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REVIEW ARTICLE

6-(Hydroxyalkyl)penicillanates as Probes for Mechanisms of β-Lactamases

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6-(Hydroxyalkyl)penicillanates have proven helpful as probes for the mechanisms of β-lactamases, enzymes of resistance for β-lactam antibiotics. The present report summarizes the concepts on design, syntheses and use of these molecules in mechanistic studies of β-lactamases.

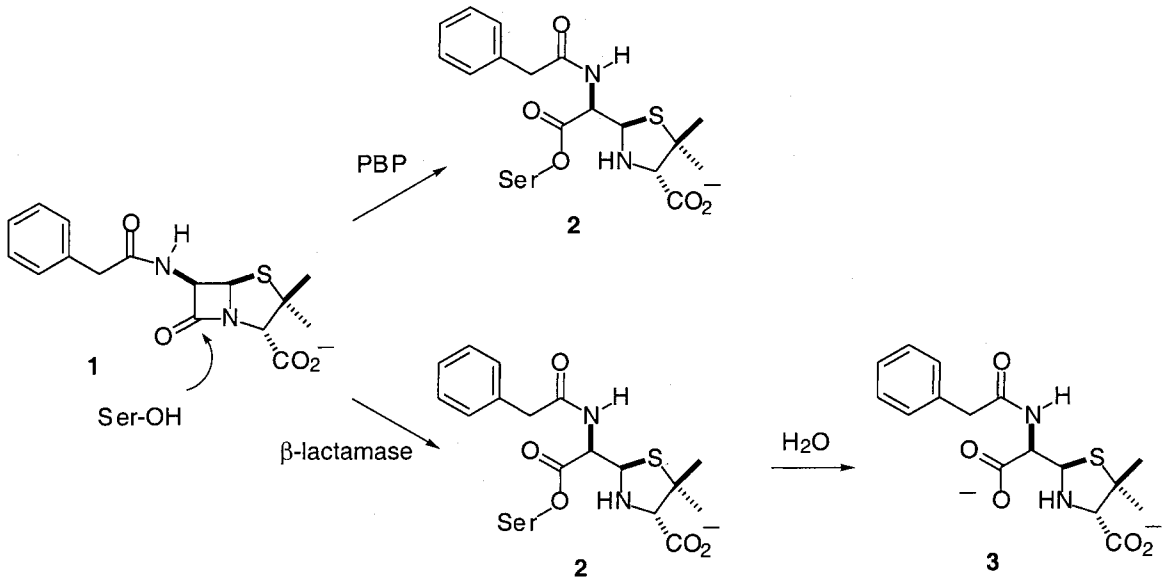
Discovery and introduction of penicillins and other β-lactam antibiotics to the clinic may arguably be the most significant contribution to medicine in the twentieth century. These antibiotics must have saved hundreds of millions of patients from death by infectious diseases.[1,2] Whereas the initial observations by ALEXANDER FLEMING and subsequent work toward isolation of preparations of the drug that came to be known as penicillin by HOWARD FLOREY and ERNST CHAIN all took place in the United Kingdom, the realities of the World War II necessitated an altruistic and selfless sharing of the information with scientists in the United States to push forward the search for this miracle drug for the war effort. Indeed, many scientists worked on the project, with incremental contributions from each, such that the collective effort made penicillin available for the first large-scale clinical use by 1942.[3] However, there were a handful of individual who were there from the beginning, stayed active in the field for the many years to come and consistently made important contributions to the science of antibiotics. One of these individuals was EDWARD ABRAHAM.

ABRAHAM's contributions to research on penicillin were numerous and it began with his elucidation of the structure of penicillin in 1943.[4] He went on to make ground-breaking discoveries on cephalosporins, molecules that to the present day remain as the most commonly used antibiotics. He also was one of the first to investigate the properties of β-lactamases, enzymes that are the most common cause of resistance to β-lactam antibiotics. Whereas none of the authors of this report were personally acquainted with EDWARD ABRAHAM, we have been the direct scientific beneficiaries of his significant contributions. It is an honor and a privilege to be invited to contribute to this tome in celebration of the work of EDWARD ABRAHAM.

