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Cyclobutanone Analogues of β-Lactams Revisited: Insights into Conformational Requirements for Inhibition of Serine- and Metallo-β-Lactamases

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Antimicrobial resistance is an emerging epidemic worldwide,[1] and β-lactamases represent the most important threat to the continued use of β-lactam antibiotics.[2] The combination of β-lactams with β-lactamase inhibitors has proven to be a very successful strategy for overcoming resistance, but the ever-increasing prevalence of extended-spectrum β-lactamases (ESBLs), carbapenemases,[3] and metallo-β-lactamases (MBLs)[4] compromises the effectiveness of current penicillins, cephalosporins, carbapenems, and mechanism-based β-lactamase inhibitors. Consequently, there is a pressing need for new antibiotics and broad spectrum β-lactamase inhibitors.[5]

Cyclobutanone analogues of β-lactams were explored as potential β-lactamase inhibitors and antibiotics in the early 1980s. Such strategies showed limited success, however, and only weak inhibition of R-TEM-2 β-lactamase, BcI β-lactamase, and R61 transpeptidase was reported. No antibiotic activity was detected in vivo for any of the cyclobutanones.[6,7] As a result, the cyclobutanone system was set aside in favor of other inhibitor templates including that of the penicillanic acid sulfones.

We have decided to re-examine the cyclobutanones because of their potential to exhibit broad-spectrum inhibition, not only of serine β-lactamases and transpeptidases but also of other penicillin-binding proteins and metallo-β-lactamases, via enzyme-bound hemiketals or hydrates (Figure 1). Herein we disclose biochemical results which demonstrate that cyclobutanones can indeed act as inhibitors of serine- and metallo-β-lactamases and indicate that inhibitory potency is closely related to previously unappreciated conformational properties of the bicyclic system. In addition, we disclose the first X-ray crystal structure of a cyclobutanone covalently bound to a β-lactamase as a serine hemiketal.

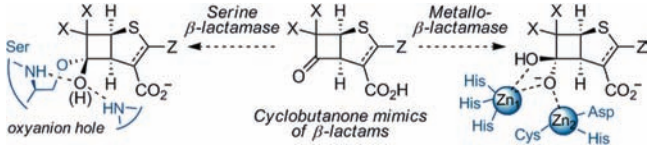
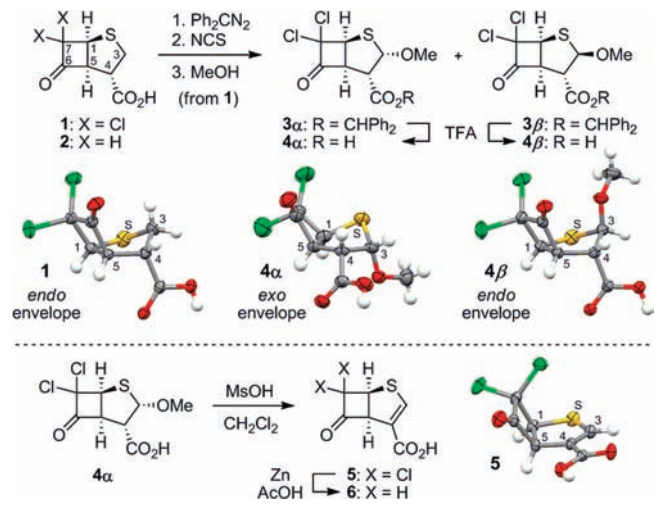


Figure 1. Cyclobutanones as potential broad-spectrum inhibitors.

Recently, we described the synthesis of 3-alkoxy-2-thiabicyclo-[3.2.0]heptan-6-one ethyl esters and discovered that anomeric effects involving the sulfur and the 3-alkoxy substituents could be exploited to control the conformation of the structure.[8] In order to investigate whether conformational preferences were important for β-lactamase inhibition, a modified synthetic route was required for the synthesis

Scheme 1. Synthesis and X-ray Structures of Cyclobutanone Analogues of Penams and Penems



of the free acid form of the 3-alkoxy cyclobutanone derivatives (Scheme 1). This strategy involved the esterification of **1** with diphenyldiazomethane, followed by chlorination with *N*-chlorosuccinimide and methanolysis. The 3-methoxy diastereomers (**3α**/**3β** = 3:1) were separated and individually treated with TFA (10% in CH_2Cl_2) to provide the free acids **4α** and **4β** without epimerization at C3.

