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β-Lactones as a New Class of Cysteine Proteinase Inhibitors: Inhibition of Hepatitis A Virus 3C Proteinase by *N*-Cbz-serine β-Lactone

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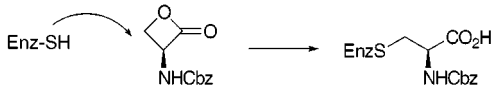
ABSTRACT



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***N*-Benzyloxycarbonyl-L-serine β-lactone (1) is shown to irreversibly inactivate the 3C cysteine proteinase of hepatitis A virus (HAV) with $k_{inact} = 0.70$ min^{-1}, $K_I = 1.84 \times 10^{-4}$ M and $k_{inact}/K_I = 3800$ M^{-1} min^{-1} at an enzyme concentration of 0.1 μM. Mass spectrometric and HMQC NMR studies using ^{13}C-labeled 1 show that the active site cysteine (Cys-172) thiol of the HAV 3C proteinase attacks the β-position (i.e. C-4) of the oxetanone ring, thereby leading to ring opening and alkylation of the sulfur. In contrast, the enantiomer of this β-lactone, 2, is a reversible competitive inhibitor ($K_i = 1.50 \times 10^{-6}$ M) at similar enzyme concentrations. The β-lactone motif represents a new class of inhibitors of cysteine proteinases.**

Considerable effort is devoted to the study and inhibition of cysteine proteinases because they are targets for development of therapeutic agents for many diseases, including viral infections, parasitic ailments, arthritis, cancer, and osteoporosis.[1] The 3C cysteine proteinases of picornaviruses attract extensive attention because they are essential for maturation and replication of pathogens such as hepatitis A virus (HAV), human rhinovirus (HRV) (a causative agent for common cold), poliovirus, foot and mouth disease virus, and encephalomyocarditis virus.[1−3] This family of viruses has a positive strand RNA genome and generates a single polyprotein that undergoes multiple proteolytic cleavages to produce the mature viral proteins. In HAV, these cleavages are initiated by the 3C proteinase,[4] an enzyme present in all picornaviruses.[3,5] Crystal structures have been reported for HAV 3C,[6] HRV 3C,[7] and poliovirus 3C proteins[8] and show that structurally these enzymes resemble trypsin-like serine proteinases.[2,3] Enzyme inhibition studies on HAV 3C and HRV 3C proteinases have employed a large variety of compounds as potential therapeutic leads, including peptide aldehydes,[9,10] peptide fluoromethyl ketones,[11] β-lactams,[12] isatins,[13] homophthalimides,[14] vinylogous esters and sul-

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(1) For reviews, see: (a) Roose, J. P.; Van Noorden, C. J. F. *J. Lab. Clin. Med.* **1995**, *125*, 433−441. (b) Otto, H.-H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133−171. (c) Thompson, S. K. *Bioorg. Med. Chem.* **1999**, *7*, vii and following articles in issue.

(2) Malcolm, B. A. *Protein Sci.* **1995**, *4*, 1439−1445.

(3) (a) Bergmann, E. M.; James, M. N. G. In *Proteinases of Infectious Agents*; Dunn, B., Ed.; Academic Press: San Diego, CA, 1999; pp 139−163. (b) Gorbalenya, A. E.; Snijder, E. J. *Perspect. Drug Discov. Design* **1996**, *6*, 64−86.

(4) Schultheiss, T.; Sommergruber, W.; Kusov, Y.; Gauss-Müller, V. *J. Virology* **1995**, *69*, 1727−1733.

(5) Dougherty, W. G.; Semler, B. L. *Microbiol. Rev.* **1993**, *57*, 781−822.

(6) Bergmann, E. M.; Mosimann, S. C.; Chernaia, M. M.; Malcolm, B. A.; James, M. N. G. *J. Virol.* **1997**, *71*, 2436−2448.

(7) Matthews, D. A.; Smith, W. W.; Ferre, R. A.; Condon, B.; Budahazi, G.; Sisson, W.; Villafranca, J. E.; Janson, C. A.; McElroy, H. E.; Gribskov, C. L.; Worland, S. *Cell* **1994**, *77*, 761−771.

