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PEPTIDYL EPOXIDES AS INHIBITORS OF α-CHYMOTRYPSIN: REMARKABLE CHANGE FROM IRREVERSIBLE TO REVERSIBLE COMPETITIVE INHIBITOR AS A CONSEQUENCE OF THE IMPROVEMENT OF BINDING AFFINITY

Dong H. Kim,* Zhi-Hong Li, and Soo Suk Lee

Center for Biofunctional Molecules and Department of Chemistry
Pohang University of Science and Technology, San 31 Hyojadong Pohang 790-600, Korea

Abstract: The replacement of the ester group in (2*S*,3*R*)-2-benzyl-3,4-epoxybutanoic acid methyl ester (**1**) with a *N*-benzoyl-2-pyrrolidylcarboxamide moiety changes the mode of inhibition for α-chymotrypsin from an active site directed inactivator to a reversible competitive inhibitor.

α-Chymotrypsin[1] is a serine endopeptidase with a primary specificity for peptide substrates containing aromatic amino acid residues at the P_1 position. The enzyme has attracted much attention as a prototypic protease: It has served as a model for the study of enzymic mechanism and development of inhibitor design principles, both of which can be applied for other serine proteases of pharmacological interest.[2] Recently,

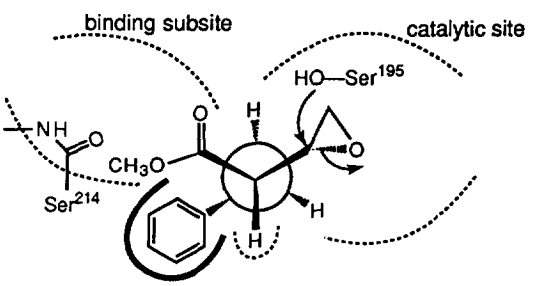
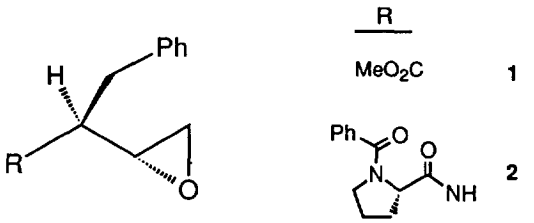


Figure 1. Schematic representation showing 2-benzyl-3,4-epoxybutanoic acid methyl ester (**1**) binds at the active site of α-chymotrypsin, in such a way that there ensues a chemical reaction to a covalently modify the Ser^{195} hydroxyl.

we have reported a novel inhibitor design principle which makes use of enzyme's substrate stereospecificity.[3] (2*S*,3*R*)-2-Benzyl-3,4-epoxybutanoic acid methyl ester (**1**), an active site directed inactivator for α-chymotrypsin is the first example which was designed under the principle. Because of its

molecular configuration at the α-position, the inhibitor binds the enzyme in such a way that its epoxide ring rather than the ester moiety rests at the catalytic region of the enzyme, thus the oxirane ring undergoes an electrophilic ring opening reaction with the catalytic hydroxyl of Ser-195 to bring about a covalent modification of the catalytic site with formation of an ether linkage (Figure 1).[3] The occurrence of the chemical modification of the Ser-195 hydroxyl by the inhibitor was subsequently substantiated by the electrospray ionization mass spectra of the inactivated enzyme and its degradation fragments.[4] The inhibitory potency of **1** expressed as k_{inact}/K_I was calculated to be 27 $M^{-1}s^{-1}$. In this communication we wish to report the result of our effort directed to exploit the design principle to obtain inhibitors of improved potency.



It was thought that the improvement of the inactivating potency of the prototype inhibitor would be achieved by lowering its K_i value through enhancement of the inhibitor binding to the enzyme, which was thought to be attained by replacing the ester moiety with a peptidyl group that interacts with auxiliary binding subsites at the active site of the enzyme. *N*-Benzoylpyrrolidylcarboxamide derivative **2** was chosen as the compound for the study in light of the report of Segel and others.[5] They showed that substrates of α-chymotrypsin having a proline at the P_2 position bind the enzyme with high affinity by virtue of its hydrophobic interactions possibly with the side chain of Ile-99 as well as a smaller entropy decrease upon binding to the enzyme due to its restricted rotational freedom, in addition to hydrogen bondings between the substrate and active site backbone residues of the enzyme.

