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A Mechanism-Based Inhibitor Targeting the DD-Transpeptidase Activity of Bacterial Penicillin-Binding Proteins

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Abstract: Penicillin-binding proteins (PBPs) are responsible for the final stages of bacterial cell wall assembly. These enzymes are targets of β-lactam antibiotics. Two of the PBP activities include DD-transpeptidase and DD-carboxypeptidase activities, which carry out the cross-linking of the cell wall and trimming of the peptidoglycan, the major constituent of the cell wall, by an amino acid, respectively. The activity of the latter enzyme moderates the degree of cross-linking of the cell wall, which is carried out by the former. Both these enzymes go through an acyl-enzyme species in the course of their catalytic events. Compound **6**, a cephalosporin derivative incorporated with structural features of the peptidoglycan was conceived as an inhibitor specific for DD-transpeptidases. On acylation of the active sites of DD-transpeptidases, the molecule would organize itself in the two active site subsites such that it mimics the two sequestered strands of the bacterial peptidoglycan *en route* to their cross-linking. Hence, compound **6** is the first inhibitor conceived and designed specifically for inhibition of DD-transpeptidases. The compound was synthesized in 13 steps and was tested with recombinant PBP1b and PBP5 of *Escherichia coli*, a DD-transpeptidase and a DD-carboxypeptidase, respectively. Compound **6** was a time-dependent and irreversible inhibitor of PBP1b. On the other hand, compound **6** did not interact with PBP5, neither as an inhibitor (reversible or irreversible) nor as a substrate.

Penicillin-binding proteins (PBPs) are important bacterial enzymes that carry out the final steps of the bacterial cell wall assembly.[1−3] Three of these important activities are the transglycosylase, DD-transpeptidase, and DD-carboxypeptidase reactions, which are performed on the surface of the bacterial plasma membrane. The transglycosylase activity catalyzes the polymerization of lipid II to give rise to the polymeric glycosidic backbone of the cell wall, repeating units of *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc). A uniquely bacterial peptide is appended to the muramic acid residue (**1**). While some structural variations to the peptide portion of the bacterial peptidoglycan are seen, that shown for **1** is typically found in all Gram-negative and some Gram-positive bacteria.

DD-Transpeptidases carry out the indispensable cross-linking step of the cell wall. The cross-linking reaction gives the structural rigidity to the cell wall that is critical for bacterial survival. The DD-transpeptidase activity swaps one amide bond for another, in the process of which the cross-linked cell wall is formed (**1** → **2** → **4**; Figure 1A). The DD-carboxypeptidase activity moderates the degree of cross-linking by removing the terminal D-Ala of the peptidoglycan (Figure 1B), hence preempting the possibility of cross-linking. Both the DD-carboxypeptidase and DD-transpeptidase activities utilize an active-site serine strategy in their mechanisms. The serine experiences acylation by the peptidoglycan (**2**) in both groups of enzymes. In DD-transpeptidases, a second strand of peptidoglycan (**3**) binds the active site to undergo the cross-linking reaction with the acyl-enzyme species (**2**), to give rise to the cross-linked cell wall (**4**). The acyl-enzyme species undergoes hydrolysis in DD-carboxypeptidases (**2** → **5**), to result in a smaller peptide.

Tipper and Strominger proposed a number of years ago that the backbone of a β-lactam antibiotic mimics the acyl-D-Ala-D-Ala portion of the peptidoglycan structure.[4] By so-doing, β-lactam antibiotics also acylate the active site serine of DD-carboxypeptidases and DD-transpeptidases. However, in contrast to the case of the peptidoglycan that acylates the active site and allows the terminal D-Ala to serve as the leaving group, acylation of the active site by β-lactams leaves the "leaving group" covalently tethered to the "acyl-enzyme" species. Hence, the enzyme is essentially irreversibly inactivated, which is the basis for how β-lactam antibiotics kill bacteria. Edified by this information, we conceived of cephalosporin **6**. This compound has been incorporated with structural features that mimic the first strand of the peptidoglycan (the red portion of **6**), hence the molecule should acylate the enzyme active site serine. However, on serine acylation, the portions of the structure of species **7** drawn in blue would mimic the second strand of the

