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Synthesis and biological evaluation of aryl phosphoramidate prodrugs of fosfoxacin and its derivatives

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ABSTRACT

Aryl phosphoramidate prodrugs of fosfoxacin derivatives **15a-b** and **8a-b** were synthesized and investigated for their ability to target bacteria. No growth inhibition was observed neither for *Mycobacterium smegmatis* nor for *Escherichia coli* on solid medium, demonstrating the absence of release of the active compounds in the bacterial cells. Investigation of the stability of the prodrugs and their multienzymatic cleavage in abiotic and biotic conditions showed that the use of aryl phosphoramidate prodrug approach to deliver non-nucleotides compounds is not obvious and might not be appropriate for an antimicrobial drug.

1. Introduction

Mycobacterium tuberculosis is a rod-shaped bacterium belonging to the family Mycobacteriaceae. Bacterial cells are surrounded by an unusual, waxy mycolic acid lipid complex coating on its cell surface. *M. tuberculosis* is the causative agent of tuberculosis (TB), which is one of the major infectious diseases in the world, affecting 10.4 million patients and responsible for 1.4 million deaths in 2016, among which 95% of the deaths were reported in developing countries [1]. Standard treatment for TB has been available for more than 60 years, but such treatment, a combination of antibiotics taken daily for six months, is constraining [2]. Moreover, its efficiency is threatened due to the increasing emergence of multidrug-resistant *M. tuberculosis* strains throughout the world. This emphasizes the urgency and necessity to develop innovative drugs towards new therapeutic targets. Isoprenoid biosynthesis in *Mycobacterium* represents such a target in this context [3,4]. Indeed, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoids, are synthesized in most bacteria including many pathogens, plant plastids, unicellular green algae and apicomplexan parasites *via* the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, which is absent in human (Fig. 1).

The deoxyxylulose 5-phosphate reductoisomerase (DXR), the second enzyme of this pathway, is effectively inhibited by fosmidomycin and its analogue FR900098, two natural phosphonohydroxamic acids **1a-b** as well as by two synthetic phosphonohydroxamic acids **2a-b** (Fig. 2) [5–9]. Unfortunately, due to a lack of uptake, fosmidomycin and its analogues are inefficient and do not inhibit the growth of mycobacteria. This result is not surprising as mycobacteria, in contrast to many other bacteria, do not possess a transporter for such molecules [10] and are surrounded by a hydrophobic waxy cell wall preventing passive diffusion into the cells, making the bacteria naturally resistant to most antibiotics.

To overcome the absence of mycobacterial cell wall crossing, and to increase oral bioavailability of these compounds, the prodrug approach was adopted. Hence, the synthesis of prodrugs of fosmidomycin **3a-b** and of its derivatives **4a-b** has been developed in which the phosphonate negative charge is masked by hydrophobic groups such as acyloxymethyl or alkoxycarbonyloxymethyl moieties (Fig. 2) [11–13]. Once the prodrug is inside the bacterial cells, the biolabile protecting group is enzymatically cleaved, releasing the parent drug in the cell and stopping the bacterial growth by inhibition of the DXR.

Numerous cell metabolites are phosphorylated compounds, but are rarely exploited as potential antibiotics due to their limited cell penetration and their sensitivity towards the hydrolytic activity of phosphatases. However, in the early 1990s, it was reported that fosfoxacin, the natural phosphate analogue of fosmidomycin, exhibited potent antibacterial activity against Gram-negative and Gram-positive bacteria [14,15]. Latter on, fosfoxacin **5a** and its *N*-acetylated analogue **5b** were shown to be more potent inhibitors than fosmidomycin on the DXR

Abbreviations: DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CPY, carboxypeptidase Y; GlpT, glycerol 3-phosphate transporter

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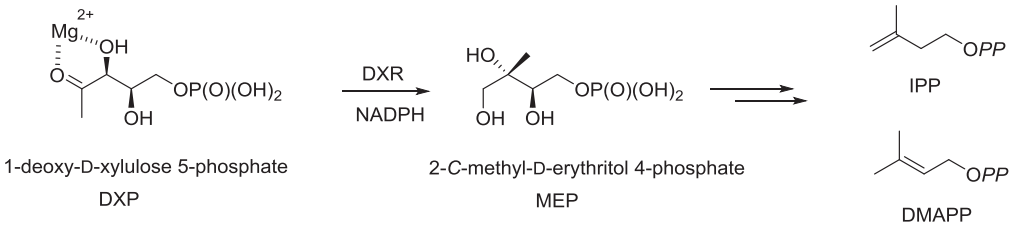


Fig. 1. Biosynthesis of IPP and DMAPP *via* the MEP pathway: second step catalyzed by the deoxyxylulose 5-phosphate reductoisomerase (DXR).

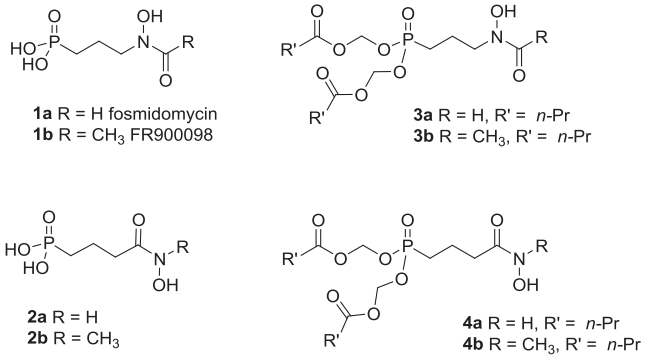


Fig. 2. Phosphonate DXR inhibitors and examples of their prodrug analogues.

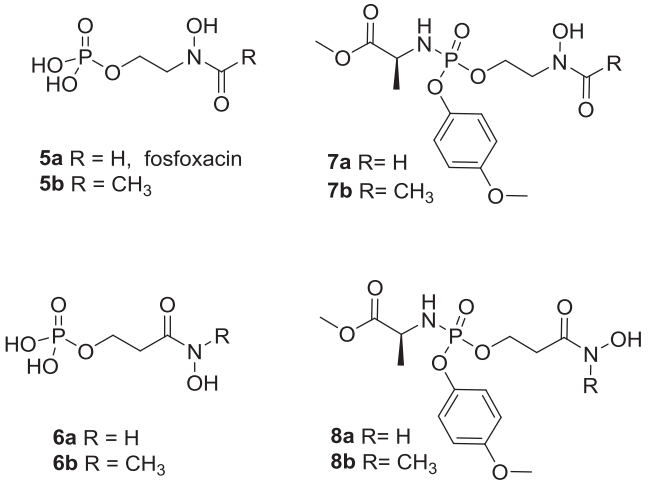


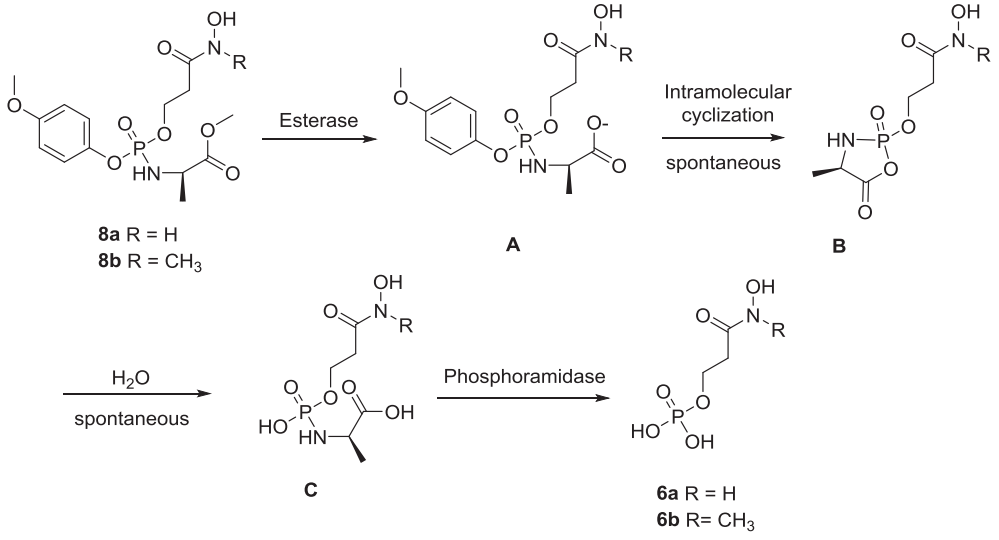
Fig. 3. Phosphate DXR inhibitors and aryl phosphoramidate prodrug analogues.

from *Synechocystis* sp. PCC6803 (Fig. 3). Recently, we reported the synthesis and biological evaluation of phosphate isosters of fosmidomycin and its analogues **6a-b** as inhibitors of *Escherichia coli* and *M. smegmatis* DXRs. We pointed out that the efficiency of phosphate compounds compared to their phosphonate homologs might be notably different towards DXRs isolated from different microorganisms [16].

