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Approaches to the simultaneous inactivation of metallo- and serine-β-lactamases

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ABSTRACT

A series of cephalosporin-derived reverse hydroxamates and oximes were prepared and evaluated as inhibitors of representative metallo- and serine-β-lactamases. The reverse hydroxamates showed submicromolar inhibition of the GIM-1 metallo-β-lactamase. With respect to interactions with the classes A, C, and D serine β-lactamases, as judged by their correspondingly low K_m values, the reverse hydroxamates were recognized in a manner similar to the non-hydroxylated N–H amide side chains of the natural substrates of these enzymes. This indicates that, with respect to recognition in the active site of the serine β-lactamases, the O=C–NR–OH functionality can function as a structural isostere of the O=C–NR–H group, with the N–O–H group presumably replacing the amide N–H group as a hydrogen bond donor to the appropriate backbone carbonyl oxygen of the protein. The reverse hydroxamates, however, displayed k_{cat} values up to three orders of magnitude lower than the natural substrates, thus indicating substantial slowing of the hydrolytic action of these serine β-lactamases. Although the degree of inactivation is not yet enough to be clinically useful, these initial results are promising. The substitution of the amide N–H bond by N–OH may represent a useful strategy for the inhibition of other serine hydrolases.

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The expression of one or more β-lactamase enzymes constitutes an unusually effective bacterial strategy for eluding β-lactam antibiotics. By the Ambler convention, the β-lactamases are grouped into four classes, A–D, with classes A, C, and D constituting serine enzymes, and class B comprised of zinc metallohydrolases. Although there are now many exceptions, the class A serine β-lactamases tend to prefer penicillins as substrates, while the class C β-lactamases prefer cephalosporins. Class D enzymes are referred to as 'oxacillinases' for their ability to preferentially hydrolyze oxacillin and structurally related penicillins. Resistant bacterial strains may produce more than one type of β-lactamase,[1] and, in the case of Gram-negative microorganisms, may also employ permeability barriers, such as reduced expression of outer membrane porins, and up-regulated efflux systems to deny antibiotic access to the periplasm, where high β-lactamase concentrations are maintained.[2]

One counterstrategy is the coadministration of an antibiotic together with a β-lactamase inhibitor. Current commercial inhibitors include clavulanic acid, sulbactam, and tazobactam, all of which are known to function as mechanism-based irreversible inactivators through formation of a stabilized acyl-enzyme. Unfortunately, these commercial β-lactamase inhibitors are only effective against class A serine enzymes. Recently, however, several research groups have reported success in devising experimental inhibitors that can effectively inactivate both class A and class C serine enzymes[3] and the Buynak group has reported an inhibitor that is able to inactivate both serine- and class B metallo-β-lactamases.[4]

The simultaneous inactivation of both a serine- and a metallo-β-lactamase by a single inhibitor may appear unattainable, due to the profound differences in enzyme mechanism and active site geometry. It should be noted, however, that since both the serine- and metallo-β-lactamases have evolved to recognize similar substrates (i.e., bicyclic β-lactam antibiotics), it is conceivable that a bicyclic β-lactam antibiotic scaffold could be modified to possess the functional characteristics of established inhibitors of both serine- and metallo-β-lactamases. Utilizing a bicyclic β-lactam as an inhibitory scaffold has several inherent advantages: (1) Maintaining a similarity between substrate (i.e., the β-lactam antibiotic) and the inhibitor should render it difficult for the microorganism to evolve resistance to the inhibitor without also sacrificing recognition of the substrate. (2) The bicyclic β-lactams are mechanistically capable of a number of fragmentations and transformations subsequent

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to acylation of the active site serine, resulting, in the case of the known inhibitors of the serine-β-lactamases, in the generation of a stabilized acyl-enzyme. Similar fragmentations, taking place subsequent to hydrolysis by a metallo-β-lactamase, may provide the inhibitor with conformational freedom to allow enhanced binding in the active site of a metallo-β-lactamase, thus generating a 'mechanism-based' metallo-β-lactamase inhibitor. (3) Such 'mechanism based' metallo-β-lactamase inhibitors would have the additional advantage of not being non-specific chelators. This feature is anticipated to improve specificity for the bacterial target and thus to reduce toxicity.