(8) Mosimann, S. C.; Cherney, M. M.; Sia, S.; Plotch, S.; James, M. N. G. *J. Mol. Biol.* **1997**, *273*, 1032−1047.

(9) Malcolm, B. A.; Lowe, C.; Shechosky, S.; McKay, R. T.; Yang, C. C.; Shah, V. J.; Simon, R. J.; Vederas, J. C.; Santi, D. V. *Biochemistry* **1995**, *34*, 8172−8179.

(10) Webber, S. E.; Okano, K.; Little, T. L.; Reich, S. H.; Xin, Y.; Fuhrman, S. A.; Matthews, D. A.; Love, R. A.; Hendrickson, T. F.; Patick, A. K.; Meador, J. W., III; Ferre, R. A.; Brown, E. L.; Ford, C. E.; Binford, S. L.; Worland, S. T. *J. Med. Chem.* **1998**, *41*, 2786−2805.

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fones,[15−17] halomethyl carbonyls,[18,19] and azapeptide compounds.[18,20,21] Some of these substances show in vitro antiviral activity in cell culture;[11,13−17] however, toxicity can be a problem for molecules that are generally reactive with thiols.[13] We now report that a motif not previously descibed for cysteine proteinase inhibition, namely the β-lactone functionality[22,23] in the enantiomeric serine derivatives **1** and **2**, can display good irreversible and reversible inhibition, respectively, of the HAV 3C enzyme.

β-Lactones occur naturally in a variety of organisms, and many possess potent biological activity.[24] The ability of thiols to open the four-membered ring by nucleophilic attack at either the carbonyl or at the β-position[25] suggests that cysteine proteinases could be irreversibly inactivated by β-lactones having correct substituents and stereochemistry (Figure 1). It seemed that *N*-Cbz-L-serine-β-lactone (**1**) would

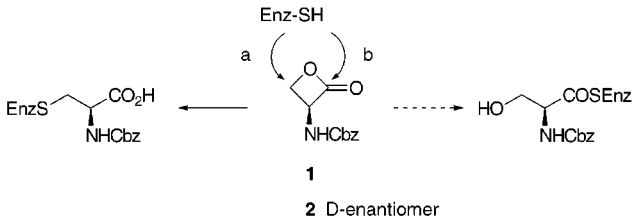


Figure 1. Possible modes of nucleophilic attack on *N*-benzyloxycarbonyl (Cbz) serine β-lactones.

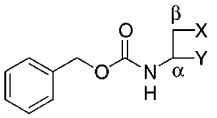
be a reasonable initial target for inhibition of the viral 3C proteinases because of its facile preparation by Mitsunobu cyclization of *N*-Cbz-L-serine,[25] its simple scaffold which permits structural variation for subsequent structure−activity studies, and its benzyl group which may mimic the P_2' phenylalanine side chain in HAV 3C substrates.[19,26] A possible concern is the susceptibility of **1** to hydrolysis since α-amino-β-lactones bearing no β-substituent display low stability in basic aqueous media.[25] However, the half-life for hydrolysis of **1** in phosphate buffer at pH 7.5 is 76 min, which is sufficiently long for enzyme inhibition studies.

Despite the absence of a P_1 glutamine side chain important for substrate recognition, **1** is a good time-dependent *irreversible* inhibitor of HAV 3C proteinase ($k_{inact} = 0.70$ min^{-1}, $K_I = 1.84 \times 10^{-4}$ M, $k_{inact}/K_I = 3800$ M^{-1} min^{-1}) at an enzyme concentration of 0.1 μM.[27] Interestingly the enantiomer **2** is a competitive *reversible* inhibitor of HAV 3C proteinase ($K_i = 1.50 \times 10^{-6}$ M) at a similar enzyme concentration.[28,29] The possibility that compound **2** may in fact be a time-dependent inhibitor but that this was not seen under the assay conditions could be eliminated because studies at other pH conditions (e.g. pH 6) and with varying concentrations of inactivator **2** also display simple competive behavior. Clearly the HAV 3C active site shows different modes of binding for enantiomers **1** and **2**, with only the former leading to permanent covalent modification of the active site (see below). Furthermore, the inhibitory properties of **1** and **2** were not affected by short exposure to 10-fold molar excess concentrations of dithiothreitol, suggesting that β-lactones of this type could be specific enzyme inhibitors that would not react inadvertently with ubiquitous biological thiols (e.g. glutathione).