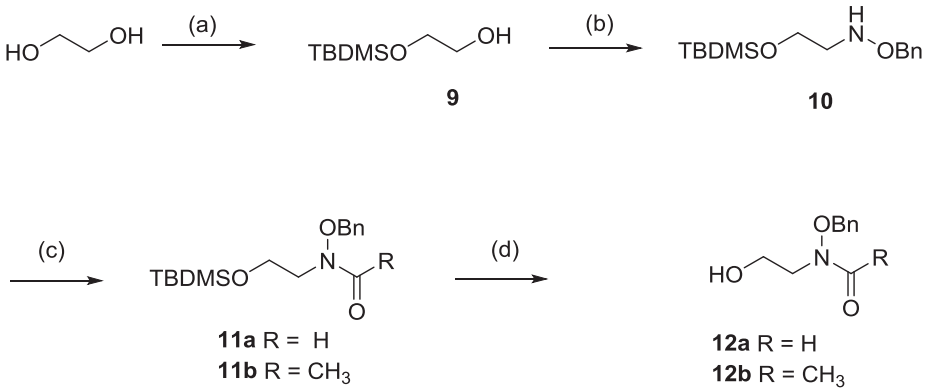
Even if the phosphate compounds are less efficient as compared to the phosphonates on *E. coli* growth inhibition, they enter into the bacteria via the GlpT/UhpT transporters. They cannot, however, cross the bacterial wall of *M. smegmatis* as this transporter is absent in Mycobacteria and passive diffusion of the negatively charged inhibitor is prevented by the hydrophobic coating of the cells. To increase the bioavailability in *E. coli* and circumvent the lack of uptake of these compounds in the mycobacteria, we designed prodrugs in which the polar phosphate group is masked. They are less hydrophilic and thus more susceptible to cross the bacterial envelope.

We chose the phosphoramidate prodrug approach to allow the penetration of fosfoxacin analogues into *Mycobacterium* spp. (Fig. 3). This method also called “ProTide approach” was introduced and developed by McGuigan. This widely used approach is based on the delivery to the target cell of monophosphorylated form nucleoside analogues and has been largely applied to improve the pharmacological properties of antiviral and anticancer agents [17–19]. To our knowledge, this phosphoramidate prodrug approach was never applied to bacteria but was once reported to improve the antiparasitic activity of 5-phospho erythronohydroxamic acid against *Trypanosoma brucei* [19].

In this work, we investigated the opportunity to use aryl phosphoramidate prodrug of fosfoxacin derivatives **7** and **8** to inhibit the growth of *M. smegmatis*, which was used as a mycobacterium model, and *E. coli* (Fig. 3). In a first attempt, we selected chemically stable aryl phosphoramidate consisting of an L-alanine methyl ester and a 4-methoxyphenyl moieties. After crossing the cell wall, prodrugs will undergo enzymatic conversions to release the parent drug. The initial step requires the action of a bacterial carboxyl esterase (Scheme 1),



Scheme 1. Potential bioactivation mechanism of phosphoramidate prodrugs **8a** and **8b**.



Scheme 2. Synthesis of the *O*-benzyl hydroxamate **12a** and **12b**. Reagents and conditions. (a) NaH, TBDMSCl, THF, 0 °C to rt, 85%; (b) (i) Tf_2O, 2,6-lutidine, DCM, −78 °C; (ii) H_2NOBn, DCM, −78 °C to rt, 65%; (c) $HCOOH/Ac_2O$, THF, rt, **11a**; Ac_2O, pyridine, rt, **11b**; (d) TBAF.3(H_2O), THF, rt, **12a** (82%), **12b** (93%).

which hydrolyses the carboxyl ester bond of compounds **8a** and **8b** to form the free carboxylate acid. This is followed by a putative nucleophilic attack of the phosphorus by the oxygen of the carboxylate group, resulting in the elimination of the aryloxy group to form a 5-membered cyclic intermediate **B**. A spontaneous hydrolysis of the cyclic phosphoramidate gives the phosphoramidate compound **C** (Scheme 1). The last step involves a hydrolysis of the P—N bond mediated by a phosphoramidase-type enzyme to release the inhibitor *i.e.* the fosfoxacin analogues **6a** or **6b** able to inhibit the DXR and consequently the bacterial growth (Scheme 1).

2. Results

2.1. Synthesis of prodrugs

The syntheses of **7** and **8** are outlined in Scheme 3. The key step to synthesize aryl phosphoramidate prodrugs **7** and **8** is the coupling of the phosphochloridate **13** with the protected hydroxamates **12** or **14**. The synthesis of the *O*-benzyl hydroxamates **12a** and **12b** were performed as outlined in Scheme 2. After a monoprotection of commercially available ethyleneglycol into the silyl ether **9**, the free alcohol was activated into the triflate with trifluoromethanesulfonic anhydride, followed by substitution with commercially *O*-benzyl hydroxylamine to give the protected hydroxylamine **10**. Formylation with the mixed acetyl/formyl anhydride generated *in situ* from a formic acid and acetic anhydride mixture lead to the *N*-formylated compound **11a** without purification as a mixture of two *Z* and *E* diastereomers in a 40:60 ratio. Acetylation with a mixture of acetic anhydride and pyridine gave the *N*-acetylated analog **11b** as the sole *E* isomer without purification. After deprotection of the silyl ether, the *O*-benzyl hydroxamates **12a** and **12b** were obtained after purification as a mixture of two *Z* and *E* diastereoisomers in a 30:70 ratio for **12a** and as the sole *E* isomer for **12b**. The *O*-benzyl hydroxamate **14** was synthesized according to previously described methods [16].

The phosphoroamidochloridate **13** was prepared from 4-methoxyphenol, phosphorus oxychloride and the L-alanine methyl ester hydrochloride according to a previously described method [20]. The phosphoroamidochloridate was then coupled with the *O*-benzyl hydroxamate **12** following conditions previously developed using *N*-methylimidazole in DCM at −78 °C [21,22]. In these conditions the desired compounds **15a-b** were obtained as a mixture of two diastereomers (δ_P = 3.05 and 3.30 ppm for **15a** and δ_P = 3.05 and 3.30 ppm for **15b**) in a 60:40 ratio due to the chirality of the phosphorus atom and contaminated with 50% byproduct in the isolated compounds (δ_P = 8.10 ppm). The byproduct might be a compound originating from an addition/elimination reaction of two L-alanine methyl ester molecules on the phosphorodichloridate, taking place throughout the synthesis of the phosphoroamidochloridate **13**. Even after several purification attempts, it was not possible to obtain the pure compounds **15a-b** and therefore the aryl phosphoramidate prodrugs **7**. No significant yield improvement of coupling reaction of compounds **16** with phosphoroamidochloridate **13** in different basic conditions was obtained. In that case, purification of the aryl phosphoramidate prodrugs **16** from the byproduct was achieved followed by the removal of the protective benzyl groups of **16a** and **16b** by catalytic hydrogenolysis with palladium over charcoal at atmospheric pressure and room temperature in methanol.

