Bioorganic & Medicinal Chemistry Letters 19 (2009) 1376–1379

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and synthesis of new hydroxyethylamines as inhibitors of D-alanyl-D-lactate ligase (VanA) and D-alanyl-D-alanine ligase (DdlB)

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ARTICLE INFO

Article history:
Received 28 November 2008
Revised 9 January 2009
Accepted 14 January 2009
Available online 19 January 2009

Keywords:
ATP-dependant ligases
De novo molecular design
Enzyme inhibitors
Antibacterial agents

ABSTRACT

The Van enzymes are ATP-dependant ligases responsible for resistance to vancomycin in *Staphylococcus aureus* and *Enterococcocus* species. The *de novo* molecular design programme SPROUT was used in conjunction with the X-ray crystal structure of *Enterococcus faecium* D-alanyl-D-lactate ligase (VanA) to design new putative inhibitors based on a hydroxyethylamine template. The two best ranked structures were selected and efficient syntheses developed. The inhibitory activities of these molecules were determined on *E. faecium* VanA, and due to structural similarity and a common reaction mechanism, also on D-Ala-D-Ala ligase (DdlB) from *Escherichia coli*. The phosphate group attached to the hydroxyl moiety of the hydroxyethylamine isostere within these systems is essential for their inhibitory activity against both VanA and DdlB.

Staphylococcus aureus and *Enterococcocus* species are Gram-positive bacteria and the major causes of nosocomial infections.[1] The glycopeptide antibiotic vancomycin[2] is used clinically as the last line of defence for the treatment of these life-threatening infections.[3] However, due to the recent emergence of vancomycin-resistant *S. aureus* and vancomycin-resistant enterococci[1,4] there is an urgent need for the development of novel antibacterial agents and agents that can overcome the resistance to vancomycin.

Gram-positive clinical isolates resistant to vancomycin are characterized by a subtle change in the structure of the peptidoglycan termini, as a result of the action of D-alanyl-D-X ligases (the Van enzymes). These enzymes replace the D-Ala-D-Ala termini with either D-Ala-D-Lac or D-Ala-D-Ser. VanA-G enzymes have been described[5] according to the particular vancomycin-resistance phenotype. Replacement of D-alanine by D-lactate eliminates a key amide NH hydrogen bond interaction with vancomycin, leading to a thousand-fold decrease in binding affinity.[6,7] The depsipeptide D-Ala-D-Lac is synthesized by the ATP-dependent depsipeptide ligase VanA.[8,9]

VanA is a 38.5-kDa protein that can use both D-lactate and D-Ala as substrates, with a concomitant pH-dependent switch between ester and peptide bond formation.[10] The catalytic mechanism is similar to that of D-Ala-D-Ala ligase (DdlB). Initially, the carboxyl of D-alanine is activated by phosphorylation by adenosine triphosphate (ATP), forming ADP and D-alanyl phosphate. This aminoacyl-phosphate is subsequently attacked by the hydroxyl group of D-lactate, which eliminates inorganic phosphate and yields the product D-Ala-D-Lac (Fig. 1).[11,12]

To date, only a few inhibitors of the Van enzymes have been described.[13,14] Phosphinate and phosphonate transition-state analogues have been reported to be reversible inhibitors of VanA.[13] Inhibitors of the Van enzymes could be used in combination with

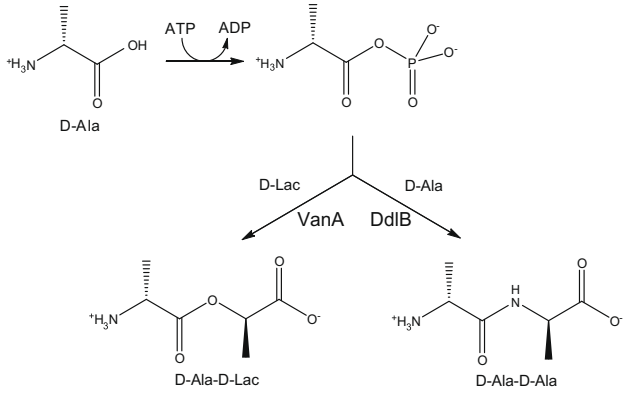

Figure 1. The mechanisms of the reaction catalyzed by VanA and DdlB.