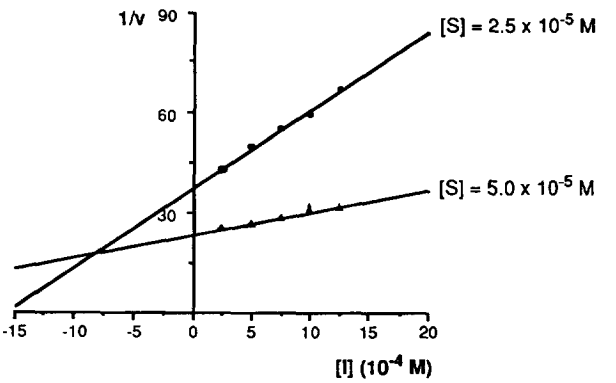


Figure 2. The Dixon plot for the inhibition of α-chymotrypsin-catalyzed hydrolysis of Suc-AAPFpNA by **2**: 0.036 M Tris buffer, pH 7.8, 0.045 M in $CaCl_2$: [E]=1.0 × 10^{-8} M, Temperature 25°C.

The designed inhibitor was synthesized by following the general method reported by Albeck and Parsky.[6] (*S*)-3-Amino-4-phenylbutene[6] (**3**) was coupled with *N*-benzoyl-L-proline using DCC and the product was oxidized with *m*-chloroperbenzoic acid to give **2**[7]. The epoxidation reaction of allylic amides has been reported to proceed stereoselectively, leading to produce peptidyl epoxides having the *threo* configuration.[6]

Contrary to the expectation, compound **2**, however, failed to exhibit a time-dependent loss of the enzymic activity, an indication of irreversible inhibition, when assayed by the procedure described previously.[8] Instead, the compound was shown to be a reversible competitive inhibitor for the enzyme having the K_I value of 0.82 mM as demonstrated by the Dixon plot[9] shown in Figure 2.

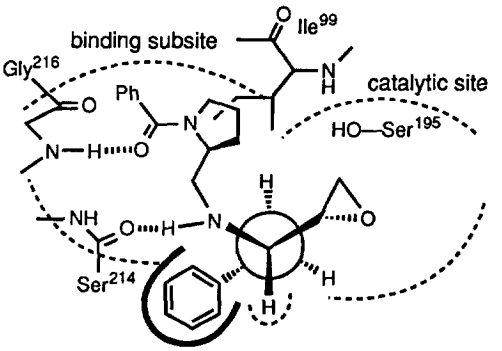


Figure 3. Schematic representation of a complex formed by binding of *N*-benzoyl-L-prolylphenylalanyl epoxide (**2**) to the active site of α-chymotrypsin.

The observed difference in the inhibitory property between **1** and **2** is surprising, but can be envisaged: As was designed and demonstrated by the kinetic analysis, **2** indeed binds the enzyme much more tightly than **1** by virtue of noncovalent interactions of the peptidyl moiety with enzyme's binding subsites as discussed above. These interactions are in addition to the hydrophobic contacts of the phenyl ring of the inhibitor to the wall of the primary binding pocket of the enzyme (Figure 3[10]). In comparison, in the complex of **1** with the enzyme there operate only hydrophobic interactions of the phenyl ring with the binding pocket. As a consequence, the C_2 carrying the oxirane moiety and the ester group in **1** can enjoy freedom of rotational movement along the C_2-C_3 axis within the boundary of the active site (Figure 1), whereas in the case of **2** no such movement is permitted because the peptidyl group is held tightly to the subsites. Because of the freedom of movement, although limited, the oxirane moiety in **1**, but not that in **2**, can approach to the hydroxyl of Ser-195 in a close enough proximity to bring about a chemical reaction, leading it to be tethered to the hydroxyl.[11] In this connection, it should be reminded that the active site of α-chymotrypsin is known to be very rigidly structured,[12] and conformational modifications are difficult to occur.

In conclusion, contrary to the expectation, **2** failed to inactivate α-chymotrysin but inhibited the enzyme in a reversible competitive fashion. The change of the mode of inhibition from affinity label to competitive inhibitor brought about by replacing the ester moiety in **1** with a peptidyl group is ascribed to the enhanced binding affinity as a result of additional binding interactions between the peptidyl of the inhibitor and the binding subsites of the enzyme. The present study demonstrates that an improvement in binding does not necessarily work advantageously in the case of irreversible inhibition of enzymes.

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