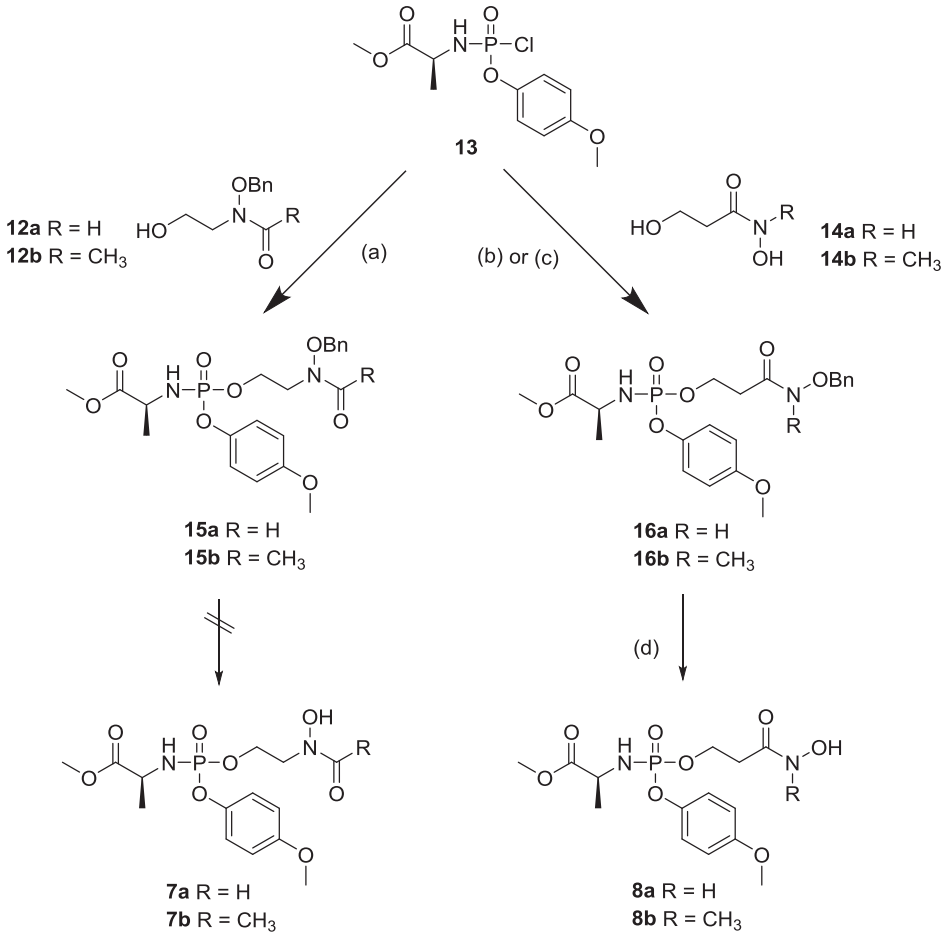
2.2. Biological properties

2.2.1. Growth inhibition of *E. coli* and *M. smegmatis* with compound *15a-b, 16a-b* and *8a-b*

Growth inhibition of *E. coli* and *M. smegmatis* induced by the synthesized aryl phosphoramidate prodrugs **8a-b** as well as by the *O*-benzyl-protected intermediates **15a-b** and **16a-b** was evaluated by the paper disc diffusion method. Isoniazid and fosmidomycin were respectively used as reference compounds for positive *M. smegmatis* and *E. coli* growth inhibition tests. None of the aryl phosphoramidate prodrugs **15a-b**, **16a-b** and **8a-b** blocked the growth of both *M. smegmatis* and *E. coli* at the highest tested dose (400 nmol/disk). The absence of growth inhibition lead to consider several hypotheses: (i) no membrane crossing of the aryl phosphoramidate prodrugs, (ii) membrane crossing of aryl phosphoramidate prodrugs followed by expulsion owing to efflux pumps, (iii) no endogenous enzymes able to release the phosphate drugs, (iv) short life time of the prodrugs in the culture medium.

2.2.2. Stability assays of the prodrugs *16a-b* and *8a-b* in the buffer used for carboxyl esterase enzymatic tests

Chemical stability of the prodrugs was evaluated by exposing them to aqueous 0.05 M triethanolamine (TEOA) buffer (pH 7.5) at 25 °C and monitored by ^{31}P NMR, a sensitive method to highlight possible modification(s) of the phosphorylated compounds (Supporting information, Fig. S1). Except for the prodrug **8b**, all the aryl phosphoramidate prodrugs are stable in TEOA over the tested reaction time (48 h). Indeed, the ^{31}P NMR spectra of these compounds showed two signals (**8a:** δ = 5.15 and 5.05 ppm; **16a**: δ = 4.85 and 4.70 ppm; **16b**: δ = 4.85 and 4.75 ppm) as the result of the presence of a mixture of diastereomers in solution due to the chirality of the phosphorus atom. Surprisingly, the *N*-methylated prodrug **8b** is unstable in the TEOA buffer. The ^{31}P NMR spectrum showed the absence of the two characteristic signals (δ = 4.85 and 4.75 ppm) of the diastereomers but the presence of two new signals at δ = 3.05 ppm and 4.25 ppm in a 85/15 ratio respectively, corresponding to metabolites with an achiral phosphorus.



Scheme 3. Synthesis of the aryl phosphoramidate prodrugs **7** and **8**. Reagents and conditions: (a) *N*-methylimidazole, DCM, −78 °C, **15a** (40%), **15b** (41%); (b) pyridine, **14a**, DCM, −40 °C, **16a** (47%); (c) triethylamine, DMAP, **14b**, DCM, −40 °C, **16b** (48%), (d) H_2, Pd/C, MeOH, rt, **8a** (77%), **8b** (92%).

*2.2.3. Esterase activity on the aryl phosphoramidate prodrugs **16a-b** and **8a-b** with CPY*

As shown in the scheme 1, the first step of the liberation of the parent drug in the cell consists of an enzymatic hydrolysis of the aminoacyl moiety releasing the free carboxylate acid **C** and involving a carboxypeptidase-type enzyme. To test the activation of the aryl phosphoramidate prodrugs into the intermediate **C**, we performed an enzymatic hydrolysis using carboxypeptidase-type enzyme and following the modifications by ^{31}P NMR. To determine whether the ester hydrolysis of the phosphoramidate prodrugs occurs or not, the compounds **16a-b** and **8a-b** were incubated with carboxypeptidase Y (CPY), and their possible modifications were monitored by ^{31}P NMR (Supplementary information, Fig. S2–S5). The assays were carried out by dissolving the compound in a H_2O/D_2O aqueous 0.05 M TEOA buffer (pH 7.5), followed by incubation with the CPY enzyme. A blank is recorded for each sample before addition of the CPY in D_2O. Upon the addition of CPY to the phosphoramidate prodrug **8a**, a fast hydrolysis occurred as two signals appeared at δ = 5.70 and 5.50 ppm. These signals correspond to the hydrolysed intermediate **B** present as two diastereomers, which were partially converted after 2 h into the aminoacyl phosphoramidate intermediate **C** according to McGuigan et al. (Scheme 1) [23]. A single signal at δ = 7.45 ppm was finally observed due to the achirality at the phosphorus atom. After 3 h, the ^{31}P NMR spectra displayed the increase of a signal at δ = −4.45 ppm concomitant with the decrease of the signal at δ = 7.45 ppm. This enzymatic assay showed a fast and complete conversion of the prodrug **8a** into the intermediate **C**. The half-life of prodrug **8a** was 20 h (Supplementary information, Fig. S2).

With the *O*-benzyl-protected precursor **16a**, addition of CPY led to similar results as previously described. The experiment showed a fast hydrolysis into the *O*-benzylated aminoacyl **C** since the initial compound disappeared after 2 h and was replaced by a compound with a ^{31}P shift at 7.40 ppm (Supplementary information, Fig. S3). Nevertheless, in contrast with previous results, longer incubation times showed a slow conversion of intermediate **C** (half-life = 2 days) into two compounds with ^{31}P shifts at −5.00 ppm and −7.50 ppm. The experiments with *O*-benzyl-*N*-methylated prodrugs **16b**, showed a fast and total hydrolysis after 2 h as the initial compound signals disappeared followed by the formation of the stable intermediate **C** (δ = 7.35 ppm). Longer incubation times did not lead to modifications of the ^{31}P NMR spectra (Supplementary information, Fig. S4).

The *N*-methylated prodrug **8b** was not stable in the buffer as two metabolites (δ = 3.05 and 4.25 ppm) are present. However, the incubation with the CPY rapidly showed an increase of the signal at 4.25 ppm with the concomitant decrease of the signal at 3.05 ppm. After 24 h of incubation the ratio was 80/20 in favour of the metabolite with a chemical shift at 4.25 ppm (Supplementary information, Fig. S5).

*2.2.4. Stability assays of the prodrugs **8a** and **8b** during M. smegmatis and E. coli cultures*

To check the stability of the prodrugs in *M. smegmatis* and *E. coli* cultures, the bacterial cells were removed from the culture medium by centrifugation at the end of the culture (24 h for *E. coli* and 40 h for *M. smegmatis*). The compounds were incubated with a mixture of resulting bacteria-free supernatants and D_2O in a 2/1 ratio and monitored over a 20 h time period by ^{31}P NMR (Supplementary information, Fig. S6-7).

The prodrug **8a** is stable in both growth media as the two signals of the diastereoisomers at δ = 5.15 and 5.10 ppm are still present in the

^{31}P NMR spectrum after 20 h (Supplementary information, Fig. S6), indicating that the bacteria do not excrete enzymes/metabolites capable of degrading the prodrugs. The spectra of the *N*-methylated **8b** in *E. coli* growth medium displayed a single signal at 3.05 ppm over the experiment time period (20 h), while the spectra of **8b** in *M. smegmatis* growth medium showed the presence of two signals at $\delta = 3.05$ and 5.20 ppm in a 3/1 ratio respectively. The latter signal decreased after 20 h in favour of the one at $\delta_P = 3.05$ ppm. The presence of this signal also observed in the TEOA buffer medium suggests that **8b** might not be stable in these conditions but is transformed into a stable compound with a phosphorus signature at 3.05 ppm (Supplementary information, Fig. S7).

2.2.5. Esterase, phosphatase and phosphoramidase activities in bacterial crude cell-free systems

Before investigating the bioactivation of the prodrugs in bacterial crude cell-free systems containing the endogenous bacterial enzymes, it was essential to test the potential esterase and phosphatase activities in both *E. coli* and *M. smegmatis* crude cell-free systems obtained by sonication of the bacterial cells. Spectrophotometric enzymatic assays for esterase activity were performed using *p*-nitrophenyl butyrate (NPB) as substrate [24]. An intense esterase activity was detected in the *M. smegmatis* crude cell-free system as shown by the release of *p*-nitrophenol (404 nmol min^{-1} mg^{-1} protein) from NPB. With a crude *E. coli* cell-free system, the esterase activity was lower, as only a 6.9 nmol min^{-1} mg^{-1} protein release rate of *p*-nitrophenol was measured.