(11) Morris, T. S.; Frormann, S.; Shechosky, S.; Lowe, C.; Lall, M. S.; Gauss-Müller, V.; Purcell, R. H.; Emerson, S. U.; Vederas, J. C.; Malcolm, B. A. *Bioorg. Med. Chem.* **1997**, *5*, 797−807.
(12) Skiles, J. W.; McNeil, D. *Tetrahedron Lett.* **1990**, *31*, 7277−7280.
(13) Webber, S. E.; Tikhe, J.; Worland, S. T.; Fuhrman, S. A.; Hendrickson, T. F.; Matthews, D. A.; Love, R. A.; Patick, A. K.; Meador, J. W.; Ferre, R. A.; Brown, E. L.; DeLisle, D. A.; Ford, C. E.; Binford, S. L. *J. Med. Chem.* **1996**, *39*, 5072−5082.
(14) Wang, Q. M.; Johnson, R. B.; Jungheim, L. N.; Cohen, J. D.; Villarreal, E. C. *Antimicrob. Agents Chemother.* **1998**, *42*, 916−920.
(15) Dragovich, P. S.; Webber, S. E.; Babine, R. E.; Fuhrman, S. A.; Patick, A. K.; Matthews D. A.; Lee, C. A.; Reich, S. H.; Prins, T. J.; Marakovits, J. T.; Littlefield, E. S.; Zhuo, R.; Tikhe, J.; Ford, C. E.; Wallace, M. B.; Meador, J. W., III; Ferre, R. A.; Brown, E. L.; Binford, S. L.; Harr, J. E. V.; DeLisle, D. M.; Worland, S. T. *J. Med. Chem.* **1998**, *41*, 2806−2818.
(16) Kong, J.; Venkatraman, S.; Furness, K.; Nimkar, S.; Shepherd, T. A.; Wang, Q. M.; Aubé, J.; Hanzlik, R. P. *J. Med. Chem.* **1998**, *41*, 2579−2587.
(17) Dragovich, P. S.; Prins, T. J.; Zhuo, R.; Webber, S. E.; Marakovits, J. T.; Fuhrman, S. A.; Patick, A. K.; Matthews D. A.; Lee, C. A.; Ford, C. E.; Burke, B. J.; Rejto, P. A.; Hendrickson, T. F.; Tuntland, T.; Brown, E. L.; Meador, J. W., III; Ferre, R. A.; Harr, J. E. V.; Worland, S. T. *J. Med. Chem.* **1999**, *42*, 1213−1224.
(18) Sham, H. L.; Rosenbrook, W.; Kati, W.; Betebenner, D. A.; Wideburg, N. E.; Saldivar, A.; Plattner, J. J.; Norbeck, D. W. *J. Chem. Soc., Perkin Trans. 1* **1995,** 1081−1082.
(19) McKendrick, J. E.; Frormann, S.; Luo, C.; Semchuk, P.; Vederas, J. C.; Malcolm, B. A. *Int. J. Mass Spectrom.* **1998**, *176*, 113−124.
(20) Venkatraman, S.; Kong, J.; Nimkar, S.; Wang, Q. M.; Aubé, J.; Hanzlik, R. P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 577−580.
(21) Yanting, H.; Malcolm, B. A.; Vederas, J. C. *Bioorg. Med. Chem.* **1998**, *7*, 607−619.
