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Evaluation of fosmidomycin analogs as inhibitors of the *Synechocystis* sp. PCC6803 1-deoxy-D-xylulose 5-phosphate reductoisomerase

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Abstract—Analogs of the antibiotic fosmidomycin, an inhibitor of the methylerythritol phosphate pathway to isoprenoids, were synthesized and evaluated against the recombinant *Synechocystis* sp. PCC6803 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). Fosfoxacin, the phosphate analog of fosmidomycin, and its acetyl congener were found to be more potent inhibitors of DXR than fosmidomycin.

1. Introduction

Isoprenoids are one of the largest natural product families with more than 35,000 reported structurally diverse compounds.[1] Despite their amazing structural diversity, all isoprenoids are derived from the common five carbon building blocks, isopentenyl diphosphate (IPP) and/or dimethylallyl diphosphate (DMAPP).[2] The biosynthesis of IPP was long believed to occur only through the mevalonate (MVA) pathway, which utilizes acetyl CoA as a starting molecule. The subsequent revelation of the methylerythritol phosphate (MEP) pathway, however, has forever changed our understanding of isoprenoid biosynthesis.[1,3] Because the MEP pathway is dominant in eubacteria and the malaria parasite *Plasmodium falciparum*, but absent in animals,[4,5] inhibition of the pathway holds promise for the development of a new class of antibiotics with minimal toxicity to humans.[6–8]

The first committed step of the MEP pathway, the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP, **1**) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **3**), is catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267; Fig. 1).[9–11] In this step, DXR catalyzes the isomerization of DXP into the intermediate, 2-*C*-methyl-D-erythrose 4-phosphate (**2**), which is subsequently reduced to MEP (**3**).[12] The isomerization requires a divalent metal ion and the reduction requires the cofactor NADPH. Shortly after the discovery of DXR, it was reported that the natural product fosmidomycin (**4**) was a potent inhibitor of the enzyme, validat-

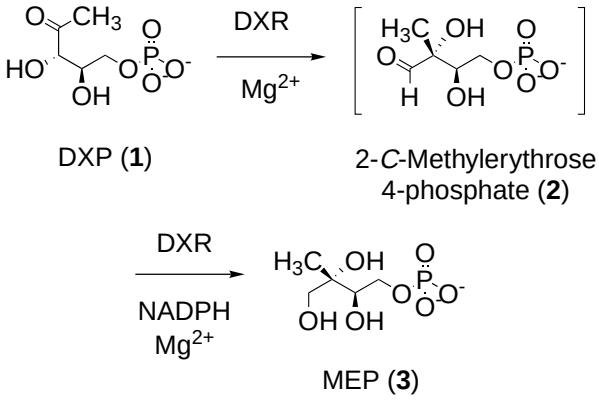

Figure 1. The conversion of 1-deoxy-D-xylulose 5-phosphate (DXP, **1**) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **3**) by deoxyxylulose 5-phosphate reductoisomerase (DXR).

Keywords: Isoprenoid biosynthesis; Fosmidomycin; Reductoisomerase; Methylerythritol phosphate pathway.
Abbreviations: DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXP, 1-deoxy-D-xylulose 5-phosphate; IPP, isopentenyl diphosphate; DMAPP dimethylallyl diphosphate; MEP, methylerythritol phosphate.
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ing the MEP pathway as a target for antibacterial agents.[13] Fosmidomycin and its close relative FR900098 (**5**) were subsequently found to be effective at curing malaria in a mouse model system.[14] This work stimulated the development of fosmidomycin as a human antimalarial treatment and clinical trials are currently in progress.[15–17]

Due to the potential for fosmidomycin as an antimalarial agent, many groups have conducted research on fosmidomycin and its mechanism of action. Recently several analogs of fosmidomycin have been reported for their inhibition of DXR or as MEP pathway inhibitors.[18–20] Prompted by the publication of these articles, we would like to report our investigations in this area. Several analogs of fosmidomycin have been synthesized and evaluated as inhibitors of the recombinant DXR from the cyanobacterium *Synechocystis* sp. PCC6803. Prior work in our laboratory has focused on the *Synechocystis* DXR[21,22] and these studies provide complementary results to research conducted on the *Escherichia coli* enzyme. Evaluation of DXR inhibition by the fosmidomycin analogs should aid in defining the structural requirements for the design of potent DXR inhibitors in conjunction with the X-ray structural data[23–27] and QSAR studies[28] that are available.

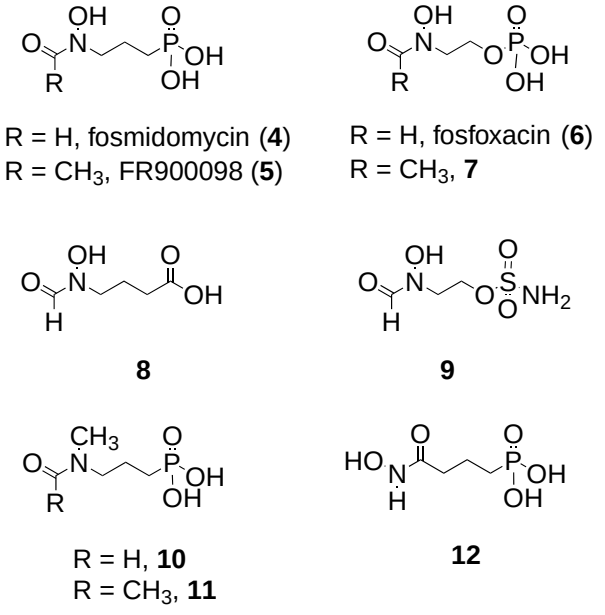
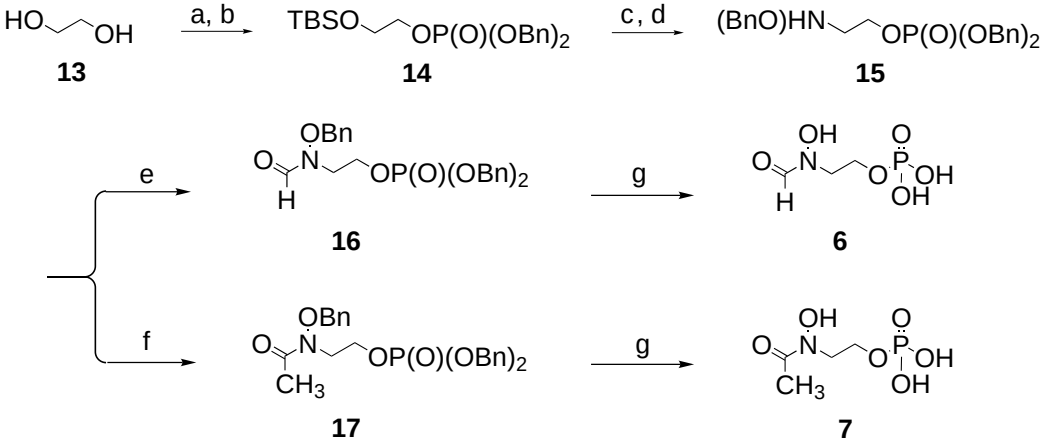


Figure 2. Fosmidomycin and its analogs.