However, the phosphatase activity measured with *p*-nitrophenyl phosphate (NPP) as the substrate, in the presence or absence of metal cations (Mn^{2+}, Mg^{2+} and Co^{+2}) was very low with a release rate of *p*-nitrophenol of 2.1 nmol $^{-1}$ min^{-1} mg^{-1} protein with the *M. smegmatis* crude cell-free system. The maximum activity (4.3 nmol min^{-1} mg^{-1} protein) was observed when $CoCl_2$ was added to the cell-free system. Concerning the cell-free system of *E. coli*, a phosphatase activity releasing *p*-nitrophenol at a 10 nmol min^{-1} mg^{-1} protein rate was determined. These low phosphatase activities are not able to cleave the phosphate of the fosfoxacin analogues **6a-b** (Scheme 1).

When the prodrug **8a** was incubated with the crude preparation of *M. smegmatis*, the same modifications on the ^{31}P NMR spectra were observed as those mediated with the CPY enzyme (Supplementary information, Fig. S8). Additionally, the prodrug **8a** was stable in a crude cell-free system from *E. coli* as only the two signals corresponding to the two diastereomers were still observed after 20 h (Supplementary information, Fig. S9). The parent drug **6a** was not formed, as no corresponding phosphorus signal was observed. Thus, no phosphoramidase activity occurred within the crude cell-free systems of either *M. smegmatis* or *E. coli*.

The *N*-methylated prodrug **8b** with the crude cell-free system of *M. smegmatis* showed a signal at 3.05 ppm complemented with four minor signals ($\delta = 7.50$, 5.20, 1.30 and -0.75 ppm). Thereafter, shifts at 7.50 and 5.20 ppm disappeared while the signal at -0.75 ppm increased. Nevertheless, the ratio of both signals at 3.05 and -0.75 ppm remained constant, even after a long incubation time (42 h) (Supplementary information, Fig. S10). In the crude cell free system of *E. coli*, prodrug **8b** showed three signals ($\delta = 3.05$, 5.20 and 1.30 ppm). Initially, the 3.05 and 5.20 ppm signals showed a 55/45 ratio. Thereafter, the shift at 5.20 ppm disappeared after 20 h (Supplementary information, Fig. S11).

3. Discussion

Recently we reported the synthesis of fosmidomycin phosphate analogues, which showed to be efficient inhibitors of the DXR. Unfortunately, the phosphate group being deprotonated and negatively charged at physiological pH, these compounds do not readily enter into cells in the absence of a transporter. To circumvent this penetration problem encountered with such hydrophilic DXR inhibitors, they have to be converted into neutral lipophilic prodrugs. Aryl phosphoramidate prodrugs represent such interesting candidates as this approach has been widely used to deliver monophosphate nucleotide derivatives into cells and to improve the pharmacological properties of antiviral and anticancer agents. However, this type of prodrugs has never been developed for antibacterial purposes. The synthesized aryl phosphoramidate prodrug **8** as well as the *O*-benzyl-protected intermediates **15a-b** and **16a-b** do not inhibit the growth of *M. smegmatis* and *E. coli* on solid medium, even at the highest tested dose (400 nmol/disk). Several hypotheses might be tested to explain this lack of activity: (i) either the prodrugs are not chemically stable in the incubation medium, (ii) or they do not cross the bacterial cell wall and/or the cytoplasmic membrane, (iii) or the bacteria lack the endogenous enzymes required to release the active free phosphate DXR inhibitors.

The correlation between the chemical stability of aryl phosphoramidate prodrugs in a buffer and the biological activity has been notably emphasized for the *in vitro* trypanocidal activity of the aryl phosphoramidate prodrugs of 2,3-*O*-isopropylidene-4-erythronohydroxamate. In the buffer TEOA, pH 7.5, prodrug **8a** as well as the *O*-benzyl-protected intermediates **15a-b** and **16a-b** are stable for 24 h whereas surprisingly, the *N*-methylated prodrug **8b** is rapidly degraded into a compound with an apparently achiral phosphorus ($\delta_P = 3.05$ ppm). The explanation for this discrepancy is not obvious as the stabilities are expected to be rather similar. The difference of the biological activities between the *N*-methylated hydroxamic acid and its non-methylated congener, is often attributed to the existence of different conformations in solution. The ratio of the conformers depends upon experimental conditions (solvent, concentration and temperature) but cannot explain the instability of **8b** in the buffer [25]. A hydroxamate can be considered as a potentially bidentate nucleophile, using either the basic N atom or the O atom in a nucleophilic reaction with a variety of electrophilic centers. They are known to be α-effect nucleophiles and as such are effective deacetylating and dephosphorylating agents. This hydrolyzing nucleophile activity is due to the anionic N-O^- form, which cannot occur with the *N*-methyl *O*-benzyl intermediate **15b**. As in water the hydroxamic acids are present as the neutral OH rather than NH form, the pKa for conversion of the *N*-OH into the negatively charged N-O^- form plays an important role for cleavage of phosphate esters as well as the substitution on the N atom. In fact, due to lone-electron pair repulsion, an *N*-methylated hydroxamic acid is more reactive in the anionic form than the NH. Therefore, the degradation of the aryl phosphoramidate prodrug **8b** might be due to a fast dephosphorylation in the buffer conditions [26–28].

The mechanism of bioactivation of the aryl phosphoramidate prodrugs involves a first enzymatic step mediated by a carboxypeptidase Y. The esterase activity occurs with prodrug **8a** and the *O*-benzyl-protected intermediates **16a-b** as they are hydrolyzed in the aminoacyl phosphoramidate intermediate **C**, which appears as a single signal at *ca.* $\delta = 7.45$ ppm in the ^{31}P NMR spectra.

However, the aminoacyl intermediate of the N-H prodrug **8a** and the NH-*O*-benzyl intermediate **16a** decomposes rapidly leading to new signals, and the disappearance of the signal for the phosphate prodrugs is observed during the experiment time course. The decomposition is probably due to the presence of the NH "acidic proton" as the aminoacyl phosphoramidate intermediate **C** corresponding to the *N*-methyl *O*-benzyl intermediate **16b** is stable over the time course. In the case of the *N*-methyl prodrug **8b,** the experiment showed an activation of the starting material with the carboxypeptidase Y into a phosphorylated compound. This result might suggest that the degraded compound ($\delta_P = 3.05$ ppm) still contains the L-alanine methyl ester in its structural backbone. These experiments indicated that the first activation step of prodrugs **8** may occur in the bacteria as long as the compound enters into the cell and is hydrolyzed by an efficient intracellular carboxypeptidase Y type enzyme.

In both growth media and as well as in *E. coli* cell-free system, prodrug **8a** was stable. However, in the *M. smegmatis* cell-free system, compound **8a** was rapidly metabolized into compounds having the

same ^{31}P NMR spectra profiles than those obtained with the CPY. These results suggest that *E. coli* does not have the appropriate intracellular and extracellular esterase in contrast with *M. smegmatis*. With the *N*-methylated prodrug **8b**, the results obtained are quite puzzling. In the growth media of *E. coli* and of *M. smegmatis* and the cell-free system of *E. coli,* the degraded form (δ_P = 3.05 ppm) was observed as the sole or major compound indicating that no esterase activity is present in these conditions. With a *M. smegmatis* cell-free system, the degraded form (δ_P = 3.05 ppm) was still present accompanied by minor metabolites, which are converted into a single metabolite (δ_P = −0.75 ppm). It is thus quite difficult to conclude on the presence of an esterase activity in *M. smegmatis*.

In the ProTides approach developed in eukaryotic cells, the enzymatic hydrolysis of the P—N bound requires an enzyme with phosphoramidase activity and it has been proposed that it belongs to the family of histidine triad nucleotide–binding proteins (Hint) [29]. In bacterial cells, Hint homologs have been annotated from sequenced genomes, but the enzymes have not been characterized. In *E. coli* a Hint homolog with a purine nucleoside phosphoramidase activity has been found, and *hint*$^-$ mutants were unable to grow [30]. In our case, no phosphoramidase activity has been detected with the cell-free system of both bacteria. Either the bacteria do not have the endogenous phosphoramidase activity or the intracellular phosphoramidase activity is substrate specific as it seems to be in *E. coli* [31].

4. Conclusion

The ProTides approach has largely been used to develop nucleoside monophosphate and monophosphonates prodrugs. This technique has proved to be efficient to deliver nucleotide therapeutics to treat viral infections and cancer. Even if a trypanocidal activity against the protozoan *Trypanosoma brucei* using non nucleotide aryl phosphoramidate prodrugs has been reported in the literature without decrypting action mechanisms of action has not be proven, no study mentioned the use of such prodrugs to target bacteria [20]. Our results suggest that the use of aryl phosphoramidate prodrug approach to deliver non-nucleotide compounds is not obvious and might not be the appropriate strategy for the design of an antimicrobial drug. We showed that two main factors must be considered: (i) the stability of the aryl phosphoramidate prodrug in the buffer and the cell free system presumably linked to the hydroxamate particular structural features, which can promote hydrolysis or/and side reactions; (ii) the presence of the release mechanism key enzymes activities *i.e.* CPY type esterase and phosphoramidase in the target microorganisms.

