

Modifications around the hydroxamic acid chelating group of fosmidomycin, an inhibitor of the metalloenzyme 1-deoxyxylulose 5-phosphate reductoisomerase (DXR)

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ABSTRACT

Fosmidomycin derivatives in which the hydroxamic acid group has been replaced by several bidentate chelators as potential hydroxamic alternatives were prepared and tested against the DXR from *Escherichia coli*. These results illustrate the predominant role of the hydroxamate functional group as the most effective metal binding group in DXR inhibitors.

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Metalloenzymes are a family of enzymes that contains in the active site one or more metal ions, which are essential for their biological function. Metals are involved in structure stabilization of the enzymes and/or in catalysis. They participate in numerous enzyme reactions including hydrolysis,¹ oxido-reduction,² isomerization³ and electron transfer reactions.⁴ Since these enzymes are mostly implied in essential metabolic pathways, their inhibition affects the growth of living organisms.⁵ Inhibitors of metalloenzymes typically contain simultaneously a group that binds to the catalytic metal ion and a moiety that mimics the substrate to which the chelating group is attached, providing the specificity of the binding.⁶

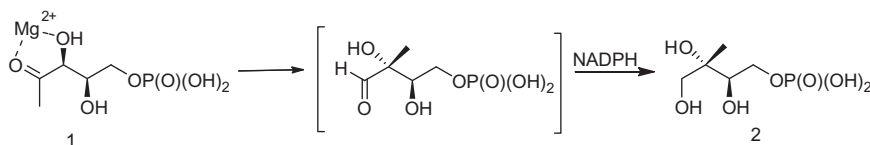
Among this family, the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), the second enzyme of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway catalyzes the Mg^{2+} triggered rearrangement of 1-deoxy-D-xylulose 5-phosphate **1** (DXP) into a non-isolable aldehyde and its concomitant NADPH-dependent reduction into 2-C-methyl-D-erythritol 4-phosphate **2** (Scheme 1). DXR is an attractive target for the development of new antimicrobials and herbicides.⁷ Indeed the MEP pathway is present in most eubacteria, in green algae, in the chloroplasts of phototrophic organisms and in some unicellular eukaryotes, but is absent in animals and fungi.⁸

Phosphonate antibiotics such as fosmidomycin **3** or FR-900098 **4** are naturally occurring potent inhibitors of the DXR (Scheme 2) and present strong antibacterial and antimalarial activity.^{9,10} Due to their moderate oral bioavailability and their fast clearance these compounds cannot be used as drugs.¹¹ In addition, many pathogens, such as *Mycobacterium tuberculosis*, are completely resistant to fosmidomycin and its analogues.¹² The crystal structure of the *Escherichia coli* DXR-fosmidomycin complex was resolved at 2.5 Å and revealed two binding sites in the catalytic domains: a specific positively charged pocket anchors by hydrogen bonds the negatively charged phosphonate and a chelating site for the binding of the divalent metal cation by the two oxygen atoms of the hydroxamate group.¹³ Based on the elucidation of crystal structures of DXR, several modifications of fosmidomycin and of its analogue FR 900098 were carried out to expand the library of potential DXR inhibitors and, most importantly, numerous analogues have been synthesized to improve the pharmaceutical properties. The modifications concern essentially the three-carbon spacer between the hydroxamate and the phosphonate groups.^{14–16} Most of them are, however, much less efficient than fosmidomycin. We replaced the phosphonate group by bioisosteric groups (sulfonate, carboxylate) but the results were also disappointing, these compounds being only weak inhibitors of the DXR.¹⁷

Hydroxamates are monoanionic bidentate chelating groups that bind Mg^{2+} with two oxygen donor atoms and form hydrogen bound within the DXR active site. Most hydroxamic acids have poor oral bioavailability and have binding affinities for metals such as zinc(II), copper(II), iron(III) and nickel(II).^{18,19} To overcome some