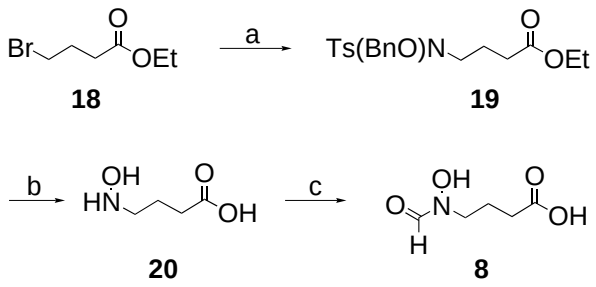
2. Results

Structurally, fosmidomycin contains a *N*-formyl, *N*-hydroxyl moiety (hydroxamate) at one end of the molecule with a phosphonate as a polar group, whereas DXP (**1**) and **2** have an α-hydroxycarbonyl group at one end and a phosphate group at the other end. Previous attempts to shorten the length of the propylene spacer compromised antibacterial activity,[29] so we decided to focus on changing the hydroxamate and polar phosphonate groups, rather than altering the spacer. The polar phosphonate head group of **4**/**5** was modified to a phosphate (**6**, **7**), a carboxylate (**8**), and a sulfamate (**9**). The *N*-formyl, *N*-hydroxyl moiety was substituted with *N*-formyl, *N*-methyl, and *N*-acetyl, *N*-methyl groups (**10**, **11**) or with the reversed hydroxamate (**12**). Figure 2 summarizes the analogs used in this study. Other investigators have also prepared analogs that replace the hydroxamate or phosphonate group with various groups.[18–20,30,31]

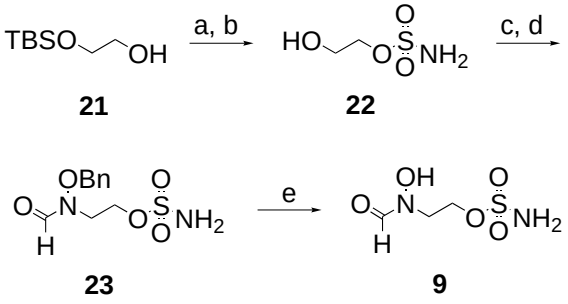
The syntheses of the analogs utilized a combination of known synthetic transformations and the synthetic routes are illustrated in Schemes 1–4. Because the main goal was to prepare the analogs for subsequent analysis as DXR inhibitors, rather than to maximize yields, focused efforts were not made to optimize the individual steps. During the course of our research, syntheses of two of the inhibitors, the carboxylate **8** and the reversed hydroxamate **12**, have been published.[18,31]



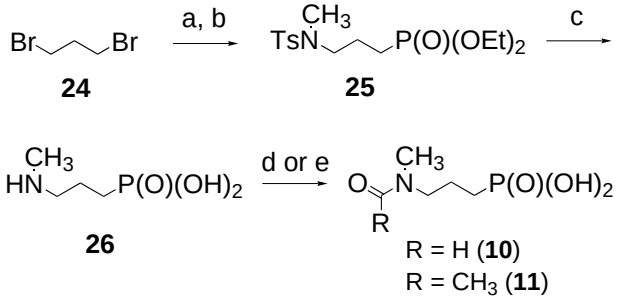
Scheme 1. Synthesis of fosfoxacin (**6**) and its acetyl congener **7**. Reagents: (a) TBSCl, DMF, TEA, 99%; (b) 1-*H*-tetrazole, $(BnO)_2PN(i\text{-}Pr)_2$, *t*-BuOOH, 79%; (c) Dowex 50, H^+, 87%; (d) Tf_2O, 2,6-lutidine; NH_2OBn, 83%; (e) 4-formyl-2-methyl-1,3,4-thiadiazole-5-thione (FMT), CH_2Cl_2; (f) Ac_2O, pyridine; (g) 10% Pd/C, H_2; 95% for (e + g) and 95% for (f + g).



Scheme 2. Synthesis of carboxylate analog **8**. Reagents: (a) NaH, HN(Ts)OBn, 99%; (b) HCl/AcOH, 1:1, 80%; (c) acetylformic anhydride, 72%.



Scheme 3. Synthesis of sulfamate analog **9**. Reagents: (a) $ClSO_2NH_2$, DMA, TEA; (b) AG-50, MeOH, 50% for two steps; (c) Tf_2O, 2,6-lutidine, $BnONH_2$, 80%; (d) FMT, CH_2Cl_2, 90%; (e) 10% Pd/C, MeOH, 50%.



Scheme 4. Synthesis of *N*-methyl analogs **10** and **11**. Reagents: (a) NaH, $NH(Ts)CH_3$, 50%; (b) NaH, $HP(O)(OEt)_2$, 70%; (c) HCl:AcOH, 1:1, 70%; (d) acetylformic anhydride, 15%; (e) Ac_2O, pyridine, 13%.

Originally, the phosphate analog of fosmidomycin (**6**) was selected for comparison of the binding effects of the phosphonate in fosmidomycin versus a phosphate, as in the substrate DXP. Surprisingly, a literature search on the phosphate analog revealed that this compound is actually a known natural product named fosfoxacin (**6**).[32] Fosfoxacin was isolated from the filtrate of *Pseudomonas fluorescens* PK-52 in 1990 and was shown to have antibacterial activity. Despite this prior report and the structural similarity to fosmidomycin, no further testing of this compound for inhibition of DXR has been reported.

In spite of the simple structures, the syntheses of the phosphate analogs **6** and **7** posed some challenges (Scheme 1). Monoprotection of ethylene glycol (**13**) and introduction of the protected phosphate group in **14** proceeded smoothly, but displacement of an activated hydroxyl group by the protected hydroxylamine required several attempts. No success was achieved with the common leaving groups such as methanesulfonyl (MsO-), *p*-toluenesulfonyl (TsO-), or via a Mitsunobu-type substitution. After this survey of several methods, transformation to the triflate with trifluoromethanesulfonic anhydride (Tf_2O) followed by displacement with *O*-benyzlhydroxylamine resulted in the formation of **15** with a reasonable yield (>80%). Intermediate **15** was N-formylated with 4-formyl-2-methyl-1,3,4-thiadiazole-5-thione (FMT) and both protecting groups were efficiently removed by catalytic hydrogenation to provide fosfoxacin (**6**). The phosphate analog (**7**) of FR900098 also was prepared from **15** in two steps.

The synthesis of the carboxylic acid analog **8** started with the introduction of *N*-Ts-*O*-benzyl-hydroxylamine to 4-bromoethylbutanoate (**18**) by a modification of a procedure previously used for the synthesis of fosmidomycin (Scheme 2).[33] The tosyl, benzyl, and ester-protecting groups of **19** were removed by treatment with acid. Formylation with the mixed acetylformic anhydride produced the desired compound **8**. During our studies there was a report on the syntheses of analog **8** and several related carboxylic acid analogs of fosmidomycin.[31] These carboxylic acid analogues, however, were not analyzed for inhibition of DXR.

The synthesis of the sulfamate analog **9** began with the monoprotected ethylene glycol **21**, which was sulfamoylated with sulfamoyl chloride (Scheme 3).[34] The intermediate **22**, formed by removal of the silyl protecting group, was converted into **23** by displacement of the triflate with the protected hydroxylamine. Formylation with FMT and final removal of the benzyl protecting group by catalytic hydrogenation provided the sulfamate analog **9**.

The *N*-methyl fosmidomycin and FR900098 analogs **10** and **11** were prepared by following a synthetic route analogous to an early synthesis of fosmidomycin, replacing *N*-tosyl-*O*-benzylhydroxylamine with *N*-methyl-*N*-tosylamine (Scheme 4).[35] The bromo groups of 1,3-dibromopropane (**24**) were displaced sequentially with *N*-methyl-*N*-tosylamine and the anion of diethylphosphite to provide **25**. Acidic deprotection provided the common intermediate **26**, which was converted to both the *N*-formyl and *N*-acetyl derivatives. The N-acylation step in both cases proceeded in unacceptably low yields, but sufficient material was obtained for evaluation as DXR inhibitors.