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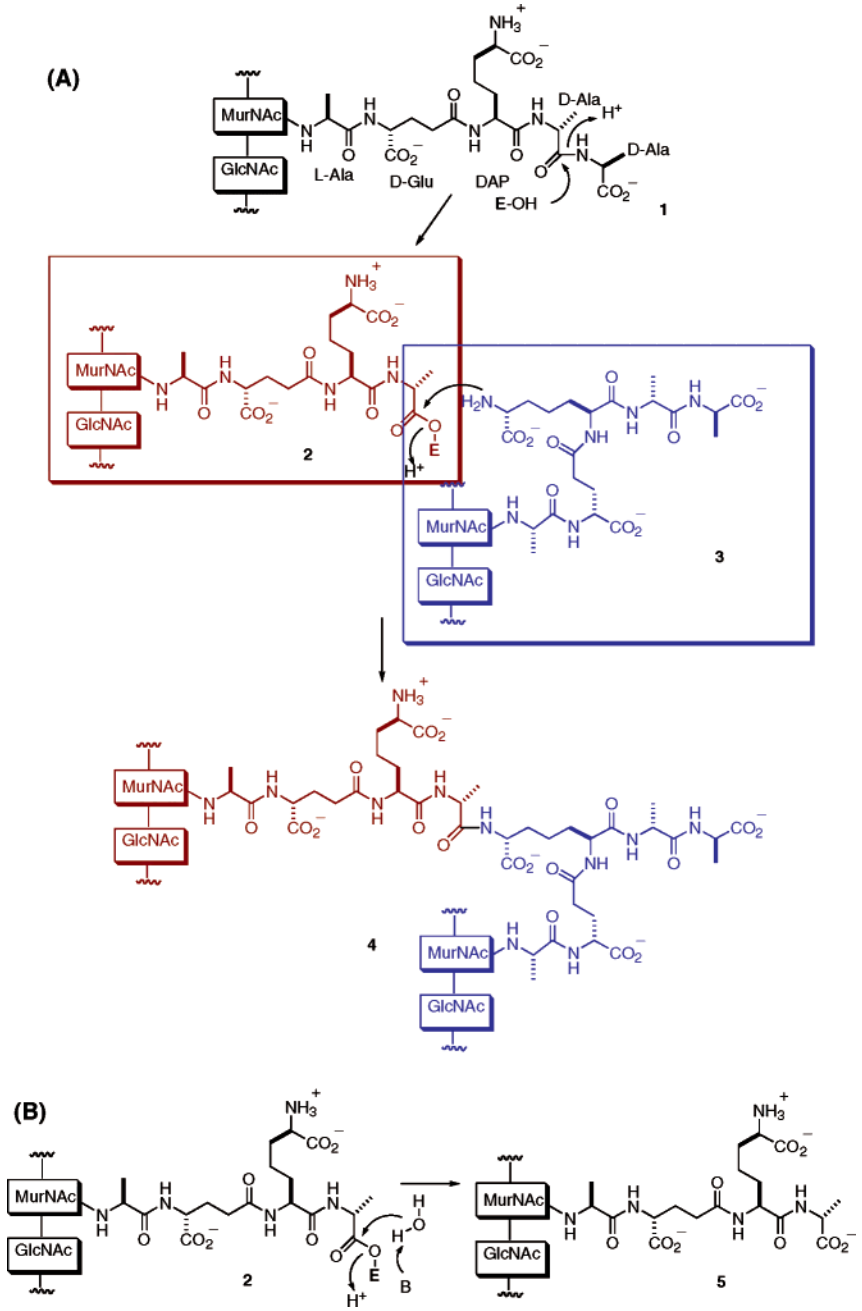
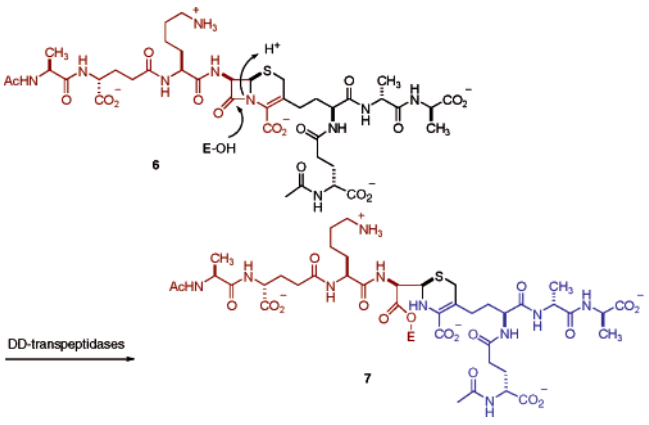


Figure 1. (A) Mechanistic steps in cross-linking of the cell wall by DD-transpeptidases. (B) Mechanistic steps in the activity of DD-carboxypeptidases.