The conformational preferences of the cyclobutanone derivatives were apparent by analysis of ^{1}H NMR spectra and confirmed with single-crystal X-ray structures. Derivatives which lack functionality at C3 and derivatives with β-methoxy substituents adopt the *endo* envelope conformation but the α-methoxy epimers favor an *exo* envelope conformation. As a consequence of the conformation of the five-membered ring, the C4 carboxylate is oriented axially in the *endo* envelope and equatorially in the *exo* envelope.

With the saturated cyclobutanone derivatives **1**, **2**, **4α**, and **4β** in hand, we also considered the potential of cyclobutanone analogues of penems or carbapenems as inhibitors. Thus, the unsaturated acid **5** was obtained by elimination of methanol from **4α** with methanesulfonic acid in CH_2Cl_2. Dechlorination of **5** with zinc in acetic acid provided acid **6** in low yield.[9]

β-Lactamases from classes A, B, C, and D were chosen for evaluation of the cyclobutanones as inhibitors. The KPCs (*Klebsiella pneumoniae* carbapenemases) are class A ESBLs that hydrolyze β-lactams of all classes and are found on transferrable plasmids. KPC-producing strains have been isolated in New York, South America, Europe, China, and very recently in Canada.[3a,10] KPC-2

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Table 1. Inhibition of β-Lactamases by Cyclobutanones[a]

ketone inhibitor	% hydrate in D_2O[b]	class A KPC-2	class B IMP-1	class C GC1	class D OXA-10
1	74[c]	76 ± 8	>1000	25 ± 3	268 ± 8
2	0[c]	117 ± 13	235 ± 14	44 ± 3	1135 ± 33
4α	>98	58 ± 2	122 ± 5	6.5 ± 1.4	156 ± 6
4β	6	99 ± 5	nd[d]	38 ± 4	547 ± 19
5	93	26 ± 2	213 ± 21	4.5 ± 0.3	370 ± 15
6	<2	170 ± 2	>500	34 ± 3	>1000

[a] IC_{50} values (μM). Inhibition was assessed by monitoring nitrocefin hydrolysis. [b] Acetone-d_6 was used as a cosolvent for solubility purposes. [c] Reference 8. [d] nd = not determined.

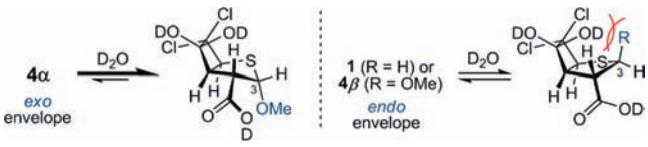

Figure 2. Cyclobutanone hydration and steric interactions at C3.

has been characterized structurally.[11] IMP-1 is a plasmid-encoded class B metallo-β-lactamase which has a wide spectrum of activity including all β-lactams except aztreonam and is found in resistant strains of *Serratia*, *Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Citrobacter*, *Achromobacter*, and *Shigella*.[3c,4a,12] GC1 is a class C enzyme from *Enterobacter cloacae* that owes its ESBL activity to a three amino acid insertion after Arg210 which gives additional conformational flexibility to the Ω loop and allows the enzyme to accommodate bulky oximino side chains of third-generation cephalosporins.[13] OXA-10 is one of the most studied class D enzymes and has been characterized by X-ray crystallography several times.[14] Interestingly, OXA β-lactamases employ a carbamylated lysine residue (Kcx70) as the general base in the turnover of β-lactams.[14b]

The cyclobutanone inhibitors found to be most potent against the serine β-lactamases KPC-2, GC1, and OXA-10 were those which are hydrated to a larger extent in aqueous solution (Table 1).[15] The dichlorocyclobutanones **1** and **5** undergo hydration to a much greater extent than their dechlorinated counterparts **2** and **6** and are also more effective as inhibitors of each serine β-lactamase.[16] Among the dichlorocyclobutanones, steric hindrance of hydration is apparent as the 3α-methoxy derivative **4α**, which adopts the *exo* envelope, undergoes hydration to a greater extent than **1** and ***4β***, which favor the *endo* envelope conformation and have greater steric bulk at C3 (Figure 2). The corresponding observation that **4α** is more potent than **1** and ***4β*** against each enzyme is consistent with the hypothesis that each β-lactamase binds more tightly the *exo* envelope conformation than the *endo* envelope. Furthermore, the unsaturated acid **5**, in which the carboxylate is fixed in an equatorial orientation, was found to be comparable in potency to **4α** with respect to the inhibition of GC1 and somewhat more potent than **4α** against KPC-2. On the other hand, **4α** and **1** were found to be more potent than **5** with respect to OXA-10, suggesting that the relatively subtle difference in orientation of the C4 carboxylate may be important for binding to the various serine β-lactamases.[17]