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doi:10.1016/j.bmcl.2009.01.034

vancomycin to eliminate the formation of the D-Ala-D-Lac termini, and thus to prevent the drop in binding affinity to vancomycin. Therefore, the aim of the present study was to design and synthesize novel small-molecule inhibitors of VanA, and to evaluate their enzyme-inhibitory activities. Ddl activity is replaced by VanA in vancomycin-resistant Gram-positive pathogens.[15] However, Ddl is an essential enzyme that is found in all bacterial pathogens, and is a drug target for antibiotics such as D-cycloserine,[15] a rarely used drug except in all but highly resistant tuberculosis infections, because of neurological side-effects.[16] Targeting Ddl could thus provide considerable therapeutic benefit. Therefore, due to the structural similarity and common reaction mechanisms of the Van and Ddl enzymes,[12] the VanA inhibitors designed here were also evaluated for inhibitory activities against *Escherichia coli* DdlB, to determine if, additionally, these molecules have broader potential efficacies against not only Gram-positive, but also Gram-negative pathogens.

To design a new class of Van and Ddl inhibitors, we applied computer-aided design using the SPROUT[17] software to a crystal structure of VanA.[18] SPROUT, a powerful suite of software for *de novo* ligand design has previously been successfully used for the development of novel inhibitors of MurD[19] and DdlB[20] from *E. coli*, of the human NK2 receptor,[21] of *Plasmodium* dihydroorotate dehydrogenase,[22,23] and of the bacterial RNA polymerase.[24] The crystallographic structure of the D-alanyl-D-lactate ligase (VanA) of *Enterococcus faecium* complexed with ADP and a phosphorylated phosphinate inhibitor has been resolved to 2.5 Å resolution,[18] where the inhibitor makes direct contacts with essentially all of the active-site residues (Fig. 2).[18]

SPROUT analysis of the VanA active site revealed a large number of residues that could have bonding interactions with a potential inhibitor. By using the SPROUT strategy, we wanted to design a small inhibitor with a reasonably high affinity for VanA. Therefore, only a small number of the active site residues were selected. In particular, due to the involvement of the imidazole ring within His244 in dictating the preference of VanA for lactate as a ligand,[18] His244 was chosen for ligand contacting. Within the high level vancomycin resistance phenotypes found in a variety of different enterococcal strains (and also Vancomycin resistant *S. aureus*) the D-Ala-D-Lac ligases all have a His244 and surrounding residues. There is general conservation of active site residues particularly with respect to the ATP site and high affinity 1st subsite for alanine. Additionally, SPROUT analysis revealed the presence of a

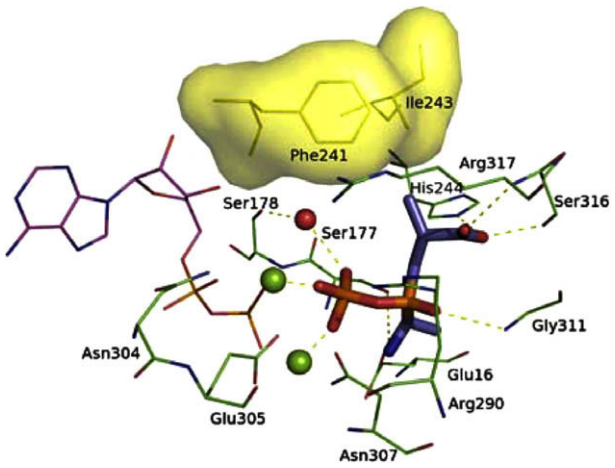


Figure 2. The bonding interactions between the phosphorylated phosphinate inhibitor and VanA based on the crystal structure 1e4e.pdb. The hydrophobic region, which does not interact with the phosphorylated phosphinate inhibitor, is presented in yellow.

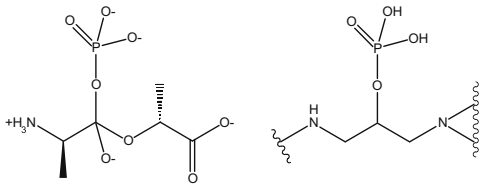


Figure 3. The structure of the high-energy transition state intermediate of the reaction catalyzed by VanA (left), and the phosphorylated HEA molecular template used for the present design of inhibitors (right).