The synthesis of the reversed hydroxamic acid derivative **12** used a synthetic route that was essentially the same as the route recently published for this same compound prepared during studies with the *E. coli* DXR.[18] Analytical data for all intermediates and the final product **12** matched well with the published data.[18]

All final compounds were tested against the recombinant *Synechocystis* DXR for inhibition. In the initial

characterization of fosmidomycin as an inhibitor of the *E. coli* DXR, it was reported that fosmidomycin showed a mixed (competitive and non-competitive with DXP) inhibition pattern with a K_i value of 37 nM.[13] Fosmidomycin and its analogs also exhibited mixed inhibition patterns with the *Synechocystis* DXR, with varying K_i values (Table 1). The initial K_i value for fosmidomycin (**4**) was found to be 58 nM, while fosfoxacin (**6**) and its acetyl congener **7** have somewhat lower K_i values of 19 and 2 nM, respectively. The carboxylate and sulfamate derivatives of fosmidomycin had much higher K_i values of 240 μM and 2.8 mM, indicating the importance of the phosphonate or phosphate group for high affinity to DXR. The *N*-methyl analogs **10** and **11** were weak inhibitors, with K_i values in the low mM range. The reversed hydroxamic acid analog **12** was an effective inhibitor of DXR, although the K_i of 4 μM is approximately 70-fold higher than the K_i for fosmidomycin.

Although the initial report characterized fosmidomycin as a mixed inhibitor of *E. coli* DXR, further studies demonstrated that fosmidomycin is actually a slow, tight-binding inhibitor of DXR.[36] Because of this determination of fosmidomycin as a slow, tight-binding inhibitor of DXR, we re-examined the inhibition of the *Synechocystis* DXR with three inhibitors, fosmidomycin (**4**), FR900098 (**5**), and the reversed hydroxamate **12**. According to this mechanism of inhibition, two different inhibition constants can be obtained. K_i describes the formation of the EI complex, while K_i^* represents conversion to the tightly bound complex and can be considered the overall inhibition constant.[37] Analyses of the progress curves for the three inhibitors indicated slow, tight-binding inhibition. The two inhibition constants are presented in Table 2 for each inhibitor. The original mixed inhibition constants determined for fosmidomycin (58 nM) and the reversed hydroxamate (4 μM) both underestimate the K_i^* values for these inhibitors (4 nM and 2.4 μM, respectively). The K_i (900 nM) and K_i^* (0.9 nM) values for FR900098 indicate that this compound is a very potent inhibitor of the *Synechocystis* DXR. A second method, which relies upon pre-incubation with the inhibitor prior to addition of the substrate, was also used to estimate the K_i^*.[38] Using this approach, DXR was pre-incubated with the various concentrations of the inhibitors for 10 minutes prior to addition of substrate. The K_i^*s were then determined by plotting the inhibitor concentration versus $1/v$.[38] The inhibition constants are shown in Table 2. As can be seen from the table, the K_i^* values determined in this manner for fosmidomycin and FR900098 are almost the same as the values obtained from the progress curves. For the hydroxamate analog, however, the K_i^* value is almost 7-fold lower.

Table 1. K_i values for fosmidomycin analogs with *Synechocystis* DXR

Compound	K_i (μM)[a,b]
4	0.057
6	0.019
7	0.002
8	240
9	2800
10	5000
11	3600
12	4

[a] Inhibition assays utilized at least four different concentrations of each analog, in triplicate.

[b] All compounds displayed mixed inhibition as determined from Lineweaver–Burk plots.

Table 2. Slow, tight-binding inhibition constants for inhibitors of *Synechocystis* DXR determined either by estimation from progress curves or by pre-incubation studies

Inhibitor	Progress curves		Pre-incubation
	K_i (nM)	K_i^* (nM)	K_i^* (nM)
Fosmidomycin (**4**)	900	4	4
FR900098 (**5**)	500	0.9	2
Hydroxamate analog **12**	5000	2400	350

3. Discussion

Fosmidomycin was the first reported inhibitor of DXR and today remains one of the most potent inhibitors of this enzyme. Although initially described as a mixed inhibitor of the *E. coli* DXR[13] and as a competitive inhibitor of the *Zymomonas mobilis* DXR,[39] subsequent work has shown that fosmidomycin is a slow, tight-binding inhibitor of DXR, with an initial phase of inhibition that is competitive with DXP and a second phase of inhibition that is non-competitive.[36] A conformational change in the enzyme has been proposed for this second phase and a recent X-ray crystal structure of the *E. coli* DXR with fosmidomycin and NADPH bound at the active site is proposed to represent this closed conformation of the enzyme.[27]

A variety of natural and synthetic analogs of fosmidomycin have been reported over the years, including the natural product FR900098, a compound which is a more potent inhibitor of the *P. falciparum* DXR than fosmidomycin.[14] In addition to fosmidomycin and FR900098, two other natural phosphonate compounds, FR-32863 and FR-33289, have been characterized, one with a double bond in the carbon backbone and one with a hydroxyl in the chain.[40] Of these natural phosphonates, fosmidomycin and FR-33289 were typically more potent as antibacterial agents than FR-900098, with FR-32863 having minimal antibacterial activity.[41] No reports have been presented for FR-32863 or FR-33289 as DXR inhibitors. The length of the carbon chain connecting the hydroxamic acid group of fosmidomycin with the phosphonate also is critical for optimal antibacterial activity. Analogs with a two-carbon chain are not effective as antibacterial agents, suggesting a poorer inhibition of DXR.[29] The same is true for methyl phosphinic acid analogs of fosmidomycin and FR-900098, which also do not have antibacterial effects.[29]

For comparison with the inhibition values with the *E. coli* enzyme,[18,36] fosmidomycin and FR900098 were both examined as inhibitors of the *Synechocystis* DXR. An initial evaluation of fosmidomycin showed

mixed inhibition of DXR with a $K_i = 57$ nM. A later examination as a slow, tight-binding inhibitor led to values of $K_i = 900$ nM and $K_i^* = 4$ nM. Both of these values compare favorably with the published values for the *E. coli* enzyme ($K_i = 215$ or 40 nM and $K_i^* = 21$ or 10 nM). The FR900098 analog was slightly more potent than fosmidomycin, with a K_i value about 2-fold less and a K_i^* value 2- to 4-fold less. The more potent nature of FR900098 is consistent with the previously observed inhibition of the *P. falciparum* DXR by FR900098.[14]

The phosphate analogs of fosmidomycin and FR900098, fosfoxacin and the acetyl congener, were determined to be more potent mixed inhibitors of the *Synechocystis* DXR than fosmidomycin, with the acetyl congener of fosfoxacin having a K_i of 2 nM. Although the K_i and K_i^* values for slow, tight-binding inhibition were not determined for these compounds, it is likely that the K_i^* values would be less than the mixed inhibition K_i values, indicating that these phosphate analogs are the most potent inhibitors reported of DXR to date. Despite its low K_i value and its associated antibacterial activity,[32] the development of fosfoxacin as a new antibacterial or antimalarial agent is likely to be limited by its unfavorable molecular properties. The phosphate group of fosfoxacin, which contributes significantly to the low K_i value, would be subjected to cleavage by phosphatases, rapidly inactivating this compound as a DXR inhibitor.

