European Journal of Medicinal Chemistry 51 (2012) 277–285

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Growth inhibition of *Mycobacterium smegmatis* by prodrugs of deoxyxylulose phosphate reducto-isomerase inhibitors, promising anti-mycobacterial agents

Sarah Ponaire, Catherine Zinglé, Denis Tritsch, Catherine Grosdemange-Billiard*, Michel Rohmer*

Université de Strasbourg/CNRS, UMR 7177, Institut Le Bel, 4 rue Blaise Pascal, 67081 Strasbourg, France

ARTICLE INFO

Article history:
Received 2 February 2012
Received in revised form
15 February 2012
Accepted 16 February 2012
Available online 25 February 2012

Keywords:
Isoprenoid biosynthesis
Prodrug synthesis
Deoxyxylulose 5-phosphate reducto-isomerase
Mycobacterium

ABSTRACT

Since *Mycobacterium tuberculosis* sets up several multiple anti-tuberculosis drug resistance mechanisms, development of new drugs with innovative target is urgent. The methylerythritol phosphate pathway (MEP) involved in the biosynthesis of essential metabolites for the survival of mycobacteria, represents such a target. Fosmidomycin **1a** and FR900098 **1b**, two inhibitors of DXR, do not affect the viability of *M. tuberculosis* cells, due to a lack of uptake. To overcome the absence of the mycobacterial cell wall crossing of these compounds, we synthesized and tested the inhibition potency of acyloxymethyl phosphonate esters as prodrugs of fosmidomycin **1a**, FR900098 **1b** and their analogs **2a** and **2b** on *Mycobacterium smegmatis*. Only the prodrugs **4b**–**6b** inhibit the bacterial growth and could be effective anti-mycobacterial agents.

1. Introduction

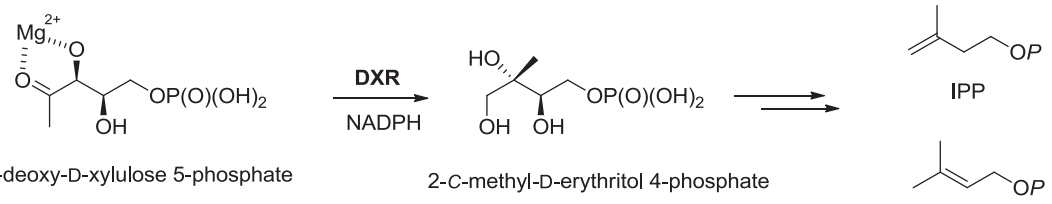
Tuberculosis is one of the major infectious diseases: around nine million new cases are annually estimated with approaching two million human deaths [1,2]. *Mycobacterium tuberculosis*, responsible for tuberculosis, owing to its unique cell wall organization, can be considered as a fortress. The development of new drugs to fight this bacterium is a difficult challenge. The first problem encountered by anti-bacterial agents is the crossing of the cell wall. In addition, this *Mycobacterium* sets up several mechanisms of resistance to major anti-tuberculosis drugs [3]. As the continuing increase of tuberculosis risk is in large part due to the development of antibiotic resistant strains, it is urgent to find other targets for the development of new anti-tuberculosis drugs than those presently used. Isoprenoid biosynthesis in *Mycobacterium* species represents such a target in this context. Isoprenoids are found in all living organisms. A number of them including bactoprenyl diphosphate required for the biosynthesis of peptidoglycan, a major cell wall component [4], and the prenyl side-chains of menaquinones involved in electron transport chains is present in mycobacteria and represents essential metabolites for their survival [5]. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoids are synthesized *via* two pathways: the mevalonate pathway, which is present in animals and humans, fungi and the cytosol of plant cells [6], and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, which has been found in plant plastids, unicellular green algae, apicomplexan parasites and in most bacteria including many pathogens such as *Mycobacterium* spp [7].

The determination of the nucleotide sequences of the genomic DNA of *Mycobacterium* spp. confirmed the presence of the sole MEP pathway in this genus of bacteria. Accordingly, all enzymes of the MEP pathway can be considered as potential targets for anti-tuberculosis drug development [8]. The knowledge of the DNA sequence also allows obtaining and studying the recombinant enzymes of this pathway.

The 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (DXR), the second enzyme of the MEP pathway [9] (Scheme 1) is inhibited by fosmidomycin (**1a**) and FR900098 (**1b**), two natural antibiotics [10], (Scheme 2) which consequently block the growth of many bacteria possessing the MEP pathway. Owing to the rapid emergence of resistance observed in the case of fosmidomycin and the fast elimination of the antibiotic in the urine [11], several groups attempted to synthesize other DXR inhibitors with improved pharmacokinetic and pharmacodynamic properties. The structures of these new molecules are all inspired from those of the antibiotics **1a** and **1b**, possessing on the one hand a phosphonate or another

* Corresponding authors.
E-mail addresses: grosdemange@unistra.fr (C. Grosdemange-Billiard), mirohmer@unistra.fr (M. Rohmer).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2012.02.031



Scheme 1. Biosynthesis of IPP and DMAPP. Second step of MEP pathway: conversion of 1-deoxy-D-xylulose 5-phosphate into 4-methyl-D-erythritol 4-phosphate by the deoxyxylulose phosphate reducto-isomerase.

isosteric moiety fitting into the phosphate-recognition site and on the other side a chelating group tightly binding the metal cation required for enzyme activity [12].

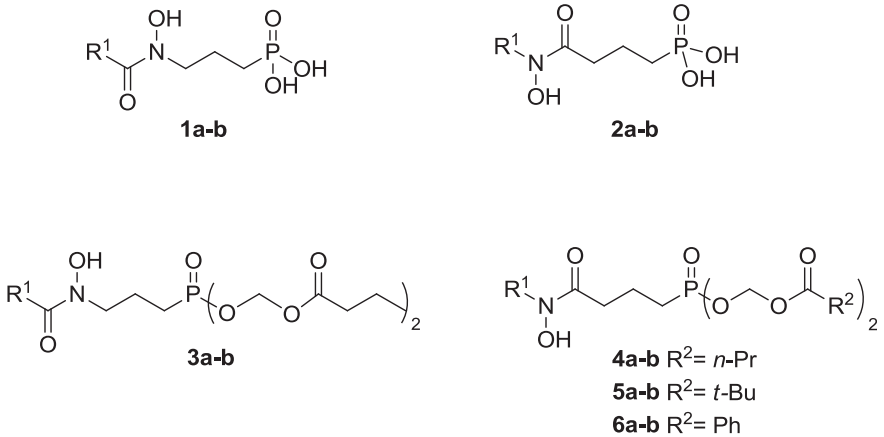
In this context, we synthesized and tested on the DXR of *Escherichia coli* two compounds (Scheme 2, **2a** and **2b**) where the metal cation-binding moiety is a hydroxamic acid group. Compound **2b** inhibits the *E. coli* DXR much like fosmidomycin and has significant anti-bacterial activity against wild type *E. coli* and even against an *E. coli* mutant resistant to fosmidomycin [13]. In the case of recombinant *M. tuberculosis* DXR, fosmidomycin **1a** and FR900098 **1b** were shown to inhibit the enzyme. The two published IC_{50} values differed, probably depending on different experimental conditions: 80 nM and 310 nM for **1a** [11,14] and 160 nM for **1b** [15]. Fosmidomycin does not inhibit the growth or affect the viability of *M. tuberculosis* cells. This lack of effect was interpreted in terms of a lack of uptake of the compound by the bacterium [16]. This fact is not too surprising. The hydrophobic cell wall of mycobacteria is an efficient barrier that prevents passive transport and makes the bacteria naturally resistant to most antibiotics. Mycobacteria cells are surrounded by a hydrophobic, waxy cell wall. Accordingly, hydrophilic compounds cross slowly this cell wall, probably *via* porins that are rare in mycobacteria, whereas lipophilic molecules penetrate by diffusion through the cell wall [17]. For that reason, anti-bacterial agents with lipophilic characteristics are more active against mycobacteria. In this context, less polar FR900098 analogs in which either the phosphonate or the hydroxamate moiety have been replaced by alternative acidic or metal coordinating groups have been prepared, but had no effect on *M. tuberculosis* growth [15,18].

A way to overcome the absence of mycobacterial cell wall crossing of fosmidomycin and its analogs is to mask the polar phosphonate moiety with hydrophobic groups. Owing to their hydrophobic character, such prodrugs may penetrate in the bacterial cells and, after enzymic conversion into the parent drug in the cells, stop the bacterial growth by inhibition of the DXR. The fact that such prodrugs of FR900098 are able to enhance *in vivo* and *in vitro* anti-malarial activity strengthens the relevance of this approach [19]. In this work, we synthesized and tested the inhibition potency of acyloxymethyl phosphonate esters as prodrugs of fosmidomycin, FR900098 and their analogs **2a** and **2b** on the non-pathogenic, fast growing *Mycobacterium smegmatis*. It is expected that the parent compounds are released from such prodrugs intracellularly in two steps: the hydrolysis of the acyl group by an esterase is followed by a rapid and spontaneous hydrolysis of the resulting hemiacetal into the free inhibitor and formaldehyde [20]. Finally, the DXR of *M. smegmatis* was cloned to check whether this enzyme is inhibited by fosmidomycin and its analogs. Such lipophilic fosmidomycin and FR900098 prodrugs have been recently described and tested as anti-bacterial agents [21].

