Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1737–1739

The inhibition of metallo-β-lactamase by thioxo-cephalosporin derivatives

Wing Y. Tsang,[a] Anupma Dhanda,[b] Christopher J. Schofield,[b] Jean-Marie Frère,[c] Moreno Galleni[c] and Michael I. Page[a,*]

[a]*Department of Chemical and Biological Sciences, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, UK*
[b]*The Dyson Perrins Laboratory and The Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK*
[c]*Centre d'Ingénierie des Protéines, Institut de Chimie B6, Université de Liège, Sart-Tilman, B-4000 Liège, Belgium*

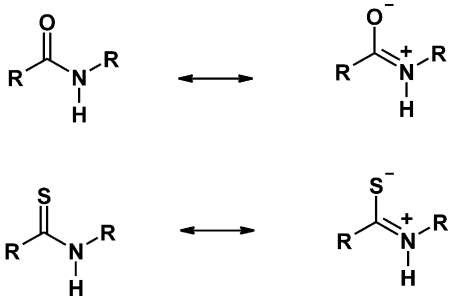
Received 5 November 2003; revised 15 January 2004; accepted 16 January 2004

Abstract—The 8-thioxocephalosporins are poor substrates for the *B. cereus* metallo β-lactamase ($k_{cat}/K_m = 61.4\ M^{-1}\ s^{-1}$) and act as weak competitive inhibitors ($K_i \sim 700\ \mu M$). The hydrolysis product of thioxocephalosporin, a thioacid, also inhibits the enzyme competitively with a $K_i = 96\ \mu M$, whereas the cyclic thioxo-piperazinedione, formed by intramolecular aminolysis of thioxocephalexin has a K_i of 29 μM.

1. Introduction

The large barrier to rotation in amides and their low chemical reactivity towards nucleophilic attack at the acyl centre has been attributed to amide resonance which is usually described in terms of charge transfer from N to O (Scheme 1). It is expected that there would be greater π charge transfer from N to S in thioamides due to the larger size of S and weaker π bond of C=S despite the smaller electronegativity of S compared with O (Scheme 1). Thioamides have a larger barrier to C–N bond rotation than amides[1] and are more easily protonated on sulfur.[2] We have recently reported the relative reactivities of the cephalosporin (**1**) group of antibiotics and their thioxo-analogues (**2**).[3] In addition to the differences in chemical reactivity, which are generally small,[3] the different charge distribution in thioamides[2,4] and their acidities[5] may cause interesting differences in their interaction with enzymes compared with their oxygen analogues.

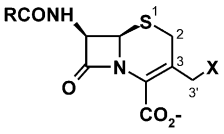
A major cause of bacterial resistance to the otherwise lethal action of β-lactam antibiotics is their ability to produce β-lactamase enzymes which catalyse the hydrolysis of the β-lactam ring.[6] One class of these enzymes contains zinc at their active site. Pratt et al. have reported that 8-thioxo-cephalosporins are poor substrates for the BC II metallo β-lactamase.[7] Given that certain thiols[8,9] and thiol esters[10] have been shown to act as potent inhibitors of the metallo β-lactamases, we were interested to examine whether the hydrolytic product thioacids or other degradation products of thioxo-lactams would act as inhibitors by binding to the metal-ion of the metallo β-lactamases.[11–13]



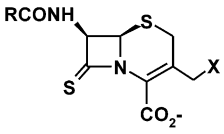
Scheme 1.

* Corresponding author. Tel.: +44-1484-47-2169; fax: +44-1484-47-2182; e-mail: m.i.page@hud.ac.uk

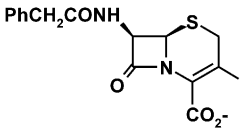
0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.01.047