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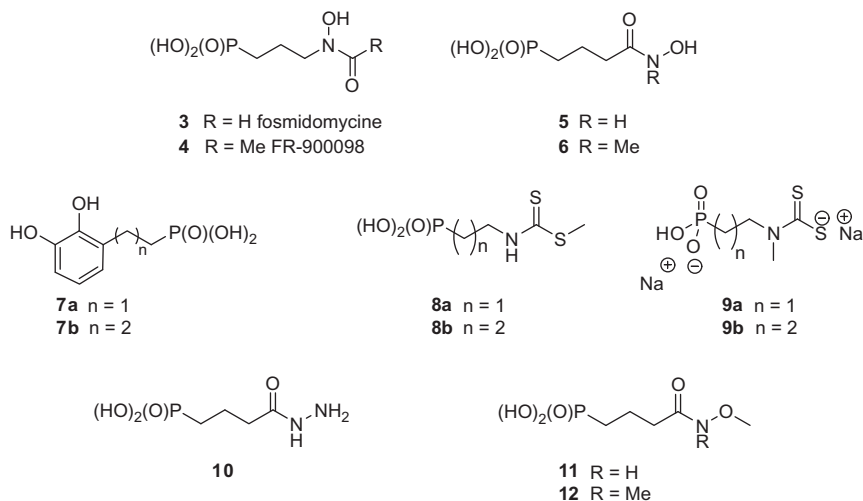
Scheme 1. Reaction catalyzed by the DXR.

of these limitations, variations of the chelating group have also been investigated based on the replacement of the hydroxamate by an hydroxyurea, a benzoxazolone, a benzoxazolethione, an oxaloylpyridone, and catechols.²⁰ In this context, we have previously reported the synthesis of two phosphonohydroxamates **5** and **6** (Scheme 2) that present a reverse hydroxamate moiety with respect to that of fosmidomycin. They show a biological activity comparable to that of fosmidomycin, particularly the *N*-methylated compound **6**, which is also efficient on a fosmidomycin-resistant *E. coli* strain.²¹ The goal of our studies was to discover an effective alternative to the hydroxamate metal binding group. We investigated accordingly the replacement of the hydroxamate group by other bidentate chelators such as a catechol,^{20c,22} a hydrazide,²³ or a dithiocarbamate group.²⁴ The modification of the spacer length was also included in this study, and the hydroxamic acid functionality was modified by alkylating the hydroxamate oxygen. All compounds were tested on the DXR of *E. coli* for potential inhibition.

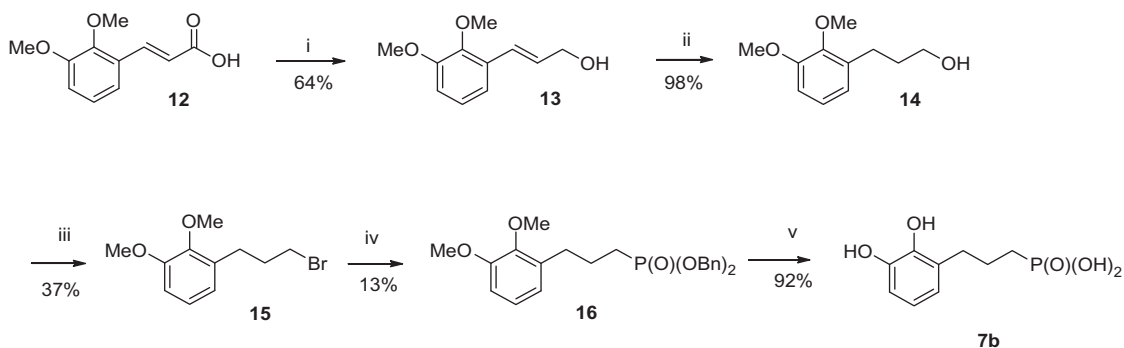
The catechol phosphonate **7a** was synthesized as previously described by J.W. Frost, starting from commercial 2,3-dimethoxy-

benzaldehyde.²² As its higher homologue 2-(2,3-dimethoxyphenyl)acetaldehyde is not commercially available, compound **7b** was prepared according to Scheme 3. The reduction of 2,3-dimethoxycinnamic acid **12** with NaBH₄ in the presence of LiCl, followed by hydrogenation, gave alcohol **14**. This compound was converted to bromide **15** by treatment with phosphorus tribromide. Nucleophilic substitution with bromide **15** with the NaH generated anion of dibenzyl phosphite gave the corresponding phosphonate **16**. The simultaneous deprotection of the methoxy and the benzyl groups was performed in one step with boron tribromide and afforded compound **7b**.

