Enzyme Inhibitors

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A De Novo Designed Inhibitor of D-Ala–D-Ala Ligase from *E. coli*

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Peptidoglycan is an essential component of the bacterial cell wall and provides the structural integrity necessary to resist internal osmotic pressure and to prevent cell lysis.[1] It is a covalent linear glycan polymer that consists of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, the latter of which have an appended pentapeptide chain. In addition to the transglycosylase and transpeptidase enzyme families, the biosynthesis of peptidoglycan involves a number of ATP-dependant ligases (namely the Mur ligases). These catalyze the assembly of the pentapeptide moiety through the successive addition of L-Ala, D-Glu, *m*-dpm or L-Lys, and D-Ala–D-Ala to UDP-MurNac by MurC, MurD, MurE, and MurF enzymes, respectively (*m*-dpm = *meso*-diaminopimelic acid; UDP = uridine-5′-diphosphoryl). An additional ATP-dependant enzyme, D-Ala–D-Ala ligase (DD-ligase) is responsible for supplying the D-Ala–D-Ala dipeptide, which is the substrate for MurF.

Inhibition of any of these essential enzymes in either Gram-positive or Gram-negative organisms leads to the loss of cell shape and integrity followed by bacterial death.[2–4] The emergence of bacterial strains that are resistant to all antibiotics in current clinical use has created an urgent need for the development of new inhibitors of peptidoglycan biosynthesis enzymes and, in particular, those that act on targets that have not been previously exploited.

As part of a structure-based design and synthesis program for the discovery of new enzyme inhibitors, we previously described the computer-aided molecular design, synthesis, and biological evaluation of novel inhibitors of the ligase responsible for D-Glu attachment, MurD.[5] Other research groups, using either library-screening methods or substrate-inspired approaches, have also reported useful inhibitors of the MurC[6–8] and MurD[6,9,10] ligases. A phosphinate-based transition-state isostere of DD-ligase has also been reported.[10]

The structure-based design of small-molecule enzyme inhibitors is a powerful tool in drug discovery, particularly if an X-ray crystal structure of the target enzyme is available. For cases in which the X-ray crystal structure includes a substrate or a known inhibitor, a substrate-inspired approach can be adopted; classical molecular modeling techniques can be used to alter the structure of the co-crystallized substrate, and a new molecule with enhanced enzyme-binding affinity can be produced. To identify different inhibitor structure types, the technique of virtual high-throughput screening (VHTS) can also be applied to the crystal structure of the enzyme in an attempt to identify new "hits" by docking the 3D structures of molecules contained in a database.[11]

However, both techniques have a number of drawbacks. The substrate-inspired approach requires a suitable enzyme–substrate co-crystal structure. Furthermore, the structures of the designed molecules will necessarily be biased toward that of the original co-crystallized small molecule, which, as is the case for many natural enzyme substrates, may not possess drug-like properties and may not be an ideal starting point. Although suitable databases allow the design of molecules by the VHTS method to focus on drug-like fragments, this technique is limited by the size and diversity of the small-molecule database; even databases that consist of millions of compounds constitute only a small fraction of the available "diversity space".

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Recent reports involving the application of de novo design indicate that this approach is a powerful alternative to these classical drug discovery approaches.[12–15] Through the use of only structural features present within the enzyme, new inhibitor designs can be built up sequentially according to the requirements of the targeted binding site. Therefore, in principle, de novo design should be a far more useful technique than VHTS, because a good de novo design program will examine structure space many orders of magnitude larger than that of most virtual libraries currently in use for this purpose. Herein, we report the application of the de novo molecular design program SPROUT[16] to the design of a novel inhibitor of DD-ligase from *E. coli*. We also describe the development of an efficient synthesis of this novel inhibitor and the enzymological characterization of its interaction with DD-ligase and a related enzyme.

SPROUT[16] is a powerful suite of software modules for de novo structure-based molecular design. SPROUT has modules for 1) characterizing regions within a protein, 2) detecting "hot-spots" in which ligand atoms are expected to form strong interactions with the protein, 3) docking molecular fragments to these sites, 4) joining these fragments with a molecular backbone to give a complete ligand, and 5) ranking the set of predicted ligands by criteria that include predicted binding energy, structural complexity, and synthetic accessibility.