The other two changes to the phosphonate head group, substitution with either a carboxylate or a sulfamate, led to large increases in the K_i values. The carboxylate analog had a K_i of 240 μM, while the sulfamate group had a K_i approximately 12-fold higher than that of **8**. The presence of two ionizable groups on the phosphonate and phosphate must play key roles in the high potency of inhibition. The phosphonate (2nd $pK_a \sim 8$) and the phosphate (2nd $pK_a \sim 7$)[42] groups can potentially bind as mono- or dianions, while the carboxylate can only be a monoanion and the sulfamate is neutral. Even if the phosphonate or phosphate binds as a monoanion, the remaining ionizable group could be involved in a key hydrogen bond. In the crystal structures, the pH of the crystallization solution was 5–6, so both phosphate and phosphonate groups would be expected to be monoanions.

The removal of the *N*-hydroxy group would clearly be expected to greatly decrease the potency of the analogs, because the compounds would no longer be able to chelate the divalent metal ion. The *N*-methyl analogs clearly demonstrate this phenomenon. With the absence of a group capable of chelating the metal ion, the inhibition constants increase to millimolar concentrations.

Initial analysis of the reversed hydroxamate analog of fosmidomycin resulted in mixed inhibition with a K_i of 4 μM. The recent report of this same analog as a slow, tight-binding inhibitor of the *E. coli* DXR with a $K_i = 169$ nM and a $K_i^* = 68$ nM,[18] however, led to a re-examination of the nature of the inhibition of **12** with the *Synechocystis* DXR. Compound **12** also displayed slow, tight-binding inhibition to the *Synechocystis* DXR, but the K_i (5 μM) and K_i^* (2.4 μM) values determined from the progress curves were much higher than those observed with the *E. coli* enzyme. Using a pre-incubation approach, however, produced a lower K_i^* value of 350 nM. This lower K_i^* value with the pre-incubation studies suggests that the conformational change associated with the slow, tight-binding inhibition by the hydroxamate takes longer than the change associated with fosmidomycin. The progress curves for the four-minute assay for **12** apparently led to this underestimation of K_i^*. Even the lower K_i^* value is approximately 5-fold higher than the K_i^* value for **12** with the *E. coli* DXR. The nature of this difference in inhibitor potency is as yet unclear, but a comparison of the sequence alignments reveals that all of the active site residues shown to act directly with either fosmidomycin or DXP in the *E. coli* crystal structures are completely conserved in the *Synechocystis* enzyme. Apparently subtle differences in how these residues align at the active sites of the *E. coli* and *Synechocystis* DXRs lead to a greater difference in inhibition between fosmidomycin and its reversed hydroxamate derivative in the *Synechocystis* DXR.

The broad range of inhibition potencies of these analogs provides information about the structural requirement for the design of better inhibitors. At the initiation of this project, it had been proposed that fosmidomycin might be mimicking the intermediate 2-*C*-methylerythrose-4-phosphate (**2**) because each had a formyl carbon and phosphorous atom separated by five chemical bonds.[13] However, the first X-ray structure of the *E. coli* DXR with fosmidomycin bound suggested that fosmidomycin binds in a position more similar to the substrate DXP than to the intermediate.[25] The analog studies add to the knowledge from the structural studies. Potent inhibitors should contain either a phosphate or phosphonate for high affinity, although the metabolic liability of the phosphate group argues for use of a phosphonate. The hydroxamate group is also highly sensitive to changes, with even minor alterations leading to a decrease in inhibition. These results along with prior work on fosmidomycin inhibition suggest that it will be difficult to improve upon the inhibition qualities of fosmidomycin and FR900098.

4. Experimental

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM 400, AC 300 or DRX 600 instruments. ^1H and ^{13}C NMR chemical shifts were reported as δ using the residual solvent signal as an internal standard ($CHCl_3$ at 7.26 ppm for ^1H NMR and 77.00 ppm for the centerline of the $CDCl_3$ triplet for ^{13}C NMR samples). Either *tert*-butanol (31.21 ppm for the CH_3) or MeOH-d_4 (49.15 ppm for the centerline of the quintet) was added as an internal standard for the samples in D_2O for referencing ^{13}C NMR spectra. Phosphoric acid (85%) was used as an external standard for ^{31}P NMR spectra (0.00 ppm). Low- and high-resolution chemical ionization and fast atom bombardment mass

spectra (CIMS and FABMS) were obtained on a Kratos MS50TC spectrometer. Electrospray mass spectra were obtained on a PerkinElmer SCIEX API3 spectrometer. Silica gel (Merck, grade 60, 220–400 mesh) was used for flash chromatography. The solvents used for the synthetic procedures were dried before use as commonly recommended.[43] Commercial grade reagents and the starting materials were purchased from Sigma–Aldrich and used without further purification. Acetylformic anhydride was prepared as described.[44] The water used in the experiments was purified through a Milli Q water purification system (Millipore). The cultured bacterial cells were disrupted by sonication with a XL2000 Microson™ (Misonix) ultrasonic cell disruptor. The bacterial culture samples were centrifuged in a Beckman JI-HS or Beckman J2-HS centrifuge at the temperature as indicated. Unless stated otherwise, synthetic reactions were performed in oven-dried glassware under a positive pressure of argon.

4.1. 2-(*tert*-Butyldimethylsilyloxy)-ethanol[45]

To a solution of ethylene glycol (**13**; 1 g, 16.1 mmol), triethylamine (TEA, 2.5 mL, 24.1 mmol), and DMAP (20 mg, 0.16 mmol) in CH_2Cl_2 (120 mL) was added TBDMSCl (2.91 g, 19.3 mmol) in 5 portions over 1 h at 0 °C. The resulting heterogeneous reaction mixture was gradually warmed to rt. The mixture was stirred for 12 h before dilution with water and CH_2Cl_2. The organic layer was washed successively with solutions of saturated aq $NaHCO_3$, saturated aq NH_4Cl, water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The pale yellow oil was purified by vacuum chromatography[46] (SiO_2, EtOAc/hexanes, 1:3) to give a colorless oil (2.9 g, 99%).

4.2. Phosphoric acid, dibenzyl 2-(tert-butyldimethylsilyloxy) ethyl ester (14)

To a solution of 2-(*tert*-butyldimethylsilyloxy)-ethanol (100 mg, 0.6 mmol) in CH_2Cl_2 (30 mL) were added 1-*H*-tetrazole (80 mg, 1.2 mmol) and dibenzyl diisopropyl phosphoramidite (390 μL, 1.2 mmol). The resulting solution was stirred for 4 h at rt under argon at rt before *tert*-butyl hydroperoxide (70% solution, 0.5 mL) was added dropwise at 0 °C. The reaction mixture was warmed to rt and stirred for 8 h. The mixture was diluted with CH_2Cl_2, and the organic layer was washed with water twice, and the combined aqueous layers were re-extracted with CH_2Cl_2 (5 mL × 3). The combined organic layer was washed with a saturated aq solution of sodium bicarbonate, water and brine, dried with $MgSO_4$, and concentrated in vacuo. The concentrate was purified by flash chromatography (EtOAc/hexanes, 1:3) to afford the desired product (196 mg, 79%). IR (neat) 1251, 1007, 765 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31–7.37 (m, 10H), 5.05 (d, $^3J_{HP}$ = 8.0 Hz, 2H), 5.04 (d, $^3J_{HP}$ = 7.9 Hz, 2H), 4.04 (dt, $^3J_{HP}$ = 7.1 Hz, J = 5.2 Hz, 2H) 3.76 (dt, J = 5.2 Hz, $^4J_{HP}$ = 1.1 Hz, 2H), 0.88 (*s*, 9H), 0.04 (*s*, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 135.88 (d, $^3J_{CP}$ = 6.9 Hz), 128.53, 128.45, 127.88, 69.18 (d, $^2J_{CP}$ = 5.5 Hz), 68.61 (d, $^2J_{CP}$ = 5.9 Hz), 62.10 (d, $^3J_{CP}$ = 7.9 Hz), 25.81, 18.29, −5.37; ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 0.16 (br s); HRCIMS calcd for $C_{22}H_{34}O_5PSi$: 437.19132. Found: 437.19076 (M+H).

