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Synthesis and biological evaluation of phosphate isosters of fosmidomycin and analogs as inhibitors of *Escherichia coli* and *Mycobacterium smegmatis* 1-deoxyxylulose 5-phosphate reductoisomerases

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Abstract

Hydroxamate analogs of fosfoxacin, the phosphate homolog of fosmidomycin, have been synthesized and their activity tested on *Escherichia coli* and *Mycobacterium smegmatis* DXRs. Except for compound **4b**, the IC_{50} values of phosphate derivatives are approximately 10 fold higher than those of the corresponding phosphonates. Although their inhibitory activity on *Escherichia coli* DXR is less efficient than their phosphonate analogs, we report the ability of

phosphate compounds to inhibit the growth of *Escherichia coli.* This work points out that the uptake of fosfoxacin and its analogues is taking place *via* the GlpT and UhpT transporters. As expected, these compounds are inefficient to inhibit the growth of *M. smegmatis* growth inhibition probably due to a lack of uptake.

Keywords: antimicrobials, fosfoxacin, fosmidomycin, DXR, GlpT and UhpT transporters

Abbreviations: DXP: 1-deoxy-D-xylulose 5-phosphate; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP: 2-*C*-methyl-D-erythritol 4-phosphate; GlpT: glycerol-3-phosphate transporter; UhpT: hexose-6-phosphate transporter; FosR: fosmidomycin resistant strain of *E. coli*

1. Introduction

In many pathogenic bacteria, the enzymes of the 2-*C*-methyl-D-erythrirol 4-phosphate (MEP) pathway, involved in the biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate, the two universal and essential units for the biosynthesis of isoprenoids are recognized as valuable targets for the development of new antimicrobial compounds.[1] Of particular interest is the enzyme 1-deoxyxylulose 5-phosphate reductoisomerase (DXR) that catalyzes the transformation of 1-deoxy-D-xylulose 5-phosphate (DXP) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) *via* an isomerization and a NADPH dependent reduction reaction.[2–4] Fosmidomycin **1a** and its close relative FR900098 **2a,** two natural retro-hydroxamate phosphonic acids are efficient inhibitors of DXR (Figure 1).[5–8] Bacteria, however, rapidly develop resistance to fosmidomycin.[9,10] In an attempt to improve the efficiency of such inhibitors, numerous analogs have been synthesized. Unfortunately, to date, the results appear rather disappointing as none of these compounds are significantly more efficient than fosmidomycin. In previous work, we described the synthesis and the DXR inhibiting activity of hydroxamate phosphonic acid **3a** and of its *N*-methylated derivative **4a** (Fig. 1). The latter compound, which is as effective as fosmidomycin on DXR, has significant antibacterial activity against wild type *Escherichia coli* and even against an *E. coli* mutant resistant to fosmidomycin.[11]

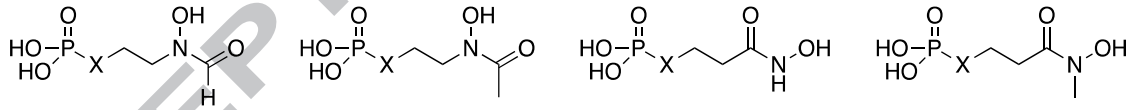


Figure 1. DXR inhibitors.

Crystallographic structures of the DXR-NADPH-fosmidomycin complex show that the phosphonate group of the inhibitor is located in the phosphate binding cleft of the substrate DXP and is bound in a similar fashion as the phosphate group. Moreover, the hydroxamate chelates the Mg^{2+} dication present in the active site.[12,13]

In the early 1990's, fosfoxacin **1b**, the phosphate analog of fosmidomycin, was isolated from the filtrate of a *Pseudomonas fluorescens* PK-52 culture and was shown to have potent

antibiotic activity against Gram-negative and Gram-positive bacteria.[14] Latter on, fosfoxacin and its *N*-acetylated analog **2b** were synthetized and evaluated as inhibitors of the DXR from *Synechocystis* sp. PCC6803. They were found to be more potent than fosmidomycin.[15] Despite these promising results, no further investigations of fosfoxacin on other microbial DXRs have been reported.

In this work, we synthesized the phosphate derivative of fosmidomycin, fosfoxacin, and several other related hydroxamate analogs (**2**-**4**, Fig. 1) in order to evaluate their effect on the activity of DXRs from *E. coli* and *Mycobacterium smegmatis*. This study also allowed us to investigate the influence of the replacement of a phosphonate with a phosphate group. As fosfoxacin was previously shown to inhibit the growth of several bacteria, it is obvious that this phosphate ester can cross bacterial envelopes, including that of *E. coli*. In this study, we also compared the efficiency of the different phosphate DXR inhibitors to inhibit the growth of *E. coli*. However, as for fosmidomycin and its phosphonate analogs, the phosphate derivatives should not be capable of crossing the bacterial wall and of inhibiting the growth of *M. smegmatis*.