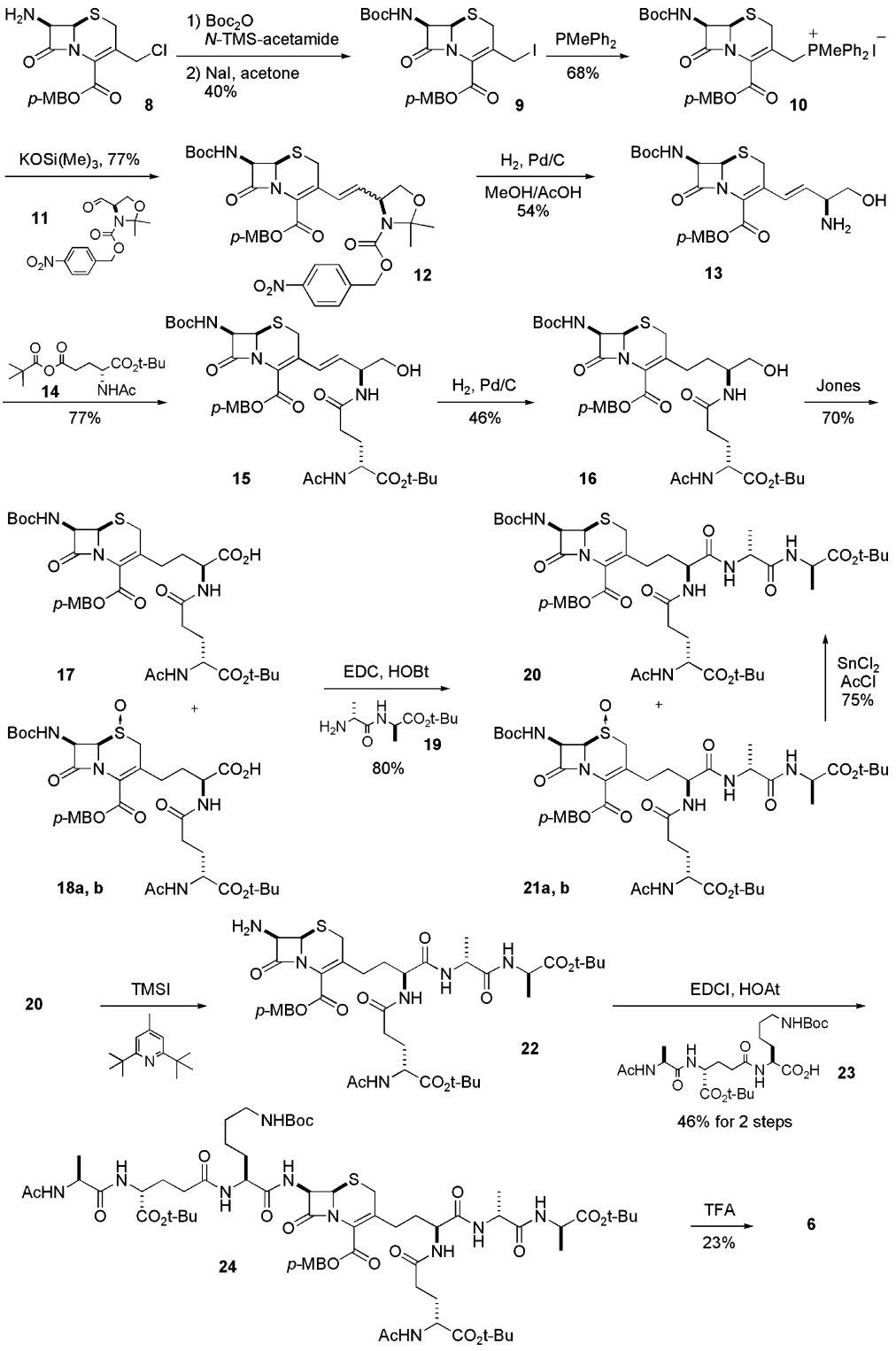
peptidoglycan sequestered within the active site of a DD-transpeptidase, on a trajectory for the cross-linking event. In light of the fact that compound **6** would have structural features that would occupy both peptidoglycan-binding subsites, we were anticipating that this molecule should be specific for DD-transpeptidases. For the same structural reason, we had anticipated that a molecule such as **6** would not be recognized by DD-carboxypeptidases, which interacts with one strand of peptidoglycan.