4.3. Phosphoric acid, dibenzyl 2-hydroxyethyl ester

Compound **14** (50 mg, 0.1 mmol) was dissolved in methanol prior to the addition of 1 g Dowex® 50Wx8-100 (H^+ form) resin. This heterogeneous mixture was stirred for 12 h at rt. The resin was removed by filtration and the filtrate was concentrated to a pale yellow oil, which was purified by flash chromatography (5% MeOH/$CHCl_3$) to provide the product as a colorless oil (32 mg, 87%). IR (neat) 3582, 1230 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.37–7.34 (m, 10 H), 5.06 (d, $^2J_{HP}$ = 8.5 Hz, 2H), 5.05 (d, $^3J_{HP}$ = 8.4 Hz, 2H), 4.08 (dt, J = 4.4 Hz, $^3J_{HP}$ = 9.4 Hz, 2H), 3.73 (dt, J = 4.4 Hz, $^4J_{HP}$ = 0.9 Hz, 2H), 2.64 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 62.01 (d, $^2J_{CP}$ = 5.3 Hz), 69.59 (d, $^2J_{CP}$ = 5.6 Hz), 69.82 (d, $^3J_{CP}$ = 6.1 Hz), 128.02, 128.62, 128.68, 135.61 (d, $^3J_{CP}$ = 6.5 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 1.19; HRFABMS calcd for $C_{16}H_{20}O_5P$: 323.10484. Found: 323.10496 (M+H).

4.4. Phosphoric acid, dibenzyl 2-benzyloxyamino-ethyl ester (15)

To a solution of phosphoric acid, dibenzyl 2-hydroxyethyl ester (67 mg, 0.21 mmol) in CH_2Cl_2 (25 mL) was added 2,6-lutidine (30 μL, 0.25 mmol) under an argon atmosphere. The solution was cooled to −78 °C before adding trifluoromethane sulfonic anhydride (39 μL, 0.21 mmol) dropwise. After the reaction mixture was stirred at −78 °C for 30 min, *O*-benzyl hydroxylamine (39 mg, 0.31 mmol) in CH_2Cl_2 was added dropwise. The solution was stirred for 1 h at −78 °C, warmed to rt, and stirred for another 2 h. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous solutions of NH_4Cl, $NaHCO_3$, water and brine, dried over $MgSO_4$, filtered, and concentrated to a pale yellow oil in vacuo. The resulting residue was purified by flash chromatography (EtOAc/hexanes, 2:1) to yield the product as a colorless oil (74 mg, 83%): IR (neat) 1253, 1011, 732 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.31–7.38 (m, 15H), 5.04 (d, $^3J_{HP}$ = 8.3 Hz, 2H), 5.03 (d, $^3J_{HP}$ = 8.3 Hz, 2H), 4.67 (*s*, 2H), 4.15 (dt, J = 5.1 Hz, $^3J_{HP}$ = 7.2 Hz, 2H), 3.07 (dt, J = 5.1Hz, $^4J_{HP}$ = 1.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 137.75, 135.76 (d, $^3J_{CP}$ = 6.2 Hz), 128.56, 128.32, 127.94, 127.82, 128.47, 128.36, 76.04, 69.35 (d, $^2J_{CP}$ = 5.4 Hz), 64.25 (d, $^2J_{CP}$ = 5.8 Hz), 51.68 (d, $^3J_{CP}$ = 7.3 Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 0.38; HRFABMS calcd for $C_{23}H_{27}O_5NP$: 428.16269. Found: 428.16377 (M+H).

4.5. Phosphoric acid, dibenzyl 2-(*N*-benzyloxy-*N*-formylamino)-ethyl ester (16)

To a solution of **15** (74 mg, 1.17 mmol) in CH_2Cl_2 (20 mL) was added FMT[47] (50 mg, 0.31 mmol) at rt. The resulting solution was stirred for 12 h. The reaction mixture was washed with water, and the aqueous

layer was re-extracted with CH_2Cl_2 (5 mL × 2). The combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated to a yellow film. The crude residue was purified by flash chromatography (EtOAc/hexanes, 1:2) to afford a colorless film (77 mg, 98%): IR (neat) 1689, 1280, 1018 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) two amide rotamers were present as a 2:1 ratio: major—8.18 (br s, 1H), 7.32–7.35 (m, 15H), 5.02 (dd, $^3J_{HP}$ = 9.8 Hz, $^2J_{HH}$ = 1.8 Hz, 4H), 4.13 (m, 2H), 3.77 (m, 2H): minor—7.87 (br s, 1H), 7.32–7.35 (m, 15H), 5.03 (d, $^3J_{HP}$ = 8.3 Hz, 2H), 5.02 (d, $^3J_{HP}$ = 8.3 Hz, 2H), 4.10 (m, 2H), 3.38 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) major—163.83, 135.63, 135.58, 134.13, 129.48, 129.11, 128.71, 128.56, 127.98, 78.15, 69.47 (d, $^2J_{CP}$ = 5.8 Hz), 63.14, 49.17: minor—158.79, 135.63, 135.58, 134.13, 129.48, 129.11, 128.71, 128.56, 127.98, 78.15, 69.25 (d, $^2J_{CP}$ = 5.3 Hz), 62.44, 45.25; ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) −0.03, 0.26; HRFABMS calcd for $C_{24}H_{27}O_6NP$: 456.15760. Found: 456.15872 (M+H).

4.6. Phosphoric acid, dibenzyl 2-(*N*-acetyl-*N*-benzyloxy-amino)-ethyl ester (17)

Compound **15** (74 mg, 1.17 mmol) was dissolved in a 1:1 mixture of Ac_2O and pyridine (25 mL), and stirred for 12 h at rt. The solvent was removed in vacuo and the residue was purified by flash silica column chromatography (EtOAc/hexanes, 1:1) to give a colorless oil (79 mg, 98%): IR (neat) 1672, 1280, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.32–7.37 (*m*, 15H), 5.02 (d, $^3J_{HP}$ = 8.2 Hz, 2H), 5.01 (d, $^3J_{HP}$ = 8.1 Hz, 2H), 4.79 (s, 2H), 4.16 (dt, $^3J_{HP}$ = 7.4 Hz, $^3J_{HH}$ = 5.6 Hz, 2H), 3.83 (br s, 2H), 2.05 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 172.93, 135.73, 135.67, 134.30, 129.28, 129.00, 128.73, 128.59, 128.39, 127.98, 76.82, 69.39 (d, $^2J_{CP}$ = 5.3 Hz), 63.72 (d, $^3J_{CP}$ = 5.4 Hz), 46.44, 20.44; ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 0.04; HRFABMS calcd for $C_{25}H_{29}O_6NP$: 470.17325. Found: 470.17204 (M+H).

