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A new type of carboxypeptidase A inhibitor: design, synthesis, and mechanistic implication

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Abstract : 2-Benzyl-3,4-epoxybutanoic acid (BEBA) which was designed rationally as an irreversible inhibitor of carboxypeptidase A on the basis of the known topology of the active site and catalytic mechanism of the enzyme indeed inactivated the enzyme very efficiently with a covalent modification at the carboxylate of Glu-270. The partition ratio of BEBA was determined to be 20.3. Of four stereoisomers of BEBA, (2*S*, 3*R*)- and (2*R*, 3*S*)-BEBA show the inhibitory activity, and the other two isomers are essentially inactive. This stereospecificity of BEBA in the inhibition was explained with a proposition of a three-dimensional representation of the active site of the enzyme. All four stereoisomers were synthesized effectively and conveniently starting with optically active 2-benzyl-2-vinylacetic acid which was obtained by a kinetic resolution of racemic methyl ester of the acid using α-chymotrypsin.

INTRODUCTION

Carboxypeptidase A (CPA, EC 3.4.17.1) is a much studied and well characterized metalloprotease, and thus serving as a prototypic enzyme for many metalloenzymes including physiologically important proteases such as angiotensin converting enzyme and enkephalinase (ref. 1,2). The structure of the native enzyme has been determined by X-ray crystallographic method to a resolution of 1.54 Å. It preferentially cleaves off the C-terminal amino acid residue that possesses an aromatic side chain from peptide substrate with L-stereospecificity (ref. 1,2). CPA has also served as a model in the development of inhibitor design strategies that can be translated to other metalloproteases of great medicinal importance (ref. 3), and accordingly a good variety of different types and potencies of inhibitors have been developed, culminating to obtaining an inhibitor of a K_i value in the range of femtomolar (ref. 4). In this report, we describe the synthesis and mechanistic implication of a new type (pseudomechanism-based

inactivator) of CPA inhibitor which we have designed rationally on the basis of the known topology of the active site as well as the functional role of binding sites and catalytic groups of the enzyme.

INHIBITOR DESIGN

CPA consists of a polypeptide chain of 307 amino acid residues and a Zn^{2+} at the active site. The Zn^{2+} present at the active site plays dual roles, serving not only as a binding group in complexing with a substrate, but also as an activating center for the scissile peptide bond: The coordinative interaction between the Zn^{2+} and the carbonyl oxygen of the scissile peptide bond of the substrate polarizes the carbonyl, causing it to be an electrophilic center (ref. 1,2). Other principal binding sites present at the active site are Arg-145 and the substrate recognition pocket of a narrow mouth and a wide interior (ref. 5,6). The guanidinium moiety of Arg-145 forms hydrogen bond bridges with the terminal carboxylate of the substrate, and the substrate recognition pocket which extends deep into the core of the enzyme molecule accommodates the side chain aromatic ring of P_1' residue of the substrate (ref. 1,2). Although it is generally believed that the carboxylate of Glu-270 plays a pivotal role in the catalytic action of CPA, the controversy as to its exact function in the catalytic mechanism has been existed. Presently, there are two prevaling explanations regarding its involvement in the catalytic mechanism: The carboxylate of Glu-270 functions either as a nucleophile which attacks the activated carbonyl carbon directly (anhydride pathway) (ref. 5), or as the promoter of a nearby water molecule for nucleophilic attack at the scissile amide bond (general base mechanism) (ref. 2,8). The guanidinium moiety of Arg-145 and the carboxylate of Glu-270 are found at or near the surface of the enzyme molecule, and the recognition pocket is invaginated deep into the core of the enzyme. The Zn^{2+} which is also found deep in the active site crevice is positioned spatially in a transoid relationship to the recognition pocket within the active site cleft (ref. 9,10). These knowledge of the active site topology and the mechanistic implications of the functional groups present at the active site allowed for a rational design of a novel type of irreversible inhibitor for CPA.