The work that will be described here takes advantage of the mechanistic knowledge that is beginning to emerge on β-lactam antibiotics, their multifaceted targeting of bacterial enzymes, and the process of acquired resistance to these antibiotics. The mechanistic knowledge has led us in designing molecular probes for these various functions.[5,6] Furthermore, we have taken these molecular probes on to studies with the proteins for which they were designed. The knowledge of the details of the interactions of these enzymes with the probes have been instrumental in investigations of the ways nature has developed protein

Dedicated to the memory of Sir EDWARD ABRAHAM.

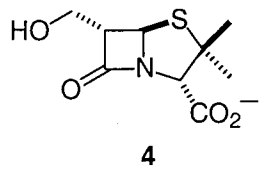
Scheme 1.



motifs for recognition of β-lactam antibiotics and of evolution of these proteins. This information ultimately guides us in refining our ability to devise novel β-lactams that are either inhibitors for the resistance enzymes or are antibiotics that can circumvent the resistance determinants entirely.[5~7]

The work in this area became possible with the availability of the first X-ray structures for penicillin-binding proteins (PBPs), the targets of β-lactam antibiotics, and β-lactamases.[8~11] The structures provided knowledge for the first time on how these drugs work and how resistance to them may arise. The vast majority of these enzymes use an active-site-serine strategy. The β-lactam antibiotic acylates the active-site serine to give an acyl-enzyme species. The approach of the serine is from the α face of the antibiotic, as exemplified by Scheme 1 for penicillin G (**1**). In the case of PBPs, the acyl-enzyme species (**2**) is stable and accounts for the irreversible inhibition of these essential bacterial enzymes by these antibiotics. On the contrary, the acyl-enzyme species for β-lactamases undergo deacylation to result in destruction of the drug (**3**) and the regeneration of the enzyme to go through the catalytic process again.

These structures were the beginning point for the design of a series of probes based on the nucleus of penicillanic acid. We reasoned that the approach of the water molecule to the ester of the acyl-enzyme intermediate could be from either the α or the β face. If we were able to design a molecule that would acylate β-lactamases, but would prevent the approach of the hydrolytic water to the ester, we would have not only an inhibitor for the resistance enzyme, but also a probe for the mechanism of the hydrolytic step. The computer-aided design based on the X-ray structure of the enzyme lead to compound **4**.[12] This compound and others that will be discussed in this report were synthesized by the general route of Scheme 2. The synthetic scheme allowed the generation of the desired stereo centers at the C6 and its side chain. The technically demanding step in the synthesis was reduction of the halide in **8**. The bromo moiety in **8** was reduced by one of two methods; one proceeded predominantly by inversion and the other by retention of configuration at C6. The products of reduction (**9** and **10**) were allowed to undergo hydrogenolysis of the ester function over palladium on carbon to obtain the desired compounds. Over a dozen molecules of this general



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Scheme 2.