In particular, we envisioned that the 7-(*N*-acylhydroxyamino) cephems **1a** and **1b**, would resemble the known inhibitory 7-(hydroxyalkyl)cephalosporins, **2**, and also posses the reverse hydroxamate (i.e., *N*-acylhydroxylamine) functionality. Like the structurally-related hydroxamic acids, reverse hydroxamates possess the ability to complex active site zinc[5] and have recently been employed as inhibitors of a number of medicinally relevant metalloenzymes, including peptide deformylase (PDF),[6] TNF-α converting enzyme (TACE),[7] matrix metalloproteinases (MMP's),[8] and 1-deoxy-D-xylulose-5-phosphate reductoisomerase[9] (DXR, a prospective drug target for selected bacteria and for the protozoan parasite *Plasmodium falciparum*, a causative agent for malaria, where the reverse hydroxamate functionality complexes manganese instead of zinc[10]). Corresponding 6β-(hydroxymethyl)penicillinates, both as sulfides,[11] **3a**, and as sulfones,[12] **3b**, have been described previously. The sulfones **3b** are superior to the sulfides **3a** as inhibitors of the serine β-lactamases. In our earlier study of 6β-(mercaptomethyl)penicillinates,[4] we also observed that the sulfones, **4b**, were superior to the corresponding sulfides **4a** as inactivators of representative serine β-lactamases. In the cephalosporin series, however, good inhibitors of the class C β-lactamases were observed to depend more on the C7 stereochemistry than on the oxidation state of the dihydrothiazine sulfur, with the unnatural 7α-(mercaptomethyl)cephalosporinates (as sulfides, sulfoxides, and as sulfones) stereochemistry, shown, being superior to the 7β isomers. Thus we likewise desired to create a sulfone analog **1b** of the cephalosporin-derived reverse hydroxamate (Fig. 1).

The synthetic routes to these materials are shown in Schemes 1–3. These routes take advantage of our earlier methodology facilitating the preparation of 7-oxocephalosporinates. More notably, as shown in Scheme 2, we were able to devise a novel method for the preparation of the 7-oximinocephem sulfones utilizing the capability of methytrioxorhenium (MTO) to catalyze the oxidation of primary amines to oximes. Unfortunately, all attempts to create sulfone analogs of reverse hydroxamates **12** and **15** were unsuccessful, possibly due to a greater acidity of the C7 hydrogen in the sulfone series, leading to an enhanced propensity for elimination of water from these reverse hydroxamates, and thus resulting in the formation of the unstable C7 *N*-acylimine analogs of the cephalosporin sulfones.

IC_{50} values of compounds **9**, **12**, **13**, **15**, **17**, **21**, and **23** were measured against VIM-2 and GIM-1, two plasmid-borne enzymes that are among the metallo-β-lactamases of the most immediate