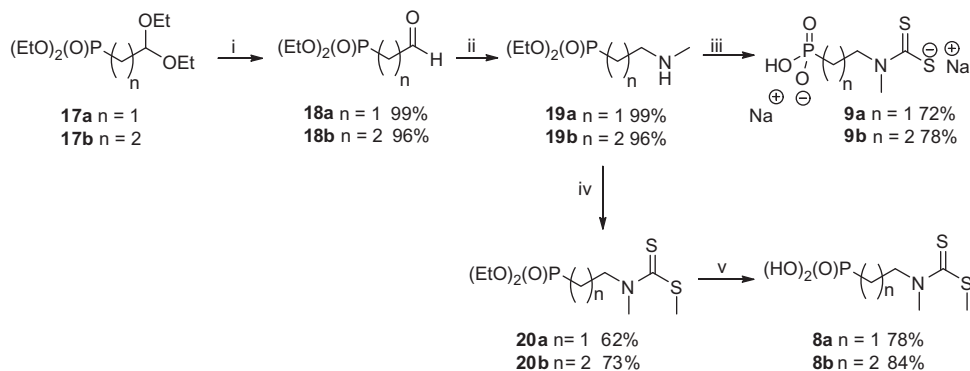
The syntheses of the sodium salt of dithiocarbamates **9a** and **9b** and their *S*-methylated derivatives **8a** and **8b** are shown in Scheme 4. Treatment of the commercially available diethyl 2,2-diethoxyethylphosphonate **17a** and its C₃ homologue diethyl 2,2-diethoxypropylphosphonate **17b** with a solution of 2 M HCl gave respectively aldehydes **18a** and **18b**. Reductive amination of the aldehydes with NaBH₃CN and methylamine hydrochloride afforded the amines **19a** and **19b**, which were converted into dithiocarbamates **9a** and **9b** by treatment with carbon disulfide in presence



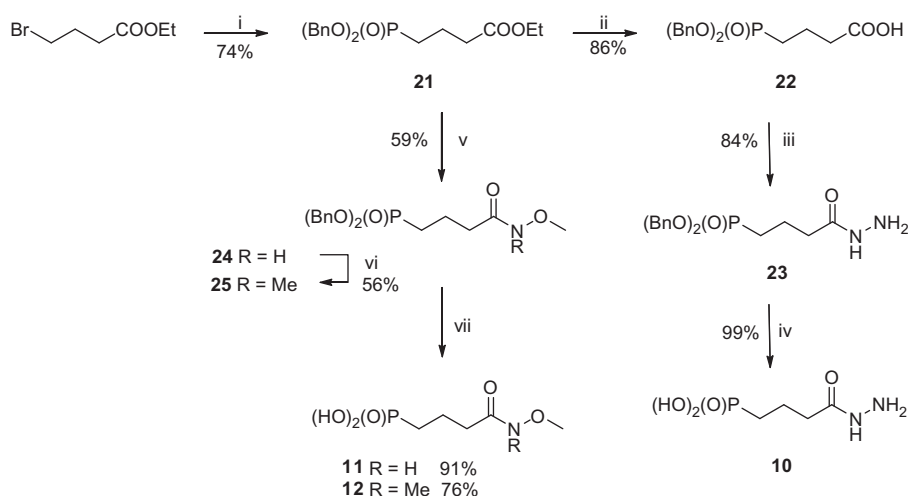
Scheme 2. Structures of fosmidomycin **3**, FR-900098 **4**, reverse phosphonohydroxamates **5** and **6** and target compounds **7–12**.



Scheme 3. Synthetic route to compound **7b**. (i) NaBH₄, LiCl, THF/EtOH, 0 °C; (ii) H₂, 10% Pd/C, MeOH; (iii) PBr₃, Et₂O reflux; (iv) NaH, THF, HP(O)(OBn)₂, 0 °C; (v) BBr₃, CH₂Cl₂/H₂O.



Scheme 4. Synthetic route towards dithiocarbamates **8a** and **8b**, **9a** and **9b**. (i) HCl 2 M aq; (ii) $\text{NH}_2\text{Me}\cdot\text{HCl}$, NaBH_3CN , MeOH, 0 °C; (iii) CS_2 , NaOH 3 M, THF; (iv) CS_2 , Et_3N , THF, 0 °C then MeI; (v) HCl 6 M aq, reflux, 2 days.



Scheme 5. Synthetic route to hydrazide **10** and O-methylated hydroxamates **11** and **12**. (i) $(\text{BnO})_2\text{P}(\text{O})\text{H}$, NaH, THF; (ii) LiOH, THF/ H_2O , rt; (iii) $\text{H}_2\text{N}-\text{NH}_2$, EtOH; (iv) H_2 , Pd/C, EtOH; (v) $\text{NH}_2\text{OMe}\cdot\text{HCl}$, AlMe_3 , THF, −40 °C; (vi) NaH, THF, MeI; (vii) H_2 , Pd/C, MeOH.