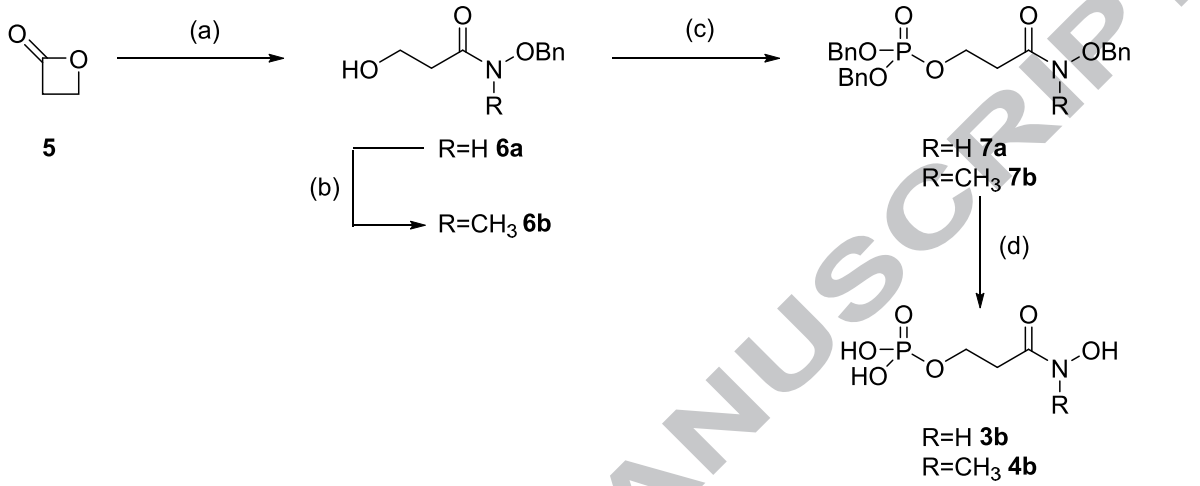
2. Results

2.1. Synthesis of phosphate derivatives 1b-4b

Fosfoxacin **1b** and its acetylated homolog **2b** were synthesized according to previously described methods.[15] The spectral data were in accordance with those published in the literature.

The syntheses of compounds **3b** and **4b** are outlined in Scheme 1. The *O*-benzyl hydroxamate derivative **6a** was obtained in one step from commercially available βpropiolactone **5** using *O*-benzyl hydroxylamine hydrochloride in presence of an excess of LiHMDS.[16] Compound **6a** was obtained as a mixture of two *Z* and *E* conformers in a 7:3 ratio, respectively, due to the restricted rotation around the C-N bond. The ^{1}H NMR spectrum of **6a** shows two signals for the CH_2CON(H)OBn protons: one major signal at 2.33 ppm corresponding to the protons of the *Z* conformer and a minor signal at 2.50 ppm corresponding to the protons of the *E* conformer.[17,18] The selective *N*-methylation versus *O*-methylation of **6a** was accomplished under basic conditions. The methyl group was introduced by reaction of **6a** with K_2CO_3 in acetone under reflux followed by addition of methyl iodide. The ^{1}H NMR spectrum of **2b** shows one signal for CH_2CON(CH_3)OBn protons at 2.62 ppm corresponding to the protons of

the *E* conformer. Introduction of dibenzyl phosphate was made using standard phosphoramidite chemistry. Alcohols **6a** and **6b** were coupled with dibenzyl *N*,*N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole to yield dibenzylphosphite derivatives, which were oxidized to phosphate (**7a** and **7b**) using *m*-chloroperbenzoic acid.[19]



Scheme **1.** Reagents and conditions. (a) (i). $H_2NOBn.HCl$, LiHMDS, THF, -78 °C (ii). TBAF.3(H_2O), THF, rt, 80 % ; (b) MeI, K_2CO_3, acetone, 60 °C, 81 % ; (c) (i). 1*H*-tetrazole, $(BnO)_2PN(iPr)_2$, DCM, rt ; (ii). *m*-CPBA, DCM, 0 °C, **7a** (60 %), **7b** (85 %) ; (d) H_2, Pd/C, EtOH, rt **3b** (99 %), **4b** (56 %).

A mixture of two *Z* and *E* conformers in a 8:2 ratio was obtained for the phosphotriester **7a,** whereas for the *N*-methylated homolog **7b** only the *E* conformer was detected in deuterated chloroform solution. Removal of the protective benzyl groups of **7a** and **7b** was performed by catalytic hydrogenolysis with palladium over charcoal at atmospheric pressure and room temperature in ethanol. The phosphate esters **3b** and **4b** required no further purification and were obtained with overall yields of 48 % and 29 %, respectively. After deprotection, as determined by ^{1}H NMR in deuterated methanol, the target compounds **3b** and **4b** were obtained as a unique conformer. For molecule **3b**, the signal at 2.45 ppm corresponding to CH_2CON(H)OH was less deshielded than the signal at 2.87 ppm corresponding to CH_2CON(CH_3)OH of the molecule **4b**, suggesting that compound **3b** was in a *Z* conformation whereas molecule **4b** was present as an *E* conformer.[17,18] To confirm the assignments of **4b**, a NOESY experiment was realized. As a result, the spectrum displayed correlations between the singlet at *ca.* 3.21 ppm assigned to the *N*-Me protons and the triplet at *ca.* 2.87 ppm corresponding of the -C**H**$_2$CONROH protons, indicating that these protons are within 5 Angstroms through space and corroborated the presence of a *E* conformer. All

phosphate compounds were tested against recombinant His-tagged DXR of *E. coli* and *M. smegmatis* for inhibition.