We disclose herein the multistep synthesis of compound **6**. Furthermore, we describe the evaluation of compound **6** with PBP1b (which exhibits the DD-transpeptidase activity) and with PBP5 (which is a DD-carboxypeptidase), both from *Escherichia coli*. As anticipated, the compound inhibited the DD-transpeptidase but was not even recognized by DD-carboxypeptidase, either as a substrate or as an inhibitor.

Compound **6** was synthesized according to the approach outlined in Scheme 1. The key intermediate **12** was obtained

Scheme 1



by the Wittig reaction of the phosphonium salt **10** with aldehyde **11** in the presence of potassium trimethylsilanolate. Compound **12** was briefly hydrogenolyzed over Pd/C in a mixture of acetic acid and MeOH. The resultant amine **13** was coupled with the mixed anhydride **14** to give compound **15**. Subsequent hydrogenation of **15** over Pd/C resulted in compound **16**. Jones oxidation of the alcohol moiety in **16** gave a mixture of the desired acid **17** and the sulfoxides **18a** and **18b**. The protected D-Ala-D-Ala-O^{t}Bu (**19**) was coupled to the mixture of **17** and **18a,b** in the presence of EDCI and HOBt, giving compounds **20** and **21a,b**. The mixture was stirred in a solution of stannous chloride and acetylchloride in DMF to allow the reduction of the undesired **21a,b** to the desired **20**. This procedure was reported previously for reduction of sulfoxides to sulfides.[5] Selective deprotection of the Boc group of **20** was achieved by

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treatment with iodotrimethylsilane (TMSI) and 2,6-di-*tert*-butyl-4-methylpyridine in acetonitrile. The resultant amine **22** was coupled with the tripeptide **23** (*N*-Ac-L-Ala-γ-D-Glu(α-O^{t}Bu)-L-Lys(ϵ-*N*-Boc)) to give **24**. A global deprotection of all the protective groups of **24** in trifluoroacetic acid produced the desired final cephalosporin **6**.

To demonstrate that compound **6** is indeed an inhibitor of DD-transpeptidase and not of DD-carboxypeptidases, we were in need of these enzymes. These proteins are difficult to study because they are anchored on the cytoplasmic membrane of bacteria by amino acid stretches at either the *N*- or *C*-terminal portions of PBPs. To obtain the proteins in soluble forms, we set out to remove the membrane anchors from PBP1b and PBP5 of *E. coli*, a DD-transpeptidase and a DD-carboxypeptidase, respectively. Both PBP1b[6] and PBP5[7] have been cloned previously, but these clones are generally not available. Hence, we undertook to clone these two proteins within our laboratory.

PBP5 is anchored to the plasma membrane via a carboxy-terminal 18-amino-acid[8] domain in its native state. To avoid accumulation of the overexpressed protein in the periplasm, where the peptidoglycan (the PBP substrate) is located, the PBP5 gene lacking the signal peptide and the anchor regions was cloned directly under the T7 promoter of the pET-24a(+) vector. The goal was achieved by PCR-amplifying the PBP5 gene from *E. coli*. The protein was expressed in the cytoplasm of *E. coli* BL21 and was purified to apparent homogeneity after one chromatographic step. The final yield was approximately 120 mg of protein per liter of culture.

A similar strategy was chosen for cloning of the *ponB* gene encoding PBP1b. The periplasmic portion of the protein lacking membrane anchor[9] was amplified by PCR and cloned under T7 promoter into pET-33Sh vector, which was derived from commercially available pET-33b(+) (Novagene). The resulting construct had the membrane anchoring motif replaced with a His-Tag label, and the protein was expressed in the cytoplasm of *E. coli*. For purification of the His-tagged PBP1b, a two-step purification was used. The final yield of the protein was 20−25 mg of protein per liter of culture.

