Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 12, pp. 1287-1292, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00216-2

(3R, 4S)-3-BENZYL-4-BROMOMETHYLOXETAN-2-ONE, A FAST ACTING ALTERNATE SUBSTRATE INHIBITOR OF α-CHYMOTRYPSIN

Dong H. Kim* and Jae Jeong Ryoo

Center for Biofunctional Molecules and Department of Chemistry,
Pohang University of Science and Technology, Pohang 790-784, Korea

Abstract : (3*R*,4*S*)-3-Benzyl-4-(bromomethyl)oxetan-2-one is a novel type of fast acting alternate substrate inhibitor for α-chymotrypsin. The hydroxyl group that is generated during the turnover process appears to be responsible for the high stability of the acyl enzyme intermediate.

Serine proteases play a vital role in a variety of important physiological processes such as digestion of food intake, blood coagulation, and fertilization[1]. Recently, these enzymes have received much attention as they are known to be implicated in the etiology of numerous diseases including emphysema[2], arthritis[3], and metastasis[4]. Of the serine proteases, α-chymotrypsin is most well studied, and has been served as a designing target in the development of novel protease inhibitors[1b,5]. A large number of these inhibitors bear an electrophilic heterocycle capable of being attacked by the side chain hydroxyl of the active site serine of the proteases to give ring-opened acyl enzyme intermediates. A certain type of the intermediates thus formed are highly stable and resist to be hydrolyzed. Accordingly, an impairment of the enzymic activity results, which remains for the lifetime of the acyl enzyme intermediates. These inhibitors are referred to as alternate substrate inhibitors.[6] On the other hand, there may be generated, during the acylation process, a chemically reactive functional group which can undergo an alkylation reaction with an accessible nucleophile at or near the active site. This covalent attachment inactivates the enzyme permanently, even after deacylation. This type of inhibitors are called mechanism-based inactivators.[7]

Recently, we have reported on the development of novel designing approaches for irreversible inhibitors of enzyme, whose inhibitory actions rest on the interaction of an oxirane ring with nucleophiles present at the active site[8]. As an extension of the study, we have designed

(3*R*,4*S*)-3-benzyl-4-(bromomethyl)oxetan-2-one (BBMO) as a potential mechanism-based inactivator for α-chymotrypsin. In binding the potential inhibitor to the enzyme, the benzyl group is accommodated in the primary recognition hydrophobic pocket of the active site, which would place the oxetane ring at the catalytic region as shown in Figure 1[9]. There, then, follows an acylation of the catalytic serine hydroxyl of the enzyme with the concomitant generation of a hydroxyl which we expected to undergo an oxirane ring formation *via* an intramolecular displacement reaction. Such a ring formation reaction is commonly observed in solution chemistry[10]. The chemically reactive oxirane thus generated was thought to be functioning as an alkylating moiety, causing to modify the enzyme covalently by reacting with an active site nucleophile possibly the imidazole of His-57 (Figure 1). However, when the designed inhibitor was evaluated as the inactivator for α-chymotrypsin, it was shown to be an alternate substrate inhibitor rather than the mechanism-based inactivator. This communication reports kinetic analyses of the inhibition to show that (3*R*,4*S*)-BBMO is a novel type of alternate subsrate inhibitor for α-chymotrypsin.

