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The effect of chain length and unsaturation on Mtb Dxr inhibition and antitubercular killing activity of FR900098 analogs

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Abstract

Inhibition of the nonmevalonate pathway (NMP) of isoprene biosynthesis has been examined as a source of new antibiotics with novel mechanisms of action. Dxr is the best studied of the NMP enzymes and several reports have described potent Dxr inhibitors. Many of these compounds are structurally related to natural products fosmidomycin and FR900098, each bearing retrohydroxamate and phosphonate groups. We synthesized a series of compounds with two to five methylene units separating these groups to examine what linker length was optimal and tested for inhibition against Mtb Dxr. We synthesized ethyl and pivaloyl esters of these compounds to increase lipophilicity and improve inhibition of Mtb growth. Our results show that propyl or propenyl linker chains are optimal. Propenyl analog **22** has an IC_{50} of 1.07 μM against Mtb Dxr. The pivaloyl ester of **22**, compound **26**, has an MIC of 9.4 μg/mL, representing a significant improvement in antitubercular potency in this class of compounds.

Keywords

Mycobacterium tuberculosis; Nonmevalonate pathway; Dxr; Antibiotic

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains one of the world's deadliest infectious diseases.[1] Emergence of multi-drug (MDR) and extensively-drug (XDR) resistant strains, as well as co-infection with HIV, has made TB both difficult and expensive to treat.[2] New TB therapies are needed to shorten treatment, be effective against all strains and metabolic states of the organism, and work well with HIV drugs. Thus, there remains a significant need for new and improved strategies against Mtb. The nonmevalonate pathway (NMP) of isoprene biosynthesis (Figure 1) is essential for Mtb survival and, as it is not present in humans, is an attractive set of targets for novel drug development.[3-5] The NMP synthesizes 5-carbon building blocks from pyruvate and glyceraldehyde-3-phosphate. These

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building blocks are the starting materials for many complex cellular metabolites. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr), is the first committed step in the NMP and is responsible for conversion of 1-deoxy-D-xylulose-5-phosphate (DXP) to 2-C-methyl-D-erythritol 4-phosphate (MEP).[6] Dxr catalyzes both a reduction and isomerization using NADPH as a cofactor.

Natural products fosmidomycin (**1**) and FR900098 (**2**) inhibit Mtb Dxr by mimicking DXP's polar character and kill many non-mycobacterial organisms reliant on this enzyme (Figure 2).[7-9] Our early work in this area showed that lipophilic analogs of **1** and **2** more effectively kill a range of bacterial strains, including Mtb.[10-12] Since that time, we and others have reported Dxr inhibitors belonging to several structural families,[11, 13-16] but very few of these have displayed potent antitubercular activity. Many of these inhibitors retain key structural features found in the parent compounds **1** and **2**: a retrohydroxamic acid, a phosphonate, and an *n*-propyl carbon chain linking the nitrogen and phosphorus atoms. In the 1980s, a series of *Streptomyces-derived* and inspired products exchanging the *n*-propyl chain for ethylene and propenyl chains were described.[17, 18] Among these, the propenyl compound was found to be comparable to the propyl analogs **1** and **2** and showed potent antibacterial activity against *B. subtilis* and *E. coli*.[18] As this work came before the discovery of Dxr as the cellular target of these inhibitors, the inhibitory activity of these carbon chain-modified analogs against the purified enzymer is largely unknown. To fill this gap and expand on the set of analogs examined, we synthesized analogs of **1** and **2**, varying the length of the carbon linker from 2-5 methylene groups. We also prepared the propenyl analog to examine the influence of unsaturation within the propyl chain. as our interest is the development of antitubercular agents working through Dxr inhibition, we evaluated these analogs as inhibitors of Mtb Dxr. To study the effects of these structural changes on antitubercular activity, the ethyl and selected pivaloyl esters were prepared. The compounds synthesized and evaluated are shown in Figure 2.

