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Isoprenoid Biosynthesis via the Methylerythritol Phosphate Pathway: Structural Variations around Phosphonate Anchor and Spacer of Fosmidomycin, a Potent Inhibitor of Deoxyxylulose Phosphate Reductoisomerase

Catherine Zinglé, Lionel Kuntz, Denis Tritsch, Catherine Grosdemange-Billiard,* and Michel Rohmer*

Université de Strasbourg/CNRS, Strasbourg, UMR 7177, Institut Le Bel, 4 rue Blaise Pascal, 67070 Strasbourg, France

grosdemange@unistra.fr; mirohmer@unistra.fr

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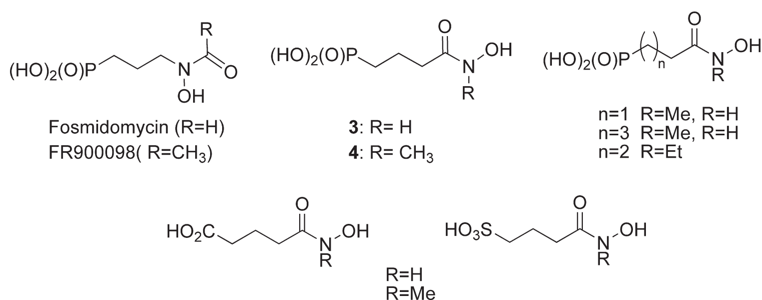

Fosmidomycin and its analogue FR-900098 are potent inhibitors of 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (DXR), the second enzyme of the MEP pathway for the biosynthesis of isoprenoids. This paper describes the synthesis of analogues of the two reverse phosphonohydroxamic acids **3** and **4**, in which the length of the carbon spacer is modified, the *N*-methyl group of **3** is replaced by an ethyl group, and the phosphate group is replaced by potential isosteric moieties, i.e., sulfonate or carboxylate functionalities. The potential of the synthesized analogues to inhibit the *E. coli* DXR was evaluated.

Introduction

The discovery of the alternative mevalonate-independent methylerythritol phosphate (MEP) pathway for the biosynthesis of isoprenoids,[1] which is present in pathogens such as the tuberculosis-causing bacterium *Mycobacterium tuberculosis* and the malaria protozoan agent *Plasmodium falciparum* but absent in humans, represents an attractive target for the design and development of new antimicrobial drugs.[2−4] Fosmidomycin **1** (Scheme 1), a phosphonate antibiotic isolated from *Streptomyces lavendulae*,[5] inhibits 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (DXR), the second enzyme of the MEP pathway of many Gram-negative bacteria, some Gram-positive bacteria,[6] and the malaria parasites.[7] The resolution at 2.5 Å of the three-dimensional structure of the *Escherichia coli* DXR-fosmidomycin complex revealed two binding sites in the catalytic domain: a first one for the chelation of a divalent metal ion (Mn^{2+}, Mg^{2+}, or Co^{2+})

*To whom correspondence should be addressed. Phone: + 33 (0)3.68.85.13.49 and + 33 (0)3.68.85.13.46. Fax: + + 33 (0)3.68.85.13.45.

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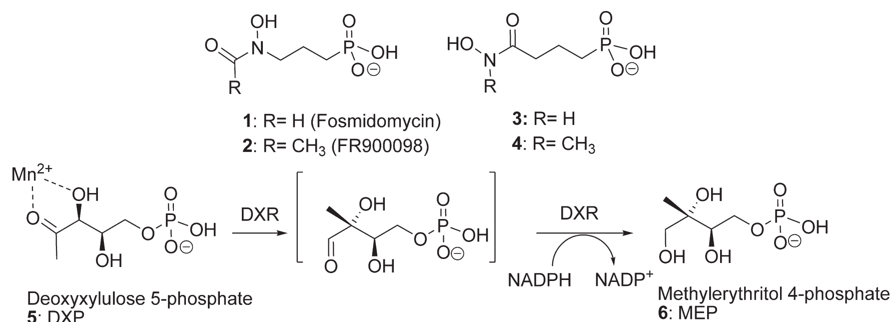
SCHEME 1. 1-Deoxy-D-xylulose 5-Phosphate Reducto-Isomerase (DXR) and Its Inhibitors (1−4)



by the two oxygen atoms of the hydroxamate moiety of the antibiotic, and a second one where the negatively charged phosphonate group is anchored by several hydrogen bonds in a specific positively charged pocket. These two functional groups involved in the antibiotic binding are connected by a spacer of three methylene groups.[8] Fosmidomycin **1** and its analogue, FR-900098 **2** (Scheme 1), are effective antimalarial agents with good tolerability and rapid onset of action, but late recrudescence and fast clearance preclude their use alone in a single drug treatment.[9] In the case of *Mycobacterium tuberculosis*, the inefficiency of fosmidomycin to inhibit its growth was due to a lack of uptake.[10]