Gratifyingly, inhibition of the metallo-β-lactamase IMP-1 was also observed, and the most potent inhibitors were **4α** and **5**. As the unsaturated acid **5** was more effective than its dechlorinated analogue **6** against the serine enzymes and IMP-1, it was somewhat surprising to find that the dechlorinated acid **2** was more potent than compound **1**. This exception to the general trend indicates that electron-withdrawing substituents like chlorines are not necessarily beneficial for the inhibition of all β-lactamases.

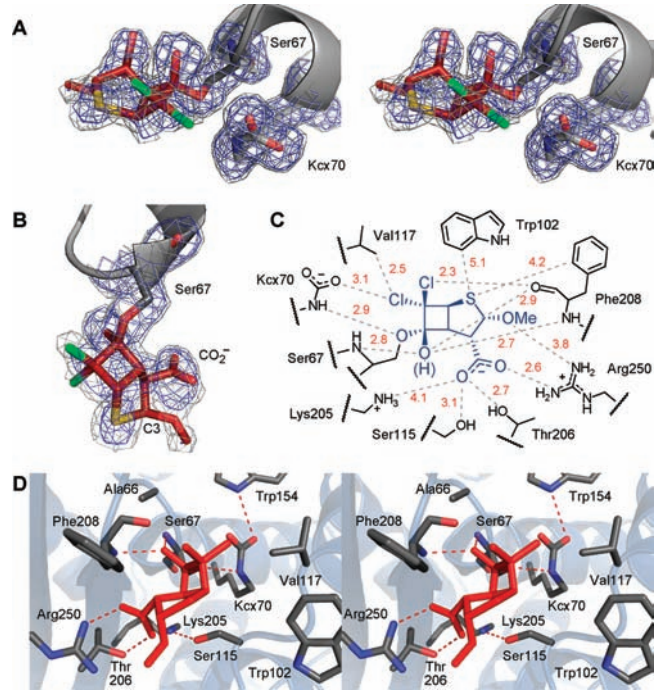

Figure 3. X-ray structure of OXA-10 complexed with cyclobutanone **4α** as a serine-bound hemiketal. (Crystallographic coordinates of the OXA-10 complex with **4α** have been deposited in the Protein Data Bank (PDB: 3LCE). (A) Stereoview of the active site with $2F_o - F_c$ electron density maps contoured at 1.0σ (blue) and 0.5σ (gray). (B) Electron density at the hemiketal linkage. (C) Interactions of OXA-10 with **4α**. (D) Alternate stereoview of the active site.

Structural insight into enzyme−inhibitor interactions was pursued by solving the X-ray crystal structure of a complex of **4α** with the class D β-lactamase OXA-10 (Figure 3). The structure was obtained by soaking preformed crystals of OXA-10 with the inhibitor and was refined to 2.0 Å resolution (Table S1, Supporting Information).[9] Two dimers are present in the asymmetric unit and, consistent with previous observations,[14b] the inhibitor is bound to only two of the four monomers (Figure S1, Supporting Information). Carbamylated Lys70 (Kcx70), which has been associated with a more highly active state of OXA-10,[18a] is observed in all four monomers.

The clear continuous electron density from the active-site serine through to and including the tetrahedral carbon of the hemiketal linkage (Figure 3, parts A and B) provides unambiguous support for the inhibition mechanism originally envisaged for such compounds (Figure 1). Recent studies with other serine-dependent hydrolytic enzymes suggest that such a hemiketal likely exists in the alkoxide form, stabilized by the functionality that defines the oxyanion hole.[19] The structure also shows that the inhibitor adopts the *exo* envelope such that the C4 carboxylate has an equatorial orientation and the 3α-methoxy group has an axial orientation. The electron density associated with the chlorine atoms is relatively weak, possibly indicating that X-ray radiation may have caused partial dechlorination of the inhibitor.[20,21]

Computational models of enzyme−inhibitor complexes were constructed in order to gain further insight into specific active site interactions and provide support for the observed inhibition (Figures S5−S8, Supporting Information).[9] These models revealed that cyclobutanone **1** must adopt its normally disfavored *exo* envelope conformation in order to appropriately orient the carboxylate for interactions with appropriate residues of each β-lactamase (Figure 4). The β-methoxy derivative ***4β*** must also adopt this conformation in order to avoid steric clashes within each active site (not shown).