4.7. Phosphoric acid, mono- [2-(*N*-formyl-*N*-hydroxy-amino)-ethyl] ester (fosfoxacin; 6)

A solution of **16** (60 mg, 0.13 mmol) in methanol (10 mL) was stirred with a catalytic amount of 10% Pd/C under a hydrogen atmosphere (1 atm) at rt overnight. The Pd/C was removed by filtration, and the filtrate was concentrated to a pale yellow film. The residue was redissolved in water (2 mL), and the aqueous layer was washed with chloroform (2 mL × 3) before being purified by CF-11 cellulose column chromatography (2.5 × 30 cm; flow rate = 1 mL/min) eluting with a continuous gradient of 0 to 1% of 0.1% TFA/THF in water. Five milliliter fractions were collected, and the fractions that were PMA-active were collected and freeze-dried to a colorless film (20 mg, 91%). ^1H NMR (400 MHz, D_2O) δ (ppm) two amide rotamers with 3:7 ratio: 8.32 (br s, minor) and 7.94 (br s, major), 3.93 (m, 2H), 3.64 (m, 2H) ^{13}C NMR (100 MHz, D_2O) δ (ppm) 160.42, 60.62 (d, $^2J_{CP}$ = 3.0 Hz), 51.16 (d, $^3J_{CP}$ = 5.0 HZ) ^{31}P NMR (121 MHz, D_2O) δ (ppm) 3.88; HRFABMS calcd for $C_3H_7NO_6P$: 184.00110. Found: 184.00101 (M−H).

4.8. Phosphoric acid mono- [2-(*N*-acetyl-*N*-hydroxy-amino)-ethyl]-ester (7)

A solution of **17** (62 mg, 0.13 mmol) in methanol (10 mL) was stirred with a catalytic amount of 10% Pd/C under a hydrogen atmosphere (1 atm) at rt overnight. The Pd/C was removed by filtration, and the filtrate was concentrated to a pale yellow film. The residue was redissolved in water, and the aqueous layer was washed with chloroform (5 mL × 3) before being applied to a CF-11 cellulose column (2.5 × 30 cm). The desired compound was eluted with a continuous gradient of 0 to 1% (0.1% TFA/THF) in water. Five milliliter fractions were collected, and the PMA-active fractions were collected and freeze-dried to a white solid (24 mg, 93%). ^1H NMR (600 MHz, D_2O) δ (ppm) two amide rotamers; 2.17 (s) and 2.16 (s), 3.78–3.83 (*m*, 2H), 4.00–4.02 (*m*, 2H); ^{13}C NMR (100 MHz, D_2O) δ (ppm) 160.95 and 164.92, 60.96 (d, $^2J_{CP}$ = 2.0 Hz), 58.53 and 51.63 (d, $^3J_{CP}$ = 6.6 Hz), 10.84; ^{31}P NMR (121 MHz, D_2O) δ (ppm) 1.53; HRFABMS calcd for $C_4H_9O_6NP$: 198.01675. Found: 198.01695 (M−H).

4.9. 4-[*N*-Benzyloxy-*N*-(toluene-4-sulfonyl)-amino]-butyric acid ethyl ester (19)

Solid sodium (30 mg, 1.23 mmol) was added to dry ethanol (45 mL) and the mixture was warmed to 60 °C. After the mixture became clear, it was cooled to rt before being treated with *N*-benzyloxy-toluene-4-sulfonamide (312 mg, 1.13 mmol) in ethanol (5 mL). The reaction mixture was stirred for 2 h at rt. To this solution was added 4-bromobutyric acid ethyl ester (**18**) (200 mg, 1.02 mmol) dropwise, and the resulting solution was refluxed for 12 h. The reaction was quenched by water and the solvent was removed in vacuo. The resulting yellow residue was dissolved in EtOAc and the organic layer was washed with saturated aq NH_4Cl, water and brine, dried over $MgSO_4$, filtered, and concentrated to a yellow oil. The residue was purified with flash chromatography (EtOAc/hexanes, 1:9) to give 403 mg of a colorless oil (>99%): IR (neat) 1734, 1364, 1171 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.74 (d, *J* = 8.3 Hz, 2H), 7.34–7.43 (m, 5H), 7.31 (d, *J* = 8.3 Hz, 2H), 5.10 (s, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 2.91 (br s, 2H), 2.41 (s, 3H), 2.33 (t, *J* = 7.43 Hz, 2H), 1.81 (p, *J* = 7.1 Hz, 2H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 172.82, 144.76, 135.18, 130.02, 129.71, 129.56, 129.47, 128.51, 128.71, 79.86, 60.44, 52.61, 31.24, 22.09, 21.59, 14.17; HRCIMS calcd for $C_{20}H_{26}O_5NS$: 392.15317. Found: 392.15365 (M+H).

4.10. 4-*N*-Hydroxyaminobutyric acid (20)

A solution of **19** (345 mg, 0.89 mmol) in a 1:1 mixture of conc. HCl and acetic acid (50 mL) was refluxed overnight. The solvent was removed in vacuo and the residue was dissolved in water (10 mL). The aqueous layer was washed with chloroform and concentrated to a brown

residue. The crude product was purified on a Dowex 50Wx8-100 resin, eluting with 2 N NH_4OH. The fractions were pooled and freeze-dried to a pale yellow film (85 mg, 81%). ^1H NMR (400 MHz, MeOH-d_4) δ (ppm) 2.99 (t, $J = 7.5$ Hz, 2H), 2.27 (t, $J = 7.4$ Hz, 2H), 1.88 (p, $J = 7.5$ Hz, 2H); ^{13}C NMR (100 MHz, MeOH-d_4) δ (ppm) 182.64, 40.42, 35.51, 24.73; HRCIMS calcd for $C_4H_{10}O_3N$: 120.06607. Found: 120.06608 (M+H).

4.11. 4-(*N*-Formyl-*N*-hydroxyamino)-butyric acid (8)

Compound **20** (15 mg, 0.12 mmol) was stirred in 500 μl of acetylformic anhydride at rt for 12 h. The reaction mixture was concentrated to a yellow brown oil and purified on a Varian Bond Elute® C_{18} cartridge eluting with gradient from 0 to 100% MeOH in H_2O. The fractions, which were $FeCl_3$-active by TLC, were pooled and freeze-dried to a pale yellow solid (13 mg, 72%). IR (neat) cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ (ppm) 1:1 mixture of amide rotamers: 7.92 and 8.25 (2s, 1H), 3.53 and 3.58 (2t, $J = 6.6$ Hz, 2H), 2.20–2.24 (m, 2H), 1.88–1.95 (m, 2H); ^{13}C NMR (100 MHz, MeOH-d_4) δ (ppm) 180.65, 164.19 and 159.79, 35.12 and 34.62, 24.71, 23.84; HRCIMS calcd for $C_5H_9NO_4$: 147.05318. Found: 147.05450 (M^+).

4.12. Sulfamic acid 2-(*tert*-butyldimethylsilyloxy)-ethyl ester

Sulfamoyl chloride was prepared as described.[48] The impure needle-shaped crystals were identified as the desired product from the IR spectrum [IR (neat, cm^{-1}) 3625, 3381, 3278, 1546, 1384, 1185, 1064, 924] which matched the reported values. To a solution of 2-(*tert*-butyldimethylsilyloxy)ethanol (90 mg, 0.51 mmol) in DMA (7 mL) was added triethylamine (86 μL, 0.61 mmol). The solution was cooled in an ice bath before adding sulfamoyl chloride (118 mg, 1.0 mmol) under a N_2 atmosphere and then stirred for 2 h at 0 °C. The reaction mixture was warmed to rt and stirred for 3 h. The reaction mixture was diluted in EtOAc and the resulting organic layer was washed successively with saturated aqueous solutions of $NaHCO_3$ and NH_4Cl, water and brine. The combined organic layer was dried over $MgSO_4$, filtered, concentrated, and the residual TEA was removed in vacuo to afford a pale yellow oil (60 mg). IR (neat) 2954, 2932, 1712, 1560, 1366, 1184, 936 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 5.43 (br s), 4.25 (t, $J = 4.8$ Hz, 2H), 3.89 (t, $J = 4.8$ Hz, 2H), 0.88 (s, 9H), 0.08 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 72.05, 61.48, 25.76, 18.26, −5.44; MS (CI): *m/z* 256.1 (M+H).