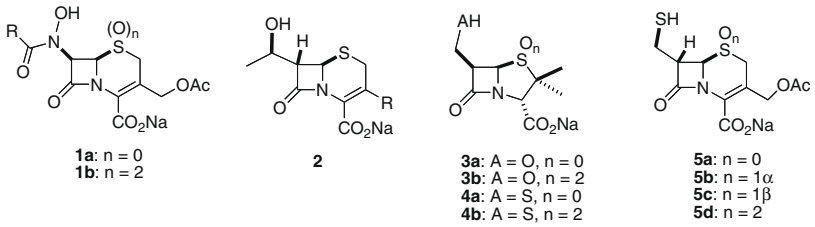
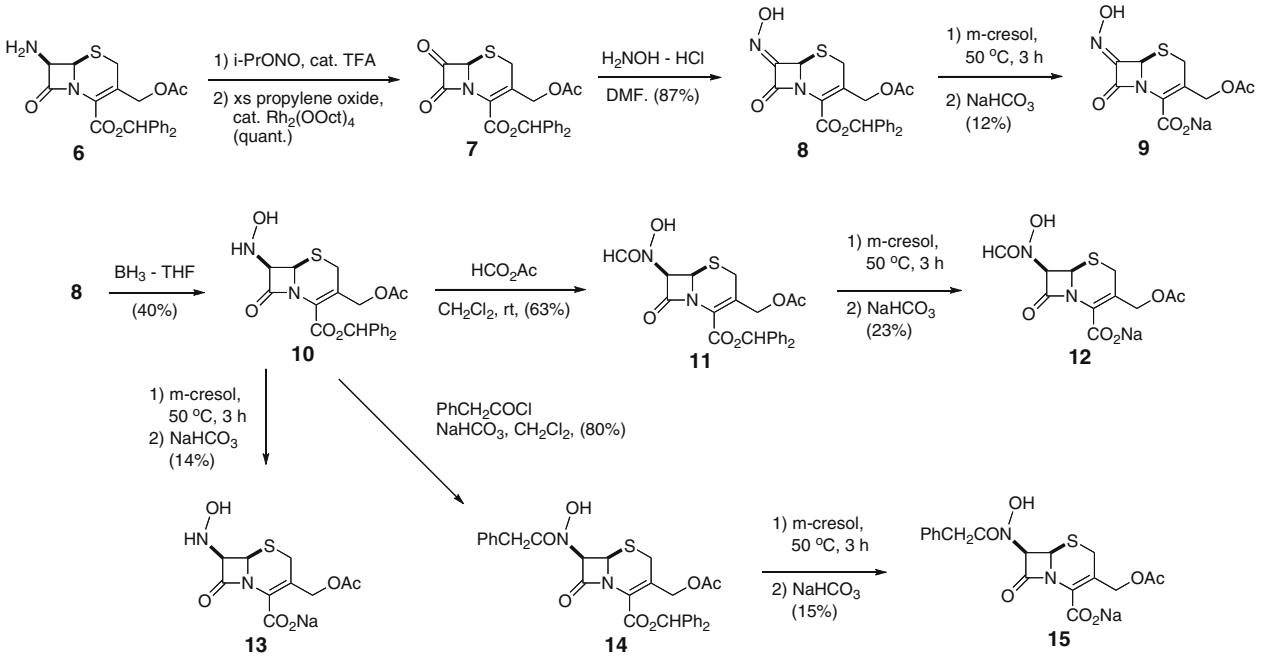
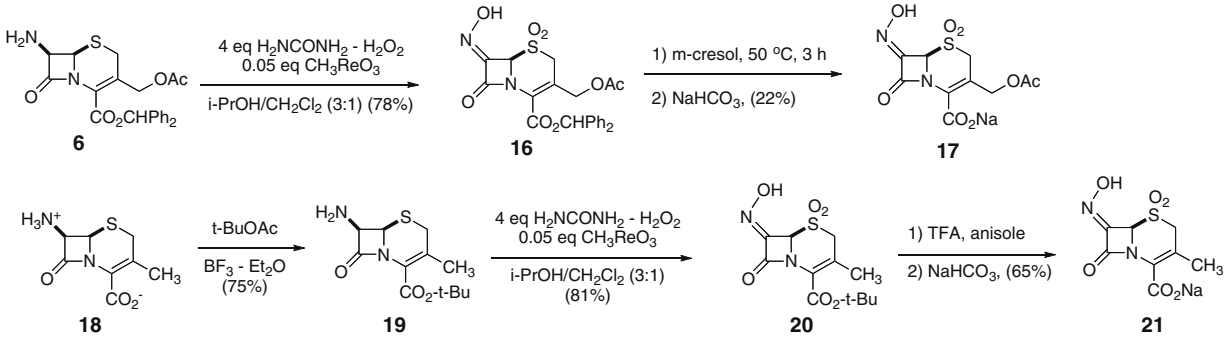


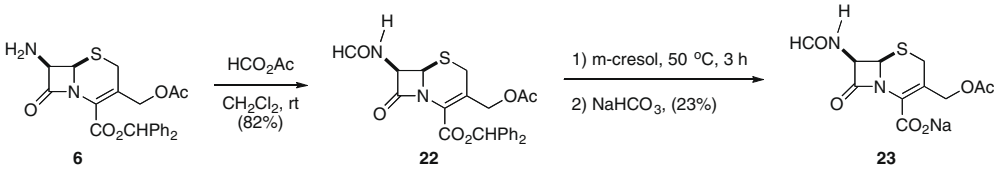
Figure 1.



Scheme 1.



Scheme 2.