Investigations are presently oriented toward the design of fosmidomycin analogues with improved pharmacological properties. Attention was mostly focused on modifications of the phosphonate group and the three-carbon spacer, yielding, on the one hand, phosphate,[11] carboxylate,[12] sulfonate,[13] sulfone,[13] or sulfamate[11−13] analogues and various phosphonate diester prodrugs[14] and on the other hand rigidified carbon spacers with a cyclopropyl[15]or a cyclopentyl ring,[16a] spacers with an aryl substituent in the α position of the phosphonate,[16] or spacers including oxa groups.[17] We previously reported the synthesis of the two reverse phosphonohydroxamic acids **3** and **4** (Scheme 1), which revealed as potent inhibitors of the *E. coli* DXR the natural antibiotics **1** and **2**. The *N*-methylated derivative **4** is much more effective than the non-*N*-methylated compound **3** and is nearly as efficient as fosmidomycin in enzyme assays.[18] The same observation has been reported for FR-900098 **2**, an analogue of fosmidomycin bearing an acetyl group in place of the formyl group.[7,8]

Although X-ray crystal structures of DXR in complex with fosmidomycin have been solved, the rational design of new potent inhibitors by docking methods is not obvious.[19] We present here the synthesis and the biological activity of analogues of the two phosphonohydroxamic acids **3** and **4**. On the one hand, the influence of the length of the carbon spacer and the replacement of the *N*-methyl group of **3** by an ethyl group on the inhibitory activity was investigated. On the other hand the phosphonate anchor of **3** and **4** was replaced by other acidic bioisosters such as a carboxylate, which displays a planar geometry, or a sulfonate sharing with the phosphonate a pyramidal geometry around the central atom.[20]

Results and Discussion

Syntheses of C_3 and C_5 Analogues of Hydroxamates 3 and 4, and of the *N*-Ethyl C_4 Analogue. The hydroxamates **11a**, **11b**, **14a**, **14b**, and **15** were obtained by using the same strategy as that utilized for the syntheses of the C_4 derivatives **3** and **4** previously described (Scheme 2).[18]

Synthesis of Carboxylate Isosters 19a and 19b. The syntheses of the carboxylic acid **19a** and of its *N*-methylated derivative **19b** are shown in Scheme 3. The first step was the introduction of the hydroxamic acid group. Treatment of

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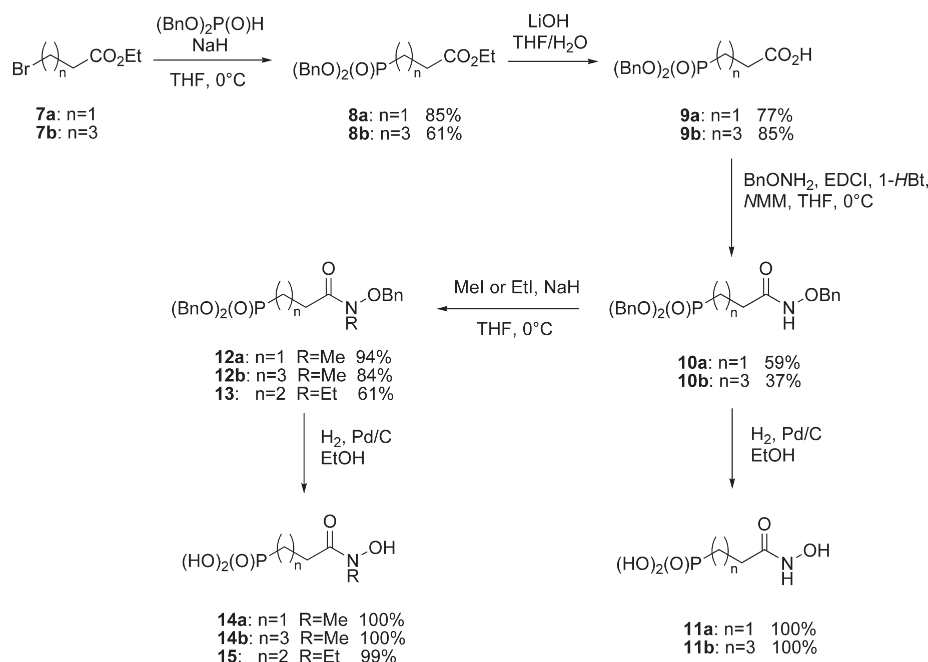
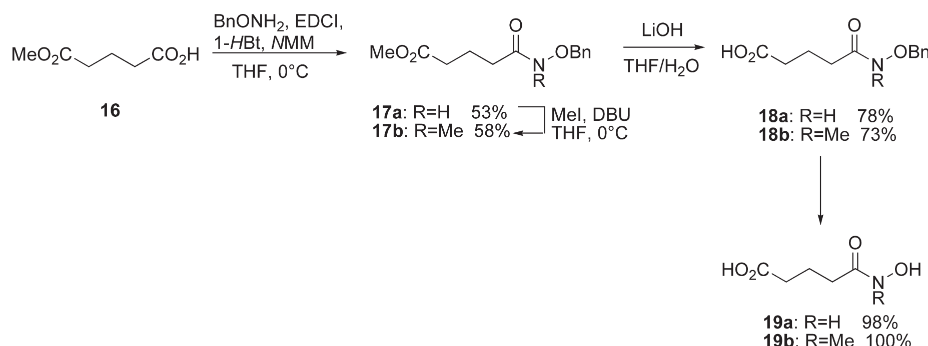
SCHEME 2. Syntheses of C_3 (11a, 14a) and C_5 (11b, 14b) Analogues of Hydroxamates 3 and 4, and of the *N*-ethyl C_4 Analogue (15)