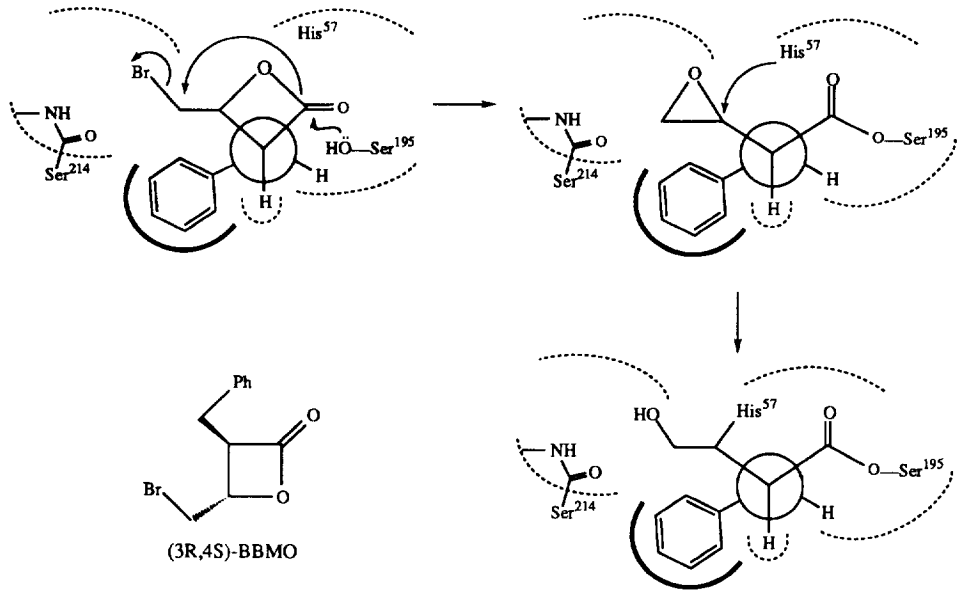

Figure 1. Rationale used in the design of (3*R*,4*S*)-BBMO as a mechanism-based inactivator for α-chymotrypsin (see explanation in the text).

The designed inhibitor was synthesized in a 62% yield by treating (*S*)-2-benzyl-2-vinylacetic acid with bromine in alkaline conditions following the method of Shibata et al.[11]

The kinetic study was carried out by following the competitive substrate assay method[6k] based on the kinetic expression (equation 1) derived by Main for the competitive inhibition that can be represented by Scheme 1.[12] In the equation, S_o and I_o represent the initial substrate and inhibitor concentrations, respectively, v_o and v are the velocity of substrate hydrolysis at the initial time and at time t. Figure 2 shows progress curves thus obtained for the inhibition of α-chymotrypsin by (3*R*,4*S*)-BBMO of different concentrations, from which values of k_{obs} were obtained directly using the computer-assisted UV spectrophotometer (Hewlett Packard Diode array spectrophotometer 8452A).

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

$$E + S \overset{K_m}{\rightleftharpoons} E{\cdot}S \longrightarrow E + P$$

Scheme 1

Equation (1) may be rearranged, after being integrated, to give equation 2 from which kinetic parameters of K_i and k_a (acylation rate constant) are obtainable: From the double reciprocal plot of k_{obs} *vs* $[I]_o$ (Figure 3), values of K_i and k_a were calculated to be 2.55 μM and 2.94 min^{-1}, respectively.

$$\ln\left(\frac{v}{v_o}\right) = \frac{-k_a K_m [I]_o}{K_i \cdot K_m + [S]_o K_i + [I]_o K_m} \cdot t = -k_{obs} t \qquad (1)$$

$$\frac{1}{k_{obs}} = \frac{1}{k_a} + \frac{K_i}{k_a}\left(1 + \frac{[S]_o}{K_m}\right)\frac{1}{[I]_o} \qquad (2)$$

The compound showed a time-dependent inhibition of the enzymic activity under pseudo first-order conditions (Figure 4). The observed inhibitory kinetics satisfies a requirement of the mechanism-based inactivation, but it conforms to an alternate substrate inhibition as well. In order to discriminate between the two possible modes of inhibition we carried out a dialysis experiment to observe a return of the enzymic activity. This return of enzymic activity clearly demonstrates that (3*R*, 4*S*)-BBMO is an alternate substrate inhibitor rather than a mechanism-based inactivator. Apparently, the acyl enzyme intermediate formed during the turnover is stable and difficultly hydrolyzed, which is supported by the low deacylation rate constant (k_d) of 0.094 min^{-1} determined by the proflavin displacement assay method of Bernhard.[13]