4.13. Sulfamic acid 2-hydroxyethyl ester (22)

The crude sulfamic acid 2-(*tert*-butyldimethylsilyloxy)-ethyl ester product (60 mg) in methanol (10 mL) was treated with 0.5 g (wet weight) AG 50W X8-100 (H^+ form) and stirred for 5 h at rt. The resin was removed by filtration and the filtrate was concentrated to a yellow oil. The crude oil was purified on a Varian Bond Elute® C18 cartridge eluting with a gradient of methanol in H_2O (0 to 100%). The product eluted at approximately 5% methanol in H_2O and was concentrated to a pale yellow oil (35 mg, 50% for two steps). IR (neat) 3343, 2974, 2926, 2889, 1449, 1380, 1180, 1088, 1049, 881 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) δ (ppm) 4.11 (t, $J = 4.8$ Hz, 2H), 3.73 (t, $J = 4.8$ Hz, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ (ppm) 72.33, 61.20; HRCIMS calcd for $C_2H_8O_4NS$: 142.01740. Found: 142.01770 (M+H).

4.14. Sulfamic acid 2-(*N*-benzyloxyamino)-ethyl ester (23)

To compound **22** (28 mg, 0.2 mmol) in dry CH_2Cl_2 (10 mL) was added 2,6-lutidine (31.9 mg, 0.3 mmol). The solution was cooled to −78 °C before adding trifluoromethane sulfonic anhydride (40 μL, 0.24 mmol) dropwise. After the reaction mixture was stirred at −78 °C for 30 min, *O*-benzyl hydroxylamine (49 mg, 0.4 mmol) was added dropwise. The solution was stirred for 1 h at −78 °C, warmed to rt, and stirred for another 2 h. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aq $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated. The resulting yellow oil was purified by flash chromatography (EtOAc/hexanes, 3:2) to yield a pale yellow oil (39 mg, 80%). IR (neat) 3343, 2974, 2926, 2889, 1449, 1380, 1180, 1088, 1049, 881, 785 cm^{-1}; ^1H NMR (300 MHz, MeOH-d_4) δ (ppm) 7.39–7.31 (m, 5H), 4.72 (s, 2H), 4.28 (t, $J = 5.3$ Hz, 2H), 3.19 (t, $J = 5.3$ Hz, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ (ppm) 129.47, 129.33, 128.83, 77.00, 67.52, 51.50.

4.15. Sulfamic acid 2-(*N*-formyl-*N*-hydroxyamino)-ethyl ester (9)

To a solution of **23** (35 mg, 0.14 mmol) in CH_2Cl_2 (10 ml) was added FMT (45 mg, 0.28 mmol) at rt, and the resulting solution was stirred for 12 h. The reaction mixture was concentrated in vacuo and purified by flash chromatography (EtOAc/hexanes, 2:1) to give a pale yellow oil (35 mg, 90%). The residue (32 mg, 0.12 mmol) was dissolved in methanol (10 mL) and a catalytic amount of 10% Pd/C was added to the solution. The heterogeneous mixture was stirred for 12 h under a slight positive pressure of H_2 gas (1 atm) at rt. The reaction mixture was filtered and concentrated to a pale yellow oil before purification on a Varian Bond Elute® C_{18} column eluting with a stepwise gradient (0–70%) methanol in H_2O. The desired product was eluted with 15% methanol/H_2O and the fractions were pooled and lyophilized to give a yellow syrup (11 mg, 50%). ^1H NMR (400 MHz, MeOH-d_4) δ (ppm) 1:2 ratio of 8.38 & 7.98 (s), 4.33 (t, $J = 5.5$ Hz, 2H), 3.92 (t, $J = 5.5$ Hz, 2H); ^{13}C NMR (100 MHz, MeOH-d_4) δ (ppm) 164.9 and 160.0, 65.95, 51.05; HRCIMS calcd for $C_3H_8N_2O_5S$ 184.01539. Found: 184.01571 (M^+).

4.16. *N*-(3-Bromopropyl)-*N*-toluene-4-sulfonamide

To a heterogeneous mixture of NaH (120 mg, 2.75 mmol) in dry THF (100 mL) was transferred a rt solution of *N*-tosyl-*N*-methyl amine (500 mg, 2.72 mmol) in THF (20 mL) via a cannula at 0 °C. One hour later, 1,3-dibromopropane (**24**; 500 mg, 2.5 mmol) was added dropwise at 0 °C. The reaction

mixture was stirred for 12 h at rt and refluxed for 3 h before quenching with water. The solvent was removed in vacuo and the crude product was dissolved in EtOAc (30 mL). The organic fraction was washed with saturated aq NH_4Cl, water and brine, dried over $MgSO_4$, filtered, and concentrated to a pale yellow oil. The residue was purified by flash chromatography (EtOAc/hexanes, 1:9) to get a colorless oil (250 mg, 34%; 65% based on recovered starting material). IR (neat) 1366, 1170 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.68 (d, $J = 8.4$ Hz, 2H), 7.33 (d, $J = 8.$ 4 Hz, 2H), 3.47 (t, $J = 6.5$ Hz, 2H), 3.13 (t, $J = 6.7$ Hz, 2H), 2.11 (dt, $J = 6.6$, 6.6 Hz, 2H), 2.75 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 143.50, 134.23, 129.74, 127.45, 48.80, 35.63, 31.28, 30.14, 21.51; HR CIMS calcd for $C_{11}H_{17}O_2N^{79}BrS$: 306.10634. Found: 306.10655 (M^+).

4.17. {3-[-*N*-Methyl-*N*-(toluene-4-sulfonyl)-amino]-propyl}phosphonic acid diethyl ester (25)

To a heterogeneous mixture of NaH (42 mg, 1.01 mmol) in dry THF (50 mL) was added diethyl phosphite (126 μL, 0.98 mmol) dropwise at 0 °C and stirred for 2 h. A solution of *N*-(3-Bromopropyl)-*N*-toluene-4-sulfonamide (250 mg, 0.82 mM, in 5 mL THF) was added to the mixture at 0 °C via a syringe. The mixture was warmed to rt and stirred for 12 h. The solvent was removed in vacuo and the residue was dissolved in EtOAc (100 mL). The organic layer was washed with a solution of saturated aq NH_4Cl, water and brine, dried over $MgSO_4$, filtered, and concentrated, before being purified by flash chromatography (3% MeOH/CH_2Cl_2) to yield 210 mg of a colorless oil (70%). IR (neat) 1162, 1245, 1350 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.65 (d, $J = 8.4$ Hz, 2H), 7.31 (d, $J = 8.4$ Hz, 2H), 4.03–4.20 (m, 4H), 3.04 (t, $J = 6.3$ Hz, 2H), 2.71 (s, 3H), 2.43 (s, 3H), 1.78-1.86 (m, 4H), 1.37 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 143.25, 134.12, 129.53, 127.16, 61.42 (d, $^2J_{CP} = 6.4$ Hz), 50.12 (d, $^3J_{CP} = 18.7$ Hz), 34.52, 22.40 (d, $J_{CP} = 142.8$ Hz), 21.28, 20.65 (d, $^2J_{CP} = 3.9$ Hz), 16.25 (d, $^3J_{CP} = 5.8$ Hz); ^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm) 32.55; HRFABMS calcd for $C_{15}H_{27}O_5NPS$: 364.13476. Found: 364.13451 (M+H).