of 3 M NaOH, a treatment that also released the free phosphonate group from the ethyl diester. The S-methylated analogues were obtained by addition of carbon disulfide on the amines **19a** and **19b** in presence of triethylamine followed by in situ addition of methyl iodide. Deprotection of the ethyl groups in acidic conditions afforded the desired compounds **8a** and **8b**. The ^1H NMR spectra of **20** and **8** display two peaks at ca. 3.20 and 3.40 ppm for the N-methyl protons corresponding, respectively, to the Z and E conformers in a ca. 60/40 ratio. In fact, it has been shown that the N-substituted dithiocarbamates are present as an equilibrium mixture of the Z and E conformations due to the restricted rotation around the dithiocarbamate C–N bond.²⁵

The synthesis of hydrazide **10** is described in Scheme 5. The first step was the introduction of the phosphonate group. Deprotection of dibenzyl phosphite with sodium hydride in THF afforded the corresponding anion, which upon reaction with the commercially available ethyl 4-bromobutanoate gave the corresponding phosphonate **21**. Hydrolysis of the ethyl ester **21** with lithium hydroxide afforded the carboxylic acid **22**, which was converted into the hydrazides **23** by treatment with hydrazine. The benzyl groups were removed by catalytic hydrogenolysis in the presence of palladium over activated charcoal at room temperature and atmospheric pressure to provide the desired hydrazide **10**.

The O-methylhydroxamate derivatives **11** and **12** were prepared from phosphonate **21** as previously described (Scheme 5).

Treatment of the ester **21** with methoxyamine hydrochloride in the presence of trimethylaluminum afforded the O-methyl hydroxamate **24**.²⁶

The N-methylation of the O-methyl hydroxamate **24** was accomplished by using methyl iodide in the presence of NaH to provide the expected compound **25**. The benzyl groups of **24** and **25** were removed by catalytic hydrogenolysis with palladium over charcoal under atmospheric pressure and at room temperature. The yields were quantitative, and no further purification of the O-methylated hydroxamates **11** and **12** was required.

The ability of the synthetic compounds **7–12** to inhibit the His-tagged recombinant *E. coli* DXR was investigated. The rate of conversion of DXP into MEP by the enzyme was determined by monitoring the NADPH consumption at 340 nm with an UV–visible spectrophotometer.²¹ Since fosmidomycin and probably most of its derivatives are slow tight-binding inhibitors of the DXR,²⁷ the IC_{50} values of the inhibitors were determined with and without pre-incubation (2 min at 37 °C) of the compounds **7–12** with the enzyme and NADPH before the reaction was started by addition of DXP. The results are summarized in Table 1.

Compared to fosmidomycin **3** and its reverse hydroxamate analogue **6**, all synthesized compounds were found to be either no or weak inhibitors (millimolar IC_{50} values) of the *E. coli* DXR (Table 1). Except for compound **11**, the pre-incubation of the enzyme with the different compounds did not improve notably the inhibition potency (maximum 1.8 fold) while, in the case of fosmidomycin

Table 1

Inhibition of recombinant His tagged *E. coli* DXR by fosmidomycin **3** and reverse *N*-methylated phosphonohydroxamate **6** and chelating analogues **7–12**

Compd	IC ₅₀ (μM) ^a without pre-incubation	IC ₅₀ (μM) ^a with pre-incubation (2 min)
3	0.25	0.032
6	0.5	0.048
7a	2000	1200
7b	ni ^b	ni ^b
8a	2800	2700
8b	ni ^b	ni ^b
9a	ni ^b	ni ^b
9b	ni ^b	ni ^b
10	1800	1000
11	11500	930
12	6900	3800

^a Mean from at least 2 different assays. Errors were <5%.

^b ni = no inhibition at 1000 μM.

and compound **6**, the enhancement was about 8 and 10 fold respectively. Compounds **7b** and **8b** having a hydrocarbon spacer with an additional methylene group showed a dramatic decrease of the affinity of DXR with regard to the compounds **7a** and **8a** having a three methylene carbon chain like fosmidomycin.¹⁷

As previously reported by Deng et al. catechol **7a** is a weak inhibitor, with regard of fosmidomycin, of the *E. coli* DXR. This compound does not fit well in the active site. Either the interactions between the catechol group and the cation or those between the phosphonate group and the enzyme do not take place. Interestingly they noted a slightly better inhibition (fivefold) with a catechol having the phosphonate chain in *beta* position of one the hydroxyl groups.²⁸ This catechol has been also reported to be the best *Mycobacterium tuberculosis* DXR inhibitor with an IC₅₀ value in the micromolar range, in a series of five compounds in which the hydroxamic acid moiety has been replaced by metal binding heterocycles.^{20c} In those cases, the catechol group adopts a better positioning in the active site leading to a tighter binding between the enzyme and the inhibitor. Compound **7b** did not inhibit the enzyme. We expected, however, that the additional methylene group would increase the flexibility of the spacer and lead to a better fitting of compound **7b** into the active site. The absence of inhibition with this compound may be explained by a steric hindrance preventing the formation of the enzyme/inhibitor complex and/or by the rigidity of the active site preventing its accommodation to larger molecule.