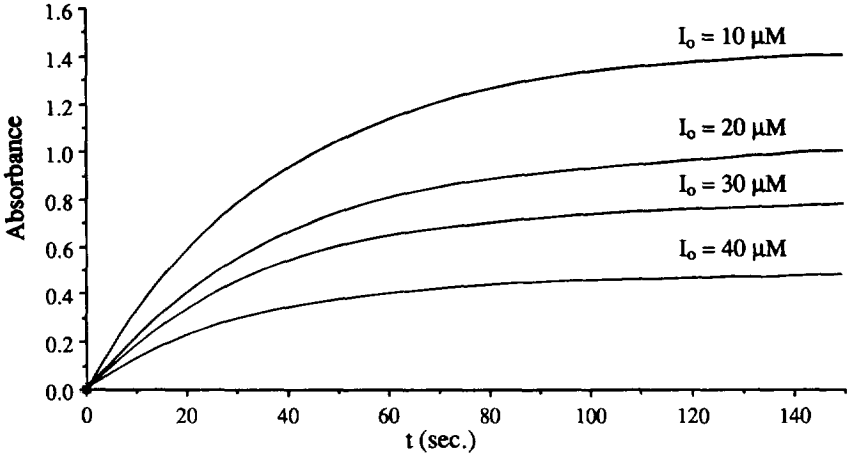

Figure 2. Progress curves for the inhibition of α-chymotrypsin by (3*R*,4*S*)-BBMO in the presence of succinyl-Ala-Ala-Phe-*p*-nitroanilide. α-Chymotrypsin (100 nM) was added to a solution of (3*R*,4*S*)-BBMO (10 – 40 μM) in 2.5% acetone-0.04% TRIS buffer, 0.5 M $CaCl_2$, pH 7.8 at 25 °C, and the hydrolysis of the substrate was followed at 400 nm.

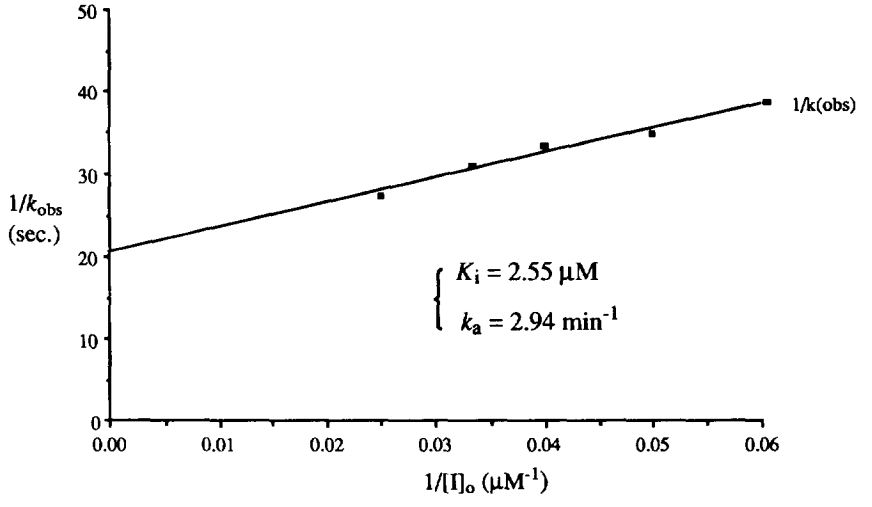

Figure 3. Determination of K_i and k_a of (3*R*,4*S*)-BBMO for the inhibition of α-chymotrypsin by the method of competitive substrate assay. A double reciprocal plot of the initial concentration of (3*R*,4*S*)-BBMO *vs* k_{obs} gives a straight line with a slope of (K_i/k_a) $(1+[S]_o/K_m)$ and a y intercept of $1/k_a$ (see equation (2)).