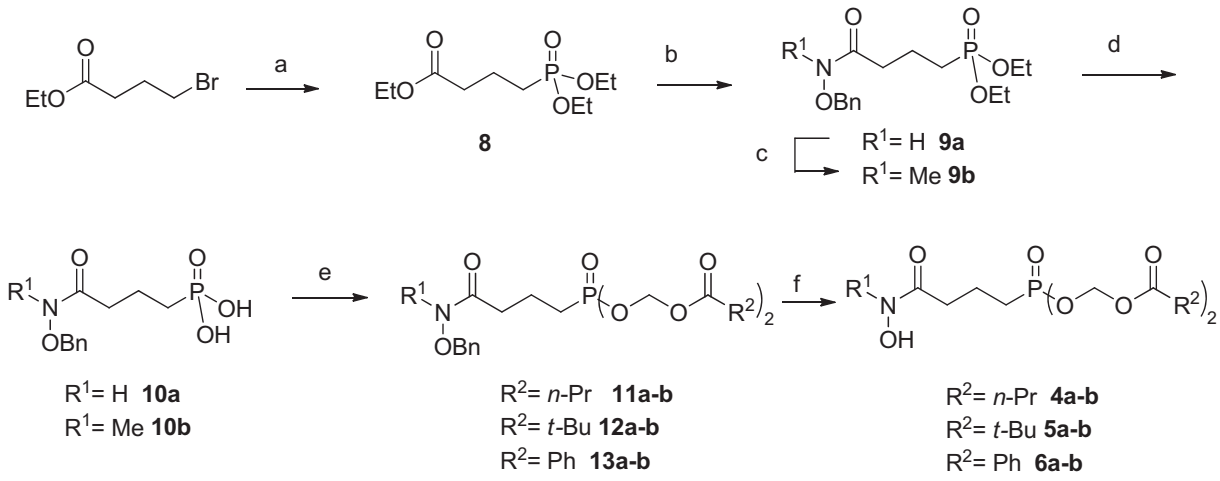
2. Results

2.1. Synthesis of the prodrugs

The propyloxymethyl ester prodrugs **3a** and **3b** of fosmidomycin (**1a**) and FR900098 (**1b**) were synthesized according to a previously described method [19,22]. The synthesis of the prodrugs **4**–**6** was achieved starting from the phosphonate **8**, which is readily accessible by nucleophilic substitution of the commercially available ethyl 4-bromobutyrate with the NaH generated anion of diethyl phosphite (Scheme 3) [12]. The *O*-benzyl hydroxamate derivative **9a** was obtained in one step from **8** using *O*-benzylhydroxylamine hydrochloride in presence of LiHMDS as described by Gissot et al. [23]. The methyl group was introduced by reaction of **9a** with NaH followed by addition of methyl iodide. Deprotection of the phosphonate group using bromotrimethylsilane led to the acid **10** and subsequent alkylation of the crude phosphonic acids with the appropriate chloromethyl esters.



Scheme 2. Chemical structures of the tested compounds. Fosmidomycin **1a**, FR900098 **1b**, phosphono-hydroxamic acids **2a** and **2b** and their acyloxymethyl phosphonate esters prodrugs **3**, **4**, **5** and **6** (**a**: $R^1 = H$; **b**: $R^1 = CH_3$).



Scheme 3. Synthesis of prodrugs **4**, **5** and **6** (**a**: $R^1 = H$; **b**: $R^1 = CH_3$). Reagents and conditions. a) $(EtO)_2P(O)H$, NaH, THF, 84%; b) $BnONH_2 \cdot HCl$, LiHMDS, THF, −78 °C, 69%; c) MeI, NaH, THF, 85%; d) 1) TMSBr, CH_2Cl_2, 0 °C; 2) H_2O, THF; e) chloroacyloxyalkyl/arylester, Et_3N, DMF, 70 °C, **11a** (26%), **11b** (45%), **12** (21%), **12b** (39%), **13a** (35%), **13b** (40%); f) H_2, Pd/C, EtOH, **4a** (95%), **4b** (94%), **5a** (94%), **5b** (92%), **6a** (96%), **6b** (93%).

Benzyl deprotection was performed by catalytic hydrogenolysis with palladium over charcoal at atmospheric pressure and room temperature. Prodrugs **4**–**6** were obtained from the *O*-benzylated precursors **11**–**13** in quantitative yields and required no further purification.

2.2. *M. smegmatis* DXR sequence

The amino acid sequence obtained from the nucleotide sequence of the DXR from *M. smegmatis* DSM43756 (ATCC 43756) was compared to the known sequence of the enzyme from *M. smegmatis* ATCC 700084 (Swiss-Prot entry A0QVH7) (Fig. S1 in supporting information). Two mutations were detected: Ala-282 and Asp-310 (numbering of DXR from *M. smegmatis* MC2 155 ATCC 700084) were respectively replaced by Pro and Glu. All sequenced clones had the two mutations, indicating that they are effective in the presently investigated strain. The sequence identity with the DXR from *M. tuberculosis* (Swiss-Prot entry P64012) is around 76%.

2.3. Kinetic properties of recombinant His-tagged DXR of *M. smegmatis*

As observed by others in the case of DXR from *M. tuberculosis*, the enzyme of *M. smegmatis* loses its activity during the dialysis step when glycerol and NaCl are omitted in the incubation buffer. The K_m for DXP of the *M. smegmatis* DXR, determined with $MgCl_2$ as metal cofactor, is 212 μM, about two fold higher than that found for the His-tagged DXR from *E. coli* [13]. Different values of K_m for the DXR of *M. tuberculosis* are reported in the literature: 42 μM [24] for the native enzyme, 47 μM [11] and 340 μM [25] for His-tagged enzymes. The discrepancy may be due to the assay conditions and/or to the instability of the enzyme.

As fosmidomycin and its derivatives are slow-binding inhibitors [13,26], the residual activities were measured after a pre-incubation of the DXR in presence of the inhibitors during 2 min. The enzymatic reaction was initiated by addition of DXP.

The IC_{50} values (mean values from two independent determinations) were determined by plotting the residual activity versus the Log of the concentration of inhibitor. Initial rates of the enzymatic reaction were used to determine the IC_{50} values for fosmidomycin, FR900098 and inhibitors **2a** and **2b** (Table 1). As shown with His-tagged DXR of *E. coli* [13], fosmidomycin and compound **2b** inhibit the DXR of *M. smegmatis* at about the same level. FR900098 seems to be a little more efficient while **2a**, as observed with the enzyme of *E. coli*, is substantially less effective. The comparison of IC_{50} values is delicate as they depend on the experimental conditions: concentration of the substrates, pre-incubation or not of the inhibitor with the enzyme, nature of the cation (Mg^{2+} or Mn^{2+}), use of His-tagged enzyme or not. In spite of these restrictions, the DXR of *M. smegmatis* seems to have less affinity for the tested inhibitors than the *E. coli* enzyme as the IC_{50} values are about tenfold higher than those reported for the *E. coli* DXR. Concerning the DXR of *M. tuberculosis*, IC_{50} values of 80 nM [11] and 310 nM [14] are given for fosmidomycin. Compounds **3b** and **4b** were tested on DXR of *E. coli* and *M. smegmatis* but no inhibition was found for a 1 μM concentration.

2.4. Growth inhibition of *M. smegmatis* with prodrugs (**3**–**6**)

Growth inhibition of *M. smegmatis* induced by the synthesized prodrugs (**3**–**6**) was evaluated by the paper disc diffusion method. Isoniazid is an effective anti-tuberculosis agent inhibiting the biosynthesis of mycolic acids, which are essential component of the mycobacterial cell wall. It is considered as a prodrug that requires activation by an endogenous catalase peroxidase for inhibiting the enoyl–acyl carrier protein [27]. This anti-bacterial agent was accordingly utilized as reference compound for a positive *M. smegmatis* growth inhibition test.

As previously mentioned in the case of *M. tuberculosis* [10,14,15] the free DXR inhibitors fosmidomycin **1a** and FR900098 **1b** do not block the growth of *M. smegmatis*. The same behavior was observed in the presence of the synthetic DXR inhibitors **2a** and **2b**.

Table 1
Inhibition of *Mycobacterium smegmatis* DXR. Assays were performed in 50 mM Tris/HCl buffer (pH 7.5) containing 3 mM $MgCl_2$ and 2 mM DTT. The concentrations of NADPH and DXP were 0.15 and 0.5 mM respectively. The enzymatic reaction was initiated by adding DXP after pre-incubation of the enzyme with the inhibitor in the presence of NADPH for 2 min at 37 °C.

Inhibitors	IC_{50} (nM)
Fosmidomycin **1a**	510 ± 30
FR900098 **1b**	320 ± 20
2a	1480 ± 40
2b	410 ± 30

All these DXR inhibitors are most probably unable to cross the cell wall and were therefore converted into less polar and more lipophilic acyloxymethyl phosphonate esters **3**–**6**. The first attempts were performed with the **2a** and **2b** derivatives **4**–**6**. None of the prodrugs **4a**, **5a** and **6a** based on the non *N*-methylated inhibitor **2a** inhibited the bacterial growth when 400 nmol were deposed on the disc. In contrast, in the presence of the *N*-methylated prodrugs **4b**, **5b** and **6b** of inhibitor **2b**, clear growth inhibition zones were observed around the impregnated paper discs (Fig. S2 in supporting information). Like isoniazid, these compounds are able to inhibit the bacterial growth. The diameters of the growth inhibition zones depended on the nature of the phosphonate masking acyl chains (Table 2).

The *n*-propyloxymethyl esters prodrug **4b** being the most effective, the similar prodrugs of fosmidomycin and FR900098 bearing the same *n*-propyl containing masking group, **3a** and **3b** respectively, were synthesized. Surprisingly, neither the prodrug of fosmidomycin **3a**, nor that of FR900098 **3b** blocked the growth of *M. smegmatis* while fosmidomycin **1a** and FR900098 **1b** inhibited like the hydroxamate **2b** the DXR of this bacterium (Table 1).

2.5. Resazurin viability test

Bacterial viability can be estimated by a colorimetric resazurin test performed in a liquid medium [28]. A color change from blue to pink indicates reduction of resazurin and is correlated with bacterial growth and viability of the cells. After an incubation of 15 min, a culture containing no inhibitor turned already pink. In the presence of isoniazid at a 10 μM concentration, the color became pink in less than 1 h. At a 20 μM concentration and above, the blue color lasted at least 5 h, but the color of the culture became after 12 h purplish, suggesting that some bacterial cells were perhaps still surviving. In the presence of the *n*-propyl prodrug **4b**, a similar phenomenon was observed. A 250 μM concentration of the prodrug had little influence on the growth of the bacteria, and the color changed to pink within 15 min. From a minimal 500 μM concentration, the growth is strongly inhibited. The color remained blue for at least 5 h and tended after to become also slowly purplish. The resazurin test confirms thus that the prodrug **4b** is capable of inhibiting the growth of *M. smegmatis*. It is, however, less efficient than isoniazid on the growth of *M. smegmatis* as the minimal inhibitory concentration (MIC) is between 250 and 500 μM for **4b** in contrast with the value between 10 and 20 μM (or 1.4–2.8 μg/mL) for isoniazid, i.e. close to the value reported in the literature for *M. tuberculosis* (7 ± 2 μg/mL) [29].

