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# Inhibition of β-Lactamases by 6,6-Bis(hydroxylmethyl)penicillanate

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β-Lactamases of classes A and C are the two most prevalent resistant determinants to β-lactam antibiotics among bacterial pathogens. Both these enzymes pursue different mechanisms for their catalytic processes, highlighted by the fact that the hydrolytic water molecule in each approaches the ester of the intermediary acyl-enzyme species from the opposite ends. 6,6-Bis(hydroxylmethyl)penicillanate was designed as an inhibitor that would impair the approach of the hydrolytic water molecule in either of these enzymes upon formation of the acyl-enzyme species. The design, synthesis, and kinetic evaluation of this inhibitor are disclosed herein.


## INTRODUCTION

β-Lactamases are bacterial resistance enzymes to β-lactam antibiotics. There are at least four different classes of these enzymes known, each of which follows a distinct catalytic mechanism in turnover of the substrates (*1–3*). β-Lactamases of classes A and C are the first and the second most common among these enzymes. The catalytic mechanisms for turnover of substrates in these enzymes go through two steps. In the first, the enzyme experiences acylation at an active site serine, and in the second step the enzyme undergoes deacylation. In their respective catalytic mechanisms, enzymes of classes A and C pursue different strategies. In class A enzymes the hydrolytic water approaches from the α face of the ester species, and in the case of class C enzymes, the approach of the hydrolytic water is from the opposite β face. The processes of promotion of the hydrolytic water in both cases are also different in the two enzymes (*4–8*).

We had shown previously that 6α-hydroxymethylpenicillanate (**1**) acylates the class A TEM-1 β-lactamase readily. However, the acyl-enzyme species did not undergo hydrolysis for a number of hours (*6,9*). We demonstrated by the determination of the X-ray structure of the acyl-enzyme species for the inhibited enzyme that the hydroxyl moiety makes a critical hydrogen bond to the hydrolytic water molecule, whereby the nucleophilicity of the water molecule is reduced (*9*). Also, the hydroxymethyl

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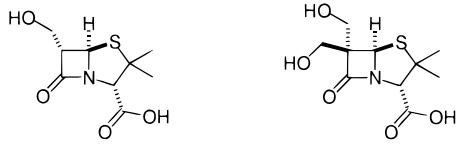
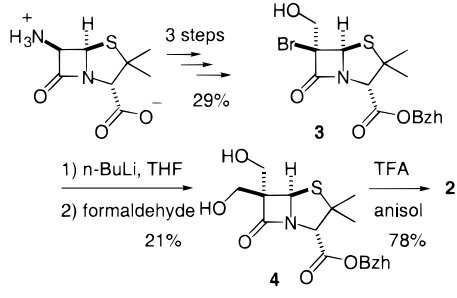
group presented a physical barrier to the approach of the water molecule at the ester carbonyl. In essence, compound **1** is able to impair the deacylation step of the catalytic process. In light of the fact that the approach of the water molecule in the class C enzymes is precisely from the trajectory opposite that of class A enzymes, we wondered if 6,6-bis(hydroxymethyl)penicillanate (**2**) would be able to acylate the active sites of these enzymes and if the two hydroxymethyl moieties would block the approach of the hydrolytic water molecule from either the α or the β direction. We present the synthesis of compound **2** in this report and in kinetic experiments with purified enzymes we demonstrate that indeed the compound is capable of such a task.