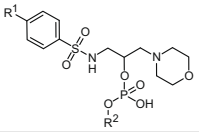
number of additional potential H-bonding sites within the putative binding cavity. Thus, Arg290, Gly311, Ser316 and Arg317 were chosen due to their close proximities to His244. In addition, Ser177, Ser178 and Asn307 were also selected as H-bond donors/acceptors. Furthermore, examination with SPROUT also revealed the presence of a small hydrophobic region (not in direct contact with the phosphorylated phosphinate inhibitor; yellow in Fig. 2) bordered by the phenyl ring of Phe241 and the isobutyl side chain of Ile243.

Hydroxyethylamines (HEAs) have been described as analogues of tetrahedral high-energy reaction intermediates of proteinases,[25,26] and have often been used for the design of potent enzyme inhibitors.[27,28] Taking advantage of our experience in the synthesis of HEAs, we wanted to use the HEA template as a basis for the design of VanA inhibitors.[29,30] To mimic the high-energy intermediate of the reaction catalyzed by VanA, we decided to use the phosphorylated HEA scaffold (Fig. 3).

In the next step, the selected phosphorylated HEA template was used to gradually build a small-molecule inhibitor using SPROUT. During this analysis, a substituted benzene ring was selected to bind in the hydrophobic region close to Phe241 and Ile243. A sulfonamide group, which has H-bond donor/acceptor properties, was introduced to link the benzene ring and phosphorylated HEA template, which was predicted by SPROUT to make contacts with His244, Gly311 and Arg317. Additionally, the design required the presence of another ring attached to the amino group of our HEA template. Application of SPROUT suggested a tetrahydrofuryl ring may be suitable due to the possible hydrogen bonding interaction with Ser177. However, for reasons of synthetic availability, we decided to use a morpholine ring, which still retains the oxygen as the H-bond acceptor.

The two most promising *de novo* designed small molecules, for which the predicted binding affinities according to SPROUT would be in the micromolar range or better (Table 1, compounds **7a** and **7b**), were selected and their predicted orientations and interactions within the VanA active site were further analyzed. Analysis of inhibitor **7a** (Fig. 4) revealed that residues His244 and Gly311

Table 1
VanA and DdlB inhibitory activities of compounds **6a, b** and **7a, b**



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Compound	R^1	R^2	% Inhibiton at 500 μM [a]	
			VanA	DdlB
6a	MeO	Ph	NI	NI
7a	MeO	H	83 (224 ± 19 μM)	83 (110 ± 28 μM)
6b	F	Ph	65	NI
7b	F	H	75 (290 ± 23 μM)	77 (136 ± 12 μM)

[a] NI = no inhibition observed; values of IC_{50} ± standard deviation are given in parentheses.

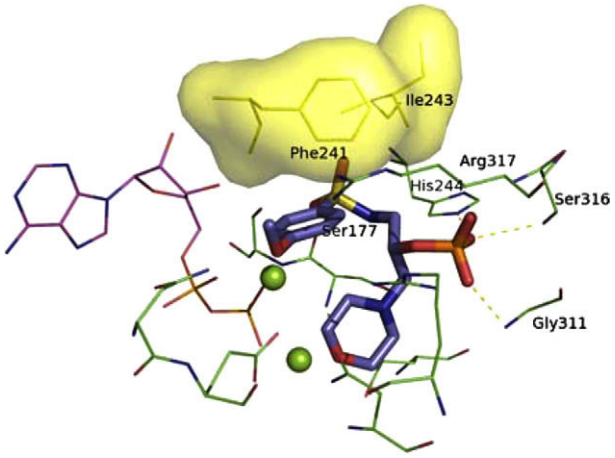


Figure 4. SPROUT designed inhibitor **7a** (blue) docked to the active site of VanA. ADP is magenta and Mg^{2+} is green.