2.2. Inhibition of *E. coli* and *M. smegmatis* H-DXR with compounds 1-4

The inhibition potency of the phosphate derivatives, characterized by their IC_{50} value was determined as described previously.[11] Assuming that the phosphate analogs act as slow binding inhibitors like fosmidomycin, they were pre-incubated with DXR during 2 min in the presence of NADPH. Residual activity was measured after initiating the enzymatic reaction by addition of DXP. The IC_{50} values are listed in Table 1.

Table 1

Inhibition of *E. coli* and *M. smegmatis* H_6-DXRs by fosmidomycin **1a,** FR-900098 **2a**, phophonohydroxamic analogs **3a** and **4a**, fosfoxacin **1b** and its analogs **2b-4b**.

Compounds	*E. coli* DXR IC_{50} (nM)	*M. smegmatis* DXR IC_{50} (nM)
Fosmidomycin **1a**	42	510*
FR-900098 **2a**	4	320*
3a	170	1480*
4a	48	410*
Fosfoxacin **1b**	342	2100
2b	77	279
3b	2600	9200
4b	46	233

* Values obtained from reference.[20]

Phosphates **2b** and **4b** are effective inhibitors against *E. coli* DXR as well as fosmidomycin **1a** with similar IC_{50} values (IC_{50} = 46 nM and 77 nM *vs* IC_{50} = 42 nM) but they are 12 to 20-fold less potent inhibitors than FR-900098 **2a**. The IC_{50} values for the fosfoxacin **1b** (IC_{50} = 342 nM) and the *N*-H analog **3b** (IC_{50} = 2600 nM) indicate that these compounds are poor inhibitors of DXR in contrast to the results obtained with *Synechocystis* sp. PCC6803 for fosfoxacin (Ki = 19 nM) and its acetylated analog **2b** (Ki = 2 nM), which were found to be more potent than fosmidomycin (Ki = 57 nM).[15] Concerning the DXR of *M. smegmatis*, phosphate compounds **2b** and **4b**, with IC_{50} values of 279 and 233 nM respectively, are the

most potent inhibitors. The phosphonate compounds fosmidomycin **1a,** FR-900098 **2a** and *N*-H phosphonohydroxamate **4a** with respectively IC_{50} values of 510 nM, 320 nM and 410 nM are slightly less efficient. Fosfoxacin and the *N*-H analog **3b** are poor inhibitors with IC_{50} values 8 and 30-fold lower than the methylated derivatives **2b** and **4b** indicating that the presence of the methyl group in compounds **2b** and **4b** has clearly a positive effect on the affinity of the enzyme for these compounds.

2.3. Growth inhibition of *E. coli* and *M. smegmatis* with compounds 1-4

The antimicrobial activity of phosphate compounds **1b-4b** was determined using the paper disc diffusion method and was compared with the antimicrobial activity of their phosphonate homologs **1a-4a**. The diameters of the inhibition zone are given with respect to the amount of inhibitor deposited on the disc (Table 2).

Table 2

E. coli growth inhibition with compounds **1**-**4** ((Supporting information Fig. S2)

Compounds	**Amount of compound/disc** (nmoles)	**Diameter of the growth inhibition zone** (mm)
Fosmidomycin **1a**	2	34
FR-900098 **2a**	2	32
3a	400	12
4a	80	30
Fosfoxacin **1b**	160	24
2b	100	27
3b	400	<10
4b	160	20

Fosmidomycin which is, with FR-900098, the most efficient inhibitor of *E. coli* growth was used as a positive control and the reference. The other DXR inhibitors had a lower effectiveness to inhibit the bacterial growth as larger amount had to be added to observe growth inhibition zones ((Supporting information Fig. S2). Clearly there is no direct relation between the capacity to inhibit the DXR and that to stop the bacterial growth; for compound

4a, a 40-fold larger amount was deposited on the paper disc to observe a diameter similar to that of fosmidomycin. The *N*-H phosphonate **3a** and *N*-H phosphate **3b** compounds were almost ineffective. As a general rule, the phosphate compounds were less efficient than the phosphonate homologs.

All DXR inhibitors were tested on a fosmidomycin resistant strain of *E. coli* (FosR).[10] Except compound **4a**, none of the inhibitors was able to affect bacterial growth (Supporting information Fig. S3).

