Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3255–3258

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PEROXIDES AS OXIDATIVE ENZYME INHIBITORS: MECHANISM-BASED INHIBITION OF A CYSTEINE PROTEASE BY AN AMINO ACID OZONIDE

Patrick H. Dussault,*[a] A. Denise George,[b] and Tony K. Trullinger[a]

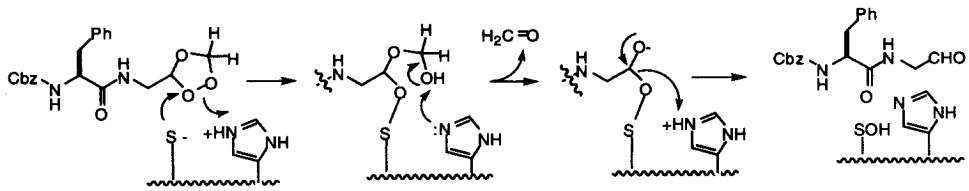
[a]*Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304, U.S.A.*
[b]*Department of Chemistry, Nebraska-Weslayan University, Lincoln, NE 68504, U.S.A.*

Received 13 August 1999; accepted 15 October 1999

Abstract: A stable ozonide derived from Cbz-*L*-Phe accomplishes rapid and stoichiometric inhibition of papain at less than 100 μM concentration under conditions where formation of the corresponding aldehyde is negligible. Oxidation of the active site thiolate by the bound peroxide is believed to lead to formation of an inactive sulfenate or sulfenic acid. Reduction of the ozonide in excess DMSO provides a convenient method for *in situ* generation of a peptide aldehyde. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Cysteine proteases regulate a wide variety of physiological functions and altered levels of protease activity may be associated with a number of disease states as well as programmed cell death.[1,2,3,4,5] The obvious therapeutic potential associated with selective inhibition of individual cysteine proteases has led to the exploration of a number of different classes of mechanism-based inhibitors.[5,6,7] However, a continuing challenge in this area is the availability of inhibitors selective for cysteine proteases in the presence of other proteases and cellular nucleophiles. In the course of a research program into the synthesis of peroxide natural products, we became interested in the whether the characteristic reactivity of the peroxide group could provide a unique basis for enzyme inactivation. In this report, we describe the synthesis of a protected amino acid ozonide and the effective inhibition of the cysteine protease papain.



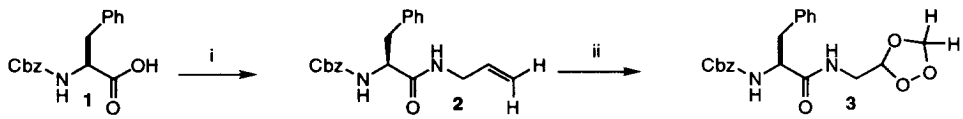
Scheme 1 Proposed Pathway for Inhibition

PII: S0960-894X(99)00563-6

The design of our initial inhibitor was based upon the premise outlined in Scheme 1. Substitution of a peroxide group in place of the amide carbonyl of a peptide substrate would place the reactive O-O linkage in the vicinity of the active site thiol/thiolate common to all cysteine proteases. Depending upon the exact structure of the peroxide, subsequent oxygen transfer would form a sulfenic acid or sulfenate. Our initial choice of substrate was an ozonide (**3**) derived from a protected phenylalanine, a core structure that has been effective for delivery of a variety of inhibitors. The ozonide (1,2,4-trioxolane) functional group was chosen as an easily generated peroxide functional group that would be relatively stable towards routine isolation and purification and selectively reactive towards the active site thiolate.[8,9]

Synthesis

Cbz-*L*-Phenylalanine (Phe) was converted to the corresponding allylamide under standard peptide linking conditions (Scheme 2).[10] Ozonolysis of the allyl amide furnished a crystalline ozonide, which could be purified by flash chromatography and stored for up to several days at refrigerator temperatures with no detectable decomposition.[11]