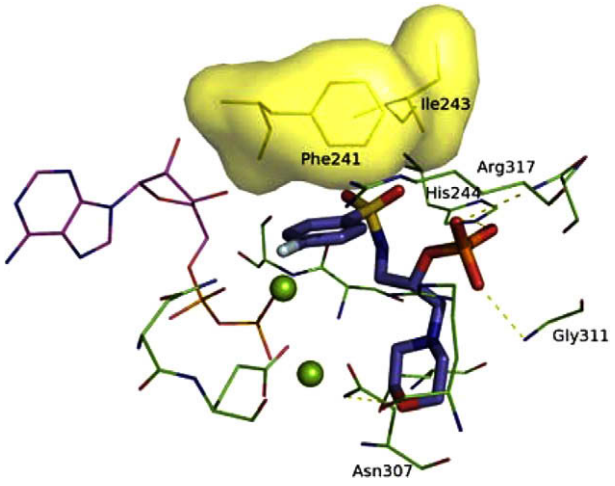
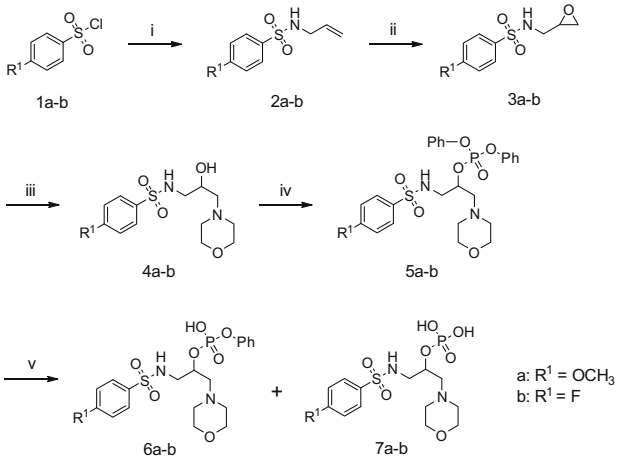


Figure 5. SPROUT designed inhibitor **7b** (blue) docked to the active site of VanA. ADP is magenta and Mg^{2+} is green.

are important in making hydrogen bonds with the phosphate group of the designed inhibitor. Additionally, a substituted benzene ring interacts with the hydrophobic region within the cavity. The morpholine ring oxygen makes a hydrogen bond with the side chain of Ser177. On the other hand, for inhibitor **7b**, the predicted interactions are with His244, Gly311 and Arg317 (involving H-bonds with phosphate groups), Asn 307 (H-bond with the morpholine moiety) and the hydrophobic region located near Phe241 (designed to interact with the aromatic ring within the inhibitor) (Fig. 5).

Following molecular design, efficient synthetic approaches to compounds **7a** and **7b** were developed and optimized to obtain these molecules in high yields (Scheme 1).[31] The synthesis started from benzenesulfonyl chlorides **1a** and **1b**, which were coupled with allylamine to give **2a** and **2b**, respectively. Epoxides **3a** and **3b** were then prepared by oxidation with *meta*-chloroperbenzoic acid and subsequently opened with morpholine in the presence of calcium trifluoromethanesulphonate ($Ca(OTf)_2$) as a catalyst,[30] to give the HEA derivatives **4a** and **4b**. The diphenylphosphate group was introduced using diphenylphosphoryl chloride, with DMAP as a catalyst/ base. Finally, deprotection of phenyl esters **5a** and **5b** was performed using hydrogenation in the presence of PtO_2. The compounds **6a** and **b** and **7a** and **b** were isolated following preparative thin-layer chromatography.



Scheme 1. Reagents and conditions: (i) allylamine, CH_2Cl_2, 0 °C to rt, 1 h, 88–91%; (ii) mCPBA, CH_2Cl_2, rt, 48 h, 73–76%; (iii) morpholine, $Ca(OTf)_2$, dioxane, MW, 120 °C, 4 min, 84–88%; (iv) diphenylphosphoryl chloride, DMAP, CH_2Cl_2, 0 °C to rt, 1 h, 75–81%; (v) H_2, PtO_2, glacial acetic acid, 4 days, then separation by preparative thin-layer chromatography using CH_3CN/H_2O/MeOH (3/1/1) as mobile phase.