3. Discussion

Fosmidomycin **1a**, FR900098 **1b** and the phosphonohydroxamate **2b** are efficient inhibitors of the DXR of mycobacteria. They do not interfere with the growth of *M. tuberculosis* for the first two ones [17] and of *M. smegmatis* for all three inhibitors, whereas they easily inhibit the growth of the Gram-negative *E. coli* [13]. Fosmidomycin is carried into the *E. coli* cell *via* the transport system of glycerophosphate and at a lower level of glucose 6-phosphate [30]. In the absence of such transporters in mycobacteria, none of the DXR inhibitors is capable of penetrating into the cell or at a too low concentration. A fosmidomycin resistant *E. coli* strain was obtained by mutation of the glycerophosphate transporter. It remained, however, sensitive to the DXR inhibitor **2b**, suggesting that this compound may be transported into the cell *via* another transporter (A. Hemmerlin, D. Tritsch, unpublished results). The mycobacterial cell wall is an extremely efficient impermeable barrier protecting the cell from passive diffusion of toxic compounds such as antibiotics. It is responsible for the natural resistance of mycobacteria toward most common antibiotics and chemotherapeutic agents [31]. Small hydrophilic compounds diffuse through porins, which are water-filled protein channels [32]. The permeation of hydrophilic compounds is low due to the limited number of porins in the mycobacterial outer membrane and to their longer size as compared to those of other bacteria. A hydrophilic free DXR inhibitor is thus probably unable to penetrate into the cell through those porins or only at such low rates that its intracellular concentration is too low to efficiently inhibit the DXR.

This permeation problem encountered with small hydrophilic enzyme inhibitors may be overcome by converting them into lipophilic prodrugs. Acyloxymethyl phosphonate esters represent interesting candidates. Once they have penetrated into the cells, hydrolysis of the ester group of the phosphonate masking group by an endogenous esterase releases a hemiketal, which cleaves spontaneously into the free DXR inhibitor and formaldehyde [20]. This concept is validated. The *N*-methylated prodrugs **4b**, **5b** and **6b** induced significant growth inhibition of *M. smegmatis*, with the prodrug **4b** with an *n*-propyl chain in the masking group being by far the most powerful growth inhibitor. The prodrugs **5b** and **6b** with respectively *t*-butyl and a phenyl group showed some growth inhibition on a *M. tuberculosis* strain with minimal inhibiting concentration in the 50–100 μg/mL and 25–100 μg/mL range [23]. Although rich in lipids, the mycobacterial cell wall represents also a strong barrier for hydrophobic molecules. For penetration, such molecules are expected to cross the cell wall through their lipid domain, but the influx is limited by the low fluidity of the lipid phase due to the presence of mycolic acids [33]. This low fluidity of the lipid domain may explain the lower efficacy of prodrugs **5b** and **6b** with a bulky pivaloyl or a rigid phenyl group as compared to compound **4b**, which presents with its *n*-propyl chain the smallest and most flexible phosphonate masking group.

The negative results obtained on the growth of *M. smegmatis* with all other prodrugs are quite puzzling. The rather similar non-methylated analogs **4a**, **5a** and **6a** do not inhibit the growth of this bacterium at the highest tested dose (400 nmol/disk). Due to the structural similarity of the non-methylated prodrugs **4a**, **5a** and **6a** and their *N*-methylated homologs **4b**, **5b** and **6b**, it is likely that the non-methylated prodrugs penetrate in the bacterial cells and are converted into the corresponding DXR inhibitor **2a**. The absence of growth inhibition with the non *N*-methylated compounds may result from a too low intracellular concentration of the DXR inhibitor 2**a**, which is in addition three to four fold less effective than its *N*-methylated homolog **2b**, to efficiently inhibit the DXR and accordingly the bacterial growth. Surprisingly, the fosmidomycin and FR900098 prodrugs **3a** and **3b** had also no effect on the growth of *M. smegmatis* at the highest tested dose (400 nmol/disk). This is in sharp contrast with the anti-bacterial activity of prodrug **2b** against *M. smegmatis*. The explanation for this discrepancy is not obvious as solubility and diffusion are expected to be rather similar for all prodrugs. The absence of growth inhibition may be ascribed to a lack of penetration of compounds **3a** and **3b**. In this case, the prodrugs may not cross the *M. smegmatis* cell wall by passive

Table 2
Growth inhibition of *Mycobacterium smegmatis*. The antimicrobial activity of isoniazid (INH) and DXR inhibitor prodrugs **4b**, **5b** and **6b** was determined using the paper disc diffusion method (see Fig. S2).

Tested compounds	Amount of prodrug/disc (nmol)	Diameter of the growth inhibition zone (mm)
Isoniazid	15	29
4b	400	23
5b	400	15
6b	400	11

diffusion, a more or less specific transporter allowing the influx of prodrugs **4b**, **5b** and **6b**. Another explanation could be that the fosmidomycin and FR900098 prodrugs or their parent compounds are expelled outside the cell by efflux pumps. Finally, the hypothesis that fosmidomycin and FR900098, generated inside the cell, are inactivated by the bacteria cannot be set aside [34]. At this stage it cannot even be excluded that the free DXR inhibitor **2b** has also another target than the DXR, the inhibition of which would lead to the arrest of bacterial growth.

When the prodrugs were tested on *E. coli*, none of them had a noticeable anti-bacterial activity, even at the highest tested amount (400 nmol/disk). Either the compounds most probably do not cross the cell wall of this bacterium, or *E. coli* does not have the esterase required to cleave the ester bond in the phosphonate masking groups of the prodrugs.

Small hydrophilic DXR inhibitors do not affect the growth of *M. smegmatis*. This problem was overcome with at least one of these inhibitors **2b**, which presents a reverse hydroxamic moiety as compared to that of FR900098. Masking the polar phosphonate group with acyloxymethyl esters resulted in prodrugs that effectively inhibit the growth of *M. smegmatis*. This approach solves apparently the problem of the impermeability of the mycobacterial cell wall toward enzyme inhibitors and will be applied to the growth inhibition of *M. tuberculosis*.

4. Experimental section

All non-aqueous reactions were run in dry solvents under an argon atmosphere. All reagents and solvents were reagent grade. After solvent extraction of an aqueous phase, the organic layers were combined, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. DXP was obtained by chemical synthesis as previously described [35]. Fosmidomycin was obtained from Dr R. J. Eilers (Monsanto, St Louis, MO, U.S.A.). Isoniazid (INH) was purchased from Acros Organics. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh) with the solvent system as indicated. TLC plates were revealed by spraying with an ethanolic solution of *p*-anisaldehyde (2.5%), sulfuric acid (3.5%) and acetic acid (1.6%) or with an ethanolic solution of phosphomolybdic acid (20%) followed by heating. ^{1}H NMR experiments were performed in $CDCl_3$, CD_3OD, D_2O or DMSO-d_6 with $CHCl_3$ (δ = 7.26 ppm), DHO (δ = 4.65 ppm), CHD_2OD (δ = 3.31 ppm), CHD_2SOCD_3 (δ = 2.50 ppm) as internal reference, ^{13}C NMR in $CDCl_3$, CD_3OD, D_2O or DMSO-d_6 with $CDCl_3$ (δ = 77.0 ppm), CD_3OD (δ = 49.0 ppm), DMSO-d_6 (δ = 39.5 ppm) as internal reference, and ^{31}P NMR in $CDCl_3$, CD_3OD, DMSO-d_6 with H_3PO_4 (δ = 0 ppm) as external reference. Most of the hydroxamates are present as two *Z* and *E* conformers in equilibrium. If only one signal is described, it is common to all conformers. The evaluation of the relative amount of the conformers was made by integration of the *N*-CH_3 and/or the CH_2–CON proton signals.

4.1. Synthesis of prodrugs

4.1.1. Ethyl 4-(diethoxyphosphoryl)butanoate (**8**)

Sodium hydride (575 mg, 24 mmol) was added to a solution of diethyl phosphite (3 g, 22 mmol) in THF (100 mL) at 0 °C. The reaction mixture was stirred for 20 min at room temperature. A solution of ethyl bromobutyrate (4.70 mL, 33 mmol) was added dropwise and the reaction mixture was stirred for 48 h. The reaction was quenched with a saturated solution of NH_4Cl and extracted with diethyl ether (3×15 mL). The solvent was removed under reduce pressure. The residue was purified by flash chromatography (EtOAc/petroleum ether, 7:3) affording (**8**) as a colorless oil (4.6 g, 18 mmol, 84%, R_f = 0.12, EtOAc/petroleum ether, 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.19 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 1,26 (t, 6H, J = 7.1 Hz, $POCH_2CH_3$), 1.69–1.89 (m, 4H, PCH_2CH_2), 2.33 (t, 2H, J = 7.2 Hz, CH_2CO), 4.04 (m, 6H, OCH_2, $POCH_2$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 14.2, 16.3 (d, J = 6.0 Hz), 18.1 (d, J = 4.3 Hz), 24.9 (d, J = 141.6 Hz), 34.4 (d, J = 15.5 Hz), 60.4, 61.4 (d, J = 6.4 Hz), 172.5; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 32.3; HRMS (EI$^+$) *m*/*z* calcd for $C_{10}H_{21}O_5PNa$ [M + Na]$^+$ 275.1019, found 275.1025.