Scheme 1 shows the synthetic route used to prepare compounds **7-9**, all with a carbon chain of 2 methylene groups. Compound **3**[19] was reacted with *N*-(diethoxyphosphoryl)-*O*-benzylhydroxyl-amine[20] in the presence of sodium hydride, sodium iodide and tetrabutylammonium bromide to form **4** (25%). Further reaction with concentrated hydrochloric acid gave **5** in quantitative yield.[21] Compound **5** was then formylated using acetic anhydride and formic acid to give **6a** (71%) or acetylated in the presence of acetyl chloride and triethylamine to give compound **6b** (52%). Hydrogenation was used to remove the benzyl group, forming **7** (58%) and **8** (38%). Treatment of **8** with bromotrimethylsilane, water, and sodium hydroxide gave the mono-sodium salt **9** in quantitative yield.

Scheme 2 was used to prepare analogs with four or five methylene groups between the nitrogen and phosphorous atoms. Dibromoalkanes **10a** and **10b** were treated with triethylphosphite in a microwave-assisted Michaelis-Arbuzov reaction to form **11a** (61%) and **11b** (64%).[22] Acetylated *O*-benzylhydroxylamine[23, 24] was treated with sodium hydride and compounds **11a** and **11b** to form intermediates **12a** (79%) and **12b** (37%). Compounds **12a** and **12b** underwent hydrogenation to form compounds **13** (34%) and **14** (49%). Deprotection of the ethyl esters gave compounds **15** and **16** in quantitative yield.

Synthesis of unsaturated FR900098 analog **22** is shown in Scheme 3. Dibromo compound **17**[25] was treated with sodium hydride to effect elimination, yielding compound **18** (41%). Boc-protected *O*-benzylhydroxylamine[26] was reacted with sodium hydride and then compound **18** to form substituted product **19** (84%). Alternately, compound **19** was prepared directly from **17** in one step using a single treatment of NaH and the amine in 41% yield. Removal of the BOC protecting group *in situ* and subsequent acetylation yielded compound **20** (70%).[27] To preserve the double bond, BCl_3 was used to remove the benzyl group of **20**,

affording compound **21** (52%).[28] Deprotection with bromotrimethylsilane gave α/β-unsaturated phosphonic acid **22** (quantitative).[29]

To assist penetration of compounds across the mycobacterial cell wall[10, 30], pivaloyl esters were prepared from two phosphonic acids (Scheme 4). Diethyl protected intermediates **12a** and **20** were treated with bromotrimethylsilane yielding compounds **23a** (87%) and **23b**[31] (quantitative). Subsequent reaction with chloromethylpivalate gave esters compounds **24a** (6%) and **24b**[32] (40%). Catalytic hydrogenation removed the benzyl group in saturated analog **24a**, yielding compound **25** (85%). Treatment with BCl_3 deprotected unsaturated analog **24b** to yield compound **26** (13%).[33]

The analogs were evaluated for inhibition of Mtb Dxr and growth of Mtb (Tables 1-3). All of the saturated compounds, with chain lengths between two and five methylene groups, inhibited Mtb Dxr to some extent (Table 1). Among these acids, compounds with three methylene groups separating the nitrogen and phosphorus atoms (that is, compounds **1** and **2**) were the most active. Not surprisingly, these compounds did not inhibit mycobacterial growth in nutrient-rich media (>200 μg/mL in 7H9), although **9** had a very slight effect when minimal media was used (150 μg/mL in GAST). The polarity of these compounds diminishes penetration of the lipophilic mycobacterial cell wall.[10, 30]

Diethyl and dipivaloyl esterification of these compounds improved antimycobacterial activity (Table 2). As previously shown, diethyl esters of **1** and **2** (**27** and **28**, respectively) are weakly potent inhibitors of Mtb growth with MIC values of 200-400 μg/mL.[10] Pivaloyl ester **29** showed improved potency with an MIC of 50-100 μg/mL, and this compound was the most potent in the saturated series. Taken together, these data show that linker chains of two, four or five methylene units are not advantageous for Mtb Dxr inhibition or inhibition of Mtb cell growth.