SCHEME 3. Synthesis of Carboxylate Isosters 19a and 19b



the commercially available monomethylglutarate **16** with *O*-benzylhydroxylamine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in THF gave the compound **17a** in 53% yield. The *N*-methylation of the *O*-benzylhydroxamate **17a** was achieved by using DBU, a softer base than sodium hydride, which led to undesired byproduct. The *N*-methylated *O*-benzylhydroxamate **17b** was obtained in 58% yield. Hydrolysis of the methyl ester was again performed in THF with lithium hydroxide dissolved in a minimum amount of water. This provided the carboxylic acid **18a** (78% yield) and the *N*-methylated analogue **18b** (73% yield).

Removal of the benzyl group by catalytic hydrogenolysis with palladium over activated charcoal at room temperature and atmospheric pressure afforded the desired carboxylic acids **19a** and **19b** in a quantitative yield.

Synthesis of the Sulfonate Isosters 25a and 25b. The synthesis of sulfonate **25a** and of its *N*-methylated analogue **25b** started with the commercially available monomethylsuccinate **20** (Scheme 4). The hydroxamate was introduced as described above, leading to **21** (56% yield). Reduction of ester **21** with $NaBH_4$−LiCl gave alcohol **22** (73% yield).

A Mitsunobu-type reaction[21] of alcohol **22** with thioacetic acid proceeded in 84% yield to the thioacetate **23a**. The *N*-methylation of the thioacetate **23a** was accomplished by using methyl iodide in the presence of NaH to provide the expected compound **23b**. The oxidation of the thioacetate **23a** and of the *N*-methylated thioacetate **23b** with peroxytrifluoroacetic acid afforded the sulfonic acids **24a** and **24b** in 98% and 93% yield, respectively.[22] The benzyl group of **24a** and **24b** was removed by catalytic hydrogenolysis with palladium over charcoal under atmospheric pressure and at room temperature leading in quantitative yield and requiring no further purification to the sulfonic acid **25a** and to its *N*-methylated **25b**.

Most NMR spectra presented complicated signal patterns. *N*-Substituted and *N*- and *O*-substituted hydroxamic acids are present as equilibrium mixtures of *Z* and *E* conformers due to the restricted rotation around the C−N bond.[23] The relative