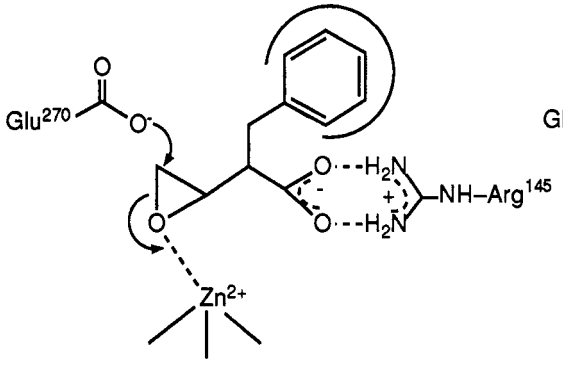


Figure 1. CPA • BEBA complex.

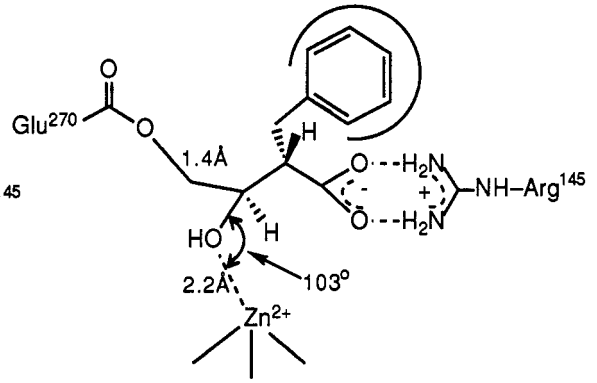


Figure 2. Inactivated CPA by BEBA

Oxiranes are stable compounds, but in the presence of a Lewis acid become labile and susceptible to ring opening. Accordingly, oxirane derivatives having structural features which are compatible with the topology of the active site would be expected to interact with the carboxylate catalytic group, leading to form a

covalent linkage (Figure 1). 2-Benzyl-3,4-epoxybutanoic acid (BEBA) has such structural features that satisfy the requirements needed for the formation of a complex with CPA at the active site. The carboxylate would form double hydrogen bonds with the guanidinium moiety of Arg-145, and the phenyl group would be accommodated by the recognition pocket. If the oxirane derivative successfully anchors the active site, the epoxide ring will be situated at a position where its oxygen atom might be able to ligate to the Zn^{2+} at the active site, causing the ring to be chemically labile. The nucleophilic attack of the carboxylate of Glu-270 on the activated oxirane ring possibly at the 3 position then result in a covalent attachment of BEBA to the enzyme with a concurrent opening of the ring (Figure 1).

BEBA was initially prepared in a lower yield by epoxidation of 2-benzyl-2-vinylacetic acid with m-chloroperoxybenzoic acid. We found later that BEBA can be prepared conveniently and in an improved yield by epoxidation of the benzyl ester of the vinylacetic acid. The benzyl ester of BEBA thus obtained is conveniently purified by a chromatographic column (silica gel). Furthermore, as benzyl esters diastereomeric BEBAs were readily separated in a racemic pair by the column. The subsequent catalytic hydrogenation using palladium on charcoal afforded BEBA as an oil.