In *E. coli*, there are two isoforms of DD-ligase (DdlA and DdlB) encoded by the genes *ddlA* and *ddlB*, with similar kinetic characteristics. The crystal structure of *E. coli* DdlB complexed with a phosphorylphosphinate inhibitor and resolved to 2.3 Å has been used to propose a catalytic mechanism for the formation of D-Ala–D-Ala.[17] To use a de novo approach for the discovery of novel DD-ligase inhibitors, we applied SPROUT to the X-ray crystal structure[18] of DD-ligase encoded by *ddlB* from *E. coli*.

The active site contains two distinct D-alanine binding sites; the N-terminal site is a high-affinity site with strict substrate specificity, whereas the C-terminal site is a lower-affinity site showing lower substrate specificity.[19] A phosphonate-based transition-state isostere is included in the crystal structure of DdlB, located within the active site. It makes direct contacts with essentially all of the residues implied by the catalytic reaction mechanism[17] (Figure 1).

To design a structurally simple inhibitor which was predicted to show high affinity for the enzyme, we wished to use only a small number of these contacts in our SPROUT design strategy. Examination of this active site with SPROUT indicated that use of the side chains of residues R255 and E15, the C^{α} backbone N–H group of G276, and the magnesium ion Mg331 should result in relatively simple designed molecular templates. Additionally, SPROUT analysis revealed the presence of a small hydrophobic region (which is not used by the phosphonate isostere) between the phenolic ring of Y216 and the alkyl side chain of L282 that we also wished to use for inhibitor design. During this work, it became apparent that the design of a molecular template which allows good contacts to be made to all these sites would require a rigid small-ring framework; the cyclopropyl-based amino acid **1**

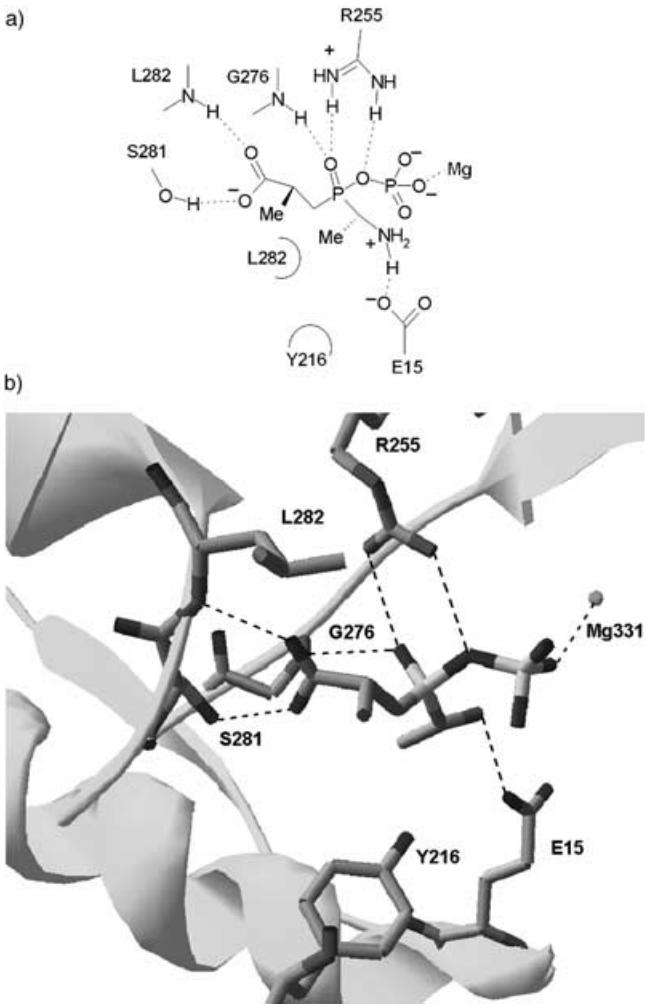


Figure 1. a) The bonding interactions between the phosphonate-based isostere and DdlB based on the crystal structure; b) view of the DdlB active site.

(Figure 2) was selected from the many suggestions that arose from the SPROUT design iterations.