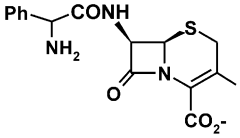
1



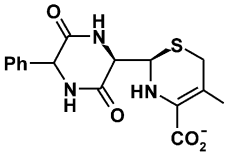
2



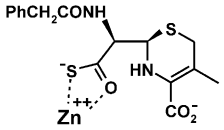
5



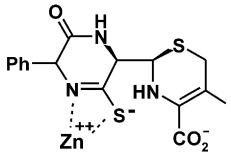
6



7



10



11

2. Results and discussion

The alkaline hydrolysis of the 3-methyl-7β(phenylacetamido)-thioxoceph-3-em-4-carboxylic acid, thioxocephalosporin (**3**), gives the ring opened thioacid (**4**) (Scheme 2) at a rate of which is just 2-fold less than that of the standard cephalosporin (**5**).[3] In terms of chemical reactivity therefore there is little difference between the thioxo- and oxo-analogues. However, cephalexin (**6**) is a cephalosporin with an amino group in the side-chain at C7 that is known to alter the degradation pathway of cephalexin in water. This occurs via both β-lactam hydrolysis and intramolecular aminolysis, the relative importance of which varies with pH.[14] Nucleophilic attack by the amino group of the C7 side chain on the β-lactam carbonyl carbon causes ring opening and formation of a piperazinedione derivative (**7**). The thioxo analogue of cephalexin, 3-methyl-7β(2-amino-phenylacetamido)-thioxoceph-3-em-4-carboxylic acid (**8**), also reacts in water with intramolecular aminolysis by the 7β-amino side chain to give the corresponding monothioxo-piperazinedione analogue (**9**) (Scheme 3).[3] It is interesting to note that the thioxo-β-lactam and its oxygen analogue react at similar rate towards hydroxide-ion catalysed hydrolysis but the intramolecular aminolysis is 10^3-fold faster with thioxo-β-lactam than that of its oxygen analogue.[3]

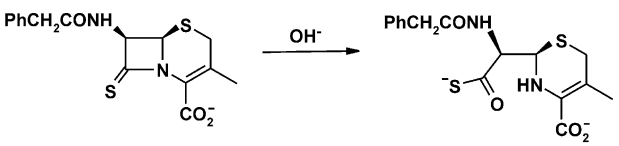
Given the propensity of sulfur to co-ordinate to zinc,[15] we were interested to compare the interaction of the three thioxo species, **3**, **4** and **9** with the zinc metallo-enzyme *Bacillus cereus* 569/H (BCII) β-lactamase.[6,8]

The enzyme catalysed hydrolysis of thioxocephalosporin (**3**) was assayed in 0.1M pH 7.0 MOPS aqueous buffer with an ionic strength of 1.0M (KCl) and 5.0×10^{-5} M $ZnSO_4$ at 30 °C in a reaction cell of total volume of 2.6 mL. containing *B. cereus* 569/H (BCII) β-lactamase (7.0×10^{-7} to 1.4×10^{-6} M) and the thioxo-β-lactam (2.0×10^{-5} M). For thioxocephalexin (**8**), the enzyme catalysed hydrolysis was carried out in the same buffer but with 1×10^{-4} M $ZnSO_4$ and 1.2×10^{-6} M of BCII in the reaction cell. The disappearance of both compounds was followed at 310 nm.

The thioxocephalosporin (**3**) and its hydrolysis product (**4**) were also tested as competitive inhibitors against the metallo-β-lactamase. The hydrolysis product was prepared by reacting the thioxocephalosporin (**3**) with sodium hydroxide and then using the neutralised solution. Their inhibitory activities were determined with 1×10^{-5} M nitrocefin as a reporter substrate in 0.1M pH 7.0 MOPS aqueous buffer with an ionic strength of 1.0M (KCl) and 5.0×10^{-5} M $ZnSO_4$ at 30 °C. The K_i values were determined by the effect of varying concentration of the compounds on the value of the second-order rate constant k_{cat}/K_m for the hydrolysis of nitrocefin using the following equation in which $(k_{cat}/K_m)_I$ and $(k_{cat}/K_m)_o$ are the observed second-order rate constants for hydrolysis of the reporter substrate in the presence and absence, respectively, of a given concentration of the inhibitor, I, and K_i is the dissociation constant of the enzyme–inhibitor complex.

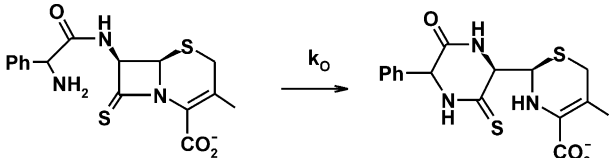
$$\left(\frac{k_{cat}}{K_m}\right)_I = \left(\frac{k_{cat}}{K_m}\right)_o \times \left(\frac{K_i}{[I]+K_i}\right)$$

The competitive inhibition of the metallo-β-lactamase by the intramolecular aminolysis product (**9**) of thioxocephalexin (**8**) was monitored by injecting the metallo-β-lactamase (1.59×10^{-5} M, 20 μl) and reporter substrate cephaloridine (0.01M, 20 μl) into reaction cells after reacting thioxocephalexin (**8**) of different concentrations (5×10^{-5} to 2×10^{-4} M) in 2 mL of 0.1M pH 7.0 MOPS aqueous buffer with 1×10^{-4} M of $ZnSO_4$ for 8 min to give **9**. The K_i value was determined using the method described above.