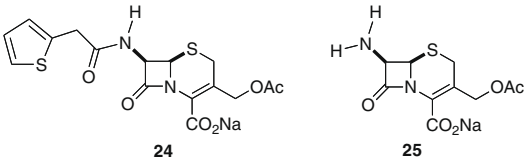


Scheme 3.

clinical significance. Recombinant enzymes were expressed and purified as described by Avison et al.[13] using 50 mM Tris, [pH 7.5], 100 μM $ZnCl_2$, 0.02% [wt/vol] sodium azide as buffer system and Q-Sepharose and Superdex 75 matrices (GE Healthcare, Uppsala, Sweden) for ion-exchange and gel filtration steps, respectively. Inhibition assays were performed in 96-well plate format in a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The chromophoric cephalosporin nitrocefin was used as a reporter substrate and its degradation monitored using absorbance at 482 nm. Assays were performed at 25 °C in 50 mM cacodylate buffer [pH 7.0], 0.1 mM $ZnCl_2$, supplemented with 100 μg/ml bovine serum albumin (BSA) to prevent denaturation of enzymes at the low concentrations employed.[14] All enzymes were pre-incubated with inhibitor for 5 min prior to addition of substrate. IC_{50} determinations employed enzyme concentrations of 1 nM and nitrocefin concentrations, respectively, of 50 μM (VIM-2) and 12.5 μM (GIM-1) (Fig. 2).

IC_{50} values for inhibition of the VIM-2 and GIM-1 enzymes by the seven compounds are shown in Table 1.

The compounds were also submitted to a kinetic evaluation with respect to their behavior as substrates of the serine-β-lactamases *Enterobacter cloacae* P99 (class C), TEM-1 (class A), and OXA-1 (class D).[15] Additional substrates **24** and **25** were incorporated for comparison purposes. Results are shown in Tables 2–4.



Of the C7 hydroxylaminocephalosporins, submicromolar inhibition of the GIM-1 metallo-β-lactamase was observed by the two reverse hydroxamates, **12** and **15**. Removal of the *N*-acyl group (compare **12** and **13**) raised this value nearly one order of magnitude, and eliminating the *N*-hydroxyl group (to leave a

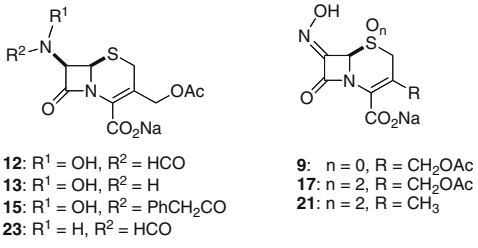


Figure 2.

Table 1
Inhibition of representative metallo-β-lactamases by 7-hydroxylaminocephems and 7-oximinocephems

Compound	VIM-2	GIM-1
IC_{50} (μM)		
12	10	0.3
13	9	2
15	2	0.3
23	35	47
9	10	15
17	270	170
21	25	>500

Table 2
Steady state kinetics parameters for the turnover of the cephalosporins **9**, **12**, **13**, **15**, **23**, **24**, and **25** by the class C P99 β-lactamase

Compound	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
12	0.32 ± 0.05	240 ± 60	1380
13	1.6 ± 0.7	1450 ± 800	1100
15	0.49 ± 0.05	5.0 ± 1.0	1.0×10^5
23	1180 ± 15	315 ± 7	3.7×10^6
24	690 ± 30	15.4 ± 1.9	4.5 ± 10^7
25	0.46 ± 0.03	290 ± 50	1590
9	2.56 ± 0.17	366 ± 30	7010
17	–	⩾200	990 ± 10
21[a]	0.54	4.5	1.2×10^5

[a] Branched pathway (partition ratio 360) to a more inert complex.

Table 3
Steady state kinetics parameters for the turnover of the cephalosporins **9**, **12**, **15**, **23**, and **24** by the class A TEM-1 β-lactamase[16]

Compound	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
12	—	⩾400	25 ± 2
15	0.215 ± 0.025	1320 ± 40	163
23	40.9 ± 1.3	680 ± 30	6.0×10^4
24	131	270	4.85×10^5
9	0.096 ± 0.002	605 ± 18	158
13[a]	—	—	2620
17[b]	—	—	1690
21[c]	0.09	2250	40

[a] Branched pathway (partition ratio 40) to a more inert complex ($k_{cat} = 1.8 \times 10^{-5}$ s^{-1}).
[b] Branched pathway (partition ratio 480) to a more inert complex.
[c] Branched pathway (partition ratio 360) to a more inert complex.