Concerning *M. smegmatis*, as anticipated, none of the compounds was able to inhibit its growth, probably the consequence of a lack of uptake (data not shown).[20,35]

3. Discussion

Over a large variety of natural and synthetic derivatives of the phosphono retrohydroxamic acid fosmidomycin, only two phosphate analogs, the natural fosfoxacin **1b** and its synthetic *N*-acetylated derivative **2b** have been reported in the literature. This is not that surprising as phosphate compounds are rarely exploited as potential antibiotics due to their limited lack of cell penetration and their sensitivity towards the hydrolytic activity of phosphatases. However, fosfoxacin, as well as its synthetic *N*-acetylated derivative, were shown to inhibit the growth of several bacteria,[14] and to be more potent inhibitors of *Synechocystis* sp. PCC6803 DXR than phosphonate homologs **1a** and **2a**.[15] Such promising results prompted us to evaluate the efficiency of fosfoxacin **1b** and its derivatives **2b**-**4b** against *E. coli* and *M. smegmatis* DXRs. Except for compound **4b**, the IC_{50} values of phosphate derivatives are noticeably higher than those of the corresponding phosphonates, with IC_{50} values approximately 10 fold higher, suggesting that the binding of these compounds to the enzyme is significantly weaker. These results are quite surprising as such a phosphate group should better fit into a phosphate binding site than a phosphonate group. Indeed, DXR has a lower catalytic efficiency for the phosphonate DXP analog (k_{cat}/K_m = 0.7 $min^{-1}\mu M^{-1}$) than for DXP, the natural substrate (k_{cat}/K_m = 24.7 $min^{-1}\mu M^{-1}$).[21] Likewise, in the case of triose phosphate isomerase, which possesses, as DXR, a flexible loop closing the active site, the phosphoglycolohydroxamate inhibitor (Ki = 3 µM) was shown to be a better inhibitor than its phosphonate analog (Ki = 159 µM).[22] Even if a phosphonate group is considered to be an isosteric phosphate group, some differences such as the pKa and the C-O-P/C-C-P bond

angles might impact their binding in the active site. The pKa_2 value of a phosphonate (7.6) being higher than that of a phosphate (6.4), indicates that the phosphate should be in dianionic form at the pH of the enzymatic assay, whereas the phosphonate should be predominantly in singly ionized form. This latter form is considered to be less favorable for an efficient binding. In addition, the C-O-P angle (118.7°) is wider than the C-C-P angle (112.1°)[23] resulting in a different setting of the inhibitors in the active site. This leads to modifications of the electrostatic and van der Waals interactions and consequently in an increase or a decrease of the affinity for the inhibitors. Moreover, the modification of the three-carbon spacer by replacing a methylene group by an oxygen atom such as in the βoxa isosters of fosmidomycin and compound **3a**, was reported to lead to a noticeable drop of the IC_{50} values for the *E. coli* enzyme while those of the **2a** and **4a** derivatives are still unchanged.[24]

Another contributing factor that may be involved in interpreting these results is the presence of a flexible loop in the DXR that has an essential role in catalysis and inhibition, shielding from the ligands the bulk solvent in the active site. [25,26] Therefore, any small but significant conformational changes (*e.g* disruption of hydrogen bound network, additional hydrogen bounds, new interactions between an inhibitor and the loop …) initiated by the binding of the substrate or an inhibitor, such as fosmidomycin *vs* fosfoxacin, might alter the closure of the loop over the active site leading to a more or less tight [DXR-NADPH-inhibitor] ternary complex.

However, it should be noted that the presence of the methyl group in compounds **2b** and **4b** as well as in the phosphonate homologs **2a** and **4a** increases the inhibitory activity against *E. coli* and *M. smegmatis* DXRs. Based on crystal structure analyses of *Plasmodium falciparum* and *M. tuberculosis* DXRs, van der Waals interactions between the methyl group of the **4a** and **2a** with the indole ring of the highly conserved tryptophan residue (*P. falciparum,* Trp 296; *M. tuberculosis*, Trp 203) from the flexible loop region might occur with the consequence of the better activity of *N*-methylated compared to *N*-H compounds.[27,28]