5. Experimental

5.1. Chemistry

5.1.1. General procedures

All non-aqueous reactions were run in dry solvents under an argon atmosphere. 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. Automated flash chromatography was performed on a Combiflash® Rf™ (Serlabo Technologies) or on a Puriflash® 215 (Interchim).

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_2HOD (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 signal multiplicity correspond to singlet, doublet, triplet and quadruplet or broad singlet. J-coupling constants are expressed in Hz.

Most of the hydroxamate are present as two *Z* and *E* conformers in equilibrium. If only one signal is described, it is common to both conformers. The evaluation of the relative amount of the conformers was made by integration of the CH_2CO or CH_2N or OCH_2DMB proton signals.

Negative or positive-mode electrospray MS were performed on a Bruker Daltonics microTOF spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal electrospray (ESI) interface. Calibration was performed using a 10 mM solution of 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}.

5.1.2. 2-((t-Butyldimethylsilyl)oxy)ethan-1-ol (**9**)

Sodium hydride (475 mg, 18.2 mmol) was suspended in THF (36 mL). The resulting mixture was cooled to 0 °C, the diol (1 mL, 17.7 mmol) was added dropwise and the mixture was stirred for 1 h. A solution of *t*-butyldimethylsilyl chloride (2.74 g, 18.0 mmol) in THF (8 mL) was added over a period of 10 min. The resulting mixture was stirred for 4 h at room temperature. A saturated aqueous solution of $NaHCO_3$ (40 mL) was added and the mixture was extracted with EtOAc (2 × 40 mL). The aqueous layer was saturated with NaCl and extracted with EtOAc (2 × 40 mL). The collected organic layers were dried over anhydrous Na_2SO_4, filtered. The solvents were removed under reduced pressure. The crude was purified by flash chromatography (EtOAc/petroleum ether, 2:8) to give the compound **9** as a colorless oil (2.48 g, 80%). Rf = 0.25 (EtOAc/petroleum ether, 1:9).^{1}H NMR (300 MHz, $CDCl_3$): δ 0.08 (6H, s, Si-CH_3), 0.91 (9H, s, *t*Bu), 1.99 (1H, bs, OH), 3.62–3.65 (2H, m, CH_2OTBDMS), 3.70–3.73 (2H, m, CH_2OH); ^{13}C NMR (75.5 MHZ, $CDCl_3$): δ_C −5.1 (Si-CH_3), 18.5 (C_4◦ of *t*-Bu), 26.1 (CH_3 of *t*Bu), 63.9 (CH_2OTBDMS), 64.3 (CH_2OH).

5.1.3. O-Benzyl-N-(2-((tert-butyldimethylsilyl)oxy)ethyl)hydroxylamine (**10**)

To a solution of **9** (424 mg, 2.40 mmol) in DCM (76 mL) was added 2,6-lutidine (0.45 mL, 3.90 mmol). The solution was cooled down at −78 °C, and trifluoromethane sulfonic anhydride (450 μL, 2.70 mmol) was added dropwise. The resulting mixture was stirred at −78 °C for 1 h, and *O*-benzylhydroxylamine (625 mg, 5.07 mmol) in DCM (6 mL) was added dropwise. The solution was stirred at −78 °C for 1 h, warmed up to room temperature and stirred for another 2 h. The reaction mixture was diluted with DCM (30 mL) and washed with a saturated aqueous solution of NH_4Cl (100 mL), a saturated solution of $NaHCO_3$ (100 mL), water (100 mL) and brine (100 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvents were removed under reduced pressure to give a pale yellow oil. The crude was purified by flash chromatography (petroleum ether → EtOAc/petroleum ether, 5:95) to give the product **10** as a colorless oil (438 mg, 65%). Rf = 0.5 (EtOAc/petroleum ether, 1:9). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.04 (6H, s, Si-CH_3), 0.88 (9H, s, *t*Bu), 3.02 (2H, t, ^3J = 5.2 Hz, CH_2N), 3.74 (2H, t, ^3J = 5.1 Hz, CH_2OTBDMS), 4.72 (2H, s, OCH_2Ph), 7.29–7.37 (5H, m, CH_{Ar}); ^{13}C NMR (125.8 MHZ, $CDCl_3$): δ_C −5.2 (Si-CH_3), 18.5 (C_4◦ of *t*Bu), 26.1 (CH_3 of *t*Bu), 54.1 (CH_2N), 59.5 (CH_2OTBDMS), 75.9 (OCH_2Ph), 127.9 (CH_{Ar}), 128.5 (CH_{Ar}), 128.6 (CH_{Ar}), 138.2 (C_{Ar}); MS (EI)$^+$: *m/z* calculated for $C_{15}H_{28}NO_2Si$ [M + H]$^+$ 282.19, found 282.19.

*5.1.4. N-(Benzyloxy)-N-(2-((tert-butyldimethylsilyl)oxy)ethyl)formamide (**11a**)*

A mixture of formic acid (2.75 mL, 73 mmol) and acetic anhydride (1.38 mL, 14.6 mmol) was stirred at room temperature during 30 min. The reagent was cooled to 0 °C and a solution of protected hydroxylamine **10** (410 mg, 1.46 mmol) in THF wad added dropwise. The reaction mixture was stirred at 0 °C during 10 min, allowed to warm up at room temperature and stirred overnight. The reaction was diluted with EtOAc and the organic layer was washed twice with water and twice with a 0.1 M aqueous solution of KOH. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The product **11a** was obtained without purification as a colorless oil (390 mg) and as a mixture of two *Z* and *E* conformers in a 40:60 ratio. Rf = 0.61 (EtOAc/petroleum ether, 1:9). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.05 (6H, s, Si-CH_3), 0.88 (9H, s, *t*Bu), 3.30 (4/10 of 2H, bs, CH_2N), 3.69 (6/10 of 2H, bs, CH_2N), 3.79 (4/10 of 2H, bs, $CH_2OTBDMS$), 4.33 (6/10 of 2H, bs, $CH_2OTBDMS$), 4.89 (6/10 of 2H, bs, OCH_2Ph), 4.98 (4/10 of 2H, bs, OCH_2Ph), 7.37 (5H, bs, CH_{Ar}), 7.95 (4/10 of 1H, bs, CHO), 8.22 (6/10 of 1H, bs, CHO); ^{13}C NMR (125.8 MHZ, $CDCl_3$): δ_C −5.2 (Si-CH_3), 18.4 (C_{4° of *t*Bu), 26.0 (CH_3 of *t*Bu), 48.1 (CH_2N), 52.1 (CH_2N), 58.6 ($CH_2OTBDMS$), 59.4 ($CH_2OTBDMS$), 76.7 (OCH_2Ph), 78.1 (OCH_2Ph), 128.7 (CH_{Ar}), 128.9 (CH_{Ar}), 129.2 (CH_{Ar}), 129.6 (CH_{Ar}), 129.7 (CH_{Ar}), 134.6 (C_{Ar}), 138.5 (C_{Ar}), 159.5 (CHO), 163.9 (CHO); MS (EI)$^+$: *m/z* calculated for $C_{16}H_{27}NO_3SiNa$ $[M+Na]^+$ 332.17, found 332.16.

*5.1.5. N-(Benzyloxy)-N-(2-((tert-butyldimethylsilyl)oxy)ethyl)acetamide (**11b**)*

To a solution of protected hydroxylamine **10** (298 mg, 1.06 mmol) in acetic anhydride (4.3 mL) was added dropwise pyridine (0.26 mL, 3.18 mmol). The reaction mixture was stirred overnight at room temperature and the solvents were evaporated to dryness under reduced pressure. The product **11b** was obtained without purification as a colorless oil (311 mg) and as the sole *E* conformer. Rf = 0.59 (EtOAc/petroleum ether 1:9). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.06 (6H, s, Si-CH_3), 0.89 (9H, s, *t*Bu), 2.09 (3H, s, $COCH_3$), 3.75–3.79 (4H, m, OCH_2CH_2N), 4.88 (2H, s, OCH_2Ph), 7.38 (5H, s, CH_{Ar}); ^{13}C NMR (125.8 MHZ, $CDCl_3$): δ_C −5.2 (Si-CH_3), 18.5 (C_{4° of *t*Bu), 20.7 ($CO\mathit{C}H_3$), 26.1 (CH_3 of *t*Bu), 49.7 (CH_2N), 59.8 ($CH_2OTBDMS$), 76.7 (OCH_2Ph), 78.1 (OCH_2Ph), 128.9 (CH_{Ar}), 129.1 (CH_{Ar}), 129.4 (CH_{Ar}), 134.8 (C_{Ar}), 173.1 ($\mathit{C}OCH_3$); MS (EI)$^+$: *m/z* calculated for $C_{17}H_{30}NO_3Si$ $[M+H]^+$ 324.20, found 324.20.

