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QUANTIFICATION OF THE EXTENT OF ATTENUATION OF THE RATE OF TURNOVER CHEMISTRY OF THE TEM-1 β-LACTAMASE BY THE α-1*R*-HYDROXYETHYL GROUP IN SUBSTRATES

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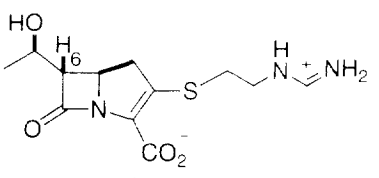
Abstract: The 6α-1*R*-hydroxyethyl group of imipenem, a clinically used carbapenem antibacterial agent, is believed to displace the hydrolytic water from its optimal position in the active site of class A β-lactamases. This interaction renders the molecule a poor substrate for these bacterial enzymes, hence preserving the antibacterial property of the antibiotic. The extent of the contribution of the 6α-1*R*-hydroxyethyl group in substrates toward stabilization of the antibiotic to the hydrolytic action of class A TEM-1 β-lactamase was studied by the synthesis and evaluation of two penicillanic acid derivatives, 6α-(1*R*-hydroxyethyl)penicillanic acid (**2**) and 6β-(1*R*-hydroxyethyl)penicillanic acid (**3**). The kinetic evaluation of the enzymic hydrolysis of these two penicillanic acid derivatives indicated that the 6α-1*R*-hydroxyethyl group imparts as much as 10^4-fold to the hydrolytic stability of the β-lactam substrate.

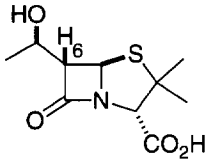
The hydrolytic function of the β-lactamases is the principal bacterial activity that accounts for the resistance to β-lactam antibiotics.[1] Over the decade from the late 1970s to the late 1980s a variety of new broad-spectrum β-lactam antibacterial agents were introduced into clinical use, including extended-spectrum penicillins (e.g., azlocillin and piperacillin), third-generation cephalosporins (e.g., cefotaxime, ceftriaxone, cefoperazone, and ceftazidime), a carbapenem (imipenem/cilastatin combination, e.g., Primaxin), a monobactam (aztreonam), and β-lactamase inactivators combined with older penicillins (e.g., clavulanate with amoxicillin or ticarcillin, and sulbactam with ampicillin). These "non-classical" β-lactam antibiotics are resistant to the hydrolytic action of β-lactamases, hence they have been used successfully in the clinic. With the availability of several crystal structures for β-lactamases, now it is becoming possible to investigate the mechanistic bases for the resistance of these antibiotics to the undesirable action of β-lactamases.

Imipenem (**1**) is an antibiotic of considerable interest, the only carbapenem antibiotic that has found clinical use. Imipenem retains its activity in the face of several examples of β-lactamase-mediated resistance to all other β-lactams, since it is a poor substrate for most β-lactamases. Furthermore, this antibiotic is often reserved as an antibiotic of the "last resort" in treatment of serious infections. The mechanism of hydrolysis of imipenem by class A β-lactamases has been the subject of investigation recently.[2-4] It has been suggested that the slow rate of turnover of this antibiotic by class A β-lactamases is due to the ability of the 6α-1*R*-hydroxyethyl group of imipenem to displace the hydrolytic water molecule from its optimal position in the active site, both in the preacylation complex,[2] as well as after acylation of the enzyme active site.[3]



1

It appeared to us that in order to quantify the contribution of the 6α-1*R*-hydroxyethyl group of imipenem on the slow enzymic hydrolysis of this β-lactam, it was necessary to have both the α- and β-isomers. Furthermore, to investigate whether the carbapenem nucleus plays a role in this attenuated rate for hydrolysis, we needed to change the nature of the nucleus itself. Hence, we envisioned compounds **2** and **3** as molecular probes, which would address these issues. These molecules are penicillanic acid derivatives incorporated with the 1*R*-hydroxyethyl group either at $C_{6\alpha}$ (i.e., **2**) or $C_{6\beta}$ positions (i.e., **3**).