4.1.2. Diethyl 4-(benzyloxyamino)-4-oxobutylphosphonate (**9a**)

A 1 M solution of LiHMDS in THF (14 mL, 13.9 mmol) was added to a solution of *O*-benzylhydroxylamine hydrochloride (2.2 g, 14 mmol) in THF (20 mL) at 0 °C for 30 min. A solution of (**8**) (3.5 g, 13.9 mmol) in THF (10 mL) was then added dropwise to the cold reaction mixture that was allowed to warm up at room temperature and stirred overnight. The reaction was quenched with a saturated aqueous NH_4Cl solution and extracted several times with EtOAc. After evaporation to dryness of the combined organic layers, the residue was purified by flash chromatography (EtOAc) yielding (**9a**) as a pale yellow oil (3.0 g, 93.0 mmol, 65%, R_f = 0.30, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.28 (t, 6H, J = 7.0 Hz, CH_3), 1.66–1.98 (m, 4H, PCH_2CH_2), 2.20 (t, 2H, J = 6.6 Hz, CH_2CO), 3.94–4.13 (m, 4H, OCH_2CH_3), 4.84 (s, 2H, CH_2Ph), 7.35 (m, 5H, Ph), 9.12 (br s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.4 (d, J = 6.0 Hz), 18.4 (d, J = 4.5 Hz), 23.9 (d, J = 140.5 Hz), 32.6 (d, J = 12.1 Hz), 61.7 (d, J = 6.4 Hz), 78.1, 128.5, 128.6, 129.1, 135.3, 170.1; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 32.5; HRMS (EI$^+$) *m*/*z* calcd for $C_{15}H_{24}NO_5PNa$ [M + Na]$^+$ 352.1284, found 352.1263.

4.1.3. Diethyl 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (**9b**)

To a solution of (**9a**) (450 mg, 1.4 mmol) in THF (26 mL) were successively added at 0 °C sodium hydride (36 mg, 1.5 mmol) and methyl iodide (130 μL, 2.0 mmol). The mixture was stirred overnight at room temperature, quenched with a saturated solution of NH_4Cl and extracted several times with AcOEt. After evaporation to dryness, the residue was purified by flash chromatography (EtOAc) yielding (**9b**) as a pale brownish oil (400 mg, 1.2 mmol, 85%, R_f = 0.17, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21 (t, 3H, J = 7.5 Hz, CH_3), 1,60–1.90 (m, 4H, PCH_2CH_2), 2.40 (t, 2H, J = 6.5 Hz, CH_2CO), 3.10 (s, 3H, CH_3N), 3.90–4.07 (m, 4H, CH_2CH_3), 4.74 (s, 2H, CH_2Ph), 7.30 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.4 (d, J = 6.0 Hz), 17.6 (d, J = 4.7 Hz), 25.0 (d, J = 140.8 Hz), 32.3 (d, J = 15.9 Hz), 61.5 (d, J = 6.4 Hz), 76.2, 128.7, 129.0, 129.3, 134.5, 173.4; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 32.5; HRMS (EI$^+$) *m*/*z* calcd for $C_{16}H_{26}NO_5PNa$ [M + Na]$^+$: 366.144, found 366.139.

4.1.4. Diethyl 3-oxopropylphosphonate [**19b**]

A solution of diethyl(3,3-diethoxypropyl) phosphonate (1 g, 3.7 mmol) in an aqueous 2 M HCl solution (10 mL) was stirred at room temperature overnight. The reaction mixture was extracted several times with $CHCl_3$. After evaporation to dryness, the residue was purified by flash chromatography (EtOAc) affording diethyl 3-oxopropylphosphonate as a colorless oil (600 mg, 3.1 mmol, 83%, R_f = 0.15, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.29 (t, 3H, J = 7.5 Hz, CH_3), 1.95–2.06 (m, 2H, PCH_2), 2.70–2.79 (m, 2H, CH_2CO), 4.01–4.12 (m, 4H, CH_2CH_3), 9.76 (t, 1H, J = 1.2 Hz, CHO); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.4 (d, J = 6.0 Hz), 18.0 (d, J = 145.9 Hz), 36.9 (d, J = 4.1 Hz), 61.8 (d, J = 6.4 Hz), 199.2 (d, J = 15.3 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 31.9; HRMS (EI$^+$) *m*/*z* calcd for $C_7H_{15}O_4PNa$ [M + Na]$^+$ 217.0600, found 217.0577.

4.1.5. Diethyl 3-(benzyloxyamino)-propylphosphonate [**19b**]

To a solution of diethyl 3-oxopropylphosphonate (200 mg, 1.0 mmol) in MeOH (8 mL) were added at room temperature in one portion *O*-benzylhydroxylamine hydrochloride (8.2 g, 52 mmol)

and sodium cyanoborohydride (650 mg, 10 mmol). The reaction mixture was stirred overnight and then evaporated to dryness. The crude was dissolved in H_2O, the pH of the solution adjusted to pH = 10, and the solution extracted several times with CH_2Cl_2. After evaporation to dryness, the residue was purified by flash chromatography (EtOAc to EtOAc/MeOH 9:1) giving diethyl 3-(benzyloxyamino)-propylphosphonate as a colorless oil (225 mg, 0.75 mmol, 73%, R_f = 0.20 EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.20 (t, 3H, J = 7.5 Hz, CH_3), 1.61–1.80 (m, 4H, PCH_2CH_2), 2.84 (t, 2H, J = 7.5 Hz, CH_2NH), 3.91–4.02 (m, 4H, OCH_2CH_3), 4.57 (s, 2H, CH_2Ph), 5.14 (br s, 1H, NH), 7.21–7.23 (m, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.3 (d, J = 6.2 Hz), 20.4 (d, J = 5.0 Hz), 23.0 (d, J = 142.1 Hz), 51.8 (d, J = 16.2 Hz), 61.2 (d, J = 6.2 Hz), 76.0, 127.5, 127.6, 128.1, 137.7; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 33.3; HRMS (EI$^+$) m/z calcd for $C_{14}H_{25}NO_4P$ $[M + H]^+$ 302.1516, found 302.1486.

4.1.6. Diethyl 3-[(N-acetyl)(N-benzyloxy)amino]propylphosphonate [19b]

To a solution of diethyl 3-(benzyloxyamino)-propylphosphonate (200 mg, 0.7 mmol) in acetic anhydride (2.6 mL) was added dropwise pyridine (133 μL) at room temperature. The reaction mixture was stirred overnight and then evaporated to dryness. The residue was purified by flash chromatography (EtOAc to EtOAc/MeOH, 9:1) affording diethyl 3-[(*N*-acetyl)(*N*-benzyloxy)amino]propylphosphonate as a colorless oil (195 mg, 0.6 mmol, 86%, R_f = 0.16, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (t, 6H, J = 7.5 Hz, CH_3), 1.60–1.71 (m, 2H, $-CH_2-$), 1.80–1.96 (m, 2H, PCH_2), 2.02 (s, 3H, CH_3), 3.64 (t, 2H, J = 7.5 Hz, CH_2N), 3.95–4.06 (m, 4H, OCH_2CH_3), 4.75 (s, 2H, CH_2Ph), 7.30 (s, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.5 (d, J = 6.2 Hz), 20.3 (d, J = 4.3 Hz), 20.5, 23.0 (d, J = 142.7 Hz), 45.6 (d, J = 18.6 Hz), 61.7 (d, J = 6.8 Hz), 76.4, 128.7, 128.9, 129.2, 134.4, 172.4; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 32.5; HRMS (EI$^+$) m/z calcd for $C_{16}H_{26}NO_5PNa$ $[M + Na]^+$ 366.1441, found 366.1406.

4.1.7. Diethyl [3-((N-benzyloxy)(N-formyl)amino)] propylphosphonate [19b]

In a round bottomed flask were added at room temperature formic acid (3.15 mL, 83 mmol) and acetic anhydride (1.6 mL, 17 mmol). After 30 min, the reaction mixture was cooled down to 0 °C, and a solution of diethyl 3-oxopropylphosphonate acid (500 mg, 1.66 mmol) in THF (3.3 mL) was added dropwise. The reaction mixture was stirred for 10 min at 0 °C and was allowed to warm up at room temperature over a 2 h period. The reaction mixture was diluted with EtOAc and successively washed with H_2O, a 0.1 M aqueous KOH solution and H_2O. The organic layer was dried, filtered and evaporated to dryness. The residue was purified by flash chromatography (EtOAc) affording diethyl [3-((*N*-benzyloxy)(*N*-formyl)amino)]propylphosphonate as a colorless oil (390 mg, 1.2 mmol, 71%, R_f = 0.10, EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (t, 6H, J = 7.5 Hz, CH_3), 1.59–1.69 (m, 2H, CH_2), 1.81–1.94 (m, 2H, PCH_2), 3.23–3.56 (m, 2H, CH_2N), 3.94–4.05 (m, 4H, CH_2CH_3), 4.76–4.88 (m, 2H, CH_2Ph), 7.29 (s, 5H, Ph), 7.90–8.12 (br s, 1H, CHO); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 16.3 (d, J = 6.2 Hz), 20.2 (d, J = 15.6 Hz), 22.8 (d, J = 141.5 Hz), 44.1 (d, J = 19.3 Hz), 61.5 (d, J = 6.8 Hz), 77.6, 128.6, 129.0, 129.3, 134.1, 163.0; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 32.1.

4.1.8. General procedure to introduce the acyloxyalkyl/phenyl group

To a solution of the diethyl phosphonate in CH_2Cl_2 (3 mL/mmol) was added dropwise at 0 °C bromotrimethylsilane (5 eq.). The reaction mixture was stirred at 0 °C for 1 h and then overnight at room temperature. The reaction was quenched by addition of H_2O (0.3 mL/mmol), and the mixture was stirred for 5 min and evaporated to dryness. The residue was dissolved in DMF (6 mL/mmol), and after addition of Et_3N (3 eq.), the reaction mixture was stirred at room temperature for 5 min. Acyloxymethyl chloride (10 eq.) was added, and the mixture was stirred at 70 °C for 5 h. Another 3 eq. of Et_3N and 5 eq. of acyloxymethyl chloride were added and the mixture was stirred overnight at room temperature. Et_2O was added to the reaction mixture and the solution was successively washed with H_2O, with a saturated solution of $NaHCO_3$ and again with H_2O. The resulting organic layer was dried, filtered and evaporated to dryness and the residue purified by flash chromatography on silica gel (Et_2O).