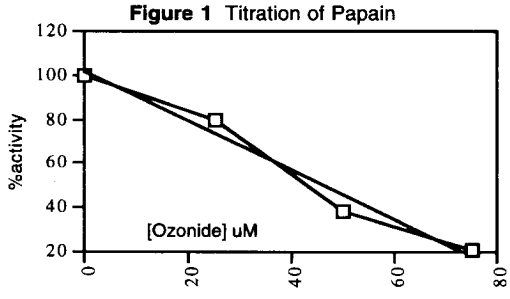
(i) HBOT, allyl amine, DCC, 0 °C to rt, 4 h, 75%; (ii)O_3, CH_2Cl_2, -78 °C, 30%.

Scheme 2 Synthesis of Amino acid Ozonide

Results and Discussion

Papain (Sigma) was activated by preincubation in 0.12 M phosphate buffer (pH 6.0, 1 mM EDTA, 10 mM cysteine) for 45 min at room temperature. An initial time-dependent inhibition was conducted at room temperature with 100 uM papain and 3 mM ozonide. Aliquots of the solution were withdrawn at 1, 5, and 10 min and assayed for the ability to hydrolyze N-α-benzoyl-*L*-arginine *p*-nitroanilide (*L*-BAPNA), monitoring the absorbance of liberated 4-nitroaniline at 410 nm.[12,13] Complete inhibition of the enzyme was observed within one minute.

Figure 1 Titration of Papain

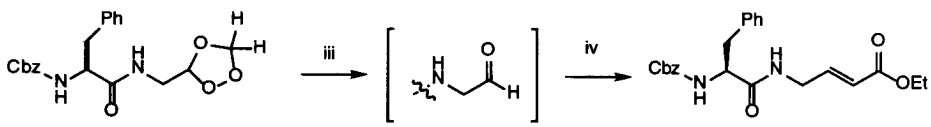


Enzyme activity was next assayed following incubation of 100 uM papain in the presence of 25, 50, and 75 uM ozonide (Figure 1). Although activity was measured at time periods of up to 10 min

after incubation, maximum levels of inhibition were once again reached within one minute. The rapid and nearly stoichometric inhibition of papain supports the mechanism-based pathway outlined in Scheme 1.

In order to establish that inhibition was derived from reaction with the ozonide, it was necessary to rule out in situ formation of peptide aldehydes, known to be potent inhibitors of cysteine and serine proteases.[14] Room temperature solutions of ozonide in CD_3CN were observed by NMR and found to be stable for hours to days. The formation of significant concentrations of aldehyde through nonenzymatic reduction by cysteine was also ruled out by a control experiment showing the half-life of ozonide to be greater than 10 min at much higher concentrations than present in the assays (5 mM ozonide, 25 mM cysteine).[14] It should be noted that peptide aldehyde may be released as a byproduct of ozonide cleavage (Scheme 1). However, as a stoichometric byproduct, aldehyde produced by this pathway could account for no more than half of the ultimate inhibition. Formaldehyde, the other expected product of ozonide breakdown, was found to have a negligible effect on enzyme activity at relevant concentrations.

Finally, we report that the amino acid ozonide provides an effective route for *in situ* generation of peptidyl aldehydes. Hanzlik has previously reported the preparation of unstable peptidyl aldehydes through methanolic ozonolysis of allylamides, followed by reductive workup.[14,15] In our case, we discovered that preparation of stock DMSO solutions of ozonide resulted in the rapid formation of a new compound, which efficiently inactivated papain. Proof of structure was achieved through dissolution of **1** in DMSO, followed by trapping with a stabilized Wittig ylide to form an unsaturated ester (Scheme 3).



(iii) 1: 9 DMSO/C_6H_6, rt, 1 h; (iv) carbethoxymethylenetriphenylphoshorane, 0 °C - rt, 12 h, 42%.