The compounds listed in Table 3 were synthesized to examine the effect of unsaturation on Mtb Dxr inhibition and cell growth. Interestingly, α/β-unsaturated compound **22** is a potent inhibitor of Mtb Dxr with an IC_{50} of 1.07 μM. Indeed, **22** is more active than parent compound **2**. While **21** and **22** do not inhibit Mtb, the more lipophilic pivaloyl ester of **22** (compound **26**) is a potent inhibitor of mycobacterial growth with an MIC of 9.4 μg/mL in rich media and 12.5 μg/mL in minimal media. To our knowledge, compound **26** displays the most potent antitubercular activity of all compounds that work through a Dxr-mediated mechanism.

Overall, the results collectively indicate that a carbon propyl or propenyl chain between the nitrogen and phosphorus atoms of fosmidomycin/FR900098 analogs yields the highest potency. Lipophilic esters of these compounds improve their antitubercular activity. α/β-Unsaturated compound **22** and its lipophilic pivaloyl ester **26** show higher potency than the parent compound FR900098 (**2**) on Mtb Dxr inhibition and antitubercular activity. These data improve our understanding of the Mtb Dxr active site and its tolerance to length variation between the phosphonate and retrohydroxamate groups. These results are significant for aiding the rational design of Mtb Dxr inhibitors using the phosphonate/retrohydroxamate scaffold and guide the development of Dxr inhibitors as antitubercular agents.

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27. Compound **20**. Acetyl chloride (1.8mL, 25.0mmol) and dry MeOH (0.5mL) were added dropwise to **19** (0.19g, 2.5mmol) in dry CH_2Cl_2 (6mL) under N_2. The reaction mixture was allowed to stir at rt for 30 min. Dry Na_2CO_3 (0.5g, 5.0mmol) and additional acetyl chloride (0.7mL, 9.8mmol) were added, and the reaction mixture was allowed to stir at rt for 3h. The reaction mixture was filtered over celite, and the solvent was removed under reduced pressure. The crude oil was purified by column chromatography using silica gel (CH_2Cl_2:MeOH, 49:1) to yield **20** (0.13g,

0.36mmol, 77%) as a clear, colorless oil. 1H NMR ($CDCl_3$, 200MHz), δ (ppm): 7.37 (s, 5H), 6.82-6.57 (m, 1H), 5.90-5.72 (m, 1H), 4.83 (s, 2H), 4.34 (bs, 2H), 4.13-3.99 (m, 4H), 2.13 (s, 3H), 1.34-1.27 (m, 6H). LCMS (ESI) m/z: 705.1 (2M+Na).