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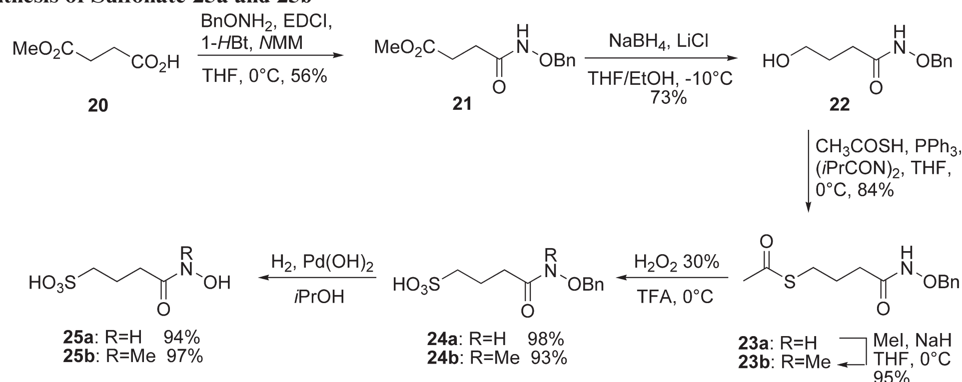
SCHEME 4. Synthesis of Sulfonate 25a and 25b



stabilities of such *Z* and *E* conformers are influenced by the structural and steric factors of the substituents on the hydroxamate moiety as well as by the nature of the solvent. Tentative assignments of the NMR signals are proposed in the Supporting Informations. They were made according to NMR studies and ab initio calculations on similar compounds reported in the literature.[24−29]

Inhibition Tests on the *Escherichia coli* Deoxyxylulose 5-Phosphate Reductoisomerase. The compounds were tested for inhibition against the recombinant *E. coli* DXR as previously described.[18] The IC_{50} values were determined with and without preincubation of the compounds with the enzyme (Table 1). Fosmidomycin and FR900098 were included as reference compounds. Slow tight-binding inhibitors such as fosmidomycin[30] can thus be ascertained because the preincubation leads to a significant decrease of the IC_{50} values (8- to 10-fold). On the one hand shortening the length of the molecule (as in **11a** and **11b**) led to a dramatic decrease of the affinity of DXR for these compounds. They probably bind only to one site, either to the divalent cation or to the phosphate-binding site. On the other hand, with compounds **14a** and **14b** having an additional methylene group, the decrease was less important, about 2-fold as compared to the IC_{50} values found for inhibitors **3** and **4**. The slow-binding mechanism was well pronounced insofar as the preincubation of the enzyme with the two compounds **14a** and **14b** resulted in a significant decrease of the IC_{50} values. Although more flexible and prone to distortions, the longer three methylene spacer apparently prevents compounds **14a** and **14b** from efficiently binding to the active site.[18]

Generally, all *N*-methyl compounds (noted **b**) were more effective inhibitors than the nonmethylated compounds (noted **a**). As suggested by the three-dimensional structure of the DXR-fosmidomycin complex, a hydrophobic interaction

TABLE 1. Inhibition of Recombinant *E. coli* DXR by Derivatives 11−15, 19, and 25

compd	X	R	*n*	IC_{50} (μM) without preincubation	IC_{50} (μM) with preincubation
Fosmidomycin **1**		H	2	0.25	0.032[a]
FR 900098 **2**		Me	2	ND[b]	0.032[c]
3	$PO(OH)_2$	H	2	1	0.17[a]
4	$PO(OH)_2$	Me	2	0.5	0.049[a]
11a	$PO(OH)_2$	H	1	1000	1000
11b	$PO(OH)_2$	Me	1	77	19
14a	$PO(OH)_2$	H	3	2.8	0.27
14b	$PO(OH)_2$	Me	3	0.9	0.11
15	$PO(OH)_2$	Et	2	8.1	6.5
19a	CO_2H	H	2	770	720
19b	CO_2H	Me	2	270	25
25a	SO_3H	H	2	1000	1000
25b	SO_3H	Me	2	564	48

[a]Reference 18. [b]ND = not determined. [c]Reference 13.

between this methyl group and the Trp212 indole group of the DXR active site may explain the increased affinity for FR-900098 **2** over fosmidomycin **1**. By comparing the IC_{50} values of compounds **4** and **15** in which the methyl group is replaced by an ethyl group, a severe decrease of the affinity for DXR was noted for derivative **15**. Preincubation of the enzyme with the inhibitor did not improve the inhibition potency. Apparently the bulky ethyl group prevents the modification of the enzyme conformation leading to the formation of a tighter enzyme/inhibitor complex observed in the case of a slow-binding mechanism.

