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INACTIVATION OF CARBOXYPEPTIDASE A BY 2-BENZYL-3,4-EPITHIOBUTANOIC ACID

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Abstract: 2-Benzyl-3,4-epithiobutanoic acid was designed and synthesized as a pseudomechanism-based inactivator for carboxypeptidase A. Designing rationale, synthesis of racemic 2-benzyl-3,4-epithiobutanoic acid having (2*S*,3*S*)- and (2*R*,3*R*)-configurations, and inhibitory kinetic parameters and their implications are reported.

Carboxypeptidase A (CPA, EC 3. 4. 17. 1) is a much studied zinc-containing monoexopeptidase serving as a prototypic enzyme for a family of zinc-containing metalloenzymes,[1] and has been used as a model enzyme for the design of metalloenzyme inhibitors.[2] Recently, we have reported a new principle for the design of a novel type of CPA inhibitors.[3] 2-Benzyl-3,4-epoxybutanoic acid (BEBA) which was designed applying the principle is a highly efficient irreversible inhibitor of CPA, inactivating the enzyme *via* a covalent modification at the catalytic carboxylate of Glu-270.[3] The designing principle may be outlined as the following: When the inhibitor binds to the enzyme, its oxirane ring would ligate to the active site zinc and thus becomes highly labile to undergo an electrophilic reaction with the carboxylate. The inhibitor thus becomes covalently attached to the carboxylate with the formation of an ester linkage (Figure 1). The covalent modification at the carboxylate was proven by the single crystal X-ray crystallographic analysis of the inactivated enzyme, which showed that the inactivating BEBA bears 2*S*,3*R*-configuration.[3b] The designing approach was based on the proposed mechanism that the carboxylate of Glu-270 serves as a nucleophile that attacks the scissile carboxamide carbon atom of the substrate to form a highly reactive anhydride intermediate.[4] However, there has been proposed an alternative explanation, in which the carboxylate functions as a base, activating a nearby zinc-bound water molecule which in turn attacks the carboxamide carbon.[5]

As an extension of the study, we have now evaluated a thiirane analog of BEBA for CPA inhibitory

activity. In this case, the thiol group that will be generated when its thiirane moiety undergoes an electrophilic reaction with the catalytic carboxylate of CPA is expected to ligate with a higher affinity than the hydroxyl of BEBA to the active site zinc, thus to improve the inhibitory activity over that of the prototypic inhibitor. It has been well demonstrated that a sulfhydryl group has a very high ligating propensity for the active site zinc.[6] As an added advantage, it is further expected that the regeneration of the catalytic carboxylate may be prevented owing to the stronger coordination of the sulfhydryl group to the active site zinc. Of four possible stereoisomers of the designed inhibitor, *i.e.*, 2-benzyl-3,4-epithiobutanoic acid (BTBA), the racemate having (2*S*,3*S*)- and (2*R*,3*R*)-configurations[7] was synthesized for the present study on the ground of the demonstrated stereochemistry with BEBA in the CPA inactivation .[3b,c, 8]

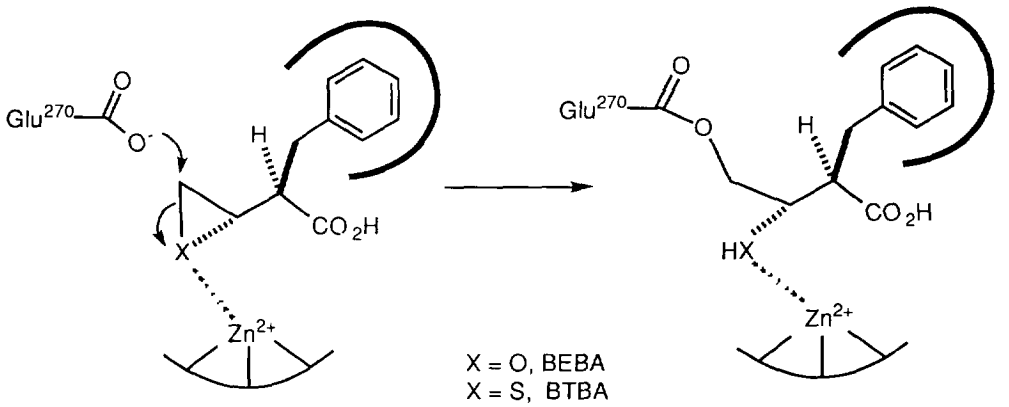
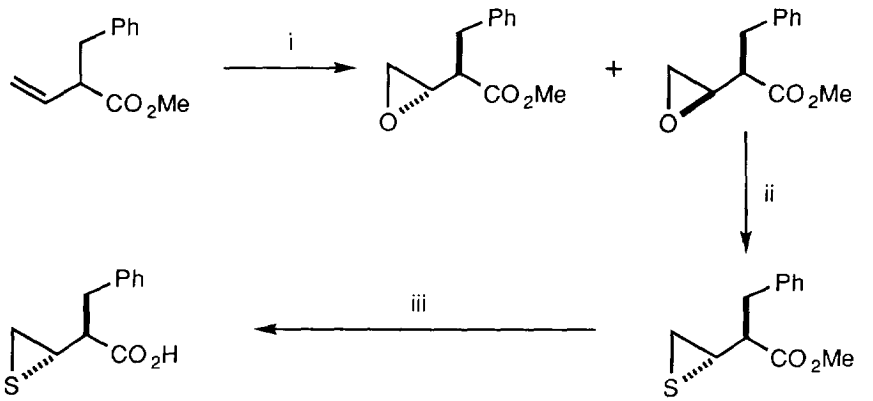


