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An In Vitro Screen of Bacterial Lipopolysaccharide Biosynthetic Enzymes Identifies an Inhibitor of ADP-Heptose Biosynthesis

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Summary

The lipopolysaccharide (LPS)-rich outer membrane of gram-negative bacteria provides a protective barrier that insulates these organisms from the action of numerous antibiotics. Breach of the LPS layer can therefore provide access to the cell interior to otherwise impermeant toxic molecules and can expose vulnerable binding sites for immune system components such as complement. Inhibition of LPS biosynthesis, leading to a truncated LPS molecule, is an alternative strategy for antibacterial drug development in which this vital cellular structure is weakened. A significant challenge for in vitro screens of small molecules for inhibition of LPS biosynthesis is the difficulty in accessing the complex carbohydrate substrates. We have optimized an assay of the enzymes required for LPS heptose biosynthesis that simultaneously surveys five enzyme activities by using commercially available substrates and report its use in a small-molecule screen that identifies an inhibitor of heptose synthesis.

Introduction

Gram-negative bacteria are growing as very important pathogens in the face of increasing antibiotic resistance. In particular, the emergence and dissemination of extended spectrum β-lactamases conferring resistance to all β-lactam antibiotics in gram-negative bacteria such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, species of *Acinetobacter*, and the *Enterobacteriaceae* [1, 2] have vaulted these previously controllable microbes to the status of extremely problematic pathogens in the same league as vancomycin-resistant gram-positive bacteria [3, 4]. Thus, there is a real clinical need for new therapeutic strategies that selectively target gram-negative organisms.

Gram-negative bacteria differ from their gram-positive counterparts in the presence of a relatively impermeable asymmetric outer membrane consisting of an outer leaflet of lipopolysaccharide (LPS) and an inner leaflet of phospholipid. The LPS layer consists of lipid A (a disaccharyl-lipid complex), a core oligosaccharide, and a repeating saccharide O-antigen (Figure 1) [5]. The core oligosaccharide can be subdivided into an inner core made of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and heptose residues and an outer core comprising hexoses and 2-acetoamido-2-deoxy-hexoses. Blockade of lipid A and Kdo biosynthesis is lethal for bacterial growth in the laboratory, while defects in the biosynthesis of heptoses results in a dramatic reduction of virulence and increased antibiotic susceptibility [6]. Opportunities to develop chemical strategies that modulate LPS biosynthesis are beneficial both as research tools to study the assembly of LPS, but also as novel antibacterial agents that can either reduce virulence or potentiate antimicrobial properties of antibiotics presently not used for gram-negative bacterial infections. Therefore, small-molecule inhibitors of LPS biosynthesis have the potential to be developed into antimicrobial agents themselves or as codrug formulations with antibiotics.

Despite this promise, there have been relatively few forays into the development of inhibitors of LPS biosynthetic enzymes, with the exception of the lipid A biosynthetic enzyme LpxC [7–9]. One reason for this lack of effort is the structural variability of the LPS within bacterial genera and species. However, the inner core and lipid A biosynthetic enzymes are generally conserved across genera and as a result are predicted to be profitable targets. Another challenge is the chemical complexity and the unavailability of the substrates of these enzymes for assays of activity.

The biosynthesis of the nucleotide-activated heptose precursors for the inner core assembly has been elucidated genetically and biochemically [6, 10–12]. In *Escherichia coli*, the first step in this pathway is the synthesis of sedoheptulose-7-phosphate, catalyzed by the transketolase TktA, followed by ketose-aldose isomerization to D-*glycero*-β-D-*manno*-heptose-7-phosphate by the LPS-dedicated enzyme GmhA (Figure 1). The sugar is then phosphorylated at the anomeric hydroxyl, trapping the product in the α configuration and generating the 1,7-bisphosphate intermediate. This reaction is catalyzed by an ATP-dependent kinase, HldE, that in *E. coli* is fused in a bifunctional enzyme with a downstream adenylyltransferase activity in the pathway. Dephosphorylation at position 7 by the phosphatase GmhB is followed by the second activity of HldE, adenylylation of the 1-phosphosugar to generate ADP-D-*glycero*-β-D-*manno*-heptose (Figure 1). Inversion of the D-stereochemistry at C6 generates the final LD-ADP-activated heptose that is a substrate for specific heptosyltransferases involved in the assembly of the LPS core.