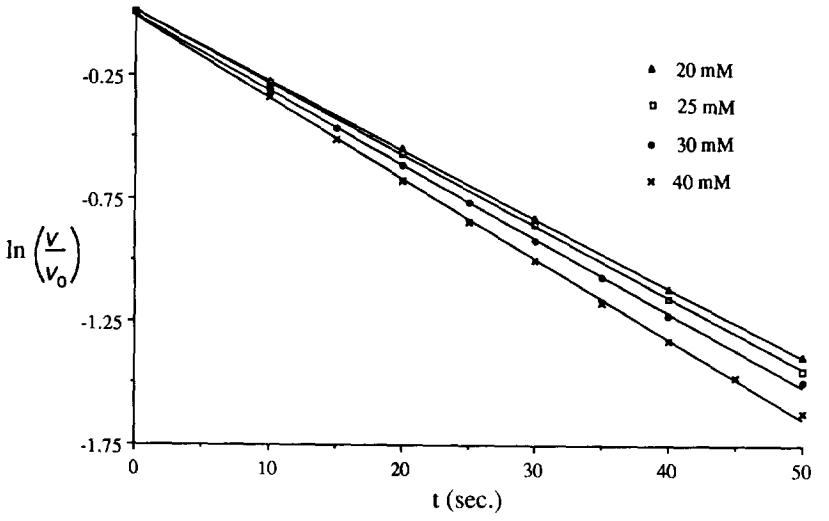

Figure 4. Time-dependent inhibition of α-chymotrypsin by (3*R*,4*S*)-BBMO

Since an X-ray structure of the acyl enzyme intermediate is not available, no definite explanation may be offered presently for the cause of the high stability of the intermediate, but it may be envisioned that the newly formed hydroxyl is responsible for the observed stability of the acyl-enzyme possibly through hydrogen bonding with various functionalities present at the active site. Reed and Katzenellenobogen suggested that hydrogen bonding between an inhibitor and enzyme is one of major binding forces which stabilizes the acyl enzyme intermediate[61]. This hydrogen bond forming propensity of the newly generated hydroxyl may also be responsible for, at least in part, its failure to undergo an oxirane ring formation reaction. However, the possibility of the hydroxyl to displace the active site water molecule that is required for the deacylation can not be eliminated[14].

In conclusion, (3*R*, 4*S*)-BBMO is a novel type of alternate substrate inhibitor of α-chymotrypsin, which inhibits the catalytic activity of the enzyme very rapidly and efficiently. The high stability of the acyl enzyme intermediate appears to be due to the hydroxyl group that is generated during the turnover process: The newly formed hydroxyl may be involved in hydrogen bond formations with various functionalities present at the active site, and/or possibly displace the water molecule required for the deacylation. Exploitation of the present observation for designing therapeutically useful inhibitors is in progress.

Acknowledgement : The authors wish to thank Dr. Du-Jong Baek for helpful discussions and the Korea Science and Engineering Foundation for the financial support of this work.