Due to the very similar topologies of the active sites of VanA and DdlB, and in light of reported activity of the phosphinate inhibitor for both VanA and DdlB,[12] we tested compounds **4a** and **b, 6a** and **b** and **7a** and **b** for their inhibitory activities against both VanA from *E. faecium* and DdlB from *E. coli*. The residual activities and IC_{50} values were determined using a pyruvate kinase/lactate dehydrogenase-coupled assay.[32] The results are presented as percentages of inhibition of VanA and DdlB in the presence of the inhibitors (Table 1), and for the most active compounds **7a** and **7b**, also as IC_{50} values. Interestingly, compounds lacking the phosphate group (**4a** and **4b**) showed no inhibitory activities against both VanA and DdlB even at 10 mM. The monophenyl phosphate esters **6a** and **6b** showed no or only moderate inhibitory activities (% inhibition = 65 at 500 μM for **6b**). Since only the phosphorylated HEA derivatives **7a** and **7b** inhibited VanA and DdlB, it can be concluded that the presence of a phosphate group is essential for inhibition of both of these enzymes. Compound **7a**, which has a methoxy group on the *para*-position of the aromatic ring, is a slightly better inhibitor of both VanA and DdlB (IC_{50} values of 224 and 110 μM, respectively). This may be due to the ability of the methoxy group to accept hydrogen bond.

During the determination of the IC_{50} values for these inhibitors, we noted that **7a** and **7b** exert hyperbolic inhibition on DdlB, whilst exerting sigmoidal inhibition on VanA (Fig. 6). This suggested that for VanA, more than one binding event accounts for the inhibitory kinetics of **7a** and **7b**, and that this second interaction was absent with DdlB. A simple explanation might be that **7a** and **7b** can occupy only a single DdlB D-alanine binding site, hence giving rise to the hyperbolic inhibition kinetics. In contrast, the relaxed specificity of the VanA site in binding the second acid substrate (D-Ala or D-lac) might allow additional interactions that are not seen with DdlB and **7a** or **7b**. In this context, it is relevant to note that an X-ray crystallographic analysis of *S. aureus* Ddl[33] revealed that an inhibitor of this enzyme (3-chloro-2,2-dimethyl-*N*-[4(trifluoro-methyl)phenyl]propanamide) binds at a position adjacent to the active site. It may be that a related situation pertains here with VanA, where inhibitors **7a** and **7b** may bind to the site occupied by one of the substrates, and in addition, also to an otherwise cryptic binding site that is not present in *E. coli* DdlB, hence giving rise to the sigmoid VanA inhibition kinetics.

In summary, we have designed and synthesized novel small-molecule inhibitors of VanA and DdlB. with IC_{50} values in the micromolar range. The results show that a phosphate attached to the hydroxyl group of an hydroxyethylamine moiety is essential

$$\%\text{Inhibition} = \left(\frac{100.[\text{Inhibitor}]^n}{K + [\text{Inhibitor}]^n}\right)$$

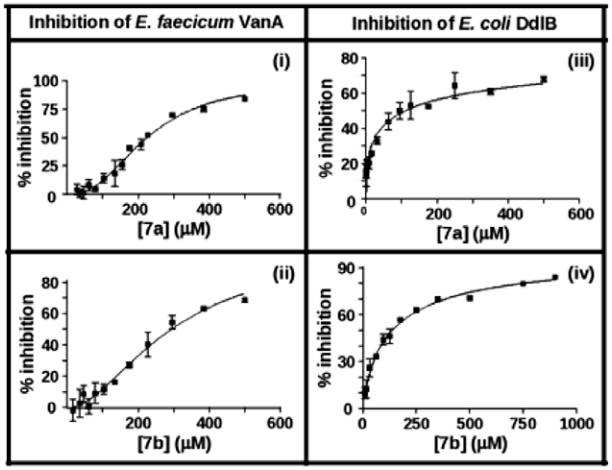


Figure 6. Dose dependence of inhibition of VanA and DdlB by compounds **7a** and **7b**. Data were fitted with Prism 4.0 by non-linear regression to the equation given above, and are means ± standard error from assays carried out in triplicate for **7a** and duplicate for **7b**.

for inhibitory activity against both VanA and DdlB. The designed compounds represent an important starting point for further optimization and modifications, to improve these inhibitory activities against VanA and DdlB. These types of inhibitors have the potential to be developed into drugs that would reverse bacterial resistance to vancomycin. Furthermore, the potency of these compounds against both VanA and DdlB suggest that it will be possible to develop broad-spectrum antimicrobials that target both Gram-negative and Gram-positive infections.

