Isoprenoid biosynthesis as a target for antibacterial and antiparasitic drugs: phosphonohydroxamic acids as inhibitors of deoxyxylulose phosphate reducto-isomerase

Lionel KUNTZ*, Denis TRITSCH*, Catherine GROSDÉMANGE-BILLIARD*, Andréa HEMMERLIN†, Audrey WILLEM*, Thomas J. BACH† and Michel ROHMER*¹

*Université Louis Pasteur/CNRS-UMR 7123, Institut Le Bel, 4 rue Blaise Pascal, 67070 Strasbourg Cedex, France and †CNRS-UPR 2357, Institut de Biologie Moléculaire des Plantes, 28 rue Goethe, 67083 Strasbourg Cedex, France

Isoprenoid biosynthesis via the methylerythritol phosphate pathway is a target against pathogenic bacteria and the malaria parasite *Plasmodium falciparum*. 4-(Hydroxyamino)-4-oxobutylphosphonic acid and 4-[hydroxy(methyl)amino]-4-oxobutylphosphonic acid, two novel inhibitors of DXR (1-deoxy-D-xylulose 5-phosphate reducto-isomerase), the second enzyme of the pathway, have been synthesized and compared with fosmidomycin, the best known inhibitor of this enzyme. The latter

phosphonohydroxamic acid showed a high inhibitory activity towards DXR, much like fosmidomycin, as well as significant antibacterial activity against *Escherichia coli* in tests on Petri dishes.

Key words: antibacterial drug, 1-deoxy-D-xylulose 5-phosphate, 1-deoxy-D-xylulose 5-phosphate reducto-isomerase, isoprenoid biosynthesis, malaria, 2-C-methyl-D-erythritol 4-phosphate.

INTRODUCTION

Isoprenoid biosynthesis proceeds via two biosynthetic pathways, depending on the organism. The long-known mevalonate pathway is found in animals, fungi, the cytoplasm of phototrophic organisms, some eubacteria and Archaea, whereas the recently discovered MEP (2-C-methyl-D-erythritol 4-phosphate) pathway is present in most eubacteria (including pathogens for humans), in unicellular green algae and in the chloroplasts of phototrophic organisms (algae, liverworts and higher plants) [1,2], as well as in some unicellular eukaryotes with an evolutionary linkage to photosynthetic phyla, such as the *Plasmodium* spp. [3] and *Prototheca wickerhamii* [4]. With such a distribution, it was soon recognized that this new pathway occurring in a number of pathogens and in higher plants would constitute a molecular target for the development of drugs, and perhaps of herbicides as well [3,5]. Among the enzymes of the MEP pathway, DXR (1-deoxy-D-xylulose 5-phosphate reducto-isomerase) is a most promising target. The enzyme catalyses the two-step transformation of 1-deoxy-D-xylulose 5-phosphate (**1**) to 2-C-methyl-D-erythritol 4-phosphate (**3**) via an intramolecular rearrangement, yielding 2-C-methyl-D-erythrose 4-phosphate (**2**), a non-isolable intermediate, which is concomitantly reduced via an NADPH-dependent reduction (Scheme 1) [6–9]. Fosmidomycin (**4**), an antibiotic active against many Gram-negative and some Gram-positive bacteria [10,11], specifically inhibits DXR. It was reported as a mixed-type inhibitor of the recombinant enzyme of *Escherichia coli* (K_i 38 nM) [12], but also described as a slow, tight-binding competitive inhibitor against DXP for the same enzyme [9]. For the same enzyme from *Zymomonas mobilis*, a pattern of competitive inhibition (K_i 0.6 μ M) was observed [13]. The antibiotic also inhibits the enzyme from higher plants [14,15]. Fosmidomycin (**4**) and the related FR-900098 (**5**) (Scheme 1) inhibit the recombinant DXR from *Plasmodium falciparum* in a dose-

dependent manner [3]. Both compounds are effective antimalarial agents, since mice infected with *Plasmodium vinckei* became free of parasites upon treatment [3]. Diester prodrugs of FR900098 presented even further improvements in antimalarial activity [16,17]. Fosmidomycin was also used for curing uncomplicated *P. falciparum* malaria in humans [18,19]. Its use as a single therapeutic agent is probably restricted by the high level of recrudescence; combination therapies would seem to be more promising [20]. Recently, the three-dimensional structure of the *E. coli* DXR–fosmidomycin complex was resolved at 2.5 Å resolution (1 Å $\equiv$ 0.1 nm) [21]. This revealed a substrate-like binding of the inhibitor, and the chelation of the Mn²⁺ cation of the active site by the hydroxamate group providing two oxygen ligands [21] (Scheme 1). Here, we present the synthesis and the biological activity of two novel potent inhibitors of the bacterial DXR, (**7**) and (**8**), both characterized by a bidentate hydroxamate chelating group.