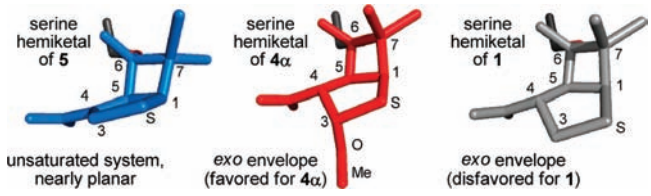

Figure 4. Conformations of enzyme-bound adducts of **5** (blue), **4α** (red), and **1** (gray) extracted from modeled complexes with GC1.[9]

Despite much research effort, the goal of a universal β-lactamase inhibitor that is effective against all classes of β-lactamases has remained elusive. Although the activity of the compounds described herein is relatively modest, these cyclobutanones represent the first type of reversible inhibitors to show moderate inhibition of all classes of β-lactamase. In addition, we are encouraged by preliminary antimicrobial assay results that indicate that cyclobutanone **5** is capable of enhancing the potency of meropenem against MBL-producing carbapenem-resistant clinical isolates.[22] It is hoped that these compounds can serve as prototypes for the discovery of more potent broad-spectrum β-lactamase inhibitors. Molecular modeling studies suggest that side chains at C3 or C7 may increase the affinity of such inhibitors for β-lactamase active sites through favorable hydrogen bonds or other noncovalent contacts. Additional crystallographic experiments aimed at probing interactions of cyclobutanones with β-lactamases and the design and synthesis of more potent broad-spectrum inhibitors based on this motif are in progress.

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Supporting Information Available: Synthetic procedures and characterization data; biochemical procedures; molecular modeling details and figures; tables of crystallographic data and four CIF files for **3α**, **4α**, ***4β***, and **5**; tables of crystallographic parameters and refinement statistics for the OXA-10 complex with **4α**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Fluorinated aldehydes and ketones are more extensively hydrated than the non-fluorinated counterparts and are also more potent inhibitors of serine- and metalloproteases. Despite the fact that these inhibitors are largely hydrated, it has been shown that inhibition occurs by binding of the aldehyde or ketone form to the active site and formation of a serine-bound hemiacetal or hemiketal. (a) Gelb, M. H.; Svaren, J. P.; Abeles, R. H. *Biochemistry* **1985**, *24*, 1813–1817. (b) Chen, R.; Gorenstein, D. G.; Kennedy, W. P.; Lowe, G.; Nurse, D.; Schultz, R. M. *Biochemistry* **1979**, *18*, 921–926.

(16) If the keto form is indeed the inhibitory species for all of the β-lactamases, then it may be appropriate to correct the IC_{50} values for the extent of hydration (Table S2, Supporting Information). The relative potency of the inhibitors follows the same trend but the differences in potency are more substantial. A reviewer is thanked for helpful comments in this context.

(17) The kinetics of inhibition of OXA-10 by **4α** were found to be complex, possibly as a consequence of the known monomer−dimer equilibrium that is affected by substrate concentration,[8] and likely also by inhibitor concentration.

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(21) Another potential contributing factor might involve disorder caused by a rapid conformational change of the inhibitor. Ab initio and DFT molecular modeling studies indicate that a model hemiketal of **4α** can adopt two slightly different *exo* envelope conformations in which the chlorines show the greatest differences in position. The low energy difference (0.1−0.3 kcal/mol) and the low energy barrier (0.4−0.8 kcal/mol) for interconversion support this hypothesis (Figures S3 and S4, Supporting Information).[9]

(22) For example, the MIC of meropenem was reduced from 64 to 16 μg/mL in the presence of **5** for an MBL-producing strain of *Chryseobacterium meningosepticum* and for an MBL-producing *Stenotrophomonas maltophilia* strain (Table S3, Supporting Information).[9]

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