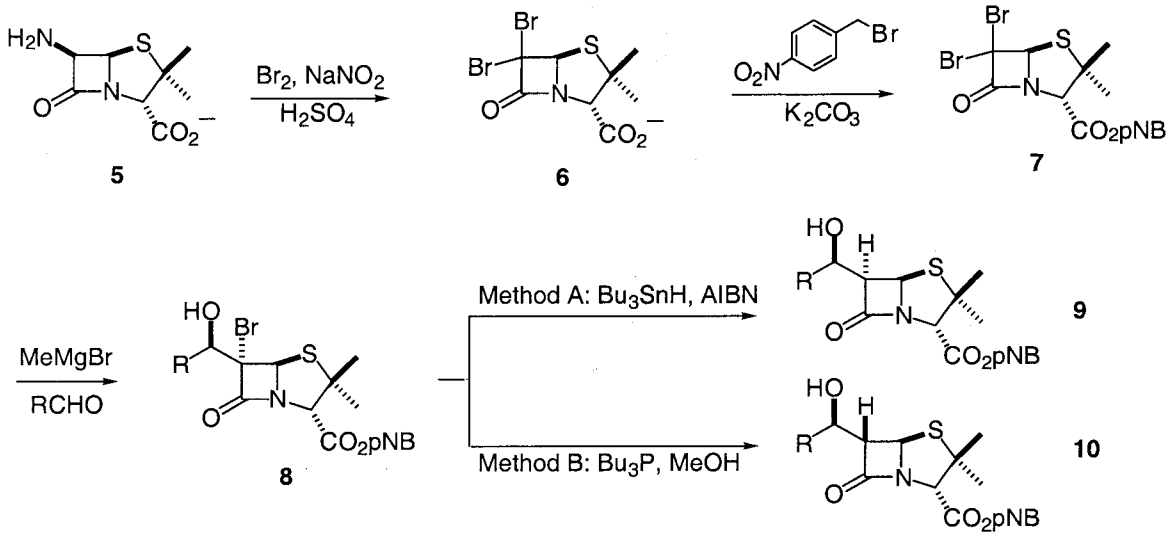
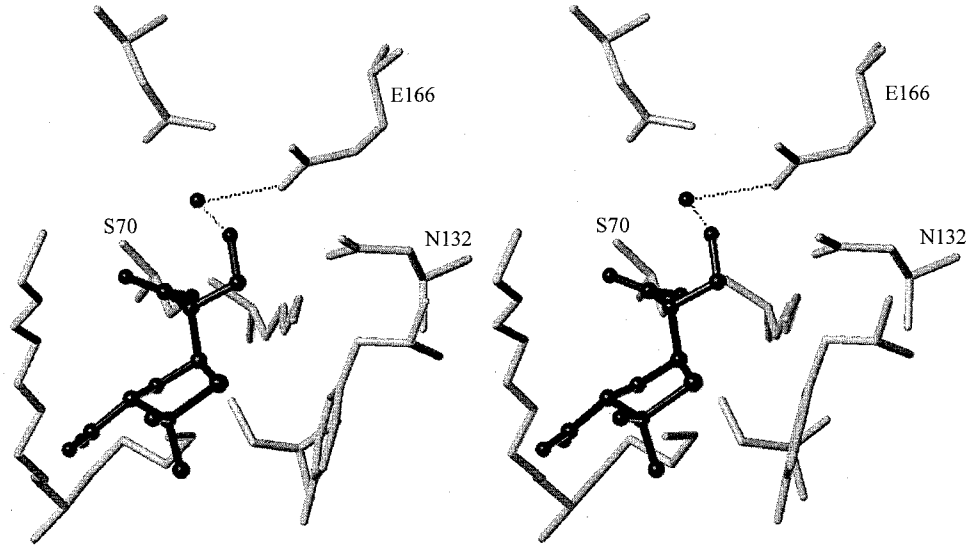


Fig. 1. Stereoview of the X-ray structure for the acyl-enzyme species of the TEM-1 β-lactamase from *Escherichia coli* inhibited by compound **4**.



Compound **4** is shown in black, and important active site residues are depicted. The hydrolytic water molecule is shown as a black sphere. Key hydrogen bonds are shown as dotted lines.

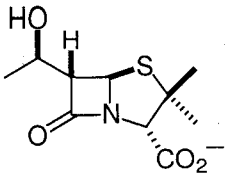
type have been synthesized.

Compound **4** inhibited the class A TEM-1 β-lactamase from *Escherichia coli* effectively. It was interesting that the kinetics of enzyme modification were biphasic, arguing for the involvement of two interconverting acyl-enzyme species, which were also predicted during the design aspect of the work. The crystal structure of the TEM-1 β-lactamase acylated by compound **4** was solved at 1.8 Å resolution (Fig. 1).[13] This structure at the time was the first X-ray structure for the acyl-enzyme species with a wild-type β-lactamases. It revealed that the hydroxymethyl group of **4** was hydrogen bonded to the hydrolytic water,

located on the α face of the active-site bound antibiotic. This hydrogen-bonding interaction attenuates the basicity of the hydrolytic water, which is promoted by Glu-166 as the general base. Furthermore, the hydroxymethyl group presented a physical barrier for the travel of the hydrolytic water to the ester moiety. Collectively, these effects accounted for excellent longevity for the acyl-enzyme intermediate. The enzyme activity did not recover from inhibition for several hours. We noted that prolonged contact between the inhibitor and the enzyme led to cracking of the crystals, which was an indication of a conformational change in the complex. Perhaps this is the necessary step for the transition from the first acyl-enzyme species to the next. The structure for this second species has not been solved.