EXPERIMENTAL

General methods

All non-aqueous reactions were run in dry solvents under an argon atmosphere. All reagents and solvents were reagent grade. TLC was performed on analytical silica-gel 60 F₂₅₄ plates (Merck) and flash chromatography on silica-gel 60 230–400 mesh (Merck) with the indicated solvent system. TLC plates were revealed by spraying with an ethanol solution of *p*-anisaldehyde (2.5 %), sulphuric acid (3.5 %) and acetic acid (1.6 %), or with an ethanol solution of phosphomolybdic acid (20 %) followed by heating. NMR spectra were recorded on Bruker AC200, AC300 or AV300 spectrometers. NMR experiments were performed in C²HCl₃ or ²H₂O using as an internal standard CHCl₃ (δ = 7.26 p.p.m.) or ²HHO (δ = 4.65 p.p.m.) for ¹H-NMR, C²HCl₃ (δ = 77.0 p.p.m.)

Abbreviations used: DTT, dithiothreitol; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reducto-isomerase; FAB, fast atom bombardment; H-DXR, His₆-tagged DXR; HRMS, high-resolution MS; LB, Luria–Bertani; MEP, 2-C-methyl-D-erythritol 4-phosphate; THF, tetrahydrofuran.

¹ To whom correspondence should be addressed (email mirohmer@chimie.u-strasbg.fr).

Scheme 1 Reaction catalysed by DXR, complexation of the Mn^{2+} cation of DXR with (a) DXP (1) or (b) fosmidomycin (4) (according to [21]), and (c) hypothetical complexation of the Mn^{2+} cation with inhibitors (7) ($\text{R}=\text{H}$) and (8) ($\text{R}=\text{CH}_3$)

Scheme 2 Synthesis of inhibitors (7) and (8)

Reagents for the chemical steps shown were as follows: (i) $(\text{BnO})_2\text{P}(\text{O})\text{H}$, NaH, THF (74%); (ii) LiOH, THF/ H_2O , room temperature (86%); (iii) BnONH_2 , 1-(3-dimethylaminopropyl)-3-ethyl carbodi-imide, 1*H*-benzotriazole, *N*-methylmorpholine, THF (66%); (iv) MeI, NaH, THF (90%); and (v) H_2 , Pd/C, EtOH (99%).

for ^{13}C -NMR and H_3PO_4 ($\delta = 0.00$ p.p.m.) for ^{31}P -NMR. Negative-mode electrospray MS was performed on a Hewlett–Packard 1100MS spectrometer using acetonitrile/water (1:1, v/v) as the solvent, and HRMS (high-resolution MS) on a ZAB-HF spectrometer with an acceleration potential of 8 keV using *m*-nitrobenzyl alcohol as matrix and xenon as the ionization gas. All compounds were found to be pure by ^1H - and ^{13}C -NMR spectroscopy.

Synthesis of inhibitors (7) and (8) (Scheme 2)

Ethyl 4-(dibenzylphosphonyl)butanoate (10)

To a solution of dibenzyl phosphite (2.02 g, 7.69 mmol, 1.5 eq.) in THF (tetrahydrofuran; 20 ml) at 0°C was added sodium hydride (184 mg, 7.69 mmol, 1.5 eq.). After 15 min, ethyl 4-bromobutyrate (9) (1 ml, 5.13 mmol, 1 eq.) was added, and the mixture

was stirred for 48 h. The reaction was quenched with a saturated solution of NH_4Cl (20 ml) and extracted with diethyl ether (3×20 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (ethyl acetate/cyclohexane, 6:4) to afford (**10**) as a colourless oil [1.42 g, 74%, R_F 0.22, ethyl acetate/cyclohexane, 7:3 (v/v)]. $^1\text{H-NMR}$ (300 MHz, C_2HCl_3): δ (p.p.m.) = 1.20 (3 H, t, $J = 7.1$ Hz, $-\text{O}-\text{CH}_2-\text{CH}_3$), 1.73–71.97 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.33 (2 H, t, $J = 7.1$ Hz, CH_2-CO), 4.08 (2 H, q, $J = 7.1$ Hz, $-\text{O}-\text{CH}_2-\text{CH}_3$), 4.90–95.10 [4 H, m, CH_2-Ph (where 'Ph' represents 'phenyl')] and 7.32 (10 H, m, Ph). $^{13}\text{C-NMR}$ (75 MHz, C_2HCl_3): δ (p.p.m.) = 14.2 ($-\text{O}-\text{CH}_2-\text{CH}_3$), 18.0 (d, $J = 5.0$ Hz, C-3), 25.3 (d, $J = 141$ Hz, C-4), 34.3 (d, $J = 16.1$ Hz, C-2), 60.4 ($-\text{O}-\text{CH}_2-\text{CH}_3$), 67.1 (CH_2-Ph), 127.9, 128.4, 128.6, 136.4 (aromatic C) and 172.5 (C-1). $^{31}\text{P-NMR}$ (121.5 MHz, C_2HCl_3): δ (p.p.m.) = 33.5. MS [FAB^+ (fast atom bombardment)] m/z : 377.0 ($M + \text{H}^+$). HRMS (FAB^+) m/z : calculated for $\text{C}_{20}\text{H}_{26}\text{O}_5\text{P}$ 377.1519, found to be 377.1518.