Inactivation of the associated genes encoding heptose biosynthesis and transferase enzymes results in increased bacterial sensitivity to antibiotics and loss of virulence [10, 13–16]. Therefore, small-molecule inhibitors of enzymes in this pathway could be leads in the development of novel antibiotics. The challenge in this approach is securing sufficient quantities of substrates for

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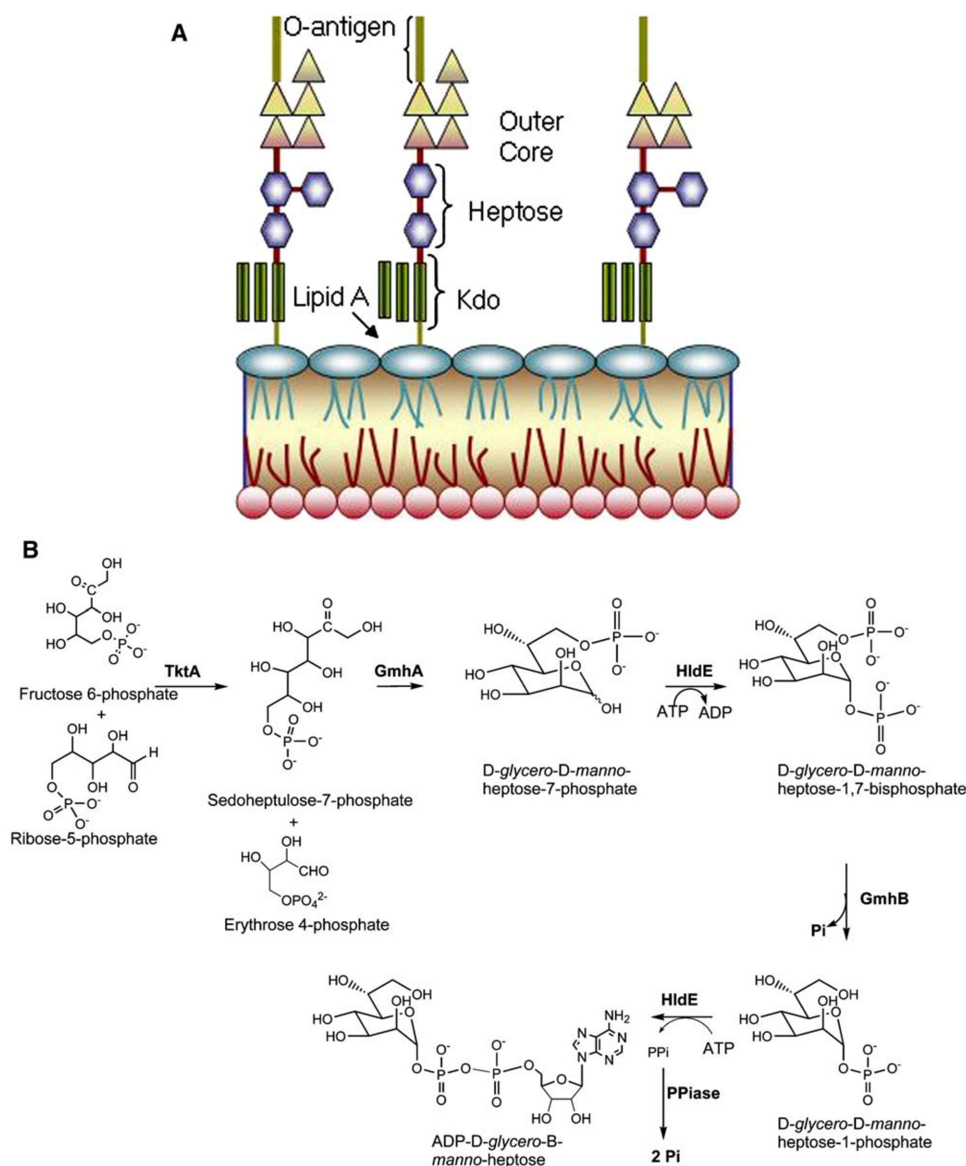