Chelating agents such as dithiocarbamate are well-known metal complexing compounds due to the presence of the anionic CS₂[−] moiety, which can coordinate mono- or bidentate cations.²⁹ In our case, the replacement of the hydroxamate with a *S*-methylated dithiocarbamate does not improve the IC₅₀ value. The relative failure of the *S*-methylated dithiocarbamate **8a** and **8b** derivatives to inhibit the DXR can be due to the soft base character of the sulfur atoms, which have a weak affinity for hard cations such as Mg²⁺. Moreover dithiocarbamates compounds are known to be unstable, and their rates of decomposition depend on the substituent on the nitrogen, pH and temperature.³⁰ In our enzyme assay conditions (pH = 7.5), the sodium salt of dithiocarbamates **9a** and **9b** decomposed releasing off carbon disulfide. Consequently, their inhibition capacity could not be determined.

Replacing the hydroxamate chelating group with a hydrazide group as in molecule **10** showed no improvement of the inhibition. The IC₅₀ value was similar to that obtained with **7a**. The reason is probably that the hydrazide group is protonated at the pH of the enzymatic assays (pH 7.5), reducing the chelating potency of the N–H with the Mg²⁺ ion.

With *O*-methylated hydroxamate **11** and its *O,N*-dimethylated derivative **12**, the inhibition, although less effective than that of

the corresponding hydroxamate **6** (2300 fold), was more efficient and showed a slow tight-binding behavior as compared to the other tested chelators. Indeed its IC₅₀ decreased about ten-fold, as observed with compounds **3** and **6**, when the inhibitor is pre-incubated for 2 min with the enzyme before starting the enzymatic reaction. In contrast, the interactions of the *O,N*-dimethylated compound **12** with DXR was less effective than all *N*-methyl hydroxamate previously described, which were more effective inhibitors than the non-methylated compounds.²¹ This is probably due to the presence of the bulky *O*-methyl group, which prevents the binding by steric hindrance.

In summary, fosmidomycin derivatives in which the hydroxamic acid group has been replaced by several bidentate chelators as potential hydroxamic alternative were prepared and tested against the DXR of *E. coli*. Even if divalent Mg²⁺ is a hard ion and prefers hard ligands with oxygen being the most preferred coordinating atom followed by nitrogen, the replacement of the hydroxamic group by hydrazine, catechol and *O*-methylated hydroxamate moieties resulted in an increase of the IC₅₀ value. These results showed that the attempts to replace the hydroxamate group by other chelating groups are ineffective and illustrate the predominant role of the hydroxamate functional group as the most effective metal binding group in DXR inhibitors.

The high inhibition potency of fosmidomycin was due to the ideal fitting of the molecule in the active site of DXR. The phosphonate group binds in the phosphate recognition site of DXP, the substrate of the enzyme, and the hydroxamate group chelates optimally to the catalytic Mg²⁺ cation. The two functional groups responsible for the inhibitor binding are linked by a three methylene spacer. Fosmidomycin is a slow-binding inhibitor; its binding to the enzyme is followed by a conformational change leading to a more stable complex.^{21,27} A preincubation of DXR with compounds **3** or **6** leads to an about 10 fold improvement of the IC₅₀ values. It is not known if the effective coordination results from the conformational change or if it takes place immediately after binding of the inhibitor. If we assume that the phosphonate group of the different potential inhibitors binds as fosmidomycin in the phosphate binding pocket, the low inhibitory potency of these molecules could result from the lack of formation of a strong coordination complex between the tested chelating groups and the cation. The absence of a strong binding could be explained by the fact that the chelating groups are not ideally placed with regard to the catalytic ion. This is probably the case for catechol derivatives, a group known to chelate Mg²⁺ ions as efficiently as a hydroxamate.^{20c} Indeed the inhibitory potency depends on the positions of the hydroxyl groups: the compound with hydroxyls in positions 3,4 are more efficient than the compound with hydroxyls in positions 2,3.²⁸ Concerning the dithiocarbamate derivatives, the problem can also come from the chelating capacity of the reactive group with regard to the Mg²⁺ ion. The latter is a hard ion, which prefers hard ligands and generally binds oxygen ligands in an octahedral coordination. Nitrogen or sulfur tends to coordinate to soft and more polarizable ions like zinc.³¹

Our results illustrate the predominant role of the hydroxamate group as the most effective bidentate metal binding group in DXR inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.09.021>.

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