4-(Dibenzylphosphonyl)butanoic acid (**11**)

To a solution of (**10**) (1 g, 2.67 mmol, 1 eq.) in THF (15 ml) was added LiOH (160 mg, 6.64 mmol, 2.5 eq.) dissolved in a minimum amount of water. The mixture was stirred overnight at room temperature ($\approx 25^\circ\text{C}$), then H_2O (20 ml) was added. The solution was extracted with ethyl acetate (15 ml). The aqueous layer was acidified with a 4 M HCl solution and extracted with ethyl acetate (3×15 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford (**11**) as colourless oil, which was not further purified (798 mg, 86%, R_F 0.36, ethyl acetate). $^1\text{H-NMR}$ (300 MHz, C_2HCl_3): δ (p.p.m.) = 1.75–71.99 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.39 (2 H, t, $J = 6.7$ Hz, CH_2-COOH), 4.91–95.11 (4 H, m, CH_2-Ph), 7.33 (10 H, m, Ph). $^{13}\text{C-NMR}$ (75 MHz, C_2HCl_3): δ (p.p.m.) = 17.7 (d, $J = 5.0$ Hz, C-3), 25.5 (d, $J = 141$ Hz, C-4), 34.0 (d, $J = 16.7$ Hz, C-2), 67.5 (CH_2-Ph), 128.0, 128.5, 128.6, 136.1 (aromatic C) and 176.9 (C-1). $^{31}\text{P-NMR}$ (121.5 MHz, C_2HCl_3): δ (p.p.m.) = 34.0. MS (FAB^-) m/z : 347.2 ($M - \text{H}^+$). HRMS (FAB^+) m/z : calculated for $\text{C}_{18}\text{H}_{20}\text{O}_5\text{P}$ 347.1054, found to be 347.1061.

Dibenzyl 4-(benzyloxyamino)-4-oxobutylphosphonate (**12**)

To a solution of the carboxylic acid (**11**) (100 mg, 0.29 mmol, 1 eq.) in THF (5 ml) were successively added 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (72 mg, 0.38 mmol, 1.3 eq.), 1*H*-benzotriazole (45 mg, 0.38 mmol, 1.3 eq.) and *N*-methylmorpholine (0.05 ml, 0.43 mmol, 1.5 eq.). The mixture was stirred for 20 min, before adding *O*-benzylhydroxylamine (69 mg, 0.43 mmol, 1.5 eq.). After stirring overnight at room temperature, the reaction mixture was diluted with ether (10 ml) and washed with a saturated solution of NaHCO_3 (10 ml), 10% HCl (10 ml), brine (10 ml) and H_2O (10 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification by flash chromatography (ethyl acetate) afforded (**12**) as a colourless oil (94 mg, 66%, R_F 0.19, ethyl acetate). $^1\text{H-NMR}$ (300 MHz, C_2HCl_3): δ (p.p.m.) = 1.67–1.98 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.13 (2 H, t, $J = 4.8$ Hz, CH_2-CO), 4.75–5.09 (4 H, m, CH_2-Ph), 7.32 (15 H, m, Ph) and 9.12 (1 H, broad s, NH). $^{13}\text{C-NMR}$ (75 MHz, C_2HCl_3): δ (p.p.m.) = 18.5 (C-2), 24.4 (d, $J = 141$ Hz, C-1), 32.7 (d, $J = 9.9$ Hz, C-3), 67.3 (CH_2-Ph), 78.1 ($\text{N}-\text{O}-\text{CH}_2-\text{Ph}$), 128.0, 128.5, 128.6, 129.1, 135.5, 136.1 (aromatic C) and 169.9 (C-1). $^{31}\text{P-NMR}$ (121.5 MHz, C_2HCl_3): δ (p.p.m.) = 33.7. MS (FAB^+) m/z : 454.0 ($M + \text{H}^+$). HRMS (FAB^+) m/z : calculated for $\text{C}_{25}\text{H}_{29}\text{NO}_5\text{P}$ 454.1783, found to be 454.1780.