KINETIC AND X-RAY CRYSTALLOGRAPHIC STUDIES

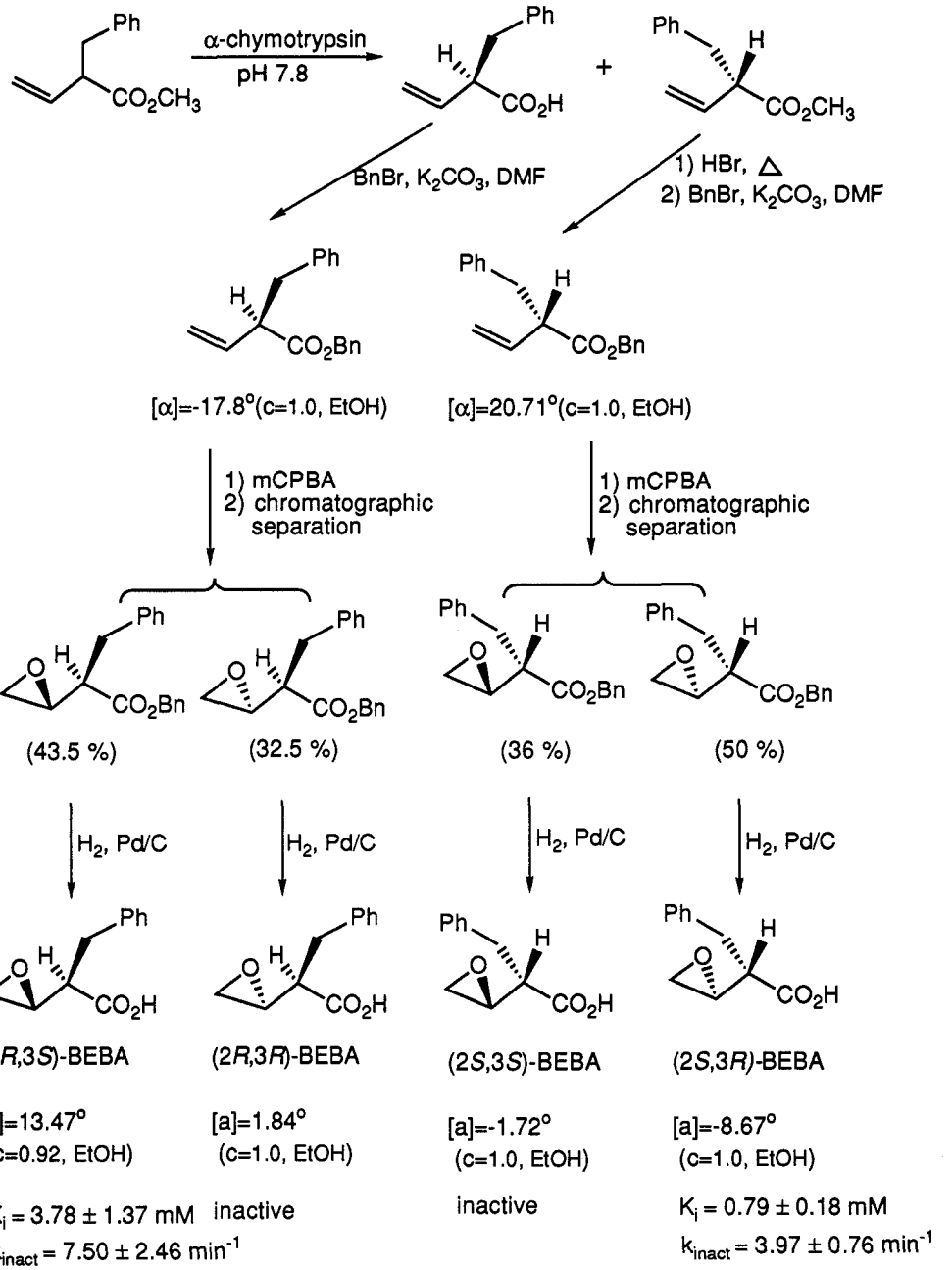
In the kinetic studies (ref. 11), BEBA showed a time-dependent loss of CPA activity and the rate of inactivation was directly proportional to the concentration of BEBA up to 1.88 μM, then became independent. The inhibitory binding constant (K_i) of BEBA was determined to be 6.25 mM. Plotting of the logarithm of the inactivation rate constants against the logarithm of concentrations of BEBA according to the method of Levy et al (ref. 12) gave a slope of 1.2, which strongly suggests that the inhibition occurs with 1:1 stoichiometry. The enzyme activity did not return after dialysis of the incubation mixture of CPA and BEBA, suggesting that the inhibition occurs irreversibly with a covalent modification possibly at the active site. The rate of the inactivation became slower in the presence of benzylsuccinic acid, a known reversible competitive inhibitor of CPA (ref. 13), which indicates that the inactivation is indeed active site directed. The partition ratio was determined to be 20.3. These kinetic data strongly suggest that BEBA indeed inactivates CPA with high efficiency via a covalent modification at the active site.