Figure 1. The Bacterial Outer Membrane and the Heptose Biosynthetic Pathway

(A) Cartoon representation of the cell envelope of gram-negative bacteria consisting of an asymmetric bilayer of phospholipid and LPS. LPS contains several conserved components, including lipid A, Kdo, and heptose.

(B) Biosynthesic pathway of activated heptose for incorporation into LPS in *E. coli*.

the enzymes for screening purposes. Here, we report an in vitro assay that overcomes the substrate accessibility barrier by recapitulating the biosynthetic pathway in vitro by using commercially available substrates to prime the assay. The utility of the assay is demonstrated in a screen of 1000 small molecules that identifies the first inhibitor of a heptose biosynthetic enzyme.

Results and Discussion

The biochemical study of ADP-heptose biosynthesis has been hampered by the lack of readily available substrates of the enzyme-catalyzed reactions. A large-scale screening campaign for inhibitors of any of these enzymes would require access to milligram to gram quantities of these rare carbohydrate substrates. We reasoned that a high-throughput screening assay bypassing the requirement for these substrates could be established by reconstituting the four enzymes and five enzymatic transformations of *E. coli* D,D-heptose biosynthesis in vitro. In this approach, the reaction is initiated with the readily available precursor sugars fructose-6-phosphate and ribose-5-phosphate in the presence of ATP. Flux through the pathway is monitored by the release of inorganic phosphate (readily visualized colorimetrically with nmol sensitivity) as a result of the activity of the penultimate phosphatase reaction catalyzed by GmhB, and by the addition of pyrophosphatase to cleave pyrophosphate generated in the final step of ADP-heptose formation catalyzed by the adenylyltransferase activity of HldE (Figure 2).

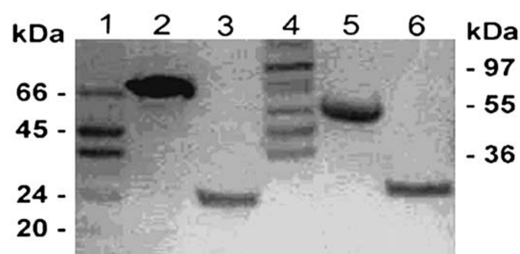

Figure 2. Overexpression of the Enzymes Required for the Biosynthesis of ADP-D-*Glycero*-β-D-*Manno*-Heptose

Lanes 1 and 4, molecular weight standards; lane 2, transketolase TktA; lane 3, phosphoheptose isomerase GmhA; lane 5, bifunctional kinase/adenylyltransferase HldE; lane 6, phosphatase GmhB.

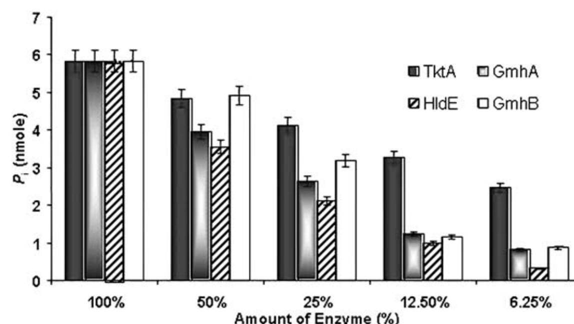

Figure 3. Validation of the In Vitro ADP-D-*Glycero*-β-D-*Manno*-Heptose Biosynthetic Pathway Assay