Compared to the phosphonate compounds, the efficiency of phosphates on the growth inhibition of *E. coli* was much lower. Several reasons may be invoked: i) the lower DXR inhibition efficiency and/or ii) the cell wall/plasma membrane crossing of the phosphate esters and/or iii) the inactivation by hydrolysis of the phosphate group by phosphatases. Nevertheless, the fact that the phosphate derivatives inhibit bacterial growth of *E. coli* shows that these compounds enter the bacteria. The hydrophilic character of the compounds suggests that they do not passively diffuse across the cell membrane. A crossing *via* a transporter is accordingly privileged, particularly *via* the glycerol 3-phosphate (GlpT)[29,30] and the hexose 6-

phosphate (UhpT) transporters.[31] These transporters allow the penetration of essential nutrients (glycerol 3-phosphate and glucose 6-phosphate) but also hydrophilic antibiotics like fosfomycin, a phosphonate antibiotic which inhibits the UDP-*N*-acetylglucosamine enoylpyruvyl transferase (MurA), an enzyme implied in the biosynthesis of peptidoglycan.[32] The import of the molecules is driven by the efflux of inorganic phosphate.[33] As already observed for fosmidomycin,[10] many resistant clones grew in the growth inhibition zone when compounds **1b**-**4b** were used. Some resistant bacteria were picked off from the inhibition zone and tested for cross-resistance with fosfomycin. All were resistant to this antibiotic indicating that a lack of uptake of the inhibitor *via* the GlpT and/or the UhpT is probably the cause.[34] The lack of uptake by mutation in the transporter system is one of the multiple causes for resistance to antibiotics. A fosmidomycin resistant strain in *Escherichia coli* (FosR) was genetically characterized and was shown to contain a transposon insertion mutation in the gene coding for the GlpT transporter.[10] Sequencing of the UhpT gene showed that it is also mutated as a transposon was inserted (Fig. S1 in supporting information). In this strain the GlpT/UhpT transporters are therefore inoperative and the fact that all phosphate derivatives were unable to prevent the growth of the bacteria, suggests that these compounds enter into the bacteria *via* these transporters. Moreover, this strain remains sensitive to compound **4a** implying that the latter follows besides a different way to penetrate into the bacteria since it was less efficient to inhibit the FosR strain than the wild type (Supporting information Fig. S4).

In the present work, we pointed out that the efficiency of a phosphate inhibitor compared to its phosphonate homolog might be notably different towards DXRs isolated from different microorganisms. Even if the phosphate derivatives were previously shown to be more potent inhibitors of *Synechocystis* DXR than their phosphonate analogs, this conclusion is not valid for all DXRs, as we have shown that they are less efficient against *E. coli* and *M. smegmatis*. . It is not obvious to rationalize these results as several changes in van der Waals and electrostatic forces at the anchoring and chelating sites as well as in the closure of the flexible loop might affect the binding of an inhibitor in the DXR active site, preventing or privileging its fixation in the DXR active site. It is important to note that the presence of the methyl group on the hydroxamate/retrohydroxamate group increases the inhibitory activity against DXR isolated from *E. coli* and *M. smegmatis* DXR as well as their phosphonate homologues.Even if the phosphate compounds are less efficient compared to the phosphonates on the growth inhibition of *E. coli*, they can enter into the bacteria *via* the GlpT/UhpT transporters. The tested DXR inhibitors cannot cross the bacterial wall of *M.*

smegmatis and to circumvent the lack of uptake of these compounds, we prepared prodrugs where the phosphate group is masked to render them less hydrophilic to allow the crossing of the bacterial envelope. Moreover such a masking should also protect the inhibitors against hydrolysis by phosphatases (Munier *et al*., unpublished results).

4. Experimental Section

4.1. General methods

All non-aqueous reactions were run in oven-dried glassware under an argon atmosphere, using dry solvents. Commercial grade reagents were purchased from Sigma-Aldrich or Acros Organics and used without further purification. Petroleum ether 40-60 °C (Sigma-Aldrich) was used for purification Flash chromatography was performed on silica gel 60 230–400 mesh with the solvent system as indicated. TLC plates were revealed under UV light (254 nM) and/or by spraying with an ethanolic solution of phosphomolybdic acid (20%) or an ethanolic solution of potassium permanganate followed by heating.

The NMR spectra were recorded on a BRUKER Avance 300 (^{1}H-NMR: 300 MHz; ^{13}C-NMR, 75.5 MHz; ^{31}P-NMR 121.5 MHz; ^{19}F-NMR 282.4 MHz) or a BRUKER Avance 400 (^{1}H-NMR: 400 MHz; ^{13}C-NMR, 100.6 MHz; ^{31}P-NMR 162 MHz) or a BRUKER Avance 500 (^{1}H-NMR: 500 MHz; ^{13}C-NMR, 125.8 MHz). ^{1}H-NMR experiments were performed in $CDCl_3$, D_2O, CD_3OD in $CDCl_3$ with $CHCl_3$ (δ= 7.26 ppm), DHO (δ= 4.79 ppm), CD_2HOD (δ = 3.31 ppm) as internal references. ^{13}C-NMR experiments were performed in $CDCl_3$ with $CDCl_3$ (δ= 77.23 ppm), CD_3OD (δ= 49.0 ppm) as internal references. For ^{31}P-NMR reference, the spectrometer had an external reference, corresponding to 80 % phosphoric acid in D_2O (δ = 0 ppm). The chemical shifts (δ) are expressed in ppm. s, d, t, q, or bs are abbreviations for multiplicity correspond to singulet, doublet, triplet and quadruplet or broad singulet. J-couplings are exposed in Hz.Negative or positive-mode electrospray MS were performed on a Bruker Daltonics microTOF spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an orthogonal electrospray (ESI) interface. Calibration was performed using a solution of 10 mM sodium formate. Sample solutions were introduced into the spectrometer source with a syringe pump (Harvard type 55 1111: Harvard Apparatus Inc., South Natick, MA, USA) with a flow rate of 5 µL min^{-1}.The compounds **1b** (fosfoxacin) and **2b** were synthesized according to Woo *et al.*[15]