Dibenzyl 4-[benzyloxy(methyl)amino]-4-oxobutylphosphonate (**13**)

To a solution of (**12**) (273 mg, 0.56 mmol, 1 eq.) in THF (5 ml) was added sodium hydride (15 mg, 0.61 mmol, 1.1 eq.) and methyl iodide (0.035 ml, 0.61 mmol, 1.1 eq.). The mixture was stirred overnight at room temperature, quenched with a saturated solution of NH_4Cl (10 ml) and extracted with ether (3×10 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Purification by flash chromatography (ethyl acetate) afforded (**13**) as a yellow oil (253 mg, 90%, R_F 0.29, ethyl acetate). $^1\text{H-NMR}$ (300 MHz, C_2HCl_3): δ (p.p.m.) = 1.73–1.99 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.45 (2 H, t, $J = 6.8$ Hz, CH_2-CO), 3.16 (3H, s, CH_3), 4.76 (2 H, s, $\text{N}-\text{O}-\text{CH}_2$), 4.90–5.09 (4 H, m, CH_2-Ph) and 7.33 (15 H, m, Ph). $^{13}\text{C-NMR}$ (75 MHz, C_2HCl_3): δ (p.p.m.) = 17.5 (d, $J = 5.0$ Hz, C-2), 25.3 (d, $J = 141$ Hz, C-1), 32.2 (d, $J = 15.3$ Hz, C-3), 33.5 (CH_3), 67.1 (CH_2-Ph), 76.2 ($\text{N}-\text{O}-\text{CH}_2$), 127.9, 128.4, 128.6, 128.8, 129.1, 129.3, 134.5, 136.5 (aromatic C) and 174.1 (C-4). $^{31}\text{P-NMR}$ (121.5 MHz, C_2HCl_3): δ (p.p.m.) = 34.0. MS (FAB^+) m/z : 468.0 ($M + \text{H}^+$). HRMS (FAB^+) m/z : calculated for $\text{C}_{26}\text{H}_{31}\text{NO}_5\text{P}$ 468.1940, found to be 468.1934.

4-(Hydroxyamino)-4-oxobutylphosphonic acid (**7**)

A solution of (**12**) (300 mg, 0.61 mmol) in ethanol (5 ml) was hydrogenolysed overnight at room temperature under an atmosphere of H_2 in the presence of palladium on charcoal (60 mg, 20%). The catalyst was removed by filtration through celite, and the filtrate was concentrated *in vacuo*. H_2O was added, and the solution was neutralized to pH 7 with 1 M NaOH and concentrated to dryness to afford (**7**) as an oil [134 mg, 99%, R_F 0.43 (isopropanol/ H_2O /ethyl acetate, 6:3:1 (by vol.)). $^1\text{H-NMR}$ (300 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 1.47–1.76 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.08 (2 H, t, $J = 6.50$ Hz, CH_2-CO). $^{13}\text{C-NMR}$ (75 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 18.5 (d, $J = 3.7$ Hz, C-2), 25.4 (d, $J = 135$ Hz, C-1), 32.6 (d, $J = 18.0$ Hz, C-3) and 171.9 (C-4). $^{31}\text{P-NMR}$ (121.5 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 30.6. MS (ES^-) m/z : 182.0 ($M - \text{H}^+$).

4-[Hydroxyl(methyl)amino]-4-oxobutylphosphonic acid (**8**)

A solution of (**13**) (213 mg, 0.42 mmol) in ethanol (5 ml) was hydrogenolysed overnight at room temperature under an atmosphere of H_2 in the presence of palladium on charcoal (42 mg, 20%). The catalyst was removed by filtration through celite, and the filtrate was concentrated *in vacuo*. H_2O was added, and the solution was neutralized to pH 7 with 1 M NaOH and evaporated to dryness to afford (**8**) as an oil [98 mg, 99%, R_F 0.72, isopropanol/ H_2O /ethyl acetate, 6:3:1 (by vol.)). $^1\text{H-NMR}$ (300 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 1.51–1.88 (4 H, m, $\text{P}-\text{CH}_2-\text{CH}_2$), 2.58 (2 H, t, $J = 7.4$ Hz, CH_2-CO) and 3.23 (3 H, s, CH_3). $^{13}\text{C-NMR}$ (75 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 19.2 (d, $J = 4.3$ Hz, C-2), 27.4 (d, $J = 132$ Hz, C-1), 32.5 (d, $J = 17.4$ Hz, C-3), 35.9 (CH_3) and 175.4 (C-4). $^{31}\text{P-NMR}$ (121.5 MHz, $^2\text{H}_2\text{O}$): δ (p.p.m.) = 26.1. MS (ES^-) m/z : 196.0 ($M - \text{H}^+$).