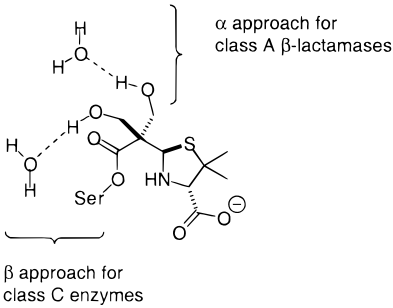

## RESULTS AND DISCUSSION

Compound **2** was synthesized according to Scheme 1. It employs an alkylation of β-bromo-α-(hydroxylmethyl)penicillanate **3** (*10*), which was synthesized from 6β-aminopenicillanate according to a literature procedure (*6,11*). The treatment of β-bromo-α-(hydroxylmethyl)penicillanate **3** with *n*-BuLi, followed by the addition of formaldehyde, gave bis(hydroxymethyl)penicillanate **4**. Compound **4** was then deprotected by trifluoroacetic acid (TFA) in the presence of anisol as an acid scavenger to afford the desired bis(hydroxymethyl)penicillanic acid **2**.

Compound **2** was evaluated with homogeneous preparations of representative members of both class A and class C β-lactamases. These were the TEM-1 β-lactamase from *Escherichia coli* and the β-lactamase from *Enterobacter cloacae* strain P99, respectively. Table 1 summarizes the kinetic measurements with these enzymes. Compound **2** showed time dependence in inactivation of both enzymes. This indicates that it is able to acylate the active site and that the acylated species enjoys longevity. This was consistent with the design concept, which envisioned that the enzymes should undergo acylation by **2** and that the hydroxymethyl groups (α or β depending on the enzyme) would impair the deacylation step (Scheme 2).




**SCHEME 1.**




**SCHEME 2.**

Compound **2** inhibited the TEM-1 $\beta$-lactamase more readily than it did the P99 enzyme (i.e, $k_{inact}$ values). In both cases, when enzyme inhibition was established, recovery of activity was extremely slow ($k_{rec}$ of the order of $10^{-3}$ to $10^{-4}$ $s^{-1}$). However, it is clear that prior to the onset of inhibition, conceivably before the establishment of the requisite hydrogen bond to the hydrolytic water molecule, some turnover takes place. The P99 $\beta$-lactamase turns over the compound roughly three-fold more readily than does the TEM-1 enzyme ($k_{cat}$ values), a factor reflected in a higher partition ratio ($k_{cat}/k_{inact}$) for the former.

Our findings here further support the proposals for the approach of the hydrolytic water molecules in the two enzymes from two opposite ends at the ester of the acyl-enzyme species. Whereas the affinities of the two enzymes for compound **2** were not sufficiently high for it to have clinical utility, this compound to our knowledge is only the second inhibitor that has been shown to inhibit both classes A and C of $\beta$-lactamases (*12–14*). In light of the fact that an inhibitor capable of inhibiting the two enzymes is currently highly sought, mechanism-based strategies such as the one disclosed here should pave the way for future developments.

## EXPERIMENTAL

$^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Mercury-400 spectrometer. Chemical shifts are reported in parts per million from tetramethylsilane on the $\delta$ scale. Infrared spectra were recorded on a Nicolet 680 DSP spectrometer. Mass spectra were recorded on Kratos MS 80RFT and MicroMass Quatro LC spectrometers. Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Thin-layer chromatography was performed with Whatman reagents on 0.25-mm silica

TABLE 1

Kinetic Parameters for Interactions of Inhibitor **1** with the TEM-1 and P99 $\beta$-Lactamases[a]

| Enzyme | $k_{cat}$ ($s^{-1}$) | $k_{inact}$ ($min^{-1}$) | $k_{cat}/k_{inact}$ | $K_i$ (mM) | $k_{rec}$ ($s^{-1}$) | $K_I$ (mM) |
|---|---|---|---|---|---|---|
| TEM-1 | 5 ± 3 | 1.1 ± 0.6 | 258 ± 28 | 0.48 ± 0.08 | (1.3 ± 0.04) × $10^{-3}$ | 15 ± 8 |
| P99 | 12 ± 5 | 0.07 ± 0.03 | 10321 ± 377 | 0.7 ± 0.1 | (5.5 ± 0.2) × $10^{-4}$ | 6 ± 3 |

[a] $K_i$ is a dissociation constant, whereas $K_I$ is a more complicated constant akin to $K_m$.