It is noteworthy that when the 6-hydroxyalkyl moiety of the penicillanate was pointing in the β face, the molecule became a mere substrate. This assertion was studied with the pair of compounds **11** and **12**.[14] In these compounds the hydroxyethyl group was introduced to the C6 position of penicillanate, but the principles described for compound **4** would be valid for the α analogue **11** as well. In essence, the hydroxyethyl group in **12** points toward the opening of the active site, leaving the approach of the hydrolytic water at the ester group unencumbered. The opposite is true for the case of compound **11**, which serves as an inhibitor for the TEM-1 β-lactamase. The enzyme could ultimately recover gradually from inhibition. The differential effect between the two molecules on the ability of enzyme to turn them over was 10^4-fold. This significant difference is incidentally at the roots of the clinical longevity of carbapenems in the face of the pathogenic organisms that express these common resistance enzymes.[15] The crystal structure for the TEM-1 β-lactamase modified by imipenem (**13**), a clinical carbapenem antibiotic, validated the mechanistic role that we attribute to the hydroxyethyl group in this complex.[16]

Three class A β-lactamases have been identified over the past few years that have acquired a broader substrate profile to include carbapenems—designated as NMC-A ("non-metallo-carbapenemase of class A"), Sme-1, and IMI-1 enzymes. Carbapenems are considered antibiotics of last resort in treatment of multiply-resistant bacteria, as described earlier. Our kinetic analysis of turnover of five β-lactams for the NMC-A β-lactamase from *Enterobacter cloacae* and the TEM-1 enzyme indicated that the NMC-A enzyme has taken a sacrifice of approximately two orders of magnitude in k_{cat}/K_m for the typical penicillin substrate, such as ampicillin, to increase its breadth of substrate profile to include imipenem, which it turns over at k_{cat}/K_m of $(3.8\pm0.7)\ 10^5\ M^{-1}s^{-1}$. Effectively, this enzyme turns over imipenem as efficiently as ampicillin. On the other hand, imipenem is a poor substrate for the common TEM-1 β-lactamase (over four orders of magnitude attenuation in k_{cat}/K_m compared to ampicillin), which explains its clinical utility in organisms that harbor this enzyme, and others like it. The NMC-A enzyme turns over 6α-(hydroxymethyl)penicillanate (**4**), the aforementioned good inhibitor of the TEM-1 enzyme, as efficiently as it does imipenem. However, as we increased the bulk of the 6α substituent further in our synthetic penicillanate derivatives (**11** and **14**), we progressively decreased turnover of the compounds, such that 6α-(hydroxypropyl)penicillanate (**14**) was only an *irreversible* inactivator for the enzyme.[17] An X-ray structure at 1.9-Å resolution for the NMC-A enzyme inhibited by 6α-(hydroxypropyl)penicillanate (an acylated enzyme species; Figure 2) has been determined. The orientation of a domain in this enzyme places the Asn-132 side chain 1 Å farther out from the edge of the active site, a dislocation that is the reason for the increased breadth of the substrate profile. The dislocation of Asn-132 away from the active site enlarged the active site slightly, but just sufficiently, for the fitting of the 6α-1*R*-hydroxyethyl group of imipenem (**13**) in the active site.



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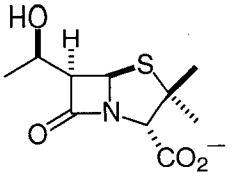

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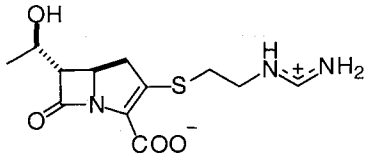

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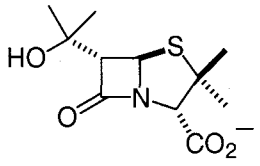