One of us recently reported the X-ray crystal structure of the closely related ligase VanA co-crystallized with the same phosphinate-based isostere as described above for DdlB.[20] In addition to the DD-ligases, VanA is present in certain vancomycin-resistant enterococci and is responsible for the formation of D-alanine–D-lactate. This unit is then incorporated into the pentapeptide chain, and it is the resulting lactate-containing peptide that binds poorly to vancomycin. We were aware that although the overall topology of the active sites of VanA and DdlB were very similar, there are also important differences, particularly in the hydrophobic region bordered by L282 and Y216 in DdlB (corresponding to R317 and H244 in VanA). We therefore used SPROUT to produce a model of our designed inhibitor **1** within the active site of VanA (Figure 3). This revealed that the three designed hydrogen-bonding contacts to E15 (E16 in VanA), R255 (R290 in VanA), and G276 (G311 in VanA) as well as binding to the magnesium ion were possible for the inhibitor **1** bound within the active site of VanA. However, VanA possesses a

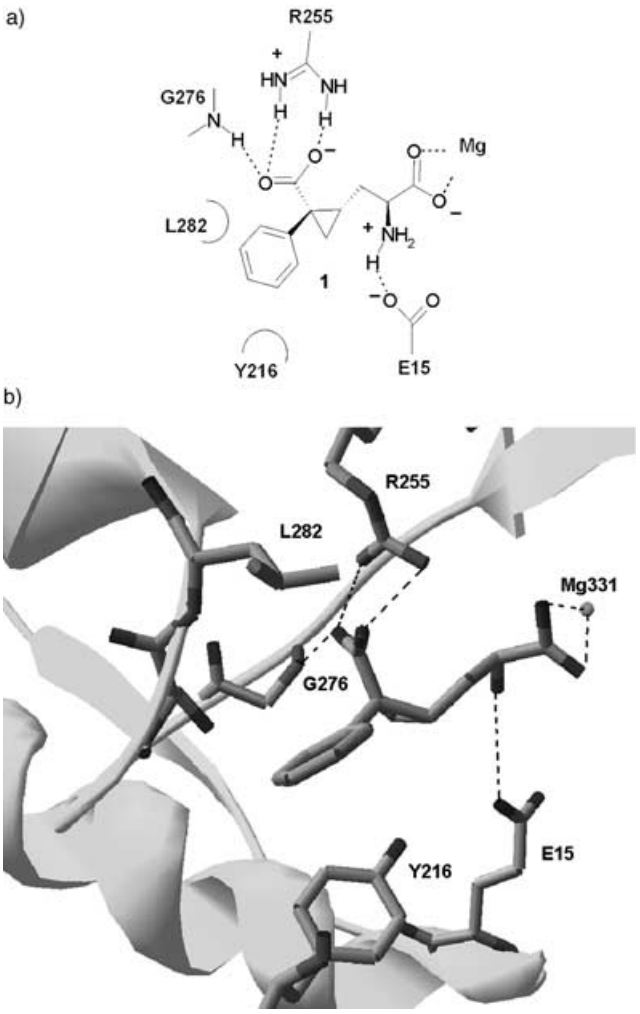


Figure 2. a) The bonding interactions of SPROUT-designed inhibitor **1**; b) view of inhibitor **1** within DdlB active site.