The cloning and purification of His-tagged DXR (H-DXR) of *E. coli* and *M. smegmatis* were previously described.[11,20] DXP was prepared according to Meyer *et al.*[36]

4.2. Synthesis of phosphate derivatives 3-4

4.2.1. *N*-(Benzyloxy)-3-hydroxypropanamide (6a)

A stirred suspension of *O*-benzylhydroxylamine hydrochloride (2.63 g, 16 mmol) in dry THF (20 mL) at -78 °C was treated with a 1 M solution of LiHMDS (65 mL, 65 mmol). After 1 h, a solution of β-propiolactone **5** (1 mL, 16 mmol) in dry THF (2 mL) was added dropwise at -78 °C. The reaction mixture was stirred at room temperature overnight. The reaction mixture was cooled at -78 °C then a saturated aqueous solution of NH_4Cl (50 mL) was slowly added and warmed to room temperature. The aqueous layer was extracted with ethyl acetate (3x70 mL), saturated with NaCl and extracted with diethyl ether (3x100 mL). The organic layers were collected and dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was dissolved in THF and treated with TBAF.$3H_2O$ (7,65 g, 24 mmol). After total consumption of starting material, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (EtOAc → EtOAc/MeOH 90:10) to give the compound **6a** (2,49 g, 80 %) as a white solid and a mixture of two conformers *Z* and *E* in a 7:3 ratio respectively. Rf = 0.19 (EtOAc/petroleum ether, 7:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 2.33 (7/10 of 2H, bs, CH_2CO), 2.50 (3/10 of 2H, bs, CH_2CO), 2.63 (7/10 of 1H, bs, OH), 2,81 (3/10 of 1H, bs, OH), 3.86 (2H, td, ^3J = 5.5 Hz, ^3J = 5.5 Hz, CH_2OH), 4.83 (3/10 of 2H, bs, OCH_2Ph), 4.94 (7/10 of 2H, bs, OCH_2Ph), 7.39 (5H, s, CH_{Ar}), 7.87 (3/10 of 1H, bs, NH), 8.31 (7/10 of 1H, bs, NH). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ(ppm) = 34.0 (CH_2CO), 35.7 (CH_2CO), 58.1 (CH_2OH), 58.7 (CH_2OH), 78.5 (OCH_2Ph), 79.7 (OCH_2Ph), 128.9 (CH_{Ar}), 129.1 (CH_{Ar}), 129.5 (CH_{Ar}), 135.3 (C_{Ar}), 135.4 (C_{Ar}), 170.2 (CO). MS $(EI)^+$ *m/z* calcd for $C_{10}H_{13}NO_3Na$ $[M+Na]^+$: 218.08, found 218.08.

4.2.2. *N*-(Benzyloxy)-3-hydroxy-*N*-methylpropanamide (6b)

To a suspension of compound **6a** (552 mg, 2.83 mmol), anhydrous K_2CO_3 (611 mg, 4.42 mmol) and anhydrous acetone (22 mL) was added iodomethane (0.90 mL, 14.5 mmol). The resulting mixture was refluxed overnight. The suspension was filtered and acetone was

removed under reduced pressure. The resulting oil was dissolved in diethyl ether (25 mL), washed with water (25 mL) and with a 10 % aqueous solution of $Na_2S_2O_3$ (2 x 25 mL). The aqueous layer was saturated with NaCl and extracted with diethyl ether (2 x 25 mL). The organic layers were collected, dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (EtOAc/petroleum ether 7:3 → EtOAc) to give **6b** as a yellow oil (480 mg, 81 %) as a sole *E* conformer. Rf = 0.43 (EtOAc/petroleum ether, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 2.62 (2H, t, ^3J = 5.2 Hz, CH_2CO), 3.05 (1H, t, ^3J = 6.0 Hz, OH), 3.22 (3H, s, NCH_3), 3.82 (2H, q, ^3J = 5.7 Hz, CH_2OH), 4.84 (2H, s, OCH_2Ph), 7.38 (5H, m, CH_{Ar}).^{13}C NMR (125.8 MHz, $CDCl_3$): δ(ppm) = 33.4 (NCH3), 34.4 (CH_2CO), 58.5 (CH_2OH), 76.5 (OCH_2Ph), 128.9 (CH_{Ar}), 129.3 (CH_{Ar}), 129.5 (CH_{Ar}), 134.3 (C_{Ar}), 174.8 (CO). MS (EI)$^+$: *m/z* calcd for $C_{11}H_{15}NO_3Na$ $[M+Na]^+$: 232.09, found 232.09.