Acknowledgments

This work was supported by the European Union FP6 Integrated Project EUR-INTAFAR (Project No. LSHM-CT-2004-512138) and the Ministry of Higher Education, Science and Technology of the Republic of Slovenia. The authors thank Dr. Chris Berrie for critical reading of the manuscript.

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31. *Spectral data for compounds* ***6–7****: 1-(4-methoxyphenylsulfonamido)-3-morpholinopropan-2-yl phenyl hydrogen phosphate (****6a****):* Yield 35%; white crystals (hygroscopic), mp 96–100 °C; IR (KBr): ν_{max} 3447, 2361, 2343, 1719, 1639, 1597, 1492, 1406, 1264, 1158, 1094, 1059, 1028, 927, 782, 640, 612, and 564 cm^{-1}; ^1H NMR (D_2O, NaOD) δ (ppm) 2.39–2.46 (m, 5H, $CH_aN(CH_2)_2$-morpholine), 2.55–2.61 (m, 1H, CH_bN), 2.76 (dd, 1H, J = 8.5 and 12.5 Hz, $NHCH_a$), 3.05 (dd, 1H, J = 3.8 and 12.5 Hz, $NHCH_b$), 3.62 (t, 4H, J = 4.5 Hz, $O(CH_2)_2$-morpholine), 3.77 (s, 3H, CH_3), 4.28–4.35 (m, 1H, CH), 6.93–7.01 (m, 4H, Ar), 7.28 (t, 1H, J = 7.3 Hz, Ar), 7.28 (dd, 2H, J = 7.8 and 7.8 Hz, Ar), 7.58–7.63 (m, 2H, Ar), NH and OH not observed, exchanged. ^{31}P NMR (D_2O, NaOD) δ (ppm) −4.21. ESI HRMS Calcd for $C_{20}H_{27}N_2O_8PSNa$ ($M+Na^+$): 509.1123. Found: 509.1138. HPLC: t_R = 15.321 min (95.7%).[34]
*1-(4-Fluorophenylsulfonamido)-3-morpholinopropan-2-yl phenyl hydrogen phosphate (****6b****):* Yield 47%; white crystals, mp 125–129 °C; IR (KBr): ν_{max} 3446, 1565, 1414, 1265, 1155, 1096, 896, 840, 760, and 552 cm^{-1}; ^1H NMR (D_2O, NaOD) δ (ppm) 2.36–2.42 (m, 4H, $N(CH_2)_2$-morpholine), 2.51–2.56 (m, 2H, CH_2 N), 2.73 (dd, 1H, J = 8.7 and 12.3 Hz, $NHCH_a$), 2.99 (dd, 1H, J = 3.2 and 12.3 Hz $NHCH_b$), 3.58 (t, 4H, J = 4.3 Hz, $O(CH_2)_2$-morpholine), 4.24–4.31 (m, 1H, CH), 6.98 (d, 2H, J = 7.8 Hz, Ar), 7.04–7.14 (m, 3H, Ar), 7.28 (dd, 2H, J = 7.5 and 7.5 Hz, Ar), 7.60–7.65 (m, 2H, Ar), NH and OH not observed, exchanged. ^{31}P NMR (D_2O, NaOD) δ (ppm) −4.24. ESI HRMS Calcd for $C_{19}H_{23}FN_2O_7PS$ ($M-H^-$): 473.0948. Found: 473.0934. HPLC: t_R = 15.68 min (95.1%).[34]
*1-(4-Methoxyphenylsulfonamido)-3-morpholinopropan-2-yl dihydrogen phosphate (****7a****):* Yield 30%; white crystals (hygroscopic), mp 119–124 °C; IR (KBr): ν_{max} 3433, 2360, 2341, 1711, 1640, 1564, 1501, 1412, 1303, 1262, 1158, 1094, 1058, 1021, 803, 668, 640, 611, and 561 cm^{-1}; ^1H NMR (D_2O, NaOD) δ (ppm) 2.08–2.15 (m, 2H, CH_2 N), 2.24–2.34 (m, 4H, $N(CH_2)_2$-morpholine), 2.66 (dd, 1H, J = 7.8 and 12.3 Hz, $NHCH_a$), 2.98 (dd, 1H, J = 3.8 and 12.3 Hz, $NHCH_b$), 3.52–3.55 (m, 4H, $O(CH_2)_2$-morpholine), 3.76 (s, 3H, CH_3), 4.00–4.05 (m, 1H, CH), 6.97 (d, 2H, J = 8.6 Hz, Ar), 7.61 (d, 2H, J = 8.6 Hz, Ar), NH and 2OH not observed, exchanged. ^{31}P NMR (D_2O, NaOD) δ (ppm) 3.95. ESI HRMS Calcd for $C_{14}H_{22}N_2O_8PS$ ($M-H^-$): 409.0835. Found: 409.0822. HPLC: t_R = 8.947 min (97.6%).[34]