4.1.9. Di(butyloxymethyl)[3-((N-benzyloxy)(N-formyl)amino)] propylphosphonate

Yellowish oil, 358 mg, 0.76 mmol, 70%, R_f = 0.14, (Et_2O); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.87 (t, 6H, J = 7.5 Hz, CH_3CH_2), 1.54 (qui, 4H, J = 7.5 Hz, CH_2CH_3), 1.70–1.90 (m, 4H, PCH_2CH_2), 2.34 (t, 4H, J = 7.5 Hz, CH_2CO), 3.53–3.61 (m, 2H, CH_2N), 4.89 (s, 2H, CH_2Ph), 5.55 (dd, 2H, J = 5.4, 9.3 Hz, OCH_2O), 5.60 (dd, 2H, J = 5.7, 9.9 Hz, OCH_2O), 7.41 (m, 5H, Ph), 7.96 (br s, 2/5 of 1H, CHO), 8.22 (br s, 3/5 of 1H, CHO); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 13.2, 17.5, 19.4, 21.9 (d, J = 141.5 Hz), 30.4, 34.9, 43.9, 64.9, 80.9 (d, J = 6.2 Hz), 128.4, 128.7, 129.5, 134.6, 162.7, 171.5; ^{31}P NMR (121.5 MHz, DMSO-d_6): δ = 33.2; HRMS (EI$^+$) m/z calcd for $C_{21}H_{32}NO_9PNa$ $[M + Na]^+$ 496.1707, found 496.1643.

4.1.10. Di(butyloxymethyl) 3-[(acetyl)(benzyloxy)amino] propylphosphonate

Pale yellow oil, 53 mg, 0.11 mmol, 46%, R_f = 0.12, (Et_2O); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, 6H, J = 7.5 Hz, CH_3CH_2), 1.60 (sex, 4H, J = 7.5 Hz, $CH_2CH_2CH_3$), 1.74–1.96 (m, 4H, PCH_2CH_2), 2.04 (s, 3H, CH_3CO), 2.29 (t, 4H, J = 7.5 Hz, CH_2CO), 3.65 (t, 2H, J = 7.5 Hz, CH_2N), 4.77 (s, 2H, CH_2Ph), 5.61 (br s, 2H, OCH_2O), 5.65 (br s, 2H, OCH_2O), 7.34 (s, 5H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.7, 18.1, 19.9 (d, J = 5.0 Hz), 24.0 (d, J = 142.1 Hz), 30.5, 35.9, 45.5 (d, J = 26.0 Hz), 76.6, 81.2 (d, J = 6.9 Hz), 128.9, 129.2, 129.4, 134.4, 172.1; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 33.5; HRMS (EI$^+$) m/z calcd for $C_{22}H_{34}NO_9PNa$ $[M + Na]^+$ 510.186, found 510.185.

4.1.11. Di(butyloxymethyl) 4-(benzyloxyamino)-4-oxobutylphosphonate (11a)

Colorless oil, mixture of two *E* and *Z* conformers in a 60:40 ratio, 75 mg, 0.16 mmol, 26%, R_f = 0.52, (Et_2O); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.92 (t, 6H, J = 7.4 Hz, CH_3CH_2), 1.62 (sex, 4H, J = 7.4 Hz, $CH_2CH_2CH_3$), 1.76–2.03 (m, 4H, PCH_2CH_2), 2.16 (t, 2/5 of 2H, J = 6.0 Hz, CH_2CON), 2.32 (t, 4H, J = 7.5 Hz, CH_2CO), 2.45 (t, 3/5 of 2H, J = 5.4 Hz, CH_2CON), 4.88 (s, 2/5 of 2H, CH_2Ph), 4.90 (s, 3/5 of 2H, CH_2Ph), 5.60 (m, 4H, OCH_2O), 7.33 (m, 5H, Ph), 8.97 (br s, 1H, NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.7, 16.9, 18.1, 24.9 (d, J = 140.9 Hz), 25.6 (d, J = 140.9 Hz), 32.4 (d, J = 21.1 Hz), 32.3 (d, J = 21.6 Hz), 35.9, 78.3, 81.2 (d, J = 6.3 Hz), 128.9, 129.3, 129.6, 134.7, 134.6, 169.7, 169.8, 172.2, 172.3; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 33.5, 33.7; HRMS (EI$^+$) m/z calcd for $C_{21}H_{32}NO_9PNa$ $[M + Na]^+$ 496.171, found 496.168.

4.1.12. Di(butyloxymethyl) 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (11b)

Colorless oil, 110 mg, 0.22 mmol, 45%, R_f = 0.55, (Et_2O); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.90 (t, 6H, J = 7.3 Hz, CH_3CH_2), 1.61 (sex, 4H, J = 7.5 Hz, $CH_2CH_2CH_3$), 1.76–1.90 (m, 4H, PCH_2CH_2), 2.30 (t, 4H, J = 7.5 Hz, CH_2CO), 2.42 (t, 2H, J = 6.5 Hz, CH_2CON), 3.13 (s, 3H, CH_3N), 4.77 (s, 2H, CH_2Ph), 5.58 (s, 2H, OCH_2O), 5.62 (s, 2H, OCH_2O), 7.33 (m, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.6, 17.1 (d, J = 4.3 Hz), 18.1, 25.8 (d, J = 140.8 Hz), 32.1 (d, J = 15.5 Hz), 35.8, 76.3, 81.1 (d, J = 6.2 Hz), 128.8, 129.1, 129.4, 135.6, 172.1, 173.9; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 33.9; HRMS (EI$^+$) m/z calcd for $C_{22}H_{34}NO_9PNa$ $[M + Na]^+$ 510.186, found 510.185.

*4.1.13. Di(pivaloyloxymethyl) 4-(benzyloxyamino)-4-oxobutylphosphonate (**12a**)*

Colorless oil, mixture of two *E* and *Z* conformers in a 70:30 ratio, 66 mg, 0.13 mmol, 21%, $R_f = 0.53$, (Et_2O); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.19$ (s, 18H, CH_3), 1.78–1.93 (m, 4H, PCH_2CH_2), 2.17 (br s, 3/10 of 2H, CH_2CON), 2.46 (t, 7/10 of 2H, $J = 6.0$ Hz, CH_2CON), 4.91 (s, 2H, CH_2Ph), 5.60 (s, 2H, OCH_2O), 5.64 (s, 2H, OCH_2O), 7.35 (m, 5H, Ph), 8.89 (br s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 17.0$, 18.3, 25.7 (d, $J = 147.2$ Hz), 25.8 (d, $J = 147.1$ Hz), 27.0, 32.3 (d, $J = 12.7$ Hz), 38.9, 78.3, 81.5 (d, $J = 6.3$ Hz), 128.9, 129.2, 129.7, 134.6, 175.3, 175.4, 177.1, 177.2; ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 33.4$, 33.6; HRMS (EI^+) *m/z* calcd for $C_{23}H_{36}NO_9PNa$ $[M + Na]^+$: 524.202, found 524.199.

*4.1.14. Di(pivaloyloxymethyl) 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (**12b**)*

Colorless oil, 80 mg, 0.15 mmol, 39%, $R_f = 0.56$, (Et_2O); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.16$ (s, 18H, CH_3), 1.75–1.87 (m, 4H, PCH_2CH_2), 2.41 (t, 2H, $J = 6.3$ Hz, CH_2CON), 3.13 (s, 3H, NCH_3), 4.77 (s, 2H, CH_2Ph), 5.58 (s, 2H, OCH_2O), 5.62 (s, 2H, OCH_2O), 7.32 (s, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 17.2$ (d, $J = 4.9$ Hz), 25.9 (d, $J = 140.2$ Hz), 26.9, 32.1 (d, $J = 16.1$ Hz), 38.8, 76.3, 81.4 (d, $J = 6.2$ Hz), 128.8, 129.1, 129.4, 134.5, 173.9, 176.9; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ (ppm) = 33.6; HRMS (EI^+) *m/z* calcd for $C_{24}H_{36}NO_9PNa$ $[M + Na]^+$ 538.222, found 538.226.

*4.1.15. Di(benzyloxymethyl) 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (**13a**)*

Colorless oil, 29 mg, 0.06 mmol, 35%, $R_f = 0.52$, (Et_2O); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.82$–1.96 (m, 4H, PCH_2CH_2), 2.42 (t, 2H, $J = 6.5$ Hz, CH_2CON), 4.75 (s, 2H, CH_2Ph), 5.86 (dd, 2H, $J = 5.1$, 9.6 Hz, OCH_2O), 5.92 (dd, 2H, $J = 5.1$, 10.8 Hz, OCH_2O), 7.35 (m, 5H, Ph), 7.38 (pseudo t, 4H, $J = 7.8$ Hz, *m*-CH), 7.54 (pseudo t, 2H, $J = 1$, 7.2 Hz, *p*-CH), 8.01 (dd, 4H, $J = 1$, 8.1 Hz, *o*-CH), 8.90 (br s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 17.4$ (d, $J = 4.3$ Hz), 25.7 (d, $J = 141.4$ Hz), 34.0 (d, $J = 16.1$ Hz), 76.5, 81.9 (d, $J = 6.8$ Hz), 128.8, 129.2, 129.3, 129.7, 130.2, 134.0, 134.1, 165.1, 171.7; ^{31}P NMR (121.5 MHz, $CDCl_3$): δ (ppm) = 34.6.