The single crystal X-ray structure at 2 Å resolution of the inactivated CPA (ref. 14) confirmed the kinetic result that the inactivation was resulted by a covalent linkage of BEBA at the active site of CPA. The electron density map shows the presence of a continuous electron density between the carboxylate oxygen of Glu-270 and ring opened BEBA at the 4-position (Figure 2). The length of the newly formed C-O bond was modeled to be 1.4 Å. The hydroxyl group at the C-3 of the covalently linked inhibitor is coordinated to Zn^{2+} having the bond length of 2.1 Å and the C—O---Zn^{2+} angle 103 ^{0}, replacing the water molecule that is coordinated to the Zn^{2+} in the native CPA. The stereochemistry of the covalently bound BEBA with the ring cleavage is established to be 2*S*, 3*R* configuration on the basis of the electron density in $2F_o$-F_c maps. We were surprised by the stereochemistry at the α-position of the bound BEBA because it corresponds to the D series, which is opposite to the established stereospecificity of CPA.

SYNTHESIS AND INHIBITORY ACTIVITY OF ALL FOUR STEREOISOMERS OF BEBA

BEBA has two stereogenic centers, and thus there exist four stereoisomers for BEBA. Although the X-ray crystal study showed that the inactivating BEBA has 2*S*, 3*R*-configuration, it was thought to be necessary to synthesize all four stereoisomers of BEBA, and evaluate their inhibitory activity against CPA. These informations are useful for the elucidation of the spatial arrangement of the binding as well as catalytic sites present at the active site of the enzyme. Racemic 2-benzyl-2-vinylacetic acid methyl ester was enzymatically

Scheme 1.



resolved using α-chymotrypsin at pH 7.8, whereby (*R*)-2-benzyl-2-vinylacetic acid ester was hydrolyzed to give the corresponding acid, leaving the (*S*)-enantiomer intact (ref. 15). The acid thus obtained was isolated, and converted to the benzyl ester by allowing to react with benzyl bromide in DMF in the presence of potassium carbonate. Epoxidation of the benzyl ester with *m*-chloroperoxybenzoic acid afforded the benzyl ester of BEBA as a mixture of diastereomers, which were separated by the chromatographic method using a silica gel column whereby the benzyl ester of (2*R*, 3*R*)-BEBA was eluted first. Each of the separated diastereomers was subjected to catalytic hydrogenation in the presence of a catalytic amount of Pd/C to afford BEBA of the corresponding stereochemistry (Scheme I). The parallel treatment of (*S*)-2-benzyl-2-vinylacetic acid which was obtained from the intact methyl ester in the enzymatic resolution of methyl 2-benzyl-2-vinylacetate (Scheme I). The absolute stereochemistry of the four diastereomers of BEBA thus synthesized was assigned on the basis of the following observations : In the TLC (silica gel, hexane (6) / ethyl acetate (1)) the benzyl ester of BEBA obtained by the epoxidation of benzyl (*S*)-2-benzyl-2-vinylacetate showed R_f value of 3.2 which is identical to that of the racemic benzyl ester of BEBA prepared from optically inactive benzyl 2-benzyl-2-vinylacetate. The racemic BEBA generated from the latter benzyl ester shows potent inhibitory activity against CPA, and as described above the X-ray crystallographic analysis of the covalently modified CPA by this racemic BEBA established that the inactivating enantiomer has the (2*S*, 3*R*)-configuration. Therefore, the BEBA obtained from (*S*)-2-benzyl-2-vinylacetic acid has the (2*S*, 3*R*)-configuration, and its enantiomer prepared from (*R*)-2-benzyl-2-vinylacetic acid by the parallel route possesses the (2*R*, 3*S*)-configuration. The stereochemistry of the remaining two isomers are then correspondingly established to be (2*S*, 3*S*) for BEBA prepared from (*S*)-2-benzyl-2-vinylacetic acid, and (2*R*, 3*R*) for the BEBA from its enantiomeric *R*-acid.