Figure 1. Schematic representation of the rationale used for designing BEBA as well as BTBA as irreversible inhibitors of CPA.

Synthesis of the designed inhibitors is shown in Scheme 1. The oxidation of 2-benzyl-2-vinylacetic acid methyl ester[9] with *m*-chloroperbenzoic acid yielded the corresponding epoxide as a diastereomeric mixture which was readily separated into two racemic products by a silica gel column chromatography method using a mixture of hexane and ethyl acetate as eluent. The *cis*-epoxide[10] thus separated was converted to the corresponding thiirane by the treatment with thiourea in methanol at room temperature. The stereochemistry at the 3-position of the product is inverted in this transformation to give *trans*-BTBA. The selective hydrolysis of the ester moiety of the methyl epithiobutanoate to give the desired compound[12] was eventually succeeded, after experiencing considerable difficulties, by treating it with a water-isopropyl alcohol solution of lithium hydroxide (3 eq.) followed by acidification with an aqueous citric acid solution.



Scheme 1. Reagents, conditions, and (yields); i) MCPBA, CH_2Cl_2, rt, 3 days (90 %); ii) thiourea, MeOH, rt, 3 days (80%); iii) 1M-LiOH, ^{i}PrOH, -10 °C → rt, 2 h, and then 10 % aq. citric acid (58 %).

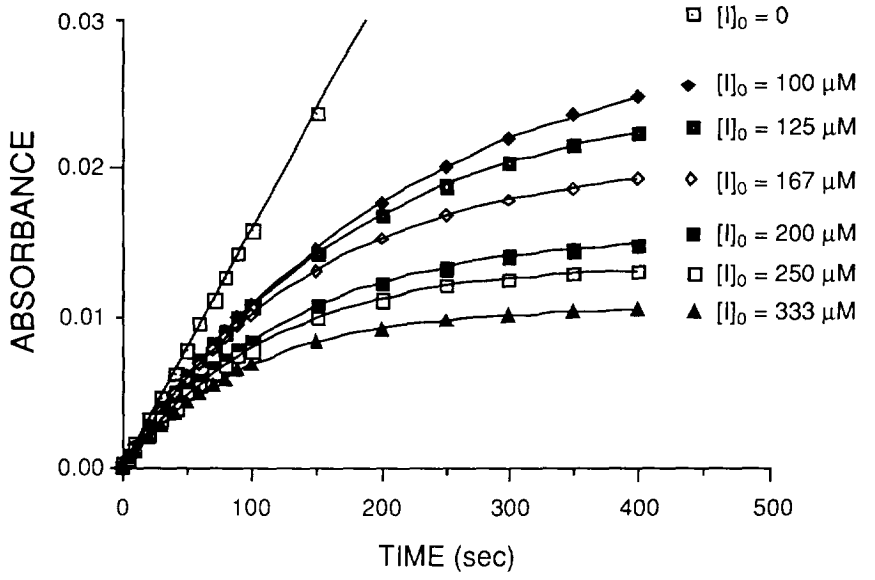


Figure 2. Inhibition of CPA by (2*S*,3*S*)- and (2*R*,3*R*)-2-benzyl-3,4-epithiobutanoic acid (BTBA) in the presence of substrate. CPA (final concentration of 5 nM) was added to a solution of the inhibitor and substrate (hippuryl-L-phenylalanine, final concentration of 500 μM at pH 7.5) (0.05 M standard Tris buffer-0.5 M NaCl, 25 °C), and the hydrolysis of the substrate was followed by UV at 254 nm.