The replacement of a phosphonate group by other acidic groups such as a carboxylate or a sulfonate has been widely studied for the comparative evaluation of their bioisosteric relationships. Considering the non-*N*-methylated compounds **19a** and **25a**, with respectively a carboxylate and a sulfonate instead of a phosphonate group (compound **3**), the inhibition was drastically decreased (about 4000- to 6000-fold). Moreover the preincubation had no effect. With compounds **19b** and **25b** the inhibition, although less effective than the corresponding phosphonate derivative **4** (500- to 1000-fold), was more efficient and showed a

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slow-binding behavior. As DXR has a rather low affinity for DXP, its natural substrate (K_M = 100−300 μM), the tight complex between DXR and compounds **1**, **2**, or **4** may essentially be ascribed to the chelation of the divalent cation by the reverse hydroxamate or the hydroxamate group of the inhibitors and to the induced conformation change after their binding to the active site. Clearly, this kind of complex did not take place with compounds **19a** and **25a**. At best, as in the case of compound **11a**, the enzyme recognized one of the two binding sites. In contrast, the interactions between DXR and compounds **19b** and **25b** were first weak, but the conformation change in the active site, probably induced by the supplementary methyl group, led to a significant tightening of the inhibition complex.

In the case of phosphonate derivatives, the two acidic groups can be involved in electrostatic interactions and hydrogen bonds. The binding mode of fosmidomycin **1** to the *E. coli* DXR revealed from the X-ray structure seemed to show that hydrogen bond formation is privileged over electrostatic interactions. Indeed, the phosphonate moiety is embedded in a network of hydrogen bonds with the phosphonate oxygen atoms acting as hydrogen acceptors and the side chains of polar amino acids such as Ser186, Ser222, Asn227, and Lys228 as hydrogen bond donors.[8a] Our results show that in the case of DXR a carboxylate or a sulfonate are not suitable surrogates of a phosphonate. The sulfonate analogue of FR900098 displayed an IC_{50} value of 23 μM and is approximately 500-fold less active than the corresponding phosphonate derivative, FR900098.[12] The reason is probably that the latter interacts in the phosphate recognition site of DXR via specific hydrogen bonds and not electrostatic interactions. Indeed, only two hydrogen bonds can be generated with a carboxylate group while a phosphonate group is able to be involved in three hydrogen bonds.[20] Structurally these two functional groups are different: a phosphonate has a pyramidal structure, and a carboxylate a planar one. If the carboxylate binds to the phosphonate recognition site, it would accommodate differently, maybe forming other hydrogen bonds. Consequently, the hydroxamate group would become unable to chelate the divalent cation present in the active site leading to a considerable decrease of the inhibition potency of the carboxylate analogue. The explanation for the relative failure of the sulfonate derivatives to inhibit the DXR is less obvious. The dibasic phosphonate group and the monobasic sulfonate share a considerable structural similarity. Both adopt a pyramidal geometry around the phosphorus and the sulfur atom. The reason seemed to be the hydrogen bonding capacity of the sulfonate and the phosphonate groups with the DXR. A crystallographic database survey of geometrical features of the hydrogen bonding interactions revealed that a sulfonic group tends to form longer interactions than carboxylic and phosphonic groups.[31] Interactions between phosphonate and hydrogen bond donors display a preference for *gauche* orientation, while those involving a sulfonate prefer an *eclipsed* geometry.[32] So subtle differences in hydrogen bonding may exist between the sulfonate and the phosphonate derivatives with the enzyme. As for carboxylate derivatives, the consequences would be an unsuitable positioning of the hydroxamate group with regard to the divalent cation and a dramatic decrease of the DXR inhibition.

In trying to understand the biosteric relationships among carboxylic, sulfonic, and phosphonic acid groups, it has been shown that a selective molecular recognition of the phosphonate group is achieved by high polar binding sites as in the DXR enzyme, whereas, strikingly, the binding sites of sulfonates display a relatively low polarity. In addition, the negative pK_a value of a sulfonic acid, which results in a constant loss of the acidic proton in any protein environment, could explain the loss of specificity of the sulfonate analogues for the active site of the DXR enzyme and the low IC_{50} values. For instance, the inhibition potency of carboxylate and sulfonate derivatives of 9-(3,3-dimethyl-5-phosphonopentyl)guanine was tested on purine nucleoside phosphorylase. The sulfonate compound binds about one-fourth as tightly as the phosphonate. The interaction of the carboxylate derivative was even much weaker (180-fold). The carboxylate group turns away from the center of the phosphate-binding site forming hydrogen bonds with other amino acids.[33]

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Supporting Information Available: General Methods and Experimental Section; ^{1}H, ^{13}C, and ^{31}P NMR spectra for compounds **8−15**; ^{1}H, ^{13}C spectra for compounds **17−25**; HSQC and HMBC spectra for compounds **24** and **25**; NOESY spectra for compounds **24b** and **25b**; and protocol for enzyme assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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