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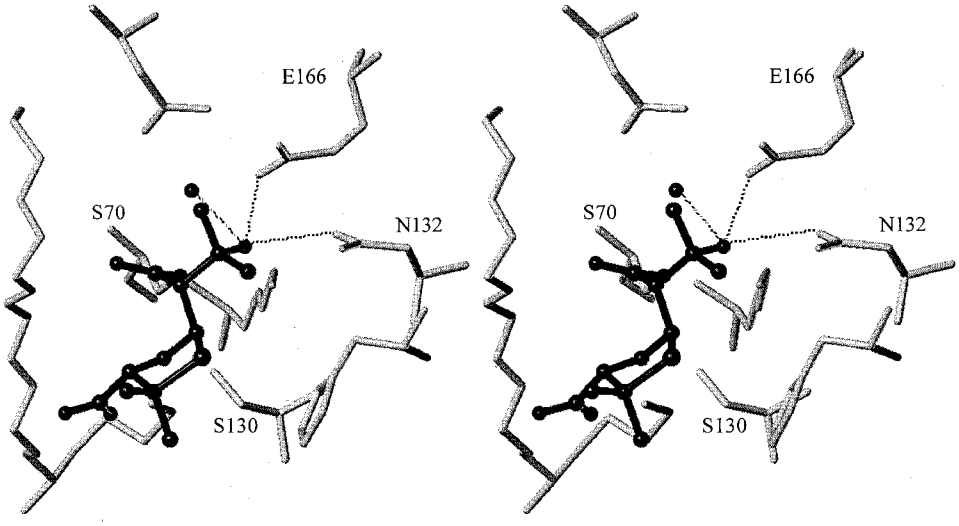
Fig. 2. Stereoview of the X-ray structure for the acyl-enzyme species of the NMCA β-lactamase from *Enterobacter cloacae* inhibited by compound **14**.

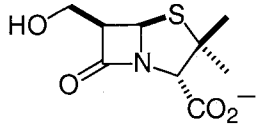


Compound **14** is shown in black, and important active site residues are depicted. The hydrolytic water molecule is shown as a black sphere. Key hydrogen bonds are shown as dotted lines.

Hydrogen bonding by the 6α-1*R*-hydroxyethyl group with the side chain of Asn-132 keeps it out of the way of the hydrolytic water, which proceeds to deacylate the acyl-enzyme intermediate of imipenem effectively.[17] The occurrence of this β-lactamase, and others like it, in clinical strains is a cause for concern. We add that selection of this enzyme in bacteria is a wonderful example of how nature would perturb the structure of an existing functional enzyme to increase the breadth of substrate profile.

The story for the class C enzymes is different. These enzymes promote the hydrolytic water from the β face of the active-site bound substrate.[18] Penicillanates have not been very helpful in investigations of the mechanism of this group of enzymes, so we will not discuss them further.

We have recently communicated our findings on the class D Oxa-10 β-lactamase from *Pseudomonas aerugenosa*.[19] This class of β-lactamases is the least studied, and our report of the X-ray structure of this enzyme was the very first for the class. We used the penicillanates **4**, **11**, **12**, and **15** as probes of the enzyme mechanism. Analysis revealed that the 6β-hydoxyalkylpenicillanates **12** and **15** were substrates for this enzyme, whereas the 6α variants (**4** and **11**) were inhibitors. Indeed, compounds **4** and **11** inhibited the enzyme in the preacylation step. This indicated that the compounds interfered with the machinery of the enzyme for the acylation step. The results collectively indicate that



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the hydrolytic water approached from the α direction for hydrolysis of the acyl-enzyme intermediate.

The X-ray structure revealed that despite the fact that the enzyme shares an overall homology with the class A enzymes, and that the hydrolytic water approached from the α direction, its mechanism of action is distinct.[19] There is no evidence for the presence of any acidic residues that would serve as the general base to promote hydrolysis of the intermediary ester, as seen in class A β-lactamases. The only plausible general base for the class D enzymes is Lys-70, which is also the residue that activates the active-site serine for acylation. So, in contrast to the cases of class A and C enzymes that employ different catalytic machineries for the acylation and deacylation steps, the enzymes of class D pursue symmetry in catalysis with Lys-70 as the basic residue for both steps of catalysis.

Considering the fact that class B enzymes are zinc-

dependent and exhibit an entirely distinct mechanism for turnover chemistry, it would appear that nature has devised at least four distinct catalytic machineries for turnover of β-lactam antibiotics.

β-Lactam antibiotics have enjoyed a major niche in antibacterial armamentarium over the past few decades. Regrettably, despite widespread resistance to these antibiotics, we currently do not have any antibiotics, and there is nothing in the therapeutic pipeline, that could replace this class of drugs. As a microbial response to the clinical success of β-lactams, one sees much diversity in sequences, structures and mechanisms of proteins that interact with these antibiotics. Meanwhile, the existing β-lactamases, of which over 250 are known currently,[5] produce fertile grounds in exploration of structural motifs that nature would develop when there is a strong selection pressure for the survival of the organism, such as presented by the clinical use of these versatile antibiotics.

Acknowledgements

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