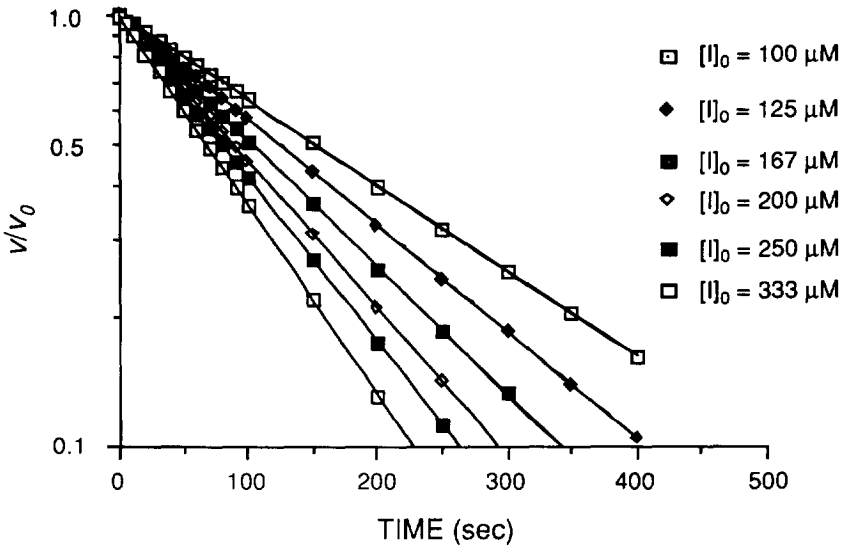


Figure 3. Semilogarithmic plots of the velocity ratio of the substrate hydrolysis *vs* time give straight lines with a slope of $-k_{obs}$ for each inhibitor concentration.

The inactivation of CPA by the BTBA was followed by the method of competitive substrate assay,[13] the method which has been developed for kinetic studies of rapid inactivators. The progress curves for CPA-catalyzed hydrolysis of hippuryl-L-Phe in the presence of various concentrations of BTBA suggested that the inhibition occurs irreversibly (Figure 2). The apparent rate constant (k_{obs}) was obtained directly from the computer-assisted UV spectrophotometer (Hewlett Packard Diode array spectrophoto- meter 8452 A).[14] Semilogarithmic plotting of v/v_0 *vs* time gives straight lines (Figure 3) in agreement with the conclusion obtained from the progress curves. Parameters of K_i and k_{inact} for the inactivation were obtained to be 94 μM and 1.31 min^{-1}, respectively, from the plot of k_{obs} *vs* $[I]_0$ in a double reciprocal fashion (Figure 4) on the basis of equation 1[15] which was derived for the inhibitory scheme shown below:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{inact}} E\text{–}I$$

$$\frac{1}{k_{obs}} = \frac{K_i}{k_{inact}}\left[1 + \frac{[S]_0}{K_m}\right]\frac{1}{[I]_0} + \frac{1}{k_{inact}} \qquad (1)$$

The above kinetic parameters compare favorably with the corresponding parameters of BEBA, *i.e.*, 86 μM and 1.59 min^{-1} for (2*S*,3*R*)-BEBA, and 155 μM and 1.11 min^{-1} for (2*R*,3*S*)-BEBA, and shows that BTBA of (2*S*,3*S*)- and (2*R*,3*R*)-configurations are also highly effective, rapidly acting pseudomechanism-based inactivators for CPA, which are much superior to known inactivators of the enzyme.[16] No significant improvement in its K_i value over those of the prototypic BEBA inactivators suggests that the sulfide in the thiirane has only a weak ligating property to the active site zinc. The observation that the inactivation rate of

CPA by BTBA is closely comparable to that of BEBA, nevertheless, indicates that the thiirane of BTBA becomes activated by the zinc upon BTBA binds CPA in consistent with the classification of BTBA as a pseudomechanism-based inactivator.[3a] Thiirane ring is known to have reduced reactivity as an electrophile compared with oxirane.[17] From the kinetic analysis it may be concluded that in the case of BTBA the two factors, *i.e.*, the stronger ligation property of the sulfhydryl group to the zinc and the lowered reactivity of the thiirane ring of BTBA towards the carboxylate nucleophile are counteracting each other, resulting in no significant improvement over BEBA in inactivating the enzyme.

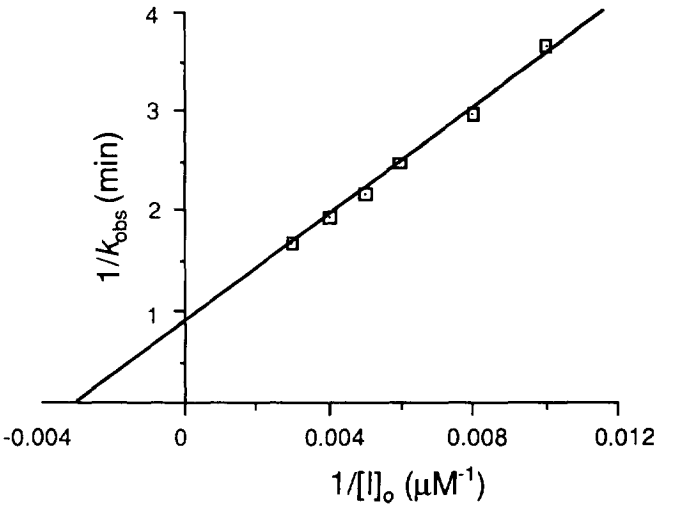


Figure 4. Double reciprocal plotting of k_{obs} *vs* $[I]_0$ gives a straight line whose y-intercept corresponds $1/k_{inact}$ and x-intercept shows $-1/K_i(1+[S]_0/K_m)$.

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7. The BTBA having the "*R*" configuration at the 2-position belongs to the "D" series.

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