Scheme 3 Generation and trapping of peptidyl aldehyde

Conclusion

A readily available and stable peroxide rapidly inactivates the cysteine protease papain. Given the close homology of the active site in many different cysteine proteases, this new class of inhibitor has high potential for inhibition of medicinally relevant proteases. In addition, the incorporation of a peroxide or ozonide within a longer peptide span able to interact with additional recognition elements may provide selectivity between different classes of cysteine proteases.

Finally, reduction of the ozonide *in situ* offers a convenient method for synthesis of peptide aldehydes. Further exploration of this chemistry will be reported in due course.

Acknowledgments: We are grateful for financial support from the Howard Hughes Medical Institute (ADG) and the NIH and for technical support from Prof. David Berkowitz.

References

1. Calkins, C. C.; Sloane, B. F. *Biol. Chem. Hoppe-Seyler* **1995**, *376*, 71.
2. Gopalan, P.; Durfresne, M.; Warner, A. H. *Biochem. Cell. Biol.* **1986**, *64*, 1010.
3. McKerrow, J. H.; Engel, J. C.; Cafrey, C. R. *Bioorg. Med. Chem.* **1999**, *7*, 639.
4. Thornberry, N. A.; Molineaux, S. M. *Protein Sci.* **1995**, *4*, 3.
5. Kirschke, H.; Barrett, A. J.; Rawlings, N. D. *Lysosomal Cysteine Proteases;* 2nd ed.; Oxford University. Oxford, 1998.
6. Shaw, E. *Advances in Enzymology* **1990**, *63*, 271.
7. Otto, H.-H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133.
8. Kuczkowski, R. L. *Chem. Soc. Rev.* **1992**, *21*, 79.
9. McCullough, K. J.; Nojima, M. In *Organic Peroxides*; Ando, W., Ed.; John Wiley & Sons: Chichester, 1992; p 661.
10. Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: New York, 1984; Vol. 21.
11. Ozonide **3**: R_f= 0.66 (40% EtOAc/hexanes); ^{1}H NMR (500 MHz, CD_2Cl_2) 7.29 (m, 10H, $C_{12}\mathbf{H_{10}}$), 6.33 (br s, 1H, OC=ON**H**CH), 5.48 (br s, 1H, C=ONHCH$_2$), 5.20 (dt, 1H, J = 20.7, 3.4 Hz, $CH_2C\mathbf{H}(OCH_2)OO$), 5.06 (s, 3H, $C_6H_5C\mathbf{H_2}O$, and OC**H**(H)O), 4.96 (s, 1H, OC**H**(H)O), 4.42 (s, 1H, NC**H**(C=O)N), 3.48 (m, 2H, NC$\mathbf{H_2}$), 3.07 (m, 2H, CHC$\mathbf{H_2}C_6H_5$); ^{13}C NMR (500 MHz, CD_2Cl_2) 171.28, 171.24, 136.5, 129.2, 128.6, 128.4, 128.1, 127.84, 127.80, 126.9, 100.5, 94.2, 66.9, 56.2, 40.1, 38.3; HRMS(FAB) calcd for $C_{20}H_{22}O_6N_2$ $(M+H)^+$: 387.1478. Found: 387.1560.
12. Initial incubations were performed by mixing 1 mL of stock papain (0.2 mM) with 1 mL of ozonide **3** (6 mM in CH_3CN at rt). Later incubations were performed similarly but with diluted stock solutions of ozonide. Assays were performed by adding 0.3 mL aliquots of the incubation to cuvettes containing 2.4 mL phosphate buffer and 0.3 mL of a 20 mM solution of (L-BAPNA in 50% CH_3CN/H_2O).
13. Arnon, R. *Meth. Enzym.* **1970**, *19*, 226.
14. Hanzlik, R. P.; Jacober, S. P.; Zygmunt, J. *Biochim. Biophys. Acta* **1991**, *1073*, 33.
15. Liu, S.; Hanzlik, R. P. *J. Med. Chem.* **1992**, *35*, 1067.