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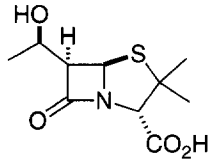

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Figure 1 (top panel) shows the structure of the acyl-enzyme intermediate for compound **2** in the active site of the TEM-1 β-lactamase. This species demonstrates that the steric interaction between the 6α-1*R*-hydroxyethyl group and the hydrolytic water, Wat-712, has displaced the water from the active site. Hence, the effect of the 6α-1*R*-hydroxyethyl group in **2** mimics that seen for imipenem.[4] For compound **3** (Fig. 1, bottom panel), the 6β-1*R*-hydroxyethyl group points to the active-site opening, and would not be expected to have any adverse effect on the rate of hydrolysis of the acyl-enzyme intermediate. Indeed, the hydrolytic water, Wat-712, does not move from its optimal position on energy minimization, and is suitably poised for attack at the ester carbonyl of the acyl-enzyme intermediate to give hydrolysis. This structure reveals that in the acyl-enzyme co.nplex for **3**, the hydroxyl of the hydroxyethyl group makes a hydrogen bond with the carbonyl of Ala-237, and that the methyl of the hydroxyethyl function, and those for Ala-237 and the $C_{2\beta}$ methyl of the penicillanate nucleus all interact favorably with each other in a hydrophobic manner. To summarize, the energy-minimized complexes predict that **2** should be a poor substrate—being unable to deacylate readily—and that **3** should be a much better substrate for the TEM-1 β-lactamase.

Compounds **2** and **3** were synthesized for analysis with the TEM-1 β-lactamase. We have reported the synthesis of compound **4** previously (pNB is the *p*-nitrobenzyl group).[5] The conversion of **4** to **5** was carried out by a slight variation of a reported procedure.[6] The critical step in the synthesis was reduction of the halide in **5**. Hence, the bromo function in **5** was reduced by one of two methods; one proceeded predominantly by inversion and the other by retention of configuration at C_6. The products of reduction (**6** and **7**) were purified by chromatography, prior to hydrogenolysis of the ester function over palladium on carbon to obtain the desired compounds **2** and **3** in 62% and 90% yields, respectively. Several protective groups for the carboxyl function were tried, and of those the *p*-nitrobenzyl group proved to be the best. This choice of ester was essential for successful purification of compounds **6** and **7**, prior to the removal of the protective group by hydrogenolysis.

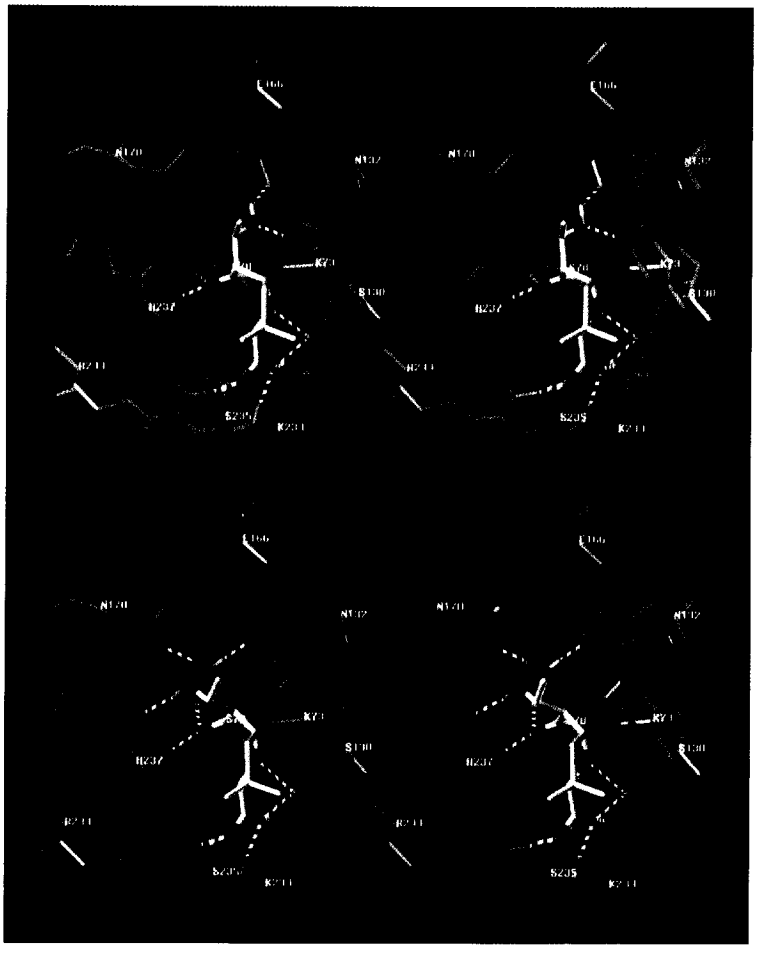
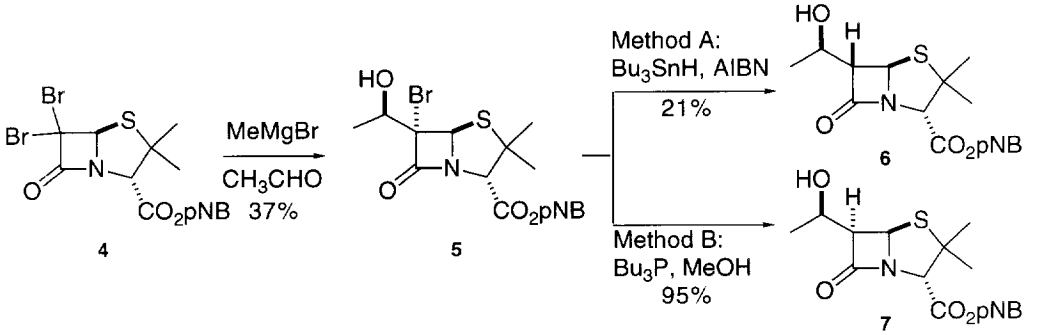
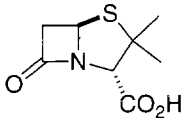