The in vitro assay was designed such that decreasing the amount of any enzyme in the pathway would result in reduced phosphate production, as detected at 660 nm by using the malachite green phosphate detection assay. Reactions were performed in duplicate with TktA (solid, black bar), GmhA (gray-scale bar), HldE (hatched bar), and GmhB (white bar) at 32 pmol, 2.1 nmol, 12 pmol, and 2.5 pmol, respectively, as 100% enzyme amount (also used as the positive control). Each enzyme was systematically analyzed by lowering the amount of one enzyme while keeping the other three enzymes at constant amounts. The error bars represent the spread between two data points.

To explore this proposal, the enzymes TktA, GmhA, HldE, and GmhB were overexpressed as hexa-His-tagged constructs in *E. coli* and purified (Figure 2). The enzymes were then coincubated initially at 500 ng levels in buffer and in the presence of 1 mM substrates to confirm substrate-dependent phosphate formation by using the endpoint phosphomolybdate-malachite green detection method [17]. The levels of enzymes were then lowered in step-wise fashion to achieve a linear dependence of phosphate release over 10 min.

With these conditions, we next determined the apparent K_m of ATP in the assay. The reconstituted pathway uses two equivalents of ATP at the kinase and adenylyltransferase steps, both catalyzed by a separate active site on the bifunctional HldE [16]. Varying ATP concentration resulted in the determination of an apparent K_m of 0.15 mM, which is a composite of both ATP-requiring steps. To identify competitive inhibitors of these ATP-dependent enzymes, we maintained ATP concentration at this level for all additional assays studies. Also, to ensure that the flux of substrates through the recapitulated biochemical pathway was sensitive to each enzymatic step, the level of each enzyme was systematically varied to enable the identification of inhibitors of each step (Figure 3). Because the screen was performed with concentrations of fructose-6-phosphate and ribose-5-phosphate that exceeded the K_m, we did not rigorously tune the concentration of TktA; nonetheless, the assay remains quite sensitive to inhibition of this enzyme (Figure 3).

The ability of this reconstituted biosynthetic pathway assay to identify inhibitors of a component enzyme was next validated with the nonhydrolyzable ATP analog β,γ-methyleneadenosine 5′-triphosphate, which was predicted to inhibit HldE kinase activity. This molecule inhibited the reaction with an IC_{50} of 5 mM, demonstrating that the assay could identify inhibitors of component enzymes.

To extend this strategy to high throughput, we optimized the assay by using an automated BioMek FX (Beckman) system. Under these conditions, a Z′ statistic of 0.7 was determined, indicating that the assay was robust and adequate for screening [18]. We next performed a screen against 1000 synthetic small molecules with protein kinase inhibitor templates at 50 μM in duplicate (Figure 4). The screen identified a compound

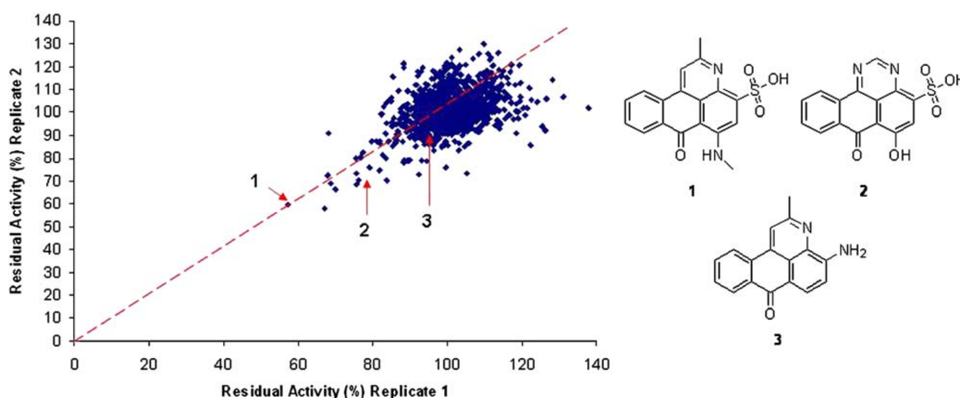

Figure 4. High-Throughput Screening of 1000 Small Molecules against the ADP-D-*Glycero*-β-D-*Manno*-Heptose Biosynthetic Pathway