*4.1.16. Di(benzyloxymethyl) 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (**13b**)*

Colorless oil, 95 mg, 0.17 mmol, 40%, $R_f = 0.50$, (Et_2O); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.82$–1.96 (m, 4H, PCH_2CH_2), 2.41 (t, 2H, $J = 6.5$ Hz, CH_2CON), 3.10 (s, 3H, CH_3N), 4.73 (s, 2H, CH_2Ph), 5.86 (dd, 2H, $J = 5.1$, 9.6 Hz, OCH_2O), 5.91 (dd, 2H, $J = 5.1$, 10.0 Hz, OCH_2O), 7.31 (m, 5H, Ph), 7.38 (pseudo t, 4H, $J = 7.8$ Hz, *m*-CH), 7.54 (pseudo t, 2H, $J = 7.2$ Hz, *p*-CH), 8.00 (dd, 4H, $J = 0.9$, 8.1 Hz, *o*-CH); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 17.5$ (d, $J = 4.6$ Hz), 26.2 (d, $J = 140.3$ Hz), 32.3 (d, $J = 15.5$ Hz), 33.8, 76.5, 82.1 (d, $J = 6.3$ Hz), 129.0, 129.1, 129.4, 129.7, 130.4, 134.2, 134.9, 165.3, 174.1; ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 34.3$; HRMS (EI^+) *m/z* calcd for $C_{28}H_{30}NO_9PNa$ $[M + Na]^+$ 578.1550, found 578.1516.

4.1.17. General procedure for the catalytic hydrogenation

The *O*-benzylated hydroxamate was hydrogenolyzed in absolute methanol (4 mL/mmol) for 1 h at room temperature over 10% palladium on charcoal. The catalyst was removed by filtration, and the reaction mixture was evaporated to dryness to give the desired compound without purification.

*4.1.18. Di(butyloxymethyl) 3-[(N-formyl)(N-hydroxy)amino] propylphosphonate (**3a**)*

Mixture of two *E* and *Z* conformers in a 50:50 ratio, yellowish oil, 76 mg, 0.19 mmol, 90%; ^1H NMR (300 MHz, DMSO-d_6): $\delta = 0.89$ (t, 6H, $J = 7.5$ Hz, CH_3CH_2), 1.57 (sex, 4H, $J = 7.5$ Hz, $CH_2CH_2CH_3$), 1.66–1.90 (m, 4H, PCH_2CH_2), 2.36 (t, 4H, $J = 7.5$ Hz, CH_2CO), 3.46 (m, 2H, CH_2N), 5.55 (dd, 2H, $J = 5.4$, 7.8 Hz, OCH_2O), 5.60 (dd, 2H, $J = 5.4$, 8.4 Hz, OCH_2O), 7.88 (s, 1/2H of 1H, CHO), 8.24 (s, 1/2H of 1H, CHO), 9.58 (s, 1/2H of 1H, OH), 10.05 (s, 1/2H of 1H, OH); ^{13}C NMR (75.5 MHz, DMSO-d_6): $\delta = 13.2$, 17.5, 19.1 (d, $J = 3.7$ Hz), 19.8 (d, $J = 3.7$ Hz), 22.3 (d, $J = 139.6$ Hz), 22.9 (d, $J = 139.6$ Hz), 35.0, 46.0 (d, $J = 20.5$ Hz), 49.0 (d, $J = 19.2$ Hz), 80.9 (d, $J = 6.2$ Hz), 157.3, 161.9, 171.6; ^{31}P NMR (121.5 MHz, DMSO-d_6): δ (ppm) = 33.5, 33.6; HRMS (EI^+) *m/z* calcd for $C_{14}H_{26}NO_9PNa$ $[M + Na]^+$ 406.1237, found 406.1217.

*4.1.19. Di(butyloxymethyl) 3-[(N-acetyl)(N-hydroxy)amino] propylphosphonate (**3b**)*

Colorless oil, 30 mg, 75.5 μmol, 92%; ^1H NMR (300 MHz, CD_3OD): $\delta = 0.97$ (t, 6H, $J = 7.5$ Hz, CH_3CH_2), 1.69 (sex, 4H, $J = 7.5$ Hz, $CH_2CH_2CH_3$), 1.82–1.97 (m, 4H, PCH_2CH_2), 2.10 (s, 3H, CH_3CO), 2.40 (t, 4H, $J = 7.5$ Hz, CH_2CO), 3.66 (t, 2H, $J = 6.0$ Hz, CH_2N), 5.64 (dd, 2H, $J = 5.4$, 8.7 Hz, OCH_2O), 5.68 (dd, 2H, $J = 5.4$, 9.0 Hz, OCH_2O); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta = 14.0$, 19.2, 20.4, 20.8 (d, $J = 4.4$ Hz), 24.5 (d, $J = 142.1$ Hz), 31.0, 36.6, 82.7 (d, $J = 6.8$ Hz), 173.5, 174.1; ^{31}P NMR (121.5 MHz, CD_3OD): $\delta = 34.1$; HRMS (EI^+) *m/z* calcd for $C_{15}H_{28}NO_9PNa$ $[M + Na]^+$ 420.139 found 420.134.

*4.1.20. Di(butyloxomethyl) 4-[(hydroxy)amino]-4-oxobutylphosphonate (**4a**)*

Colorless oil, 135 mg, 0.27 mmol, 95%, $R_f = 0.25$, (EtOAc); ^1H NMR (300 MHz, CD_3OD): $\delta = 0.97$ (t, 6H, $J = 7.4$ Hz, CH_3CH_2), 1.67 (sex, 4H, $J = 7.4$ Hz, $CH_2CH_2CH_3$), 1.79–2.01 (m, 4H, PCH_2CH_2), 2.31 (t, 2H, $J = 7.1$ Hz, CH_2CON), 2.40 (t, 4H, $J = 7.2$ Hz, CH_2CO), 5.64 (dd, 2H, $J = 4.2$, 9.0 Hz, OCH_2O), 5.69 (dd, 2H, $J = 4.3$, 9.3 Hz, OCH_2O); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 14.0$, 19.2, 19.5 (d, $J = 5.2$ Hz), 26.5 (d, $J = 140.9$ Hz), 36.3 (d, $J = 16.7$ Hz), 36.6, 82.7 (d, $J = 6.9$ Hz), 173.6, 177.6; ^{31}P NMR (121.5 MHz, CD_3OD): $\delta = 33.9$; HRMS (EI^-) *m/z* calcd for $C_{14}H_{25}NO_9P$ $[M - H^+]$ 382.126 found 382.122.

*4.1.21. Di(butyloxomethyl) 4-[(hydroxy(methyl)amino]-4-oxobutylphosphonate (**4b**)*

Colorless oil, 69 mg, 0.17 mmol, 94%, $R_f = 0.23$, (EtOAc); ^1H NMR (300 MHz, CD_3OD): $\delta = 0.97$ (t, 6H, $J = 7.4$ Hz, CH_3CH_2), 1.67 (sex, 4H, $J = 7.4$ Hz, $CH_2CH_2CH_3$), 1.78–2.02 (m, 4H, PCH_2CH_2), 2.40 (t, 4H, $J = 7.4$ Hz, CH_2CO), 2.58 (t, 2H, $J = 7.1$ Hz, CH_2N), 3.19 (s, 3H, NCH_3), 5.64 (dd, 2H, $J = 5.4$, 8.7 Hz, OCH_2O), 5.67 (dd, 2H, $J = 5.4$, 8.7 Hz, OCH_2O); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta = 14.0$, 18.6 (d, $J = 4.4$ Hz), 19.2, 26.5 (d, $J = 140.8$ Hz), 33.2 (d, $J = 17.4$ Hz), 36.4, 36.6, 82.7 (d, $J = 6.2$ Hz), 173.5, 174.7; ^{31}P NMR (121.5 MHz, CD_3OD): δ (ppm) = 34.1; HRMS (EI^+) *m/z* calcd for $C_{15}H_{28}NO_9PNa$ $[M + Na]^+$ 420.1394, found 420.1360.

*4.1.22. Di(pivaloyloxomethyl) 4-[(hydroxy)amino]-4-oxobutylphosphonate (**5a**)*

Colorless oil, 39 mg, 0.09 mmol, 94%, $R_f = 0.21$, (EtOAc); ^1H NMR (300 MHz, CD_3OD): $\delta = 1.25$ (s, 18H, CH_3), 1.81–2.30 (m, 4H, PCH_2CH_2), 2.33 (t, 2H, $J = 7.1$ Hz, CH_2CON), 5.67 (dd, 2H, $J = 5.4$, 7.2 Hz, OCH_2O), 5.71 (dd, 2H, $J = 5.4$, 7.2 Hz, OCH_2O); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 19.6$ (d, $J = 5.1$ Hz), 26.5 (d, $J = 141.4$ Hz), 27.4, 36.3 (d, $J = 17.2$ Hz), 39.9, 83.0 (d, $J = 6.9$ Hz), 175.1, 177.4, 178.3; ^{31}P NMR (121.5 MHz, CD_3OD): δ (ppm) = 33.8; HRMS (EI^-) *m/z* calcd for $C_{16}H_{29}NO_9P$ $[M - H^+]$ 410.157, found 410.156.

*4.1.23. Di(pivaloyloxomethyl) 4-[(hydroxy(methyl)amino]-4-oxobutylphosphonate (**5b**)*

Mixture of two *E* and *Z* conformers in a 90:10 ratio, colorless oil, 51 mg, 0.12 mmol, 92%, $R_f = 0.26$, (EtOAc); ^1H NMR (300 MHz, CD_3OD): $\delta = 1.24$ (s, 18H, CH_3), 1.79–2.02 (m, 4H, PCH_2CH_2), 2.28 (t, 9/10 of 2H, $J = 7.1$ Hz, CH_2CON), 2.58 (t, 1/10 of 2H, $J = 6.9$ Hz, CH_2CON)*, 2.70 (s, 9/10 of 3H, NCH_3), 2.71 (s, 1/10 of 3H, NCH_3), 5.65

(dd, 2H, $J = 5.4$, 7.5 Hz, OCH_2O), 5.69 (dd, 2H, $J = 5.4$, 7.2 Hz, OCH_2O); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 19.8 (d, J = 5.2 Hz), 26.4, 26.6 (d, $J = 140.3$ Hz), 27.4, 33.2 (d, $J = 16.7$ Hz), 36.8 (d, $J = 17.2$ Hz), 39.9, 83.0 (d, J = 6.3 Hz), 175.2, 175.3, 178.2; ^{31}P NMR (121.5 MHz, CD_3OD): δ = 33.8, 34.1; HRMS (EI^+) m/z calcd for $C_{17}H_{32}NO_9PNa$ $[M + Na]^+$: 448.171, found 448.167.