4.2.3. General procedure for phosphorylation of the alcohol group

To a solution of alcohol (1 equiv.) in dry DCM (48 mL/mmol) was successively added 1*H*-tetrazole (0.45 M, 2 equiv.) and dibenzyl *N*,*N*-diisopropylphosphoramidite (1.8 equiv.). After the total consumption of the starting material (TLC), the reaction mixture was cooled at 0°C then treated with 3-chloroperbenzoic acid (2.2 equiv.), which was slowly added as solid in small portions. The reaction mixture was warmed to room temperature and stirred overnight. DCM was added and the organic layer was washed with 10 % aqueous solution of $Na_2S_2O_5$, saturated aqueous solution of $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography.

4.2.3.1. Dibenzyl (3-((benzyloxy)amino)-3-oxopropyl) phosphate (7a). After purification by flash chromatography (EtOAc/petroleum ether 9:1 to EtOAc) the product was obtained as a colorless oil (450 mg, 60 %) and as a mixture of two conformers *Z* and *E* in a 8:2 ratio respectively. Rf = 0.43 (EtOAc/petroleum ether, 9:1) ^{1}H NMR (500 MHz, $CDCl_3$): δ(ppm) = 2.32 (8/10 of 2H, bs, CH_2CO), 2.70 (2/10 of 2H, bs, , CH_2CO), 4.25 (2H, bs, CH_2OP), 4.86 (2H, bs, OCH_2Ph), 4.99-5.06 (4H, m, $POCH_2Ph$), 7.33-7.35 (15H, m, CH_{Ar}), 7.72 (2/10 of 1H, bs, NH), 8.342 (8/10 of 1H, bs, NH). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ(ppm) = 34.7 (CH_2CO), 62.9 (CH_2OP), 63.9 (CH_2OP), 69.5 ($POCH_2Ph$), 69.7 ($POCH_2Ph$), 78.4 (OCH_2Ph), 79.6 (OCH_2Ph), 128.3 (CH_{Ar}), 128.8 (CH_{Ar}), 129.4 (CH_{Ar}), 135.4 (C_{Ar}), 135.7 (C_{Ar}), 167.5 (CO).^{31}P NMR (121.5 MHz, $CDCl_3$): δ(ppm) = -1.07, -1.13 HR MS (EI)$^+$: *m/* calcd for $C_{24}H_{26}NPO_6Na$ $[M+Na]^+$: 478.1390, found 478.1339.

4.2.3.2. Dibenzyl (3-((benzyloxy)(methyl)amino)-3-oxopropyl) phosphate (7b). After purification by flash chromatography (EtOAc/petroleum ether, 5:5 to EtOAc/Petroleum ether 8:2) the product was obtained as a colorless oil (666 mg, 85 %) as the sole conformer *E*. Rf = 0.35 (EtOAc/petroleum ethe,r 5:5).^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) = 2.73 (2H, t, ^{3}J = 6.6 Hz, CH_2CO), 3.18 (3H, s, NCH_3), 4.31 (2H, td, ^{3}J = 6.7 Hz, ^{3}J = 6.7 Hz, CH_2OP), 4.78 (2H, s, OCH_2Ph), 5.03 (2H, dd, ^{2}J = 12.4 Hz, $^{3}J_{P\text{-}H}$ = 7.9 Hz, $POCH_2Ph$), 5.06 (2H, dd, ^{2}J = 12.4 Hz, $^{3}J_{P\text{-}H}$ = 7.9 Hz, $POCH_2Ph$), 7.31-738 (15H, m, CH_{Ar}).^{13}C NMR (125.8 MHz, $CDCl_3$): δ(ppm) = 33.1 (d, ^{3}J = 6.5 Hz, CH_2CO), 33.6 (NCH_3), 63.5 (d, ^{2}J = 5.3 Hz, CH_2OP), 69.4 (d, ^{2}J = 5.3 Hz, $POCH_2Ph$), 76.6 (OCH_2Ph), 128.2 (CH_{Ar}), 128.6 (CH_{Ar}), 128.7 (CH_{Ar}), 128.9 (CH_{Ar}), 129.3 (CH_{Ar}), 129.5 (CH_{Ar}), 134.4 (C_{Ar}), 136.0 (d, ^{3}J = 7.0 Hz, $C_{Ar}CH_2OP$), 171.6 (CO).^{31}P NMR (121.5 MHz, $CDCl_3$): δ (ppm) = -1.18 HR MS (EI)$^{+}$: *m/z* calcd for $C_{25}H_{28}NPO_6Na$ $[M+Na]^{+}$: 492.1546, found 492.1522.