28. Compound **21**. A solution of **20** (0.117g, 0.34mmol) in dry CH_2Cl_2 (6.0 mL) was cooled to -50°C and BCl_3 (1.4 mL, 1M in CH_2Cl_2) was added dropwise, and the reaction mixture was allowed to stir for 2h. The reaction was quenched with saturated $NaHCO_3$ (aq, 9.0 mL) and allowed to warm to rt. The aqueous solution was extracted with CH_2Cl_2. The organic fractions were combined, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The resulting crude residue was purified using an Isolera Flash Chromatography system and a silica column (EtOAcMeOH, 49:1) to yield **21** (45mg, 0.18mmol, 52%) as a light yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 1.28 (t, 6H), 2.15 (s, 3H), 4.02 (q, 4H),4.43 – 4.25 (m, 2H), 5.84 (t, J = 18.8 Hz, 1H), 6.83 - 6.52 (m, 1H), 9.88 (bs, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 16.30, 20.36, 50.47 (d, J = 27.2 Hz), 62.37 (d, J = 7.0 Hz), 120.02, 147.62, 172.66. LCMS (ESI) m/z 252.1 (M+H).
29. Compound **22**. N,O-bis(trimethylsilyl)trifluoroacetamide (0.18mL, 0.67mmol) was added to **21** (0.03g, 0.12mmol) in CH_2Cl_2 (0.60mL) under N_2. The reaction mixture was allowed to stir at rt for 20 min. The reaction mixture was cooled to 0 °C, and bromotrimethylsilane (0.18mL, 1.34mmol) was added dropwise. The reaction was allowed to warm to rt and was stirred overnight under N_2. Ethyl bromide and excess silylating agent were removed under reduced pressure, and the residue was dissolved in aqueous NaOH (0.68mL, 7.8mg/mL) and stirred overnight. The mixture was extracted between H_2O and CH_2Cl_2. The aqueous portions were combined, and the solvent was removed by lyophilization to give **22** (0.03g, 0.12mmol, quant.) as a yellow solid. ^{1}H NMR (400 MHz, D2O) δ (ppm): 2.36 (s, 3H), 4.61 – 4.46 (m, 2H), 6.19 – 6.06 (m, 1H), 6.56 – 6.43 (m, 1H). ^{13}C NMR (101 MHz, D_2O) δ (ppm): 19.34, 50.81 (d, J = 23.7 Hz), 126.91, 137.79, 174.18. HRMS (ESI) m/z calcd. for $C_{10}H_{18}N_2NaO_{10}P_2$ (2M + Na]): 411.0328, found: 411.0334.
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31. Compound **23b**. Trimethylsilylbromide (1.75mL, 11.7mmol) was added dropwise to a stirring solution of **20** (0.5g, 1.5mmol) in dry CH_2Cl_2 (20mL) under N_2 at 0°C. The reaction mixture was allowed to warm to room temperature. After 3.5 h, the mixture was evaporated to dryness, dissolved in dry CH_2Cl_2, and evaporated to dryness again (×3). The resulting residue was stirred overnight in water (3mL) and NaOH (5.5mL, 3mmol, aq.). After 20 hours, the aqueous mixture was washed with CH_2Cl_2. The organic portion was separated, and the water was removed by lyophilization to give **23b** (0.53g, quant.) as white crystals. ^{1}H NMR ($CDCl_3$, 400MHz), δ (ppm): 2.28 (s, 3H), 4.53 (s, 2H), 5.17 (s, 2H), 6.07-6.15 (m, 1H), 6.30-6.40 (m, 1H), 7.65-7.68 (m, 5H). LCMS (ESI) m/z: 286 (M_{acid}+H), 571 ($2M_{acid}$+H), 856 ($3M_{acid}$+H).
32. Compound **24b**. Chloromethylpivalate (2.15mL, 15mmol) was added to a stirred solution of **23b** (0.49g, 1.5mmol) and triethylamine (0.45mL, 3mmol) in DMF (40mL). The reaction mixture was heated to 60°C for 16 hours. Water (50mL) was added, and the aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude oil was purified by column chromatography using silica gel and CH_2Cl_2/EtOAc to yield **24b** (0.22g, 28%). 1H NMR ($CDCl_3$, 400MHz), δ (ppm): 1.20 (s, 18H), 2.12 (s, 3H), 4.33 (bs, 2H), 4.82 (s, 2H), 5.65 (d,/= 12.8 Hz, 4H), 5.80-5.89 (m, 1H), 6.70-6.81 (m, 1H), 7.35-7.38 (m, 5H). LCMS (ESI) m/z: 536 (M+Na).
33. Compound **26**. BCl_3 (1M in CH_2Cl_2, 0.88mL) was added dropwise to a stirred solution of **24b** (190mg, 0.37mmol) in dry CH_2Cl_2 (5mL) under N_2 at -78°C. After 10 hours, the reaction mixture was poured into satd. $NaHCO_3$ (aq.) and was extracted with CH_2Cl_2. The organic layers were combined and washed with brine, dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude oil was purified by column chromatography using EtOAc, CH_2Cl_2, and MeOH. The oil was further purified over a silica plug, washed with hexanes and CH_2Cl_2, and then eluted with EtOAc to give **26** as a pale yellow oil (21mg, 13.4%). 1H NMR ($CDCl_3$, 400MHz), δ (ppm): 1.22 (s, 18H), 2.19 (s, 3H), 4.41 (s, 2H), 5.61-5.69 (m, 4H), 5.87-5.97 (m, 1H), 6.71-6.84 (m, 1H), 8.61 (s, 1H). ^{13}C NMR (CDCl3, 100MHz), δ (ppm): 20.47, 26.93, 38.88, 50.30 (d,/= 26.5 Hz), 81.70 (d,/= 5.3 Hz), 117.95 (d,/= 188.2 Hz), 148.50, 172.87, 177.30. LCMS (ESI) m/z: 446 (M+Na), 847 (2M+H), 869 (2M+Na).