Figure 1. The stereoview of the energy-minimized structures for the acyl-enzyme intermediates for compounds **2** (top panel) and **3** (bottom panel) bound in the active site of the TEM-1 β-lactamase. The amino-acid residues are shown uniformly in green. Compounds **2** and **3** are color-coded according to atom types (yellow for sulfur, red for oxygen, white for carbon, dark blue for nitrogen, and cyan for hydrogen). Hydrogen atoms are shown only when there is a hydrogen bond. Hydrogen bonds are shown as dashed yellow lines. The hydroxyl of the hydroxyethyl group is indicated in each panel by a red arrow. The hydrolytic water (Wat-712) is displaced by the hydroxyl of the hydroxyethyl group of **2** from the active site, but it is retained for the acyl-enzyme intermediate for **3**. The hydrolytic water is poised for attack at the ester carbonyl by being positioned directly above it. The details of the procedures for energy minimization were similar to those reported by us previously.[4,5,7]



Compound **2** was an exceedingly poor substrate for the TEM-1 enzyme; indeed, the turnover rate was so poor that we were able to evaluate only the ratio k_{cat}/K_m at 27 $M^{-1}s^{-1}$. Compound **2** is actually a somewhat poorer substrate for β-lactamase than is imipenem (K_m = 27 μM, k_{cat} = 0.04 s^{-1}, k_{cat}/K_m = 1480 $M^{-1}s^{-1}$).[3] A comparison of the rate of turnover of penicillanic acid (**8**) by the same enzyme is meaningful here. Compound **8** is hydrolyzed by the TEM-1 β-lactamase with k_{cat}/K_m at 6.6 x 10^4 $M^{-1}s^{-1}$.[7] On the other hand, **3** is a reasonably good substrate (k_{cat} = 0.4 s^{-1}, K_m = 1.6 μM, k_{cat}/K_m = 2.8 x 10^5 $M^{-1}s^{-1}$, K_s = 13.5 μM, k_2 = 3.7 s^{-1}, and k_3 = 0.5 s^{-1}). In terms of the values for k_{cat}/K_m, it would appear that the presence of the 6β-1*R*-hydroxyethyl group in **3** has no appreciable effect on the rate of enzymic hydrolysis of this substrate, as the determination for compound **8** is roughly the same. The measurements for compound **3** show that both the acylation and deacylation steps are slow processes. Whereas deacylation is somewhat slower than acylation in this case, there appears not to be a unique rate-limiting step in turnover of **3**. From the values for k_{cat}/K_m it is evident that the attenuation of the rate of turnover due to interactions of the 6α-1*R*-hydroxyethyl group with the enzyme is approximately 10^4-fold. These results clearly indicate that there is no special feature in the carbapenem *nucleus* to impart resistance to turnover by β-lactamase, except the 6α-1*R*-hydroxyethyl group. As shown here, the 1*R*-hydroxyethyl group at $C_{6\alpha}$ in **2**—and not at $C_{6\beta}$, as in **3**—on a penicillanic acid nucleus also makes the penicillanic acid derivative a poor substrate for β-lactamase.



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