4.18. (3-*N*-Methylamino-propyl)-phosphonic acid (26)

Compound **25** (250 mg, 0.68 mmol) was dissolved in a 1:1 mixture of acetic acid and concd HCl (30 mL), and refluxed overnight. The solvent was removed under reduced pressure and the yellow residue was purified by cation exchange chromatography using Dowex 50X8-100 and eluting with 1 N NH_4OH. The fractions that were stained with PMA on TLC were pooled and freeze-dried to a white solid (72 mg, 70%). ^1H NMR (300 MHz, MeOH-d_4) δ (ppm) 2.98 (t, $J = 6.1$ Hz, 2H), 2.59 (s, 3H), 1.86–1.97 (m, 2H), 1.58–1.68 (m, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ (ppm) 50.59 (d, $^3J_{CP} = 16.12$ Hz), 33.37, 26.08 (d, $J_{CP} = 132.5$ Hz), 21.16 (d, $^2J_{CP} = 3.2$ Hz); ^{31}P NMR (121 MHz, MeOH-d_4) δ (ppm) 22.19; HRFABMS calcd for $C_4H_{13}O_3NP$: 154.06331. Found: 154.06315 (M+H).

4.19. [3-(*N*-Formyl-*N*-methyl-amino)-propyl]-phosphonic acid (10)

A solution of compound **26** (30 mg, 0.19 mmol) in acetylformic anhydride (10 ml) was stirred at rt for 12 h. The solvent was removed in vacuo and the residue was purified on a Varian Bond Elute® C_{18} column eluting with a stepwise gradient of MeOH (0–100%) in water. The product eluted at 15% MeOH, along with minor impurities. The fractions were pooled, concentrated, and basified with $NaHCO_3$ before being applied onto an AG1-X10 (100–200 mesh, Cl^- form) ion exchange column. The desired product eluted with 1 N NH_4OH, but a yellow contaminant remained. The concentrated residue was purified further by C18 silica chromatography eluting with water to give 6 mg of a white solid after lyophilization (15%). ^1H NMR (400 MHz, D_2O) δ (ppm) 1:1 mixture of amide rotamers 8.39 and 7.96 (s, 1H), 3.23–3.33 (m, 2H), 1.99 and 1.96 (2 s, 3H), 1.61–1.71 (m, 2H), 1.36–1.47 (m, 2H); ^{13}C NMR (100 MHz, D_2O), δ (ppm) 1:1 mixture of amide rotamers 165.2 and 160.7, 51.95 (d, $^3J_{CP} = 18.9$ Hz) and 48.72 (d, $^3J_{CP} = 19.7$ Hz), 36.58 and 33.69, 25.44 (d, $J_{CP} = 134.5$ Hz), 22.22 (d, $^2J_{CP} = 3.0$ Hz) and 21.39 (d, $^2J_{CP} = 3.8$ Hz); ^{31}P NMR (121 MHz, D_2O) δ (ppm) 26.29 and 25.87; MS (ESI): *m/z* 182.1 (M+H), 204.0 (M+Na).

4.20. [3-(*N*-Acetyl-*N*-methyl-amino)-propyl]-phosphonic acid (11)

Compound **26** (30 mg, 0.19 mmol) was dissolved in a 1:1 mixture of acetic anhydride/pyridine (6 mL) and stirred at rt for 5 h. The solvent was removed in vacuo, and the residue was purified on a Varian Bond Elute® C_{18} column eluting with a stepwise gradient MeOH (0–100%) in water. The product eluted at 15% MeOH along with minor impurities. The fractions were pooled, concentrated and basified with $NaHCO_3$, before being applied onto an AG1-X10 (100–200 mesh, Cl^- form) ion exchange column. The desired product eluted with 1 N NH_4OH, but a yellow contaminant was also present. The concentrated residue was purified further by C_{18} silica chromatography eluting with water to give 5 mg of a white solid after lyophilization (13%). ^1H NMR (400 MHz, D_2O) δ (ppm) 1:1 mixture of amide rotamers 3.23–3.33 (m, 2H), 2.77 and 2.92 (2s, 3H), 1.96 and 1.99 (2s, 3H), 1.61–1.71 (m, 2H), 1.36–1.47 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ (ppm) 1:1 mixture of amide rotamers 174.49 and 174. 37, 51.95 (d, $^3J_{CP} = 18.9$ Hz) and 48.72 (d, $^3J_{CP} = 19.7$ Hz), 36.58 and 33.69, 25.44 (d, $J_{CP} = 134.2$ Hz) and 25.14 (d, $J_{CP} = 134.5$ Hz), 22.22 (d, $^2J_{CP} = 3.0$ Hz) & 21.39 (d, $^2J_{CP} = 3.8$ Hz), 21.29 and 20.66; ^{31}P NMR (121 MHz, D_2O) δ (ppm) 25.87 and 26.29; MS (ESI): *m/z* 196.0 (M+1), 218.0 (M+Na).

4.21. Expression of DXR

The target enzyme, DXR from *Synechocystis* sp. PCC6803, was overproduced in *E. coli* as a N-terminal 6× His-tagged form and purified for the inhibition studies as described previously.[21]

4.22. Enzyme assay

Activity of the recombinant DXR and inhibition by analogs were tested by monitoring the rate of consumption of NADPH at 340 nm.[10] The 500 mL total volume for each assay consisted of 100 mM Tris–HCl (pH 7.5), 1 mM $MnCl_2$, 0.2 mM NADPH, and varying concentrations of DXP and inhibitors. Each assay was initiated by adding DXR and the decrease in absorbance at 340 nm was monitored for 4 min. Inhibition assays were done with at least four different concentrations of each inhibitor, and each concentration was tested in triplicate. Kinetic data were analyzed using Trinity® Enzyme Kinetics v. 1.2 software.

4.23. Enzyme inhibition studies (for slow-tight binding inhibition)

Progress curves for slow, tight-binding inhibition analysis were obtained by initiating the reaction by adding the enzyme to the reaction mixture. The decrease in the absorbance at 340 nm was monitored for 4 min. The progress curves were fitted to:

$$A = v_s t + (v_o - v_s)(1 - e^{-kt})/k,$$

where A is the absorbance at any time t, v_0 and v_s are the initial and final steady-state rates, respectively, and k is the apparent first-order rate constant for the establishment of the final steady-state equilibrium. The curves were fitted by the nonlinear least squares, using GraphPad Prism software. The inhibition constants were estimated from v_0 and v_s by nonlinear regression using the Enzyme Kinetics program from Trinity Software.

The pre-incubation studies to determine the slow, tight-binding inhibition were initiated by incubating the enzyme (3 nM) for 10 min at 37 °C in 50 mM Tris–HCl buffer, 1 mM $MnCl_2$, 1 mg/mL BSA, 0.2 mM NADPH, and varying concentrations of fosmidomycin, FR900098, or the hydroxamate analog. After the incubation time, DXP at 0.2 mM was added and the activity was assayed by monitoring the decrease in absorbance at 340 nm as described in the standard assay for DXR. The K_i^*s were calculated from the X-intercept of the Dixon plot ($1/V$ vs [I]).[38]

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