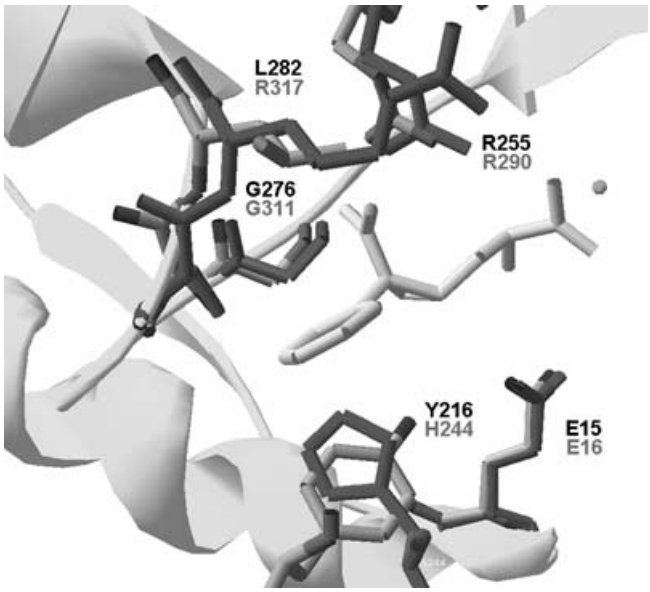
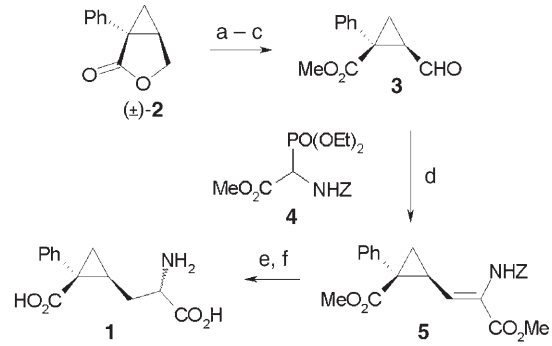


Figure 3. SPROUT-designed DdlB inhibitor **1** in the active site of VanA showing contacting residues. The corresponding residues in DdlB are superimposed (VanA residues in darker shading, DdlB numbering shown above VanA numbering).



Scheme 1. Synthesis of inhibitor **1**: a) $SOBr_2$, MeOH, 93%; b) NaI, Me_2CO, 98%; c) DMSO, iPr_2NEt, 63%; d) **4**, DBU, 87%; e) H_2, Pd/C, MeOH, 33%; f) LiOH, MeOH, 63%. DBU = 1,8-diazabicyclo-[5.4.0]undec-7-ene.

much narrower hydrophobic region between H244 and R317 than the equivalent region in DdlB (between L282 and Y216) due to protrusion of the imidazole ring of VanA H244 into the cavity (Figure 3). In fact, SPROUT analysis of the inhibitor **1**–VanA complex indicated that this inhibitor should bind only very poorly to VanA. Following synthesis, we therefore planned to measure the affinity of inhibitor **1** with both DdlB and VanA and test the selectivity of our inhibitor predicted by these computational studies.

A short synthesis of cyclopropane **1** was developed and is summarized below (Scheme 1). Ring opening of the readily available racemic lactone **2**[21] followed by halogen exchange and oxidation afforded aldehyde **3**. Olefination with phosphorane **4** followed by catalytic reduction of the double bond with concomitant *N*-deprotection and final ester hydrolysis yielded the desired inhibitor **1** as an inseparable 1:1 mixture of diastereomers.[22]

Enzymological evaluation[23] of designed inhibitor **1** was performed with recombinant *E. coli* DdlB and VanA enzymes. In keeping with our modeling predictions, this molecule (as a diastereomeric mixture) inhibits the activity of DdlB with an apparent K_i value of 12.5(±0.1) μM, whereas it had no inhibitory activity against VanA. The apparent K_i value of cycloserine for DdlB, a known inhibitor of this enzyme, was 1.4 μM. Clearly, the designed template is for a single enantiomer of absolute stereochemistry shown in Figure 2. In these proof-of-principal studies, however, we chose to perform biological evaluation with a readily available diastereomeric mixture containing this designed template along with equimolar amounts of three other stereisomeric versions of this inhibitor. Therefore, although the measured binding affinity of this mixture to DdlB is very encouraging, it is likely that the actual designed enantiomer **1** is an even more potent inhibitor of DdlB. It should be noted that the production of inhibitors that are active at the low micromolar concentration range after pure de novo design is quite typical of the current state of this approach; of course, inhibitors active at this level provide an excellent starting

point for further optimization. Development of more accurate functions for the estimation of binding affinity remains a key requirement for further progress in the de novo design of even more potent inhibitors.

In summary, it appears that SPROUT-based de novo design holds tremendous potential for the rapid discovery of selective inhibitors of DdlB and related enzymes. We believe that this approach is complementary to the use of high-throughput screening and is particularly attractive for cases in which such screening methodology is not available or in which access to large collections of library compounds of sufficient molecular diversity is limited.

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