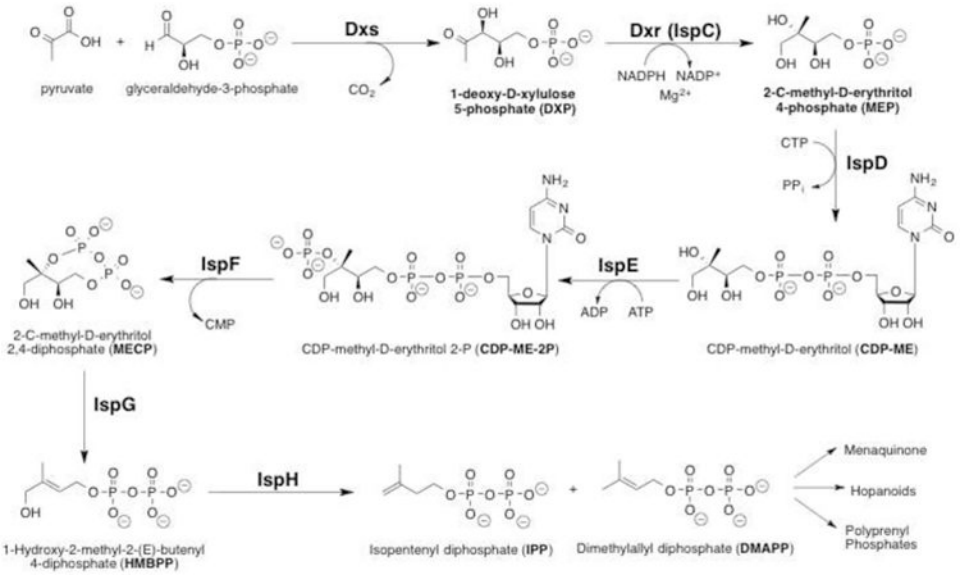


Figure 1.

Nonmevalonate Pathway of Isoprenoid Biosynthesis. Dxr (IspC) mediates the conversion of DXP to MEP in the second step.

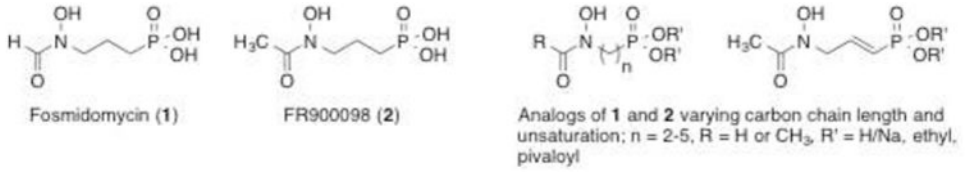
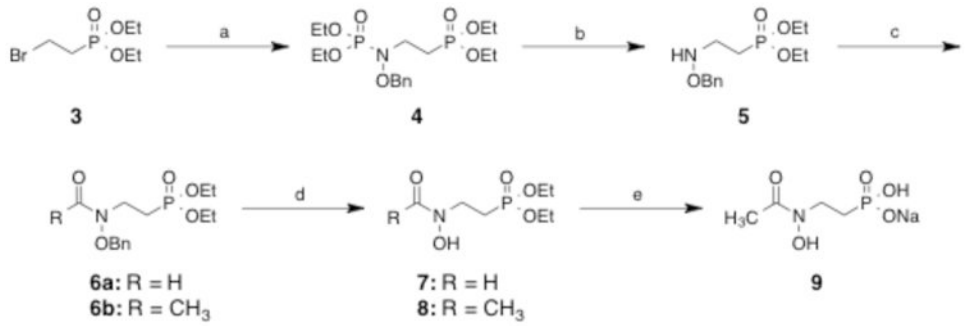
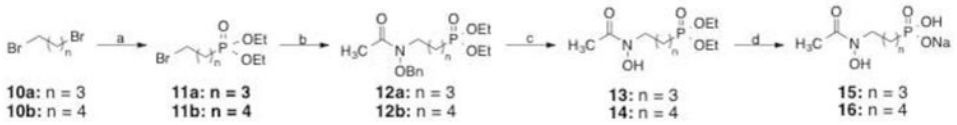