*5.1.6. N-(Benzyloxy)-N-(2-hydroxyethyl)formamide (**12a**)*

The silyl ether **11a** (376 mg, 1.21 mmol) in THF (18 mL) was treated with tetra-*n*-butylammonium fluoride (633 mg, 2.42 mmol). The reaction was monitored by TLC (EtOAc) and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (EtOAc) to give **12a** as a colorless oil (224 mg, 82%) and as a mixture of two *Z* and *E* conformers in a 30:70. Rf = 0.51 (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.70–3.80 (4H, m, OCH_2CH_2N), 4.90 (2H, bs, OCH_2Ph), 7.38 (5H, s, CH_{Ar}), 7.99 (3/10 of 2H, bs, NH), 8.25 (7/10 of 2H, bs, NH); ^{13}C NMR (125.8 MHZ, $CDCl_3$): δ_C 48.8 (CH_2N), 53.7 (CH_2N), 58.3 (CH_2OH), 60.1 (CH_2OH), 76.6 (OCH_2Ph), 78.3 (OCH_2Ph), 128.7 (CH_{Ar}), 129.0 (CH_{Ar}), 129.5 (CH_{Ar}), 128.8 (CH_{Ar}), 134.2 (C_{Ar}), 159.7 (CHO), 164.5 (CHO); MS (EI)$^+$: *m/z* calculated for $C_{10}H_{13}NO_3K$ $[M+K]^+$ 234.05, found 234.05.

*5.1.7. N-(Benzyloxy)-N-(2-hydroxyethyl)acetamide (**12b**)*

The silyl ether **11b** (311 mg, 0.81 mmol) in THF (12 mL) was treated with tetra-*N*-butylammonium fluoride (424 mg, 1.62 mmol). The reaction was monitored by TLC (EtOAc) and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (EtOAc) to give **12b** as a colorless oil (147 mg, 93%) and as the sole *E* conformer. Rf = 0.25 (EtOAc/petroleum ether, 7:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.13 (3H, s, $COCH_3$), 3.81 (4H, s, OCH_2CH_2N), 4.87 (2H, s, OCH_2Ph), 7.38 (5H, s, CH_{Ar}); ^{13}C NMR (125.8 MHZ, $CDCl_3$): δ_C 20.6 ($CO\mathit{C}H_3$), 50.8 (CH_2N), 61.3 (CH_2OH), 77.1 (OCH_2Ph), 129.0 (CH_{Ar}), 129.2 (CH_{Ar}), 129.5 (CH_{Ar}), 134.3 (C_{Ar}), 174.3 ($\mathit{C}OCH_3$); MS (EI)$^+$: *m/z* calculated for $C_{11}H_{16}NO_3$ $[M+H]^+$ 210.11, found 210.11.

*5.1.8. (4-Methoxy)phenyl (methoxy-L-alaninyl)phosphorochloridate (**13**)*

To a solution of phosphorus oxychloride (0.75 mL, 8 mmol) and 4-methoxyphenol (1.00 g, 8 mmol) in dry diethyl ether (30 mL) was added at −78 °C triethylamine (1.12 mL, 8 mmol). The resulting mixture was warmed up to room temperature and stirred overnight. The reaction was monitored by ^{31}P NMR. The tetraethylammonium chloride was filtered under argon and the filtrate was concentrated under reduced pressure. The resulting yellow oil was used for the next step without further purification. ^{31}P NMR (162 MHz, $CDCl_3$): δ_P 4.45. To (4-methoxy)phenyl phosphorodichloridate was dissolved in dry DCM (31 mL) was added L-alanine methyl ester hydrochloride (1.07 g, 7.65 mmol). The suspension was cooled to −78 °C and triethylamine (2.4 mL, 16.9 mmol) was added dropwise. The mixture was stirred at −78 °C for 15 min and stirred overnight at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was poured into Et_2O (30 mL). The tetraethylammonium chloride was filtered off. The filtrate was evaporated to dryness under reduced pressure to give a yellow oil **13**, which was used for the next step without further purification. ^{31}P NMR (162 MHz, $CDCl_3$): δ_P 8.25, 8.60.

*5.1.9. Methyl((2-(N-(benzyloxy)formamido)ethoxy)(4-methoxyphenoxy) phosphoryl)-L-alaninate (**15a**)*

To a solution of **12a** (222 mg, 1.14 mmol), the phosphochloridate **13** (807 mg, 2.62 mmol) in dry DCM (20 mL) was added at −78 °C *N*-methyl imidazole (0.45 mL, 5.7 mmol). The mixture was warmed up at room temperature and monitored by TLC and ^{31}P NMR and quenched with MeOH. The organic layer was washed with 0.5 M aqueous solution of HCl (20 mL) and the aqueous layer was extracted twice with DCM (2 × 20 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. A purification by flash chromatography gave a mixture (213 mg) of the desired compound **15a** and a byproduct (*) in a 50:50 ratio respectively, which was determined by NMR spectroscopy. The product **15a** was obtained as a mixture of two diastereoisomers in a 60:40 ratio. Rf = 0.35 (EtOAc/petroleum ether, 3:7). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.31–1.38 (6/9 of 9H, m, NCHCH_3^*, 3/9 of 9H, m, NCHCH_3), 3.45 (2H, m, NCH_2), 3.68–3.76 (9/15 of 15H, m, OCH_3^*, 6/15 of 15H, m, OCH_3), 3.98–4.07 (2/3 of 3H, m, NCHCH_3^*, 1/3 of 3H, m, NCHCH_3), 4.14 (4/10 of 2H, bs, $POCH_2$), 4.23 (6/10 of 2H, bs, $POCH_2$), 4.67 (4/10 of 2H, bs, OCH_2Ph), 4.81 (6/10 of 2H, bs, OCH_2Ph), 6.77–6.82 (1/2 of 2H, m, CH_{Ar}^*, 1/2 of 2H, m, CH_{Ar}), 7.07–7.12 (1/2 of 2H, m, CH_{Ar}^*, 1/2 of 2H, m, CH_{Ar}), 7.37 (5H, m, $CH_{Ar(Bn)}$), 7.81–7.85 (4/10 of 1H, bs, CHO), 8.19–8.21 (6/10 of 1H, bs, CHO); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 21.1–21.4 (NCH$\mathit{C}H_3^*$ and NCH$\mathit{C}H_3$), 45.3 (NCH_2), 46.5 (NCH_2), 49.9–50.4 (NCHCH$_3^*$ and NCHCH$_3$), 52.6 ($CO_2\mathit{C}H_3^*$ and $CO_2\mathit{C}H_3$), 55.8 (ArO$\mathit{C}H_3^*$ and ArO$\mathit{C}H_3$), 61.9 ($POCH_2$), 62.5 ($POCH_2$), 76.9 (OCH_2Ph), 78.4 (OCH_2Ph), 114.7 ($CH_{Ar(PMP)}$), 121.3 ($CH_{Ar(PMP)}$), 128.8 ($CH_{Ar(Bn)}$), 129.0 ($CH_{Ar(Bn)}$), 129.4 ($CH_{Ar(Bn)}$), 129.5 ($CH_{Ar(Bn)}$), 129.7 ($CH_{Ar(Bn)}$), 134.3 (C_{Ar}), 144.5 (C_{Ar}), 156.5 ($\mathit{C}_{Ar}OCH_3$), 164.1 (CHO), 164.2 (CHO),173.7 ($CH_3\mathit{C}O$), 174.1 ($\mathit{C}O_2CH_3$), 174.3 ($\mathit{C}O_2CH_3$); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ_P 3.05, 3.30, 8.10*; HRMS (EI)$^+$: *m/z* calculated for $C_{21}H_{27}N_2O_8PNa$ $[M+Na]^+$ 489.1397, found 489.1376.

*5.1.10. Methyl((2-(N-(benzyloxy)acetamido)ethoxy)(4-methoxyphenoxy) phosphoryl)-L-alaninate (**15b**)*

To a solution of **12b** (277 mg, 1.32 mmol), the phosphochloridate **13** (935 mg, 3.0 mmol) in dry DCM (24 mL) was added at −78 °C *N*-methyl imidazole (0.53 mL, 6.6 mmol). The mixture was warmed up at room temperature and monitored by TLC and ^{31}P NMR and quenched with MeOH. The organic layer was washed with 0.5 M aqueous solution