Residual activity (%) duplicate values for each compound are plotted on opposite axes. Duplicates are considered to be in agreement if they fall on a line through the origin with a slope of 1 (red, dashed line). The positions of the structurally related compounds **1**, **2**, and **3**, are shown on the graph.

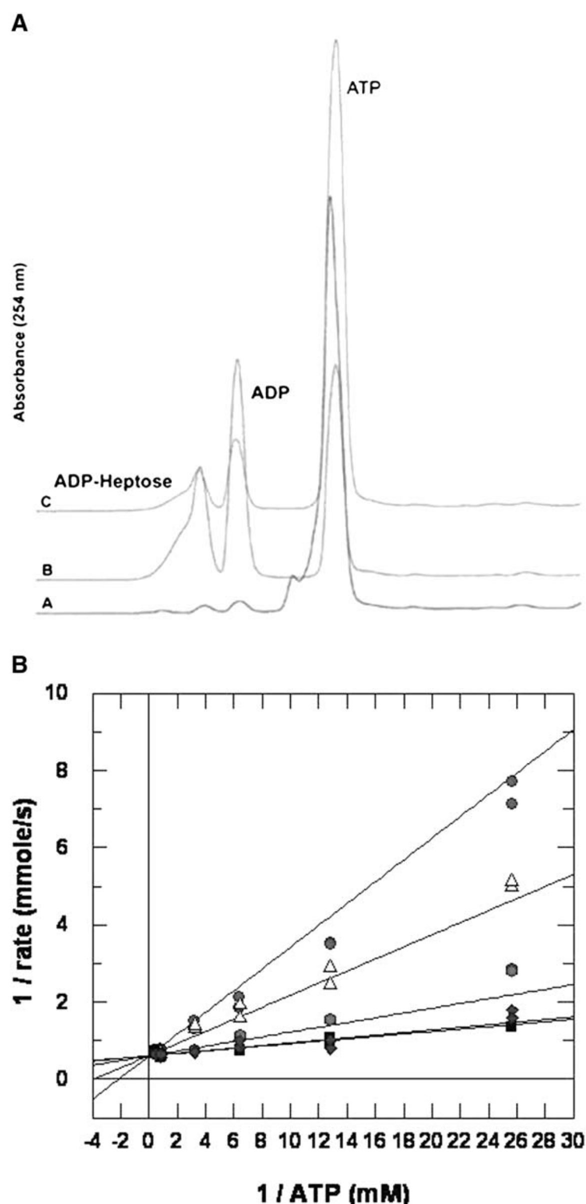

Figure 5. Characterization of Inhibitory Properties of Compound 1

(A) Paired-Ion Chromatographic analysis of the effects of compound 1 on the production of ADP-D-*glycero*-β-D-*manno*-heptose. Trace A, ATP control. Trace B, complete reaction after 10 min of incubation. Trace C, complete reaction with 500 μM compound 1.

(B) Double reciprocal plot of effect of increasing concentration of compound 1 on HldE activity. The K_i for 1 is 63 ± 8 μM.

(**1**, 2-methyl-6-methylamino-7-oxo-7H-naphtho[1,2,3-de] quinoline-4-sulfonic acid) that reduced pathway activity by ~40%. An analog of this hit, compound **2** (6-hydroxy-7-oxo-7H-benzo[e]perimidine-4-sulfonic acid), showed ~20% inhibition, while compound **3** (4-amino-2-methyl-naphtho[1,2,3-de]quinolin-7-one), was not an inhibitor, suggesting that the sulfonic acid moiety was likely important for activity.