References and Notes

1. (a) Dixon, M.; Webb, E. C. *Enzymes*, 3rd ed. Academic Press, New York, 1979; pp 301-307. (b) Fischer, G. *Natural Product Reports* **1988**, 465-495.
2. Janoff, A. *Ann. Rev. Respi. Dis.* **1985**, *132*, 417-433.
3. (a) Krantz, A. *Ann. Rep. Med. Chem.* **1993**, *28*, 187-196. (b) Hiasta, D. J.; Pagani, E. D. *Ann. Rep. Med. Chem.* **1994**, *29*, 195-204.
4. Henkin, J. *Ann. Rep. Med. Chem.* **1993**, *28*, 151-160.
5. Walpole, C. S. J.; Wrigglesworth, R. *Natural Product Reports* **1989**, 311-346.
6. (a) Henderson, R. *J. Mol. Biol.* **1970**, *54*, 341-354. (b) Robillard, G. T.; Powers, J. C.; Wilcox, P. E. *Biochemistry* **1972**, *11*, 1773-1784. (c) Zimmerman, M.; Morman, H.; Mulvey, D.; Jones, H.; Frankshu, R.; Ashe, B. M. *J. Biol. Chem.* **1980**, *255*, 9848-9851. (d) Ashe, B. M.; Clark, R. L.; Jones, H.; Zimmerman, M. *J. Biol. Chem.* **1981**, *256*, 11603-11606. (e) Weidman, B.; Abeles, R. H. *Biochemistry* **1984**, *23*, 2373-2376. (f) Gupton, B. F.; Carroll, D. L.; Tuhy, P. M.; Kam, C.-M.; Powers, J. C. *J. Biol. Chem.* **1984**, *259*, 4279-4287. (g) Ringe, D.; Seaton, B. A.; Gelb, M. H.; Abeles, R. H. *Biochemistry* **1985**, *24*, 64-68. (h) Harper, J. W.; Hemmi, K.; Powers, J. C. *Biochemistry* **1985**, *24*, 1831-1841. (i) Gelb, M. H.; Abeles, R. H. *J. Med. Chem.* **1986**, *29*, 585-589. (j) Sofia, M. J.; Katzenellenbogen, J. A. *J. Med. Chem.* **1986**, *29*, 230-238. (k) Baek, D.-J.; Reed, P. E.; Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry*, **1990**, *29*, 4305-4311. (l) Reed, P. E.; Karzenellenbogen, J. A. *J. Med. Chem.* **1991**, *34*, 1162-1176. (m) Reed, P. E.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1991**, *266*, 13-21.
7. (a) Chakaravarty, P. K.; Kraft, G. A.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1982**, *257*, 610-612. (b) Moorman, A. R.; Abeles, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 6785-6786. (c) Westkaemper, P. B.; Abeles, R. H. *Biochemistry* **1983**, *22*, 3256-3264. (d) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046-15053. (e) Gelf, M. H.; Abeles, R. H. *Biochemistry* **1984**, *23*, 6596-6604. (f) Groutes, W. C.; Stanga, M. A.; Brubaker, M. J. *J. Am. Chem. Soc.* **1989**, *111*, 1931-1932. (g) Skiles, J. W.; Fucks, V.; Leonard, S. F. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 69-72. (h) Edwards, P. D.; Meyers, E. F. Jr.; Vigayalakshmi, J.; Tuthill, P. A.; Andisik, D. A.; Gomes, B.; Strimper, A. *J. Am. Chem. Soc.* **1992**, *114*, 1854-1863. (i) Chabin, R.; Green, B. G.; Gale, P.; Maycock, A. L.; Weston, H.; Dorn, C. P.; Finke, P. E.; Hagmann, W. K.; Hale, J. J.; MacCoss, M.; Shah, S. K.; Underwood, D.; Doherty, J. B.; Knight, W. B. *Biochemistry* **1993**, *32*, 8970-8980.
8. (a) Kim, D. H.; Kim, K. B. *J. Am. Chem. Soc.* **1991**, *113*, 3200-3202. (b) Yun, M.; Park, C.; Kim, S.; Nam, D.; Kim, S. C.; Kim, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 2281-2282. (c) Kim, D. H.; Li, Z.-H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2297-2302. (d) Kim, D. H.; Kim, Y. M.; Li, Z.-H.; Kim, K. B.; Choi, S. Y.; Yun, M.; Kim, S. *Pure Appl. Chem.* **1994**, *66*, 721-728.
9. Kim, D. H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1313-1318.
10. Lewars, E. G. *Comprehensive Heterocyclic Chemistry*; Katritzky, A. R.; Rees, C. W. Ed.; Pergamon Press: Oxford, 1984; Vol 7, p. 115.
11. (a) Shibata, I.; Toyota, M.; Baba, A.; Matsuda, H. *J. Org. Chem.* **1990**, *55*, 2487-2491. (b) Black, T. H.; Huang J. *Tet. Lett.* **1993**, *34*, 1411-1412.
12. Main, A. R. *Essays in Toxicology*; Hays, W. J. Ed. Academic Press: New York, 1973; Vol. 4, pp. 59-105.
13. Bernhard, S. A.; Lee, B. F.; Tashjian, Z. H. *J. Mol. Biol.* **1966**, *18*, 405-420.
14. (a) Fersht, A. *Enzyme Structure and Mechanism*; 2nd ed., Freeman; New York, 1984; pp. 408-411. (b) Singer, P. T.; Smalås, A.; Carty, R. P.; Mangel, W. F.; Sweet, R. M. *Science*, **1993**, 259, 669-673.

(*Received in Japan* 13 *March* 1995; *accepted* 8 *May* 1995)