4.1.24. Di(benzyloxymethyl) 4-[(hydroxy)amino]-4-oxobutylphosphonate (**6a**)

Mixture of two *E* and *Z* conformers in a 60:40 ratio, colorless oil, 216 mg, 0.45 mmol, 96%, R_f = 0.23, (EtOAc); ^1H NMR (300 MHz, CD_3OD): δ = 1.78–2.05 (m, 4H, PCH_2CH_2), 2.21 (t, 2/3 of 2H, J = 7.2 Hz, CH_2CON), 2.59 (t, 1/3 of 2H, J = 6.9 Hz, CH_2CON), 5.87–5.99 (m, 4H, OCH_2O), 7.47 (pseudo t, 4H, J = 8.0 Hz, *m*-CH), 7.61 (dd, 2H, $J = 1.1$, 7.8 Hz, *p*-CH), 8.00 (dd, 4H, $J = 1.5$, 8.1 Hz, *o*-CH); ^{13}C NMR (75 MHz, CD_3OD): δ = 17.9 (d, J = 5.6 Hz), 27.3 (d, $J = 142.5$ Hz), 26.6 (d, $J = 139.7$ Hz), 32.8 (d, $J = 16.8$ Hz), 81.8 (d, $J = 6.8$ Hz), 127.5, 128.4, 129.5, 133.7, 164.8; ^{31}P NMR (121.5 MHz, CD_3OD): δ = 33.9, 34.2; HRMS (EI^+) m/z calcd for $C_{20}H_{22}NO_9PNa$ $[M + Na]^+$ 474.092, found 474.087.

4.1.25. Di(benzyloxymethyl) 4-[hydroxy(methyl)amino]-4-oxobutylphosphonate (**6b**)

Mixture of two *E* and *Z* conformers in a 60:40 ratio, colorless oil, 59 mg, 0.13 mmol, 93%, R_f = 0.26, (EtOAc); ^1H NMR (300 MHz, CD_3OD): δ = 1.81–2.08 (m, 4H, PCH_2CH_2), 2.23 (t, 2/3 of 2H, J = 7.2 Hz, CH_2CON), 2.53 (t, 1/3 of 2H, J = 6.9 Hz, CH_2CON), 2.64 (s, 2/3 of 3H, CH_3N), 3.14 (s, 1/3 of 3H, CH_3N), 5.87–5.99 (m, 4H, OCH_2O), 7.47 (pseudo t, 4H, J = 8.0 Hz, *m*-CH), 7.63 (pseudo td, 2H, $J = 1.1$, 7.8 Hz, *p*-CH), 8.02 (dd, 4H, $J = 1.5$, 8.1 Hz, *o*-CH); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta = 18.6$ (d, $J = 4.0$ Hz), 19.6 (d, $J = 5.2$ Hz), 26.5 (d, $J = 140.3$ Hz), 26.6 (d, $J = 139.7$ Hz), 33.1 (d, $J = 17.1$ Hz), 36.3, 36.7 (d, $J = 16.7$ Hz), 83.4 (d, $J = 6.9$ Hz), 129.9, 130.1, 131.1, 135.2, 166.3, 174.6, 175.2; ^{31}P NMR (121.5 MHz, CD_3OD): δ = 34.3, 34.6; HRMS (EI^+) m/z calcd for $C_{21}H_{24}NO_9PNa$ $[M + Na]^+$ 488.108 found 488.102.

4.2. Molecular biology general procedures

4.2.1. Microorganisms and culture conditions

M. smegmatis DSM43756 (ATCC 19420) was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (Braunschweig, Germany). Bacteria were grown aerobically at 37 °C in a liquid medium containing 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$ and 0.4% D-glucose. They were plated on Petri dishes containing 0.05% yeast extract, 0.05% malt extract, 0.1% $CaCO_3$ and 0.1% D-glucose and 1.6% agar [36].

4.2.2. Isolation of mycobacterial genomic DNA

Genomic DNA was isolated essentially as described [37]. Cells from a 50 mL overnight culture were harvested by centrifugation. The pellet was resuspended in solution I (1 mL, 25% sucrose, 50 mM Tris/HCl pH 8.0, 50 mM EDTA, 500 μg lysozyme) and incubated overnight at 37 °C. Solution II (1 mL, 100 mM Tris/HCl pH 8, SDS 1%, 400 μg proteinase K) was then added. After incubation for 4 h at 55 °C, DNA was extracted with phenol–chloroform and precipitated by ethanol. The precipitate was dissolved in 10 mM Tris/HCl, 1 mM EDTA pH 8 buffer (500 μL) and a ribonuclease solution (10 μL, 10 mg/mL) was added. After incubation of 15 min at 37 °C, insoluble was discarded by centrifugation. Genomic DNA was extracted with phenol-chloroform and precipitated by ethanol. It was redissolved in 10 mM Tris/HCl, 1 mM EDTA buffer (pH 8, 100 μL).

4.2.3. Cloning of mycobacterial DXR

The gene coding for DXR was amplified from the genomic DNA using the forward primer (5′-GGGAATTCCATATGACCACCTCAGCCGCATCCGG-3′) and the reverse primer (5′-CCCAAGCTTGGGAATTCCTCATTTGGTGACGAGCCCTCTTC-3′). *Nde*I (simple underlining) restriction site was introduced into the forward primer; *EcoR*I (single underlining) and *Hin*dIII (double underlining) restriction sites were introduced into the reverse primer. The primers were drawn according to the sequence of *M. smegmatis* str. MC2 155 (accession number CP000480). PCR amplification was performed in a reaction mixture (20 μL), which included of each primer (20 pmol), template DNA (0.5 pmol), 3% DMSO and 2× PhusionTM High Fidelity PCR Master system (Finnzymes, Finland) using a step cycle (30 cycles) program of 98 °C for 0.1 min, 60 °C for 0.05 min and 72 °C for 0.22 min. The purified PCR product was poly(dA) tailed with TAQ polymerase before cloning into pGEMT-easy (Promega) and sequencing. The *dxr* gene was subcloned in an engineered pBAD plasmid [13]. The insert obtained after digestion with *Nde*I and *EcoR*I restriction enzymes was ligated into the mutated vector digested with the same enzymes. The resulting plasmid, pBAD-DXR_{myco} designed for obtaining an N-terminal His-tagged enzyme was transformed into XL1-blue *E. coli* cells (Stratagene Europe).

4.2.4. Overexpression and purification of mycobacterial DXR

For overexpression of the *dxr* gene, bacteria were grown at 30 °C in Luria–Bertani medium containing ampicillin (100 μg mL^{-1}). Induction was started at the mid-exponential phase by adding L-arabinose (0.05%, w/v). After additional growth for 4 h, cells were harvested by centrifugation and broken by powdering in a mortar in the presence of liquid N_2 in a 50 mM Tris/HCl buffer (pH 8) containing 250 mM NaCl and 5 mM 2-mercaptoethanol. The recombinant protein was purified using Ni^{2+}-spin columns (Qiagen). The columns were washed with the same buffer, containing 10 mM, 50 mM and 100 mM imidazole for each respective wash (600 μL). Mycobacterial DXR was eluted with 300 mM, 500 mM and 750 mM imidazole in the buffer in three successive steps (150 μL). The fractions with highest DXR activity were pooled and dialyzed against a 50 mM Tris/HCl buffer (pH 7.5) containing 100 mM NaCl, 10% glycerol and 2 mM dithiothreitol (DTT) by repeated centrifugal ultrafiltration with Centricon 30 concentrators (Millipore). The protein concentration was determined by the method of Bradford, with BSA as the standard [38]. A molecular mass of 41 kDa for the DXR subunit was utilized in kinetic calculations.

4.2.5. Determination of the enzymatic activity

The DXR enzymatic activity was determined at 37 °C in 50 mM Tris/HCl buffer, (pH 7.5) containing 3 mM $MgCl_2$ and 2 mM DTT. The concentrations of NADPH and DXP were 0.15 and 0.5 mM respectively. Initial rates were measured by following the decrease of the absorbance at 340 nm due to the oxidation of NADPH into $NADP^+$ (Uvikon 933; Kontron Instruments). The kinetic constants (V_{max} and K_m) were calculated from a double-reciprocal plot of enzymatic rate versus DXP concentration (0.05–0.5 mM). The reaction was initiated by adding the His-tagged DXR (2.25 μg).

4.2.6. DXR inhibition assay

The IC_{50} concentrations were determined for each compound. The enzymatic reaction was initiated by adding DXP after pre-incubation of the enzyme with the inhibitor in the presence of NADPH for 2 min at 37 °C. Assays were performed in 50 mM Tris/HCl buffer (pH 7.5) containing 3 mM $MgCl_2$ and 2 mM DTT. The concentrations of NADPH and DXP were 0.15 and 0.5 mM respectively. The reaction medium contained inhibitors at various concentrations, depending on the inhibitor: from 0.1 to 0.6 μM for **1a**, **1b** and **2b**, from 0.2 to 2 μM for **2a**. Prodrugs **3b** and **4b** were tested at a concentration of 1 μM.

4.2.7. Antimicrobial activity of prodrugs

The antimicrobial activity against *M. smegmatis* was determined using the paper disc diffusion method. Plates (10 cm-diameter) were inoculated with bacteria (120 μL) of a tenfold-diluted overnight culture. Prodrugs, not soluble in water, were dissolved at a concentration of 100 mM in DMSO. Fosmidomycin **1a**, **2a** and **2b** were dissolved in water at the same concentration, INH at 7.5 mM. Except INH (2 μL), paper discs (Durieux no. 268, diameter 6 mm) were impregnated with each solution (4 μL) and placed on Petri dishes. Growth inhibition was examined after 24 h incubation at 37 °C.