As expected, (2*S*, 3*R*)-BEBA exhibited potent and time-dependent inhibitory activity against CPA with a $K_i = 0.79 \pm 0.18$ mM which compares favorably with the previous value of 6.25 mM obtained with diastereoisomeric mixture of BEBA (ref. 11). We are surprised, however, at the observation that (2*R*, 3*S*)-BEBA also inhibits CPA with a K_i value of 3.78 ± 1.37 mM. It is even more surprising to find that CPA binds preferentially the inhibitor having D configuration, i.e. (2*S*, 3*R*)-BEBA, as revealed by the X-ray crystal structure of the inactivated CPA, even if the difference in binding affinity between the two enantiomeric isomers is only marginal. The enzyme activity did not return upon dialysis of the incubation mixture of CPA and each of the active isomers. The other two isomers had essentially no inhibitory activity.

MECHANISTIC IMPLICATION AND THREE-DIMENSIONAL SCHEMATIC REPRESENTATION OF THE ACTIVE SITE

The results of present study are consistent with the anhydride mechanism for the enzymic action of CPA because no covalent modification is expected to occur at the Glu-270 by the alternative mechanism, in which the carboxylate of Glu-270 functions as a general base activating the nearby water molecule. In the latter pathway, 2-benzyl-3,4-dihydroxybutanoic acid is expected to be formed. Recently, Mock and Zhang (ref. 16) proposed a new general base mechanism which comprises the terminal carboxylate of substrate as the catalytic site for the enzymatic hydrolysis of CPA. The carboxylate of substrate enters the coordination sphere of the Zn^{2+} while the Zn^{2+} bound water molecule is concurrently transposed to the scissile carbonyl of the substrate. Thus, it is the substrate carboxylate that serves to deprotonate the zinc bound water,

promoting a nucleophilic attack, in a *si*-fashion, at the scissile peptide carbonyl which is activated by the ligating to the Zn^{2+}. The present study, however, does not support the conjectured mechanism of CPA by Mock and Zhang.

The observed stereochemical specificity of BEBA as an inhibitor of CPA may be envisioned by invoking a three-dimensional schematic representation for the active site of CPA as shown by Figure 3. In the proposed model the primary recognition pocket which is invaginated deep into the core of the enzyme molecule is shown with a bold line. The guanidinium moiety of Arg-145 and the carboxylate of Glu-270 are at or near the surface of the molecule. The spatial arrangement of other important binding and catalytic groups is indicated. The Zn^{2+} at the active site is shown with bold letters to indicate that it is present deep in the core of the enzyme molecule. The epoxide oxygen in both isomers of (2*S*, 3*R*)- and (2*R*, 3*S*)-BEBA is oriented within an effective distance (slightly over 2.0 Å) for the ligation to the Zn^{2+} to be taken place (ref. 17) when these isomers bind the active site (Figure 3), whereas the epoxide oxygen of inactive isomers is

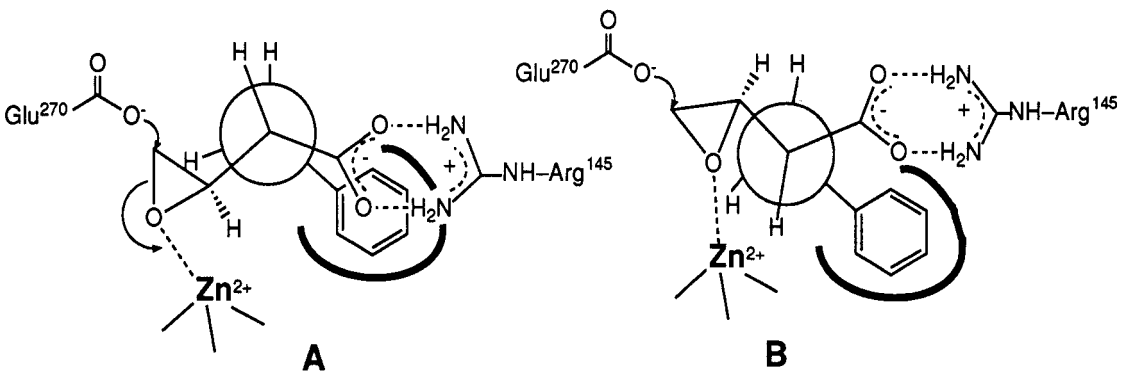


Figure 3. Schematic representation of the active site of CPA that is complexed respectively with the irreversible inhibitor of (2*S*, 3*R*)-BEBA (3A) and (2*R*, 3*S*)-BEBA (3B), both of which proceed to modify the carboxylate of Glu-270 covalently with the cleavage of the oxirane ring.