(22) Tetrahydrolipstatin (Orlistat) inactivates human pancreatic lipase by acylating a serine hydroxyl of the enzyme: (a) Lüthi-Peng, Q.; Märki, H. P.; Hadváry, P. *FEBS Lett.* **1992**, *299*, 111−115. (b) Hadváry, P.; Sidler, W.; Meister, W.; Vetter, W.; Wolfer, H. *J. Biol. Chem.* **1991**, *266*, 2021−2027. (c) Other β-lactones may also acylate related lipases: Kocienski, P. J.; Pelotier, B.; Pons, J.-M.; Prideaux, H. *J. Chem. Soc., Perkin Trans. 1* **1998**, 1373−1382 and references therein. (d) β-Lactones such as the antibiotic F-244 (1233A) may acylate a cysteine residue of HMG CoA synthase: Romo, D.; Harrison, P. H. M.; Jenkins, S. I.; Riddoch, R. W.; Park, K.; Yang, H. W.; Zhao, C.; Wright, G. D. *Bioorg. Med. Chem.* **1998**, *6*, 1255−1272. Mayer, R. J.; Louis-Flamberg, P.; Elliott, J. D.; Fisher, M.; Leber, J. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 610−616.
(23) Omuralide, the β-lactone derived from lactacystin, has been shown to inhibit the 20S proteosome by O-acylation of a threonine residue: (a) Corey, E. J.; Li, W. D. Z. *Chem. Pharm. Bull.* **1999**, *47*, 1−10. (b) Corey, E. J.; Li, W. D. Z.; Nagamitsu, T.; Fenteany, G. *Tetrahedron* **1999**, *55*, 3305−3316.
(24) For reviews of natural β-lactones, see: (a) Lowe, C.; Vederas, J. C. *Org. Prep. Proced. Int.* **1995**, *27*, 305−346. (b) Pommier, A.; Pons, J. M. *Synthesis* **1995**, *7*, 729−744. (c) For a review of syntheses of β-lactones, see: Yang, H. W.; Romo, D. *Tetrahedron* **1999**, *55*, 6403−6434.
(25) For a discussion of reactions and leading references, see: (a) Pansare, S. V.; Huyer, G.; Arnold, L. D.; Vederas, J. C. *Org. Synth.* **1991**, *70*, 1−9. (b) Pansare, S. V.; Arnold, L. D.; Vederas, J. C. *Org. Synth.* **1991**, *70*, 10−17. (c) Arnold, L. D.; Kalantar, T. H.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7105−7109. (d) Arnold, L. D.; May, R. G.; Vederas, J. C. *J. Am. Chem. Soc.* **1988**, *110*, 2237−2241.
(26) (a) Jewell, D. A.; Swietnicki, W.; Dunn, B. M.; Malcolm, B. A. *Biochemistry* **1992**, *31*, 7862−7869.
(27) Kinetic constant were obtained as described in the following: Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245−3249.
(28) Segel, I. H. In *Enzyme Kinetics*; John Wiley & Sons: New York, 1975; pp 125−135.
(29) The wild-type HAV 3C has 219 amino acids with a molecular weight of 24 kilodaltons, exists as an active monomer, and has a noncatalytic external cysteine (Cys24) as well as the active site Cys172. Our studies employed the recombinant C24S enzyme which has catalytic properties indistinguishable from the wild type (see ref 21). Ideal peptide substrates mimic the 2B/2C junction of the large precursor polyprotein with glutamine preferred at the P_1 residue: the k_{cat} of this enzyme is typically about 1.8 s^{-1} with a K_m of 2.1 mM at pH 7.5; see ref 2.