of HCl (20 mL) and the aqueous layer was extracted twice with DCM (2 × 20 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Several purifications by flash chromatography and preparative TLC gave a mixture (31 mg) of the desired compound **15b** and a byproduct (*) in a 50:50 ratio respectively, which was determined by NMR spectroscopy. The product **15b** was obtained as a mixture of two diastereoisomers in a 60:40 ratio. Rf = 0.38 (EtOAc/petroleum ether, 3:7). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.37 (2/3 of 9H, m, NCHCH_3*, 2/3 of 9H, m, NCHCH_3), 2.07 (6/10 of 2H, bs, CH_3CO), 2.08 (4/10 of 2H, bs, CH_3CO), 3.41–3.46 (2H, m, NCH_2), 3.68–3.76 (9/15 of 15H, m, OCH_3*, 6/15 of 15H, m, OCH_3*) 3.98–4.07 (2/3 of 1H, m, NCHCH$_3$*, 1/3 of 1H, m, NCHCH$_3$), 4.21–4.29 (2H, m, $POCH_2$), 4.79 (4/10 of 2H, s, OCH_2Ph), 4.81 (6/10 of 2H, s, OCH_2Ph), 6.77–6.82 (1/2 of 2H, m, CH_{Ar}*, 1/2 of 2H, m, CH_{Ar}), 7.07–7.12 (1/2 of 2H, m, CH_{Ar}*, 1/2 of 2H, m, CH_{Ar}), 7.37 (5H, m, $CH_{Ar(Bn)}$); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 20.6 (CH_3CO), 21.1–21.4 (NCHCH$_3$* and NCHCH$_3$), 46.5 (NCH_2), 46.7 (NCH_2), 49.9–50.4 (NCHCH$_3$* and NCHCH$_3$), 52.6 (CO_2CH_3* and CO_2CH_3), 55.8 (ArOCH$_3$* and ArOCH$_3$), 62.9 ($POCH_2$), 76.9 (OCH_2Ph), 114.6–114.7 ($CH_{Ar(PMP)}$), 121.3–121.4 ($CH_{Ar(PMP)}$), 128.9 ($CH_{Ar(Bn)}$), 129.2 ($CH_{Ar(Bn)}$), 129.5 ($CH_{Ar(Bn)}$), 129.9 ($CH_{Ar(Bn)}$), 144.2–144.5 (C_{Ar}), 156.5 ($C_{Ar}OCH_3$), 173.7 (CH_3CO), 174.1 (d, $^3J_{C\text{-}P}$ = 6.8 Hz, CO_2CH_3), 174.3 (d, $^3J_{C\text{-}P}$ = 7.1 Hz, CO_2CH_3); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ_P 3.05, 3.30, 8.10*; HRMS (EI)$^+$: *m/z* calculated for $C_{22}H_{30}N_2O_8P$ $[M+H]^+$ 481.1734, found 481.1762.

5.1.11. N-(Benzyloxy)-3-hydroxypropanamide-3-[(4-methoxy)phenyl (methoxyalaninyl) phosphoramidate] (16a)

Alcohol **14a** (200 mg, 1.02 mmol) was dissolved in anhydrous pyridine (2.8 mL). The mixture was cooled to −40 °C and compound **13** (627 mg, 2.04 mmol) in toluene (2.5 mL) was added dropwise. The reaction was stirred to −40 °C for 30 min and warmed up to room temperature overnight. The pyridine was evaporated under reduced pressure and the crude product was purified by flash chromatography (EtOAc/cyclohexane, 9:1) to give **16a** as a colorless oil (222 mg, 47%) and as a mixture of two diastereomers in a 50:50 ratio and two conformers *Z* and *E* in a 80:20 ratio. Rf = 0.28 (EtOAc/petroleum ether, 8:2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.31 (5/10 of 3H, d, ^3J = 6.8 Hz, NCHCH_3), 1.35 (5/10 of 3H, d, $^3J_{H\text{-}H}$ = 7.0 Hz, NCHCH_3), 2.42 (8/10 of 2H, bs, CH_2CO), 2.70 (2/10 of 2H, bs, CH_2CO), 3.52 (5/10 of 1H, m, NH), 3.67–3.73 (6H, m, OCH_3), 3.79 (5/10 of 1H, m, NH), 3.91–4.03 (1H, m, NCHCH$_3$), 4.28–4.38 (2H, m, $POCH_2$), 4.85–4.91 (2H, s, OCH_2Ph), 6.78–6.80 (2H, m, CH_{Ar}), 7.06–7.09 (2H, m, CH_{Ar}), 7.33–7.36 (5H, m, $CH_{Ar(Bn)}$), 9.24 (5/10 of 1H, bs, N(H)OBn), 9.37 (5/10 of 1H, bs, N(H)OBn); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 21.1 (NCHCH$_3$), 35.2 (CH$_2$CO), 35.8 (CH$_2$CO), 50.3 (NCHCH$_3$), 50.5 (NCHCH$_3$), 52.8 (CO_2CH_3), 55.8 (ArOCH$_3$), 63.4 ($POCH_2$), 63.6 ($POCH_2$), 78.4 (OCH_2Ph), 78.5 (OCH_2Ph), 114.8 ($CH_{Ar(PMP)}$), 121.2–121.4 ($CH_{Ar(PMP)}$), 128.8–129.0 ($CH_{Ar(Bn)}$), 129.3–129.4 ($CH_{Ar(Bn)}$), 135.4–135.7 ($C_{Ar(Bn)}$), 144.2 ($C_{Ar}OP$), 156.9 ($C_{Ar}O$ CH_3), 167.8 (CONH), 174.2 (d, $^3J_{C\text{-}P}$ = 6.8 Hz, CO_2CH_3), 174.5 (d, $^3J_{C\text{-}P}$ = 6.9 Hz, CO_2CH_3); ^{31}P NMR (162.0 MHz, $CDCl_3$): δ_P 2.70, 3.00; MS (EI)$^+$: *m/z* calculated for $C_{21}H_{27}N_2O_8PNa$ $[M+Na]^+$ 489.14, found 489.14.

5.1.12. N-(Benzyloxy)-3-hydroxy-N-methylpropanamide-3-[(4-methoxy) phenyl (methoxyalaninyl) phosphoramidate] (16b)

To a solution of alcohol **14b** (194 mg, 0.93 mmol), triethylamine (0.15 mL, 1.02 mmol), DMAP (57 mg, 0.47 mmol) in dry DCM (2.8 mL) was treated with a solution of compound **13** (860 mg, 2.8 mmol) in DCM (2.2 mL) at −40 °C. The resulting mixture was warmed up to room temperature and stirred overnight. A saturated aqueous solution of NH_4Cl (10 mL) was added and the aqueous layer was extracted with DCM (2 × 15 mL). The organic layers were collected, dried over anhydrous Na_2SO_4, filtered and solvents were removed under reduced pressure. The crude product was purified by flash chromatography (EtOAc/cyclohexane, 9:1) to give **16b** as a colorless oil (213 mg, 48%) and as a mixture of two diastereomers in a 50:50 ratio. Rf = 0.23 (EtOAc/petroleum ether, 6:4).^{1}H NMR (400 MHz, $CDCl_3$): δ 1.35 (5/10 of 3H, d, ^3J = 7.0 Hz, NCHCH_3), 1.39 (5/10 of 3H, d, ^3J = 7.1 Hz, NCHCH_3), 2.66–2.88 (2H, m, CH_2CO), 3.19 (5/10 of 3H, s, NCH_3), 3.20 (5/10 of 3H, s, NCH_3), 3.70–3.72 (3H, m, OCH_3), 3.75 (3H, m, OCH_3), 4.00–4.08 (1H, m, NCHCH_3), 4.31–4.48 (2H, m, $POCH_2$), 4.76–4.81 (2H, m, OCH_2Ph), 6.79–6.81 (2H, m, CH_{Ar}), 7.09–7.014 (2H, m, CH_{Ar}), 7.34–7.39 (5H, m, $CH_{Ar(Bn)}$); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 21.3 (NCHCH$_3$), 33.2 (CH$_2$CO), 33.6 (NCH_3), 50.3 (NCHCH$_3$), 50.4 (NCHCH$_3$), 52.6 (CO_2CH_3), 55.8 (ArOCH$_3$), 62.9 ($POCH_2$), 76.6 (OCH_2Ph), 114.7 ($CH_{Ar(PMP)}$), 121.4–121.5 ($CH_{Ar(PMP)}$), 128.9 ($CH_{Ar(Bn)}$), 129.3 ($CH_{Ar(Bn)}$), 129.5 ($CH_{Ar(Bn)}$), 134.4 ($C_{Ar(Bn)}$), 144.5–144.6 ($C_{Ar}OP$), 156.6 ($C_{Ar}O$ CH_3), 171.8 (d, $^4J_{C\text{-}P}$ = 9.4 Hz, CONCH$_3$), 174.2 (d, $^3J_{C\text{-}P}$ = 7.4 Hz, CO_2CH_3), 174.3 (d, $^3J_{C\text{-}P}$ = 7.7 Hz, CO_2CH_3); ^{31}P NMR (162.0 MHz, $CDCl_3$): δ_P 2.75, 2.90; MS (EI)$^+$: *m/z* calculated for $C_{22}H_{29}N_2O_8PNa$ $[M+Na]^+$ 503.16, found 503.16.