4.2.4. General procedure for catalytic hydrogenolysis:

The protected alcohol (1 equiv.), palladium on charcoal (10 % mol) in absolute ethanol (22 mL/mmol) was hydrogenolyzed at room temperature and at atmospheric pressure under an atmosphere of H_2. After the total consumption of starting material (TLC) the catalyst was removed by filtration over celite and the reaction mixture was evaporated to dryness to give the desired compound without purification.

4.2.4.1. 3-(Hydroxyamino)-3-oxopropyl dihydrogen phosphate (3b). The product was obtained without purification, as colorless oil, (19 mg, 99 %) and as a sole conformer *Z*. ^{1}H NMR (500 MHz, CD_3OD): δ(ppm) = 2.45 (2H, t, ^{3}J = 5.7 Hz, CH_2CO), 4.21 (2H, td, ^{3}J = 5.9 Hz, ^{3}J = 5.9 Hz, CH_2OP). ^{13}C NMR (125.8 MHz, CD_3eOD): δ(ppm) = 35.2 (d, ^{3}J = 7.8 Hz, CH_2CO), 63.6 (d, ^{2}J = 4.2 Hz, CH_2OP), 169.8 (CO). ^{31}P NMR (121.5 MHz, CD_3OD): δ(ppm) = 0.0. HR MS (EI)$^{+}$: *m/z* calcd for $C_3H_7NPO_6$ $[M-H]^{+}$: 184.0005, found 184.0011.

4.2.4.2. 3-(Hydroxy(methyl)amino)-3-oxopropyl dihydrogen phosphate (4b). The product was obtained without purification, as colorless oil, (11.5 mg, 56 %) and as a sole conformer *E*. ^{1}H NMR (500 MHz, CD_3OD): δ(ppm) = 2.87 (2H, t, ^{3}J = 6.2 Hz, CH_2CO), 3.21 (3H, s, NCH_3), 4.23 (2H, td, ^{3}J = 6.2 Hz, ^{3}J = 6.1 Hz, CH_2OP). ^{13}C NMR (125.8 MHz, CD_3OD): δ (ppm) = 34.4 (d, ^{3}J = 7.0 Hz, CH_2CO), 36.3 (NCH_3), 63.5 (d, ^{2}J = 5.2 Hz, CH_2OP), 172.8

(CO). ^{31}P NMR (121.5 MHz, CD_3OD): δ (ppm) = 0.24. HR MS $(EI)^+$: *m/z* calcd for $C_4H_9NPO_6$ $[M-H]^+$: 198.0162, found 198.0169.

4.3. His-tagged DXR activity[11]

The assays were performed at 37°C in a 50 mM Tris/HCl buffer pH 7.5 containing 3 mM $MgCl_2$ and 2 mM DTT. The concentrations of DXP and NADPH were 480 µM and 160 µM respectively. The decrease of absorbance at 340 nm due to NADPH oxidation was monitored to determine the initial rates. The retained values were the average of at least two measurements. The relative average deviation must be lower than 4%.

4.4. Inhibition of His-tagged DXR

H-DXR was pre-incubated during 2 min in the presence of the inhibitors (**1**-**4**) at different concentrations and NADPH. DXP was then added to measure the residual activity. The inhibitory potential of the tested compounds was quantified by determining the IC_{50} values. They were obtained by plotting the percentage of residual activity versus the Log of inhibitor concentration.

4.5. Bacterial growth inhibition

The antimicrobial activity of each inhibitor of H-DXR was tested by the paper disc diffusion method on *Escherichia coli* XL1 Blue growing in a LB-agar medium. The paper discs (6 mm) were impregnated with the different compounds at indicated amount. The Petri dishes were incubated overnight at 37°C.

4.6. Sequence of hexose 6-phosphate transporter gene (*UhpT*) of *E. coli* XL1Blue WT and FosR[37]

4.6.1. Genomic DNA preparation. Two overnight cultures (3 mL) of *E. coli* XL1-Blue WT and FosR were centrifuged in 1.5 mL Eppendorf tubes. The pellets were washed with autoclaved H_2O (100 µL). After centrifugation and elimination of the supernatants, the pellets were re-suspended in H_2O (100 µL) and the suspensions were heated for 4 min in a boiling water bath. After centrifugation the supernatants containing the genomic DNA were taken off and stored -20°C until PCR processing.

4.6.2. PCR and sequence determination. The amplification of whole and partial *UhpT* gene was performed by PCR using the High Fidelity PCR Master Kit (Roche) and several combinations of primers (F1-R1, F1-R2, F2-R1). The list of primers is given in Table S1. An aliquot of the DNA solution was diluted 50 fold and 2 to 6 µL of that dilution/50 µL of PCR reaction mixture was tested for amplification. The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, primer annealing at 57 °C for 1 min, and primer extension at 68 °C for 1 min. The reaction mixture was held at 68 °C for 7 min and then cooled to 4 °C. The PCR fragments were purified after agarose electrophoresis using the NucleoSpin clean-up kit from Macherey-Nagel (Germany). Their sequences were determined by the IBMP DNA sequencing platform (Strasbourg, France).

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Supplementary data

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