To probe the importance of the β-lactone ring for HAV 3C inhibition, serine, homoserine, and γ-lactone analogues were prepared (Table 1). The acyclic derivatives **3**, **4**, **7**, and **8** at a concentration of 100 μM show no significant inhibition of HAV 3C proteinase. However, the lack of inhibition could potentially be due to the anionic charge of the carboxylate at pH 7.5. Hence, γ-lactones **5** and **6** were prepared and tested against HAV 3C proteinase. γ-Lactones are well-known to undergo ring opening as a result of nucleophilic attack at the carbonyl, but they can react with nucleophiles at the γ-position.[30] However, **5** and **6** at 100 μM concentration failed to show any inhibition of this enzyme.

Table 1. Serine and Homoserine Derivatives as Inhibitors of HAV 3C Proteinase



entry	X	Y	α-confign	IC_{50} (μM)[a]
1	O—CO		*S*	35
2	O—CO		*R*	6
3	OH	CO_2Li	*S*	≫100
4	OH	CO_2Li	*R*	≫100
5	H_2CO—CO		*S*	≫100
6	H_2CO—CO		*R*	≫100
7	CH_2OH	CO_2Li	*S*	≫100
8	CH_2OH	CO_2Li	*R*	≫100

[a] IC_{50} conditions: 0.1 μM HAV 3C, 10 μM Dabsyl-GLRTQSFS-Edans, 2 mM EDTA, 0.1 mg/mL BSA, 100 mM K_3PO_4 at pH 7.5, 1% DMF.

The kinetic observations with **1** are consistent with a rapid covalent inactivation of HAV 3C proteinase. To confirm this, electrospray mass spectrometry was used to examine the enzyme after treatment with **1**. The spectra of the uncomplexed enzyme and the HAV 3C-**1** complex are shown in Figure 2. The mass difference between the enzyme−inhibitor complex and the uninhibited enzyme (219 Da) is within experimental error of the calculated mass of the inhibitor (221 Da). In parallel with these studies, a control dialysis experiment was done on the HAV 3C-**1** complex and uninhibited HAV 3C. After dialysis for 8 h at 4 °C, the uninhibited enzyme retained activity, but the HAV 3C-**1** complex showed no recovery of proteinase activity, thereby suggesting that **1** may be covalently attached rather than tightly held in a noncovalent complex.

To determine the type of adduct formed between **1** and the HAV 3C enzyme by NMR spectroscopy, **1** was synthesized with ^{13}C (99% isotopic purity) at the β-position from the corresponding commercially available labeled L-serine. Model thioether and thioester compounds were generated to assist in ascertaining whether enzymatic thiolate attack on **1** proceeds at the β-position to give a thioether or at the carbonyl of the β-lactone to form a thioester.[22,31] Since it is known that serine or threonine residues in proteins can act as nucleophiles to open β-lactones by attack at carbonyl to give acylated products,[23,32,33] the chemical shifts of model serine ester and ether derivatives were also examined.[31] The