Table 4
Steady state kinetics parameters for the turnover of the cephalosporins **9**, **12**, **15**, **17**, **23**, and **24** by the class D OXA-1 β-lactamase

Compound	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
12	—	⩾400	75 ± 1
13	—	⩾400	66 ± 2
15	0.121 ± 0.004	60 ± 5	2020
23	1.02 ± 0.06	323 ± 28	3160
24	1.81 ± 0.17	13.9 ± 3.6	8.51×10^4
9	—	⩾400	88 ± 3
17	—	⩾200	990 ± 10

simple formamide) raised the IC_{50} value more than two orders of magnitude (compare **12** and **23**). Of the oximes, the best inhibitor was the oxime sulfide **9**. The two sulfones examined, **17** and **21**, were substantially poorer inhibitors. This observed pattern of SAR's is consistent with a role for the 7-position reverse hydroxamate (or hydroxylamino) group in the observed inhibition (inactivation not demonstrated) of the GIM-1 metalloenzyme. The VIM-2 enzyme was inhibited less strongly, although here **15** was again the most effective inhibitor.

By contrast, none of the reverse hydroxamates could be characterized as potent inhibitors of the serine enzymes, but several might be characterized as inhibitory substrates. Some enlightening features of their interactions with the enzymes, however, can be gleaned from the data. As shown in Table 2, and as expected, the simple (non-*N*-hydroxyl) amides **23** and **24** were excellent substrates, as judged by the k_{cat}/K_m values, with **24** being superior largely due to a lower K_m value, presumably due to recognition of the relatively hydrophobic C7 side chain. The N-hydroxylated reverse hydroxamate **15** exhibited a K_m value of 5 μM, substantially smaller than either of the two non-N-hydroxylated substrates. The k_{cat} value for this compound, however, was reduced by 1000-fold, relative to the structurally related **24**, thus implying a potential for excellent recognition, followed by a substantial slowing of hydrolysis. Likewise, if one compares the structurally related N-hydroxylated-*N*-formyl compound **12** with its non-hydroxylated counterpart **23**, one sees a similar effect of excellent recognition of the reverse hydroxamate, demonstrated by the low K_m value of **12** and accompanied by a decrease in k_{cat} by a factor of more than 3500 due to the *N*-hydroxyl group. On consideration of the interactions with the class A TEM-1 enzyme in Table 3, and also with the class D enzyme OXA-1 in Table 4, one sees a similar decrease in k_{cat} of the N-hydroxylated cephem **15** relative to the non-hydroxylated **24**, although in the cases of these two enzymes, the reverse hydroxamate is recognized somewhat more poorly than the simple amide.

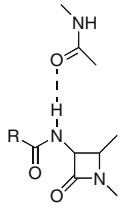


Figure 3.

It is widely appreciated that the C7 amide N–H of cephalosporins and the corresponding C6 amide N–H of penicillins represent crucial elements for substrate recognition by all serine classes of β-lactamases. As shown in Figure 3, these recognition features bind tightly to the backbone carbonyl oxygen residue 237 of class A β-lactamases and to the corresponding carbonyls of class C and D enzymes.

Replacement of N–H by N–OH can, apparently have structural consequences. For example, molecular dynamics simulations of the tetrahedral intermediate formed during hydrolysis of the acyl-enzyme derived from reaction of **15** with the ampC (class C) β-lactamase[17] showed, irrespective of whether the *E* or *Z* hydroxamic acid configuration was chosen, an instability of the adduct leading to displacement of the oxyanion from the oxyanion hole. Such a conformational change could readily lead to slow hydrolysis of **15** by class C β-lactamases (Table 2). Diversion of acyl-enzymes into conformationally less reactive species is well known to lead to inhibitory complexes of β-lactamases.[18]

Incorporation of the N–OH motif into β-lactam side chains may thus represent a general route to inhibitory substrates of both serine and metallo-β-lactamases. Its complementation with other inhibitory motifs, such as a 1-position leaving group (e.g., the sulfone of sulbactam, the enolic oxygen of clavulanate, or the dihydrothiazole sulfur of faropenem), a third generation cephalosporin side chain, or with other functionality consistent with mechanism-based inactivation of the serine β-lactamases, may enhance its effectiveness and lead to broad spectrum β-lactamase inhibitors.

Acknowledgments

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