To identify the enzymatic activity blocked by compound **1**, the reaction components of assays performed in the presence and absence of the compound were separated by reverse-phase HPLC while monitoring the absorbance at 254 nm. This reports on the presence of the adenine group and therefore is a direct measure of ADP-D-*glycero*-β-D-*manno*-heptose production and consumption of ATP. In the absence of **1**, the reaction mixture contains residual substrate ATP, product ADP from the HldE kinase activity, and ADP-D-*glycero*-β-D-*manno*-heptose (Figure 5). The distribution of peak areas of ATP:ADP:ADP-D-*glycero*-β-D-*manno*-heptose in the absence of inhibitor is 4:3:3. If **1** blocked either GmhB or the adenylyltransferase activity of HldE, the ATP:ADP ratio should approximate that found in the absence of inhibitor. On the other hand, if **1** inhibits TktA, GmhA, or the kinase activity of HldE, the levels of ATP would be much more significant than in the absence of inhibitor. In the presence of compound **1**, the ratio of ATP:ADP:ADP-D-*glycero*-β-D-*manno*-heptose is 8:1:1, indicating that TktA, GmhA, or the kinase activity of HldE are the targets.

We eliminated TktA and GmhA as targets by increasing the levels of these enzymes up to 5-fold each, and we saw no change in IC_{50}. By using an excess of TktA and GmhA to produce the HldE kinase substrate D-*glycero*-β-D-*manno*-heptose-7-phospate in situ and the canonical pyruvate kinase/lactate dehydrogenase couple to measure ADP production, we determined that **1** is a competitive inhibitor of the HldE kinase and has a K_i of 63 ± 8 μM (Figure 5). This confirmed that HldE kinase was the target of **1**, and that this compound is the first reported inhibitor of a heptose biosynthetic enzyme.

Significance

Our results demonstrate for the first time that ADP-heptose biosynthesis is a tractable target for high-throughput screening of inhibitors of LPS biosynthesis. This assay uses readily available substrates and overexpressed enzymes to enable probing of an important biochemical pathway that otherwise would be highly challenging to study. By carefully optimizing the assay in vitro, the identification of novel inhibitors of enzyme function is possible, and, thus, there is potential to advance antibiotic research. The utility of the assay was demonstrated by the identification of the first inhibitor of heptose biosynthesis. This research opens the possibility of developing new antimicrobial agents or adjuvants that act against gram-negative pathogens.

Experimental Procedures

Protein Preparation

The genes encoding TktA, GmhA, GmhB, and HldE were amplified by PCR from *E. coli* chromosomal DNA (see the Supplemental Experimental Procedures available with this article online) and were cloned into appropriate vectors to create N-terminal His6 fusions. The amplified genes were sequence verified, and the plasmids were used to transform *E. coli* BL21 (DE3), which was used to overexpress each protein. Proteins were purified over immobilized metal affinity chromatography by using an imidazole gradient. Some proteins were further purified by using a Q Sepharose anion exchange step. Proteins were analyzed by SDS polyacrylamide gel electrophoresis.

Phosphate Detection Assay

One phosphate is released directly by GmhB, and two additional phosphates are generated after the HldE adenylyltransferase reaction in the presence of pyrophosphatase. Assays were performed in 96-well plates in the presence of 0.1 U of yeast pyrophosphatase

in 20 mM HEPES (pH 8.0), 10 mM MgCl, 10 mM KCl, 1 mM fructose-6-phosphate, 140 μM ATP, 100 μM thiamine pyrophosphate, 0.1% Tween 20, 5% dimethylsulfoxide, and purified TktA, GmhA, GmhB, and HldE in a final volume of 100 μl. The reaction was initiated by the addition of ribose-5-phosphate (final concentration 1 mM). The reaction was incubated for 10 min at room temperature and was quenched by adding 5% trichloroacetic acid at 4ºC. The amount of phosphate released was measured at 660 nm after the addition of 200 ml of a mixture of ammonium molybdate (4.2%) and malachite green hydrochloride (0.045%) in 4 M HCl.