much more displaced from the Zn^{2+} (Figure 4). The carboxylate and phenyl groups of (2*S*, 3*R*)-BEBA are oriented in a partially eclipsed conformation in this mode of binding. Such a conformation of the BEBA is, however, not at all unreasonable considering the relative energy state of the conformer : In the case of *N*-acetyl-L-phenylalanine the energy difference between the conformer having the two groups (the carboxylate and the phenyl) in partially eclipsed orientation and that of more stable gauche conformation is calculated to be only about 2~3 Kcal/mol (ref. 18). Furthermore, there are several literature precedents which show the two groups in the CPA bound ligands to have such partial eclipsed conformation in X-ray crystal structure (ref. 2, 19). The epoxide ring which is activated through the ligation to the Zn^{2+} is then subjected to a nucleophilic attack by the carboxylate of Glu-270, leading to a covalent attachment of the BEBA to the enzyme with a concomitant ring cleavage (Figure 2).

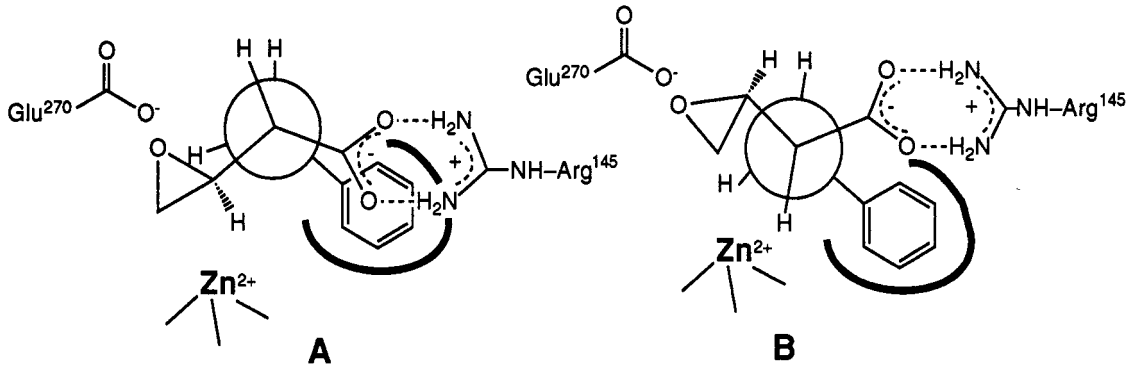


Figure 4. In the complex of CPA with the inactive stereoisomer of BEBA, i.e., (2*S*,3*S*)-BEBA (4A) and (2*R*,3*R*)-BEBA (4B) the oxirane oxygen fails to ligate to the Zn^{2+}.

SUMMARY AND CONCLUSION

In summary, BEBA is a potent inactivator of CPA, which was designed rationally on the basis of the known topology of the active site and mechanistic roles of the functional groups involved in the catalytic action. As shown by the X-ray crystal structure of the inactivated CPA, BEBA inhibits the enzyme irreversibly by covalently attaching to the carboxylate of Glu-270. Of four stereoisomers of BEBA, (2*S*, 3*R*)- and (2*R*, 3*S*)-BEBA are active as the inhibitor of CPA, and the other two are essentially inactive. An explanation is offered for the stereospecificity of BEBA in the inactivation of CPA by invoking a three-dimensional representation of the active site of CPA. All four stereoisomers of BEBA were synthesized efficiently and conveniently starting with optically active 2-benzyl-2-vinylacetic acid which was obtained by a kinetic resolution of racemic methyl ester of the acid using α-chymotrypsin.

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