*1-(4-Fluorophenylsulfonamido)-3-morpholinopropan-2-yl dihydrogen phosphate (****7b****):* Yield 40%; white crystals, mp 130–135 °C; IR (KBr): ν_{max} 3431, 2361, 2342, 1717, 1640, 1593, 1496, 1406, 1327, 1294, 1272, 1240, 1157, 1091, 1030, 982, 842, 775, 668, 640, 612, and 550 cm^{-1}; ^1H NMR (D_2O, NaOD) δ (ppm) 2.21-2.48 (m, 6H, CH_2 $N(CH_2)_2$-morpholine), 2.74 (dd, 1H, J = 8.2 and 12.0 Hz, $NHCH_a$), 3.07 (dd, 1H, J = 4.1 and 12.0 Hz, $NHCH_b$), 3.63 (t, 4H, J = 3.9 Hz, $O(CH_2)_2$-morpholine), 4.09–4.14 (m, 1H, CH), 7.21 (dd, 2H, J = 8.8 and 8.8 Hz, Ar), 7.74 (dd, 2H, J = 5.7 and 8.0 Hz, Ar), NH and 2OH not observed, exchanged. ^{31}P NMR (D_2O, NaOD) δ (ppm) 3.95. ESI HRMS Calcd for $C_{13}H_{19}FN_2O_7PS$ ($M-H^-$): 397.0635. Found: 397.0640. HPLC: t_R = 8.920 min (97.9%).[34]
32. Pyruvate kinase/lactate dehydrogenase coupled assay (PK/LDH pathway) used ADP for the formation of pyruvate from phosphoenolpyruvate (PEP) by the action of PK. LDH then catalyses the reduction of pyruvate to lactate by NADH, so by using this assay, ligase activity can be measured spectrophotometrically by following a decrease in absorbance of NADH at 340 nm, concommitant with its oxidation to NAD^+. For DdlB, all of the assays were carried out in a final volume of 200 μL and initially contained 100 mM Hepes, 10 mM $MgCl_2$, 10 mM KCl, pH 7.5, 10% (v/v) DMSO with or without the compound, 2 mM PEP, 0.2 mM ATP, 1.972 units/mL PK, 2.46 units/mL LDH, 150 μM NADH and 4.3 mM D-Ala. Assays were followed spectrophotometrically at 37 °C, at 340 nm, and the reaction was initiated by the addition of DdlB (1.2 μg/mL), which gave an initial slope of −0.210 ± 0.032 (n = 6, uninhibited reaction). For VanA, the assays were carried out in the same manner as for DdlB above, except that the concentrations of the substrates in this case were 8 mM D-Ala, 1.78 mM D-Lac, and the reaction was initiated by the addition of VanA (37.5 μg/mL), which gave an initial slope of −0.215 ± 0.019 (n = 6, uninhibited reaction).
33. Liu, S.; Chang, J. S.; Herberg, J. T.; Horng, M.-M.; Tomich, P. K.; Lin, A. H.; Marotti, K. R. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15178.
34. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV-vis detector (254 nm), using a Luna C18 column (4.6 × 250 mm) at a flow rate of 1 mL/min. The eluant was a mixture of 0.1% TFA in water (A) and acetonitrile (B). The gradient was from 10% B to 80% B in 30 min (for compounds **6a** and **6b**) or 5% B to 95% B in 15 min (for compounds **7a** and **7b**).