Primary Screen

A high-throughput screen of 1000 small molecules from ChemDiv (San Diego, CA) was performed in 96-well plates in duplicate by using a Beckman-Coulter Biomek FX Liquid Handler (Fullerton, CA) and a Molecular Devices SpectraMax Plus[384] plate reader (Sunnyvale, CA). Compounds were dissolved in dimethysulfoxide (∼50 μM final concentration) and preincubated with all enzymes and reagents except ribose-5-phosphate for 8 min at room temperature. The reaction was initiated by the addition of ribose-5-phosphate at 1 mM final concentration and was monitored as described above.

Secondary Screen

HldE activity in the presence and absence of compound **1** was determined by coupling the production of ADP with the lactate dehydrogenase-dependent oxidation of reduced NADH in the presence of phosphoenol pyruvate (PEP) and pyruvate kinase/lactate dehydrogenase. The assay mixture was comprised of 0.30 mM NADH, 3 mM PEP, 2.5 U pyruvate kinase, 2.8 U lactate dehydrogenase, 1 mM ribose-5-phosphate and fructose-6-phosphate, 5 nmol GmhA, 0.125 nmol TktA, 300 μM ATP, 20 mM HEPES (pH 8.0), 10 mM MgCl, and 40 mM KCl to make a 100 μl final volume in 96-well microtiter plates. The mixture was incubated in duplicate for 10 min in the presence of the inhibitor, spanning seven concentrations ranging from 0.004 to 1.5 mM. The reaction was initiated by the addition of 300 μM ATP, and the oxidation of NADH was monitored at 340 nm. Data were fit to the four-parameter Equation 1 by using GraFit [19] to calculate the IC_{50} value:

$$y = \frac{A - D}{1 + (I/IC_{50})^S} + D, \quad (1)$$

where A = minimum response plateau, D = maximum response plateau, I = concentration of inhibitor, and S = slope factor. The K_i value and mode of inhibition were determined by using the same ADP assay under identical conditions. Briefly, seven ATP concentrations, ranging from 0.04 to 2.5 mM, were used with four compound **1** concentrations, ranging from 0.004 to 5 mM. The mode of inhibition was assessed by visualization of double reciprocal plots and was statistically supported by application of the F-test. The K_i for competitive inhibition was determined by the fitting of Equation 2 by using GraFit [19] software:

$$\nu = V_{max} S / (K_m [1 + I/K_{is}] + S). \quad (2)$$

Supplemental Data

Supplemental Data including details of oligonucleotide primers are available at http://www.chembiol.com/cgi/content/full/13/4/437/DC1/.