Figure 2.
Fosmidomycin (**1**), FR900098 (**2**) and the analogs prepared in this work.



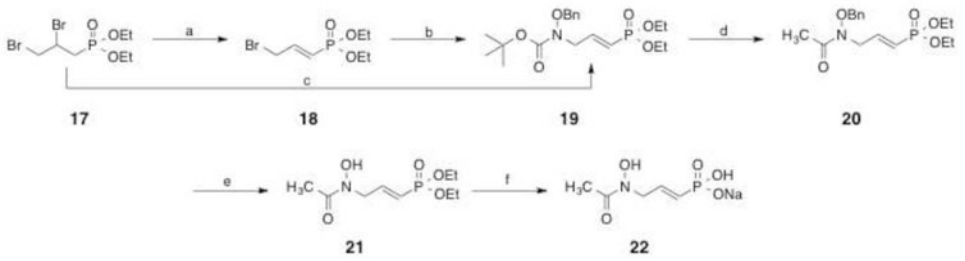
Scheme 1.

Reagents and conditions: (a) $(EtO)_2P(O)NHOBn$, NaH, Nal, TBABr, THF, reflux, 18 h; (b) HCI, EtOH, reflux, 5 min; (c) AcCI, TEA, CH_2CI_2, rt, 18 h or Ac_2O CH_2O_2, THF, rt, 2 h; (d) H_2, 10% Pd/C, MeOH, 18 h; (e) (i) TMSBr, BSTFA, CH_2CI_2, 0 °C to rt, 18 h; (ii) H_20, rt, 18 h, (iii) NaOH aq., rt, 18 h.



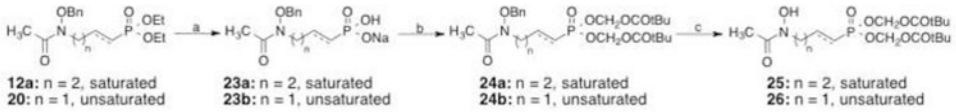
Scheme 2.

Reagents and conditions: (a) $P(OEt)_3$, microwave 20%, 10-15 min; (b) BnONHAc, NaH, Nal, THF, reflux, 18 h; (c) H_2, 10% Pd/C, MeOH, rt, 18 h; (d) (i) TMSBr, CH_2CI_2, 0 °C to rt, 18 h; (ii) H_20, rt, 18 h; (iii) NaOH aq., rt, 18 h.



Scheme 3.

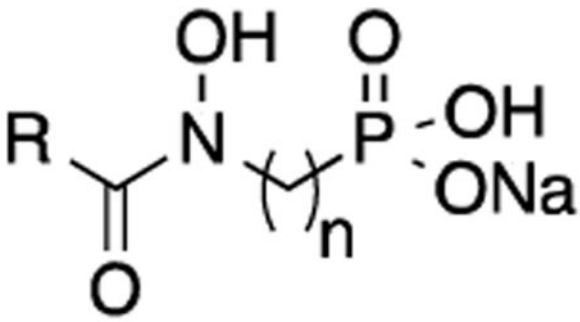
Reagents and conditions: (a) NaH, THF, 60 °C, 18 h; (b) BocNHOBn, NaH, THF, rt, 18 h; (c) BocNHOBn, NaH, Nal, THF, rt, 18 h; (d) (i) AcCI, MeOH, CH_2CI_2, rt, 30 min; (ii) AcCI, Na_2CO_3, CH_2CI_2, rt, 3 h; (e) BCI_3, CH_2CI_2, -50 °C, 2h; (f) (i) TMSBr, BSTFA, CH_2CI_2, 0 °C to rt, 18 h; (ii) H_2O, rt, 18h, (iii) NaOHaq., rt, 18 h.