4.2.8. Resazurin test [28]

A fresh preculture of *M. smegmatis* was diluted to McFarland standard 0.5 (OD_{600} ~0.132). The OD was measured against a blank containing the growth medium to take in account the turbidity due to the presence of $CaCO_3$ in suspension in it. The inoculums (2 mL) were prepared by adding the bacterial suspension (0.2 mL) to the growth medium (1.8 mL) containing the inhibitors isoniazid (INH) or **4b**. INH is soluble in water and the stock solution (5 mM) was filter sterilized. The final concentration of INH was 10–40 μM, that of **4b** 250–1000 μM. The tubes were then incubated during 24 h at 37 °C under agitation. A growth control containing no antibiotic and a blank without bacteria were included in the test.

The resazurin viability test was performed in 96-well plates. To fresh medium (150 μL) was added the inoculum (50 μL). Assays were performed in duplicate. A 0.01% filter sterilized resazurin solution (30 μL) was then added. Sterile water (200 μL) was added to all perimeter wells to avoid evaporation during the incubation. Plates were covered, sealed with Parafilm® and incubated at 37 °C. The evolution of the color was visually followed during 12 h.

Acknowledgments

The authors are indebted to Jean-Daniel Sauer, Maurice Coppé and Dr. Lionel Allouche for running the NMR spectra and Hélène Nierengarten, for measuring the mass spectra. This work was supported by a grant from the “Agence Nationale de la Recherche” (Grant Nb ANR-06-BLAN-0291-01).

Appendix A. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2012.02.031.

References

[1] C. Dye, Lancet 367 (2006) 938–940.
[2] C. Dye, K. Lönnroth, E. Jaramillo, B.G. Williams, M. Raviglione, Bull. World Health Organ. 87 (2009) 683–691.
[3] S.H. Gillespie, Antimicrob. Agents Chemother. 46 (2002) 267–274.
[4] B.A. Wolucka, M.R. McNeil, E. Hoffmann, T. Chojnacki, P.J. Brennan, J. Biol. Chem. 269 (1994) 23328–23335.
[5] M.D. Collins, T. Pirouz, M. Goodfellow, D.E. Minnikin, J. Gen. Microbiol. 100 (1977) 221–230.
[6] (a) N. Qureshi, J.W. Porter, in: J.W. Porter, S.L. Spurgeon (Eds.), Biosynthesis of Isoprenoid Compounds, vol. 1, John Wiley and Sons, New York, 1981, pp. 1–46;
(b) I. Buhaescu, H. Izzedine, Clin. Biochem. 40 (2007) 575–584.
[7] (a) S. Rosa-Putra, A. Disch, J.M. Bravo, M. Rohmer, FEMS Microbiol. Lett. 164 (1998) 169–175;
(b) M. Rohmer, Nat. Prod. Rep. 16 (1999) 565–574;
(c) M. Rohmer, C. Grosdemange-Billiard, M. Seemann, D. Tritsch, Curr. Opin. Invest. Drugs 5 (2004) 154–162;
(d) M. Rohmer, Pure Appl. Chem. 79 (2007) 739–751.
[8] (a) H. Eoh, A.C. Brown, L. Buetow, W.N. Hunter, T. Parish, D. Kaur, P.J. Brennan, D.C. Crick, J. Bacteriol. 189 (2007) 8922–8927;
(b) H. Eoh, P.J. Brennan, D.C. Crick, Tuberculosis 89 (2009) 1–11;
(c) P. Narayanasamy, H. Eoh, P.J. Brennan, D.C. Crick, Chem. Biol. 17 (2010) 117–122.
[9] T. Kuzuyama, S. Takahashi, M. Takagi, H. Seto, J. Biol. Chem. 275 (2000) 19928–19932.
[10] (a) M. Okuhara, Y. Kuroda, T. Goto, M. Okamoto, H. Terano, M. Kohsaka, H. Aoki, H. Imanaka, J. Antibiot. 33 (1980) 13–17;
(b) M. Okuhara, Y. Kuroda, T. Goto, M. Okamoto, H. Terano, M. Kohsaka, H. Aoki, H. Imanaka, J. Antibiot. 33 (1980) 24–28.
[11] R.K. Dhiman, M.L. Schaeffer, A.M. Bailey, C.A. Testa, H. Scherman, D.C. Crick, J. Bacteriol. 187 (2005) 8395–8402.
[12] C. Zinglé, L. Kuntz, D. Tritsch, C. Grosdemange-Billiard, M. Rohmer, J. Org. Chem. 75 (2010) 3203–3207 and references therein.
[13] L. Kuntz, D. Tritsch, C. Grosdemange-Billiard, A. Hemmerlin, A. Willem, T.J. Bach, M. Rohmer, Biochem. J. 386 (2005) 127–135.
[14] L.M. Henriksson, T. Unge, J. Carlsson, J. Åqvist, S.L. Mowbray, T.A. Jones, J. Biol. Chem. 282 (2007) 19905–19916.
[15] M. Andaloussi, L.M. Henriksson, A. Wieckowska, M. Lindh, C. Björkelid, A.M. Larsson, S. Suresh, H. Iyer, B.R. Srinivasa, T. Bergfors, T. Unge, S.L. Mowbray, M. Larhed, T.A. Jones, A. Karlén, J. Med. Chem. 54 (2011) 4964–4976.
[16] A.C. Brown, T. Parish, BMC Microbiol. 8 (2008) 78.
[17] (a) J. Trias, V. Jarlier, R. Benz, Science 258 (1992) 1479–1481;
(b) H. Engelhardt, C. Heinz, M. Niederweis, J. Biol. Chem. 277 (2002) 37567–37572.
[18] M. Andaloussi, M. Lindh, C. Björkelid, S. Suresh, A. Wieckowska, H. Iyer, A. Karlén, M. Larhed, Bioorg. Med. Chem. Lett. 21 (2011) 5403–5407.
[19] (a) T. Verbrugghen, P. Cos, L. Maes, S. Van Calenbergh, J. Med. Chem. 53 (2010) 5342–5346;
(b) R. Ortmann, J. Wiesner, A. Reichenberg, D. Henschker, E. Beck, H. Jomaa, M. Schlitzer, Bioorg. Med. Chem. Lett. 13 (2003) 2163–2166.
[20] J.P. Krise, V.J. Stella, Adv. Drug Deliv. Rev. 19 (1996) 287–310.
[21] E. Uh, E.R. Jackson, G. San Jose, M. Maddox, R.E. Lee, R.E. Lee, H.I. Boshoff, C.S. Dowd, Bioorg. Med. Chem. Lett. 21 (2011) 6973–6976.
[22] (a) A. Reichenberg, J. Wiesner, C. Weidemeyer, E. Dreiseidler, S. Sanderbrand, B. Altincicek, E. Beck, M. Schlitzer, H. Jomaa, Bioorg. Med. Chem. 11 (2001) 833–835;
(b) T. Kurz, K. Schlüter, U. Kaula, B. Bergmann, R.D. Walter, D. Geffken, Bioorg. Med. Chem. 14 (2006) 5121–5135;
(c) K. Schlüter, R.D. Walter, B. Bergmann, T. Kurz, Eur. J. Med. Chem. 41 (2006) 1385–1397;
(d) T. Kurz, C. Behrendt, M. Pein, U. Kaula, B. Bergmann, R.D. Walter, Arch. Pharm. (Weinheim) 340 (2007) 661–666;
(e) J. Wiesner, R. Ortmann, H. Jomaa, M. Schlitzer, Arch. Pharm. (Weinheim) 340 (2007) 667–669;
(f) T. Haemers, J. Wiesner, D. Giessmann, T. Verbrugghen, U. Hillaert, R. Ortmann, H. Jomaa, A. Link, M. Schlitzer, S. Van Calenbergh, Bioorg. Med. Chem. 16 (2008) 3361–3371.
[23] A. Gissot, A. Volonterio, M. Zanda, J. Org. Chem. 70 (2005) 6925–6928.
[24] A. Argyrou, J.S. Blanchard, Biochemistry 43 (2004) 4375–4384.
[25] L.M. Henriksson, C. Björkelid, S.L. Mowbray, T. Unge, Acta Crystallogr., Sect. D Biol. Crystallogr. 62 (2006) 807–813.
[26] A.T. Koppisch, D.T. Fox, B.S. Blagg, C.D. Poulter, Biochemistry 41 (2002) 236–243.
[27] T. Scior, I.M. Morales, S.J.G. Eisele, D. Domeyer, S. Laufer, Arch. Pharm. (Weinheim) 335 (2002) 511–525.
[28] A. Martin, M. Camacho, F. Portaels, J.C. Palomino, Antimicrob. Agents Chemother. 47 (2003) 3616–3619.
[29] L.M. McMurry, P.F. McDermott, S.B. Levy, Antimicrob. Agents Chemother. 43 (1999) 711–713.
[30] Y. Sakamoto, S. Furukawa, H. Ogihara, M. Yamasaki Biosci, Biotechnol. Biochem. 67 (2003) 2030–2033.
[31] (a) P.J. Brennan, Tuberculosis 83 (2003) 91–97;
(b) P.J. Brennan, H. Nikaido, Annu. Rev. Biochem. 64 (1995) 29–63;
(c) V. Jarlier, H. Nikaido, FEMS Microbiol. Lett. 123 (1994) 11–18.
[32] M. Niederweis, Mol. Microbiol. 49 (2003) 1167–1177.
[33] J. Liu, C.E. Barry III, G.S. Besra, H. Nikaido, J. Biol. Chem. 271 (1996) 29545–29551.
[34] (a) P.E. Almeida da Silva, A. Von Groll, A. Martin, J.C. Palomino, FEMS Immunol. Med. Microbiol. 63 (2011) 1–9;
(b) G.E. Louw, R.M. Warren, N.C. Gey van Pittius, C.R.E. McEvoy, P.D. Van Helden, T.C. Victor, Antimicrob. Agents Chemother. 53 (2009) 3181–3189.
[35] O. Meyer, J.-F. Hoeffler, C. Grosdemange-Billiard, M. Rohmer, Tetrahedron 60 (2004) 12157–12166.
[36] S.R. Putra, A. Disch, J.M. Bravo, M. Rohmer, FEMS Microbiol. Lett. 164 (1998) 169.
[37] V. Pelicic, J.-M. Reyrat, B. Gicquel, Mol. Microbiol. 20 (1996) 919–925.
[38] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.