Acknowledgments

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References

1. Walsh, T.R., Toleman, M.A., Poirel, L., and Nordmann, P. (2005). Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. *18*, 306–325.
2. Jacoby, G.A., and Munoz-Price, L.S. (2005). The new β-lactamases. N. Engl. J. Med. *352*, 380–391.
3. Canton, R., Coque, T.M., and Baquero, F. (2003). Multi-resistant Gram-negative bacilli: from epidemics to endemics. Curr. Opin. Infect. Dis. *16*, 315–325.
4. Walsh, F.M., and Amyes, S.G. (2004). Microbiology and drug resistance mechanisms of fully resistant pathogens. Curr. Opin. Microbiol. *7*, 439–444.
5. Raetz, C.R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu. Rev. Biochem. *71*, 635–700.
6. Valvano, M.A., Messner, P., and Kosma, P. (2002). Novel pathways for biosynthesis of nucleotide-activated *glycero-manno*-heptose precursors of bacterial glycoproteins and cell surface polysaccharides. Microbiol. *148*, 1979–1989.
7. Kline, T., Andersen, N.H., Harwood, E.A., Bowman, J., Malanda, A., Endsley, S., Erwin, A.L., Doyle, M., Fong, S., Harris, A.L., et al. (2002). Potent, novel in vitro inhibitors of the *Pseudomonas aeruginosa* deacetylase LpxC. J. Med. Chem. *45*, 3112–3129.
8. Onishi, H.R., Pelak, B.A., Gerckens, L.S., Silver, L.L., Kahan, F.M., Chen, M.H., Patchett, A.A., Galloway, S.M., Hyland, S.A., Anderson, M.S., et al. (1996). Antibacterial agents that inhibit lipid A biosynthesis. Science *274*, 980–982.
9. Pirrung, M.C., Tumey, L.N., Raetz, C.R., Jackman, J.E., Snehalatha, K., McClerren, A.L., Fierke, C.A., Gantt, S.L., and Rusche, K.M. (2002). Inhibition of the antibacterial target UDP-(3-O-acyl)-N-acetylglucosamine deacetylase (LpxC): isoxazoline zinc amidase inhibitors bearing diverse metal binding groups. J. Med. Chem. *45*, 4359–4370.
10. Brooke, J.S., and Valvano, M.A. (1996). Molecular cloning of the *Haemophilus influenzae gmhA* (*lpcA*) gene encoding a phosphoheptose isomerase required for lipooligosaccharide biosynthesis. J. Bacteriol. *178*, 3339–3341.
11. Kneidinger, B., Graninger, M., Puchberger, M., Kosma, P., and Messner, P. (2001). Biosynthesis of nucleotide-activated D-*glycero*-β-D-*manno*-heptose. J. Biol. Chem. *276*, 20935–20944.
12. Kneidinger, B., Marolda, C., Graninger, M., Zamyatina, A., McArthur, F., Kosma, P., Valvano, M.A., and Messner, P. (2002). Biosynthesis pathway of ADP-L-*glycero*-β-D-*manno*-heptose in *Escherichia coli*. J. Bacteriol. *184*, 363–369.
13. Harper, M., Cox, A.D., St Michael, F., Wilkie, I.W., Boyce, J.D., and Adler, B. (2004). A heptosyltransferase mutant of *Pasteurella multocida* produces a truncated lipopolysaccharide structure and is attenuated in virulence. Infect. Immun. *72*, 3436–3443.
14. Hiratsuka, K., Logan, S.M., Conlan, J.W., Chandan, V., Aubry, A., Smirnova, N., Ulrichsen, H., Chan, K.H., Griffith, D.W., Harrison, B.A., et al. (2005). Identification of a D-*glycero*-D-*manno*-heptosyltransferase gene from *Helicobacter pylori*. J. Bacteriol. *187*, 5156–5165.
15. Kim, C.H. (2003). A *Salmonella typhimurium rfaE* mutant recovers invasiveness for human epithelial cells when complemented by wild type *rfaE* (controlling biosynthesis of ADP-L-*glycero*-β-D-*manno*-heptose-containing lipopolysaccharide). Mol. Cells *15*, 226–232.
16. McArthur, F., Andersson, C.E., Loutet, S., Mowbray, S.L., and Valvano, M.A. (2005). Functional analysis of the *glycero-manno*-heptose 7-phosphate kinase domain from the bifunctional HldE protein, which is involved in ADP-L-*glycero*-β-D-*manno*-heptose biosynthesis. J. Bacteriol. *187*, 5292–5300.
17. Cogan, E.B., Birrell, G.B., and Griffith, O.H. (1999). A robotics-based automated assay for inorganic and organic phosphates. Anal. Biochem. *271*, 29–35.
18. Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. *4*, 67–73.
19. Leatherbarrow, R.J. (2000). Grafit, Version 4.0.12 (http://www.erithacus.com/grafit/).