3 4

Scheme 2.



8 9

Scheme 3.

The hydrolysis of 3-methyl-7β(phenylacetamido)-thioxoceph-3-em-4-carboxylic acid (**3**), but not thioxocephalexin (**8**), was catalysed by the Zn^{2+}-dependent metallo-β-lactamase 569/H from *Bacillus cereus* (BCII). A first-order disappearance of the substrate (**3**) was observed and no saturation kinetics seen up to 0.2 mM concentrations of the thioxo-cephalosporin. The second-order rate constant for the enzyme-catalysed hydrolysis (k_{cat}/K_m) of (**3**) was 61.4 M^{-1} s^{-1} at pH 7.02 in 0.1M MOPS buffer, 1% dioxan-water (v/v) with an ionic strength of 1.0M (KCl) at 30 °C, can be compared with a reported value of 3.69×10^3 M^{-1} s^{-1} for the O-analogue (**5**).[14] It is thought that an important role of the active site zinc-ion in catalysis is to enhance the rate of formation of and to stabilise the tetrahedral intermediate by Lewis acid co-ordination to the β-lactam oxygen. The significantly reduced rate of hydrolysis of the S-derivative probably results from a poor productive binding to the enzyme because of the larger sulfur atom and longer C=S bond, although stronger binding of the sulfur anion to the zinc-ion may have been anticipated.[15] Thioxocephalexin (**8**) showed no enzyme catalysed hydrolysis because of the fast competing intramolecular aminolysis and k_{cat}/K_m is estimated to be less than 100 M^{-1} s^{-1}.

Although the thioxocephalosporin (**3**) was hydrolysed by BCII, it also acts as a competitive inhibitor of the enzyme against the hydrolysis of more reactive substrates. For example, monitoring enzyme activity against nitrocefin, it shows a $K_i = 720$ μM, compatible with its high K_m. Presumably the sulfur of the β-lactam of (**3**) only weakly coordinates to the active site zinc. The hydrolysis product of (**3**), the thioacid (**4**), acts as a better competitive inhibitor of the metallo-enzyme BCII with a $K_i = 96$ μM. The thioacid anion also probably binds to the active site zinc (**10**), more effectively than the thioxo-β-lactam possibly reflecting observations that certain thiols are potent inhibitors of MBLs.[8–10]

Thioamides have not previously been reported as inhibitors of zinc proteases — and yet their increased acidity gives them the potential to co-ordinate to metal ions. The pK_a of (**9**) in water at 30 °C is 10.1, similar to that for thiols. Indeed, the thioamide (**9**) does act as a competitive inhibitor of BCII with a $K_i = 29$ μM. We assume that it is the ionised form which binds to the active site zinc as illustrated in (**11**).

Diketopiperazine libraries have been used extensively in screening for small molecules with biological activity.[16] In the case of metallo-enzymes the present results suggest it may be productive to include the readily prepared thioxo analogues of diketopiperazines within libraries used for screening.