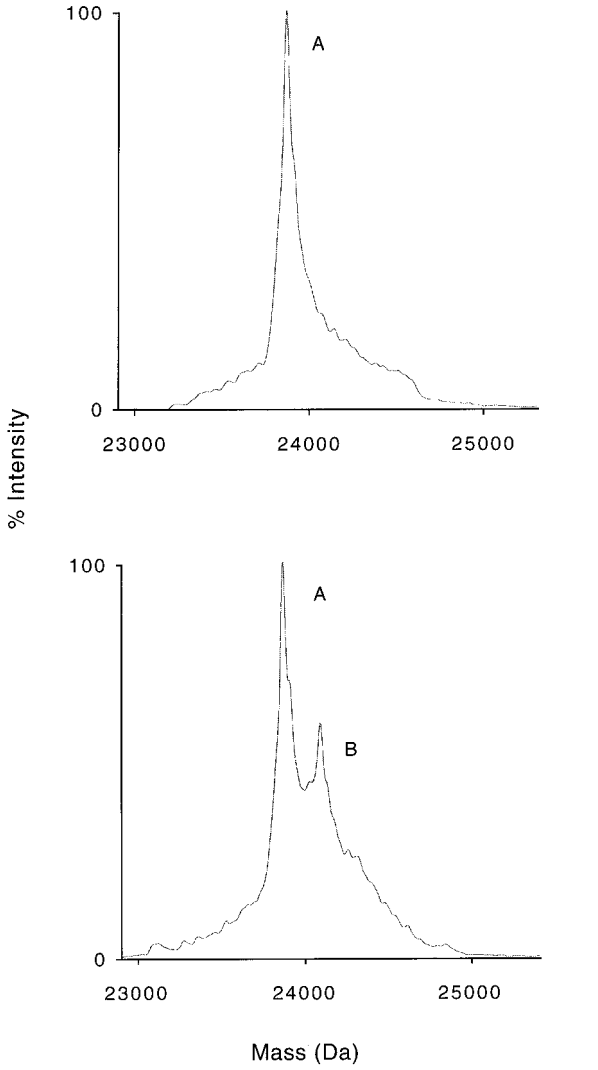


Figure 2. Electrospray mass spectra of HAV 3C proteinase (top spectra: A = 23880 Da) and HAV 3C-**1** enzyme inhibitor complex (bottom spectra: A = 23882 Da; B = 24101 Da) obtained on a Micromass ZabSpec Hybrid Sector-TOF instrument.

(30) (a) For an example of water attack at the γ-position of homoserine lactone, see: Schwab, J. M.; Ray, T.; Ho, C.-K. *J. Am. Chem. Soc.* **1989**, *111*, 1057−1063. (b) For examples of thiol attack at the γ-position of γ-lactones, see: Vaughan, W. R.; Baumann, J. B. *J. Org. Chem.* **1962**, *27*, 739−741 and references therein. (c) For general discussions of ring opening of γ-lactones, see: Aldridge, D. C.; Nicholson, S.; Taylor, P. J. *J. Chem. Soc., Perkin Trans. 2* **1997,** 1929−1938. Huisgen, R.; Ott, H. *Tetrahedron* **1959**, *6*, 253−267.

(31) Model compound, (β-carbon chemical shift): *N*-(benzyloxycarbonyl)-[3-^{13}C]-L-serine β-lactone, **1**(β-^{13}C), (68 ppm); *N*-(benzyloxycarbonyl)-*S*-methyl-L-cysteine **10**, (37 ppm); *N*-(benzyloxycarbonyl)-L-serine ethyl thioester **11**, (64 ppm); *N*-(benzyloxycarbonyl)-L-serine methyl ester **12**, (62 ppm); *N*-(benzyloxycarbonyl)-*O*-methyl-D-serine **13**, (73 ppm). An Inova 600 Varian NMR spectrometer was used to obtain HMQC spectra.

(32) Kim, D. H.; Ryoo, J. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1287−1292.

(33) Corey, E. J.; Li, W.; Nagamitsu, T. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1676−1679.

heteronuclear multiple quantum coherence (HMQC) spectra (Supporting Information) of **1**(β-^{13}C) shows a cross-peak at 68 ppm for the labeled methylene carbon; upon the addition of HAV 3C proteinase this signal disappears and a new peak appears at 40 ppm on the carbon chemical shift axis. This signal for the enzyme inhibitor complex, i.e., HAV 3C-**1**(β-^{13}C), clearly demonstrates formation of a thioether and thus *alkylation* of the active site cysteine by attack at the β-carbon of **1**. The observed chemical shift is in good agreement with that of the β-carbon of *N*-(benzyloxycarbonyl)-*S*-methyl-L-cysteine (37 ppm).

In summary, HAV 3C cysteine proteinase is inactivated by β-lactone **1** via nucleophilic ring opening of the oxetanone ring by the cysteine thiolate at the β-carbon. In contrast, the enantiomer **2** is not an inactivator, but rather binds competitively in the active site more strongly than **1**, presumably because its β-methylene carbon is not correctly placed with respect to the required trajectory for an incoming cysteine thiolate nucleophile. Preliminary results (data not shown) with HRV-14 3C proteinase, an enzyme that has similar substrate specificity to HAV 3C[26] and is a potential therapeutic target for the common cold, show that β-lactones **1** and **2** have comparable potency. Additional studies on structure−activity relationships for β-lactone inhibition of viral cysteine proteinases are in progress.

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Supporting Information Available: Experimental procedures for chemical transformations, mass spectrometry of HAV 3C-**1** inhibitor complex, HMQC spectroscopy of HAV 3C-**1**(β-^{13}C) inhibitor complex, enzymatic analysis, and X-ray structural information for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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