Both PBP1b[10] and PBP5[11,12] are known to function in their nonmembrane-anchored forms without involvement of other proteins. With the availability of these two PBPs in our hands, we attempted to evaluate compound **6** with them in enzymological experiments. Specifically, we investigated whether the compound inhibited these PBPs or served as a substrate to them. As it turned out, compound **6** was an inactivator of PBP1b, but it neither served as a substrate to PBP5 nor inhibited it (concentrations of up to 5 mM). Hence, PBP5 did not recognize compound **6** as a suitable molecule for binding in the active site. However, as anticipated by design, compound **6** not only was recognized by the active site of PBP1b but also served as an inactivator of the enzyme.

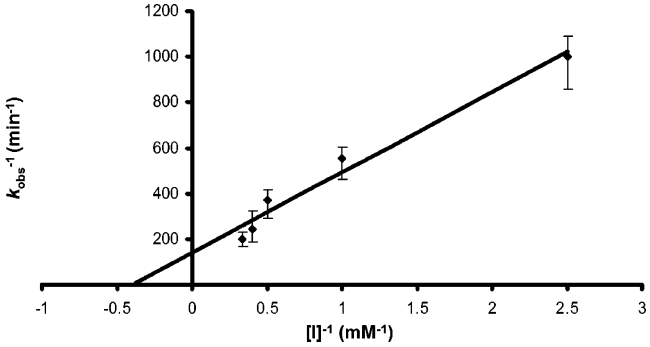

Figure 2. Double-reciprocal plot of the observed rate constants for enzyme inactivation *versus* the concentrations of inhibitor **6**.

Incubation of PBP1b with compound **6** resulted in time-dependent loss of activity. A series of the experiments were carried out at different inhibitor concentrations (0.4−3.0 mM), at a fixed concentration of the protein. At certain time intervals, aliquots were removed, diluted, and incubated with bocillin to determine the amount of the active enzyme remaining by fluorescence measurements of the bocillin-bound enzyme by SDS−PAGE. The double-reciprocal plot of the observed rate constants for enzyme inactivation (k_{obs}) as a function of inhibitor concentration (Figure 2)[13] was plotted to determine the first-order inactivation rate constant ($k_{inact} = 0.0072 \pm 0.0007$ min) and the inhibitor constant ($K_i = 2.5 \pm 0.8$ mM).

At first glance, the millimolar value for the inhibitor constant gives the impression that the inhibitor might not conform well to the requirements of the active site for binding. This is not so, for the following reason. Many important metabolites are present in millimolar concentrations; hence the enzymes that process them have evolved to experience saturation with these substrates in the same concentration range. For example, of the 21 enzymes of the glycolytic and tricarboxylic acid pathways, 10 exhibit K_m values in the millimolar range. The nascent peptidoglycan is readily available to the membrane-anchored PBPs in the periplasmic space. The high effective local concentration of peptidoglycan would facilitate its processing by the PBPs for favorable entropic reasons. Indeed, we have evidence that the PBPs that have been studied in this report process their peptidoglycan substrates with K_m values in the millimolar range (unpublished data); hence the argument that has been presented above holds in their cases.

We conclude that compound **6** is an effective mimic of the peptidoglycan structure, whereby it acylates only the active site of the DD-transpeptidase (PBP1b). On enzyme acylation, species **7** should occupy both substites of the active site, resulting in effective inhibition of the enzyme. Availability of cephalosporin **6** should be a boon to structural biological studies of DD-transpeptidase. The knowledge of the structure of the acyl-enzyme species **7** would help elucidate the manner in which DD-transpeptidases sequester the two strands of the polymeric peptidoglycan in the active site, an event that is critical for

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(14) Compound **6** was designed based on the peptidoglycan structure for Gram-negative bacteria, since this is the most common version in nature. Because Gram-negative bacteria have an outer membrane that excludes molecules larger than 700 Da, we did not expect that cephalosporin **6** (molecular weight of 985 Da) would be able to reach to the surface of the inner membrane of Gram-negatives, where PBPs are. Hence, cephalosporin **6** has no significant antibacterial activity.

survival of all bacteria. Such elucidation of the mechanism soon would pave the way in devising the next generations of antibiotics (β-lactam and otherwise) that would manifest their activity by inhibition of the events that lead to cell wall cross-linking.[14]

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Supporting Information Available: Experimental procedures, including those for cloning of the two PBPs used in this study, their purification, synthetic protocols, and characterization of all new compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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