5.1.13. Methyl((3-(hydroxyamino)-3-oxopropoxy)(4-methoxyphenoxy) phosphoryl)-L-alaninate (8a)

The protected alcohol **16a** (29 mg, 62 μmol), palladium on charcoal (10% mol) in absolute methanol (1.4 mL) was hydrogenolyzed at room temperature and at atmospheric pressure under an atmosphere of H2. After total consumption of starting material (TLC) the catalyst was removed by filtration over celite and the solvent was removed under reduced pressure. The final product **8a** was obtained without purification as a colorless oil (18 mg, 77%) and as a mixture of two diastereomers in a 55:45 ratio. Rf = 0.58 (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.33 (3H, m, NCHCH_3), 2.52 (2H, bs, CH_2CO), 3.68–3.70 (3H, m, OCH_3), 3.74 (3H, m, OCH_3), 3.92–4.00 (1H, m, NCHCH_3), 4.30–4.35 (2H, m, $POCH_2$), 6.79–6.82 (2H, m, CH_{Ar}), 7.06–7.09 (2H, m, CH_{Ar}), 10.0 (1H, bs, N(H)OH); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 20.7 (NCHCH$_3$), 32.3 (CH$_2$CO), 36.0 (NCH_3), 50.3 (NCHCH$_3$), 50.5 (NCHCH$_3$), 52.7 (CO_2CH_3), 52.8 (CO_2CH_3), 55.8 (ArOCH$_3$), 63.5 ($POCH_2$), 114.7 ($CH_{Ar(PMP)}$),114.8 ($CH_{Ar(PMP)}$), 121.2 ($CH_{Ar(PMP)}$), 121.3 ($CH_{Ar(PMP)}$), 121.4 ($CH_{Ar(PMP)}$), 121.5 ($CH_{Ar(PMP)}$), 144.1 ($C_{Ar}OP$), 156.8 ($C_{Ar}OCH_3$), 167.8 (CONCH$_3$), 167.9 (CONCH$_3$), 174.6 (d, ^3J = 5.6 Hz, CO_2CH_3), 174.8 (d, ^3J = 6.6 Hz, CO_2CH_3); ^{31}P NMR (162.0 MHz, $CDCl_3$): δ_P 2.90, 3.40; HRMS (EI)$^+$: *m/z* calculated for $C_{14}H_{21}N_2O_8PNa$ $[M+Na]^+$ 399.0928, found 399.0918.

5.1.14. Methyl ((3-(hydroxy(methyl)amino)-3-oxopropoxy)(4-methoxyphenoxy)phosphoryl)-L-alaninate (8b)

The protected alcohol **16a** (23 mg, 48 μmol), palladium on charcoal (10% mol) in absolute methanol (1 mL) was hydrogenolyzed at room temperature and at atmospheric pressure under an atmosphere of H_2. After total consumption of starting material (TLC) the catalyst was removed by filtration over celite and the solvent was removed under reduced pressure. The final product **8b** was obtained without purification as a colorless oil (18 mg, 92%) and as a mixture of two diastereomers in a 50:50 ratio and of two *Z* and *E* conformers in a 20:80 ratio respectively. Rf = 0.51 (EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.35 (5/10 of 3H, d, ^3J = 6.9 Hz, NCHCH_3), 1.39 (5/10 of 3H, d, ^3J = 6.4 Hz, NCHCH_3), 2.70 (2/10 of 2H, bs, CH_2CO), 2.92 (8/10 of 2H, bs, CH_2CO), 3.18 (8/10 of 2H, s, NCH_3), 3.29 (2/10 of 2H, s, NCH_3), 3.71 (5/10 of 3H, s, OCH_3), 3.72 (5/10 of 3H, s, OCH_3), 3.78 (3H, m, OCH_3), 3.98 (1H, m, NCHCH$_3$), 4.37–4.45 (2H, m, $POCH_2$), 6.82 (2H, dd, ^4J = 8.9 Hz, ^3J = 1.4 Hz CH_{Ar}), 7.07–7.10 (2H, m, CH_{Ar}), 9.16 (1H, bs, N(CH_3)OH);^{13}C NMR (125.8 MHz, $CDCl_3$): δ_C 20.0 (NCHCH$_3$), 32.2 (CH$_2$CO), 33.3 (CH$_2$CO), 36.0 (NCH_3), 50.3 (d, ^2J = 8.6 Hz, NCHCH$_3$), 52.8 (pd, ^5J = 3.4 Hz CO_2CH_3), 55.8 (ArOCH$_3$), 62.9 ($POCH_2$), 64.5 ($POCH_2$), 114.8 ($CH_{Ar(PMP)}$), 121.2 ($CH_{Ar(PMP)}$), 121.3 ($CH_{Ar(PMP)}$), 121.4 ($CH_{Ar(PMP)}$), 121.5 ($CH_{Ar(PMP)}$), 144.1 ($C_{Ar}OP$), 156.9 ($C_{Ar}OCH_3$), 170.5 (CONCH$_3$), 170.6 (CONCH$_3$), 174.1 (d, ^3J = 7.3 Hz, CO_2CH_3), 174.2 (d, ^3J = 7.8 Hz, CO_2CH_3); ^{31}P NMR (162.0 MHz, $CDCl_3$): δ_P 2.85, 3.10; HRMS (EI)$^+$: *m/z* calculated for $C_{15}H_{23}N_2O_8PNa$ $[M+Na]^+$

413.1084, found 413.1048.

5.2. Bacterial growth conditions

M. smegmatis DSM43756 (ATCC 19420) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Bacteria were grown aerobically at 30 °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% $CaCO_3$, 0.1% D-glucose and 1.6% agar [32]. *E. coli* XL1-blue was grown in a liquid LB medium or on a solid LB-agar medium at 37 °C.

5.3. Antibacterial activity of prodrugs

The antibacterial activity of the prodrugs was tested on *M. smegmatis* and *E. coli* XL1-blue by the paper disc diffusion method. Plates were inoculated with bacteria (120 µL) of a tenfold-diluted overnight culture. Prodrugs, not soluble in water, were dissolved at a 100 mM concentration in DMSO. Isoniazid and fosmidomycin were used as positive controls for *M. smegmatis* and *E. coli* respectively. Paper discs (Durieux no. 268, diameter 6 mm) were impregnated with the prodrug solutions (8 µL, 800 nmoles) or with a 5 mM isoniazid or a fosmidomycin (2 µL, 10 nmol) solution. Growth inhibition was examined after 24–48 h incubation.

5.4. Stability assays of the prodrugs in buffered solution

The stability of the prodrugs in a 50 mM triethanolamine (TEOA) buffer (600 µL final volume, pH 7.5) containing D_2O (200 µL) was studied over 48 h using ^{31}P NMR. The prodrugs were dissolved in DMSO at a concentration of 100 mM and diluted in the reaction medium to 3 mM.

5.5. Stability assays of the prodrugs towards carboxypeptidase Y

The hydrolysis of the prodrugs by carboxypeptidase Y (Sigma-Aldrich) was studied using ^{31}P NMR. The experiments (600 µL final volume) were carried out in a TEOA 50 mM pH 7.5 containing D_2O (200 µL). The concentration of the prodrugs was 3 mM. Spectra were recorded after addition of the enzyme (0.04 mg) and the hydrolysis was followed over 48 h.

5.6. Stability assays of the prodrugs **8a** and **8b** during M. smegmatis and E. coli cultures

E. coli XL1-blue was grown in LB medium (50 mL) for 24 h. The culture was centrifuged (15 min at 7000 rpm), and the supernatant was filtered through a 0.22 µM sterile unit (Millex Millipore). *M. smegmatis* was grown in medium (50 mL) during 40 h, and the culture was then processed as described above.

The stability of the prodrugs in both resulting supernatants was followed over 48 h using ^{31}P NMR. The prodrugs (3 mM) were incubated with a mixture of used growth medium (400 µL) and D_2O (200 µL).

5.7. Esterase and phosphatase activity in bacterial crude cell-free extracts

Cells from a 500 mL culture were harvested by centrifugation (7000 rpm, 15 min) and the pellet was frozen. Cells (about 1 g) were resuspended in a 50 mM TEOA buffer (2 mL, pH 7.5) and sonicated (5 × 30 s with 2 min cooling in ice). After centrifugation (14 000 rpm, 25 min), the supernatant was collected. Protein concentration was measured by the method of Bradford using bovine serum albumin (BSA) as a standard.

The stability of the prodrugs towards enzymes of both crude extracts was studied using ^{31}P NMR. The experiments (600 µL final volume) were carried out in a TEOA 50 mM buffer (pH 7.5) containing D_2O (200 µL) and crude cell-free system (150 µL). The concentration of the prodrugs was 3 mM. Spectra were recorded after addition of the crude cell-free system and the enzymatic reaction was followed over 20 h.

5.8. Esterase activity in crude cell-free systems of M. smegmatis and E. coli

The esterase activity in the crude cell-free systems was measured with 4-nitrophenyl butyrate (Sigma) as substrate. The assays (1 mL final volume) were performed at 30 °C in a 50 mM TEOA buffer (pH 7.5). The concentration of the substrate was 1 mM. The reaction was initiated by addition of crude cell-free system (5 µL) and followed at 405 nm with an Uvikon 933 spectrophotometer. The enzymatic activity was followed by the release of *p*-nitrophenol ($\varepsilon = 18000\ M^{-1}\ cm^{-1}$) and expressed in µmoles min^{-1}mg $protein^{-1}$.

5.9. Phosphatase activity in crude cell-free systems of M. smegmatis and E. coli

The phosphatase activity in the crude extracts was measured with 4-nitrophenyl phosphate (Sigma) as the substrate. The assays (1 mL final volume) were performed at 30 °C in a 50 mM TEOA buffer (pH 7.5). The influence of the presence of 5 mM dication ($MgCl_2$, $MnCl_2$ and $CoCl_2$), on the hydrolysis, was also investigated. The enzymatic activity was determined as for the esterase activity.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103012.

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