References and notes

1. Wiberg, K. B.; Rush, D. J. *J. Am. Chem. Soc.* **2001**, *123*, 2038. Wiberg, K. B.; Rush, D. J. *J. Org. Chem.* **2002**, *67*, 826. Lauvergnat, D.; Hiberty, P. C. *J. Am. Chem. Soc.* **1997**, *119*, 9478.
2. Min, B. K.; Lee, H.-J.; Choi, Y. S.; Park, J.; Yoon, C-J.; Yu, J.-A. *J. Mol. Struct.* **1998**, *471*, 283.
3. Tsang, W. Y.; Dhanda, A.; Schofield, C. J.; Page, M. I. *J. Org. Chem.* **2004**, *69*, 339. Jiang, Z. X. D. Phil thesis, University of Oxford, 1997.
4. Wiberg, K. B.; Rablem, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 2201.
5. Bordwell, F. G.; Ji, G.-Z. *J. Am. Chem. Soc.* **1991**, *113*, 8398.
6. Page, M. I.; Laws, A. P. *Chem. Commun.* **1998**, 1609.
7. Murphy, B. P.; Pratt, R. F. *Biochem. J.* **1989**, *258*, 765.
8. Bounaga, S.; Laws, A. P.; Galleni, M.; Page, M. I. *Biochem. J.* **1998**, *331*, 703. Bounaga, S.; Laws, A. P.; Galleni, M.; Page, M. I. *Bioorg. Med. Chem.* **2001**, *9*, 503.
9. Concha, N. O.; Janson, C. A.; Rowling, P.; Pearson, S.; Cheever, C. A.; Clarke, B. P.; Lewis, C.; Galleni, M.; Frere, J.-M.; Payne, D. J.; Bateson, J. H.; Abdel-Meguid, S. S. *Biochemistry* **2000**, *39*, 4288.
10. Payne, D. J.; Bateson, J. H.; Gasson, B. C.; Khushi, T.; Proctor, D.; Pearson, S. C.; Reid, R. *FEMS Microbiol. Lett.* **1997**, *157*, 171.
11. Gilpin, M. L.; Fulston, M.; Payne, D.; Cramp, R.; Hood, I. *J. Antibiot.* **1995**, *48*, 1081. Walter, M. W.; Felici, A.; Galleni, M.; Soto, R. P.; Adlington, R. M.; Baldwin, J. E.; Frère, J.-M.; Gololobov, M.; Schofield, C. J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2455. Goto, M.; Takahashi, T.; Yamashita, F.; Koreeda, A.; Mori, H.; Ohta, M.; Arakawa, Y. *Biol. Pharm. Bull.* **1997**, *20*, 1136. Toney, J. H.; Fitzgerald, P. M. D.; Grover-Sharma, N.; Olson, S. H.; May, W. J.; Sundelof, J. G.; Vanderwall, D. E.; Cleary, K. A.; Grant, S. K.; Wu, J. K.; Kozarich, J. W.; Pompliano, D. L.; Hammond, G. G. *Chem. Biol.* **1998**, *5*, 185. Greenlee, M. L.; Laub, J. B.; Balkovec, J. M.; Hammond, M. L.; Hammond, G. G.; Pompliano, D. L.; Epstein-Toney, J. H. *Bioorg. Med. Chem. Lett.* **1999**, 2549. Toney, J. H.; Cleary, K. A.; Hammond, G. G.; Yuan, X.; May, W. J.; Hutchins, S. M.; Ashton, W. T.; Vanderwall, D. E. *Biorg. Med. Chem. Lett.* **1999**, *9*, 2741. Walter, M. W.; Valladares, M. H.; Adlington, R. M.; Amicosante, G.; Baldwin, J. E.; Frére, J.-M.; Galleni, M.; Rossolini, G. M.; Schofield, C. J. *Bioorg. Chem.* **1999**, *27*, 35. Nagano, R.; Adachi, Y.; Imamura, H.; Yamada, K.; Hashizume, T.; Morishima, H. *Antimicrob. Agents and Chemother.* **1999**, *43*, 2497. Yang, K. W.; Crowder, M. W. *Arch. Biochem. Biophys.* **1999**, *368*, 1.
12. Payne, D. J.; Bateson, J. H.; Gasson, B. C.; Proctor, D.; Khushi, T.; Farmer, T. H.; Tolson, D. A.; Bell, D.; Skett, P. W.; Marshall, A. C.; Reid, R.; Ghosez, L.; Combret, Y.; Marchand-Brynaert, J. *Antimicrob. Agents and Chemother.* **1997**, *41*, 135.
13. Hammond, G. G.; Huber, J. L.; Greenlee, M. L.; Laub, J. B.; Young, K.; Silver, L. L.; Balkovec, J. M.; Pryor, K. D.; Wu, J. K.; Leiting, B.; Pompliano, D. L.; Toney, J. H. *FEMS Microbiol. Lett.* **1999**, *179*, 289.
14. Bundgaard, H. *Arch. Pharm. Chem. Sci. Ed.* **1976**, *4*, 25.
15. Hancock, R. D.; Martell, A. E. *Adv. Inorg. Chem.* **1995**, *42*, 89.
16. Szardenings, A. K.; Burkoth, T. S.; Lu, H. H.; Tien, D. W.; Campbell, D. A. *Tetrahedron* **1997**, *53*, 6573. Goodfellow, V. S.; Laudeman, C. P.; Gerrity, J. I.; Burkard, M.; Strobel, E.; Zuzack, J. S.; McLeod, D. A. *Mol. Divers.* **1996**, *2*, 97.