reversible inhibitor for CPA which is known to bind to the active site of CPA,[15] indicating that the inactivation is active site directed (Figure 4). Partition ratio (k_{cat}/k_{inact}) which reflects the efficiency of an inactivator was determined for both inhibitors by the titration method[13] and are included in Table 1.

These kinetic analyses taken together strongly suggest that both are indeed inactivators for CPA as they were designed, and the carboxylate of Glu-270 is the most likely site of the covalent modification. The high partition ratios exhibited by both inactivators suggest that the initial intermediates (**1**) in the inactivation pathway are susceptible to hydrolytic cleavage by water with generation of 3-amino-2-benzyl-4-hydroxybutanoic acid and free CPA.

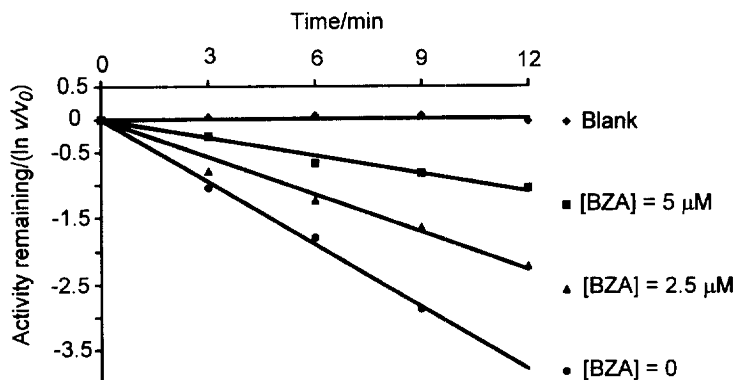


Figure 4. Inactivation of CPA by (α*S*,4*R*)-**BOOA** in the presence of varied concentrations of benzylsuccinic acid (BZA).

Table 1. Kinetic parameters in the inactivation of CPA by (α*S*,4*R*)- and (α*R*,4*S*)-**BOOA**.

Inhibitor	K_I (mM)	k_{inact} (min^{-1})	k_{inact}/K_I ($M^{-1}\,s^{-1}$)	Partition ratio
(α*S*,4*R*)-**BOOA**	1.65	0.115	1.52	94
(α*R*,4*S*)-**BOOA**	0.9	0.075	1.39	120

In conclusion, in this study we have demonstrated that (α*S*,4*R*)– and (α*R*,4*S*)–**BOOA** are a new type of irreversible inhibitors for CPA. The inhibitors were designed rationally on the basis of the established active site topology of the enzyme and a proposed catalytic mechanism for the enzymic reaction. In the design the unique property of the oxazolidinone heterocycle was exploited as a latent inactivating species. The inactivating chemistry is unprecedented and it is remarkable that both inhibitors which are in an enantiomeric relationship inactivate the enzyme equally well. The design principle may bear generality, enabling design of oxazolidinone containing inactivators for other zinc-containing proteolytic enzymes.

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8. Apparently, in the absence of the zinc-bound water molecule which is displaced by the oxazolidinone moiety, the carboxylate of Glu-270 becomes to function as a nucleophile.
9. On the basis of the X-ray structural data of CPA inactivated by (2*S*,3*R*)- and (2*R*,3*S*)-BEBA as well as that of the native CPA in which the hydrophobic pocket and the zinc ion are situated in a *transoid* relationship, we envision that the other stereoisomers would be difficulty accommodated by the active site of CPA
10. All new compounds described in this report have been fully characterized. (αS,4R)-BOOA: mp 178-179 ℃; $[\alpha]_D = -2.4°$ (c = 0.5, EtOH); IR (KBr) 3277 (NH), 3500-2500 (acid OH) 1716 (C=O), 1700 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.75 (2H, m), 2.85 (1H, d of d), 3.97 (1H, quintet), 4.24 (1H, d of d), 4.37 (1H, t), 7.16-7.30 (5H, m) 7.93 (1H, s), 12.45 (1H, s); MS *m/z* EI 191 ($M^+ - CO_2$), 235 (M^+); Anal. Calcd. for $C_{12}H_{13}NO_4$: C, 61.29; H, 5.53; N, 5.96. Found: C, 61.05; H, 5.54; N, 5.91.
11. (αR,4S)-BOOA: mp 178-179 ℃; $[\alpha]_D = +2.4°$ (c = 0.5, EtOH).
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