Scheme 4.

Reagents and conditions: (a) (i) TMSBr, CH_2CI_2, 0 °C to rt, 3-18 h; (ii) H_2O, rt, 18 h for 23a or H_2O, NaOH, rt, 18 h for 23b; (b) chloromethylpivalate, 60 °C, TEA/DMF/6-16 h; (c) H_2, 10% Pd/C, THF, rt, 18 h for 25 or BCI_3, CH_2CI_2, -70 °C, 10 h for 26.

Table 1
Effect of chain length on Mtb Dxr inhibition and Mtb MIC

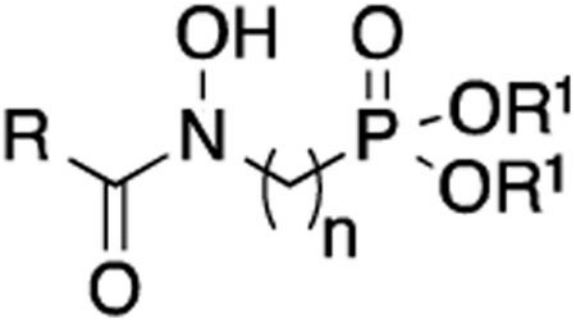


Compound	R	n	Mtb Dxr IC_{50}, μM (% inh at 100 μM)	MIC, μg/mL 7H9 (GAST)
Fosmidomycin (**1**)	H	3	0.44	>500
FR900098 (**2**)	CH_3	3	2.39	>500
9	CH_3	2	(74%)	>200 (t50)
15	CH_3	4	(80%)	>200 (>200)
16	CH_3	5	(86%)	>200 (>200)

Mtb = *Mycobacterium tuberculosis*; IC_{50} = inhibitory concentration at 50%; inh = inhibition; MIC = minimum inhibitory concentration; 7H9 = rich media; GAST = minimal media

Table 2

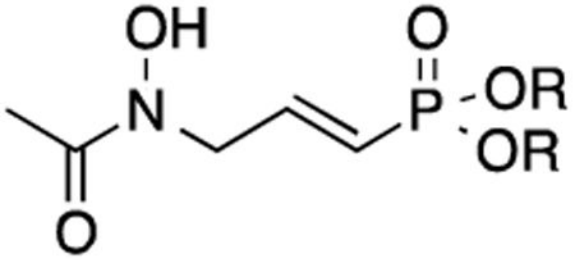
Effect of esterification on Mtb MIC



Compound	R	R^1	n	MIC, μg/mL 7H9 (GAST)
27	H	CH_2CH_3	3	400
7	H	CH_2CH_3	2	>500
8	CH_3	CH_2CH_3	2	>500
28	CH_3	CH_2CH_3	3	200-400
29	CH_3	$CH_2OCOtBu$	3	50-100
13	CH_3	CH_2CH_3	4	>200 (75)
25	CH_3	$CH_2OCOtBu$	4	≥200 (150)
14	CH_3	CH_2CH_3	5	>200 (200)

Table 3

Effect of unsaturation on Mtb Dxr inhibition and Mtb MIC



Compound	R	Mtb Dxr IC_{50}, μM	MIC, μg/mL 7H9 (GAST)
22	H/Na	1.07	>200 (150)
21	CH_2CH_3	ND*	>200 (150)
26	$CH_2OCOtBu$	ND	9.4 (12.5)

*ND = not determined