Construction of a plasmid encoding H-DXR (His₆-tagged DXR)

Genomic DNA was isolated from *E. coli* using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The gene coding for DXR (accession number AB013300) was amplified from the genomic DNA using the forward primer HEcRIF (5'-GGGAATTCCA-TATGCACCATCACCATCACCATAAGCAACTCACCATTCT-GGGC-3') and the reverse primer EcRIR (5'-CCAAGCTTGG-TCAGCTTGCGAGACGCATCACC-3'). *Nde*I and *Hind*III restriction sites were introduced into HEcRIF and EcRIR respectively (shown by single underlining). To facilitate further protein purification, a sequence coding for six histidine residues in

HEcRIF primer (shown doubly underlined) was introduced to generate a recombinant N-terminally His₆-tagged enzyme. PCR amplification was performed in a 50 µl reaction mixture, which included 50 pmol of each primer, 0.5 pmol of template DNA and 2× Hifi master mix (Boehringer Roche, Mannheim, Germany) using a step cycle (28 cycles) program of 94°C for 45 s, 52°C for 45 s and 72°C for 75°C for 1 min. The *Nde*I restriction site of the pBAD-GFPuv plasmid (accession number U62637 from ClonTech) at position 4927 bp was removed by site-directed mutagenesis, and the purified PCR product was digested with *Nde*I and *Hind*III restriction enzymes and ligated into the mutated vector digested with the same enzymes. The resulting plasmid, mpBAD-(His)₆-DXR_{coli}, was transformed into XL1-blue *E. coli* cells (Stratagene Europe).

Purification of H-DXR

For overexpression of the *dxr* gene, bacteria were grown at 30°C in LB (Luria–Bertani) medium containing ampicillin (100 µg · ml⁻¹). Induction was started at the mid-exponential phase by adding L-arabinose (0.1 %, w/v). After additional growth for 4 h, cells were harvested by centrifugation and broken by powdering in a mortar in the presence of liquid N₂ in 50 mM Tris/HCl buffer, pH 8, containing 250 mM NaCl and 5 mM 2-mercaptoethanol. The recombinant protein was purified using Ni²⁺-spin columns (Qiagen). The columns were washed twice with the same buffer, containing 10 mM and 50 mM imidazole for each respective wash. H-DXR was eluted with 300 mM and 500 mM imidazole in the buffer in two successive steps. The pooled fractions were dialysed against 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM DTT (dithiothreitol) by repeated centrifugal ultrafiltration with Centricon 30 concentrators (Millipore). The protein concentration was determined by the method of Bradford, with BSA as the standard [22]. A molecular mass of 44 kDa for the DXR subunit was utilized in kinetic calculations.

Determination of the enzymic activity

DXP was obtained by chemical synthesis (O. Meyer, J. F. Hoeffler, C. Grosdemange-Billiard and M. Rohmer, unpublished work). The DXR enzymic activity was determined at 37°C in 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM MgCl₂ and 2 mM DTT. The concentrations of NADPH and DXP used were 0.15 and 0.5 mM respectively. Initial rates were measured by following the decrease in the absorbance at 340 nm due to the oxidation of NADPH (Uvikon 933; Kontron Instruments). The kinetic constants (*V*_{max} and *K*_m) were calculated from a double-reciprocal plot of enzymic rate versus DXP concentration (0.05–0.5 mM). The reaction was initiated by adding H-DXR (2.25 µg).

Inhibition studies

Fosmidomycin was obtained from Dr R. J. Eilers (Monsanto, St Louis, MO, U.S.A.). Fosfomycin (phosphonomycin) was purchased from Fluka. Inhibitor concentrations in the stock solutions were verified by spectrophotometric phosphorus determination [23]. Initial kinetic analyses were carried out to determine the IC₅₀ concentrations. In a first series of tests, the enzymic reaction was initiated by the addition of enzyme (2.25 µg). In a second series, it was initiated by adding DXP after pre-incubation of the enzyme with the inhibitor in the presence of NADPH for 2 min and at 37°C. Assays were performed in 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM MgCl₂ and 2 mM DTT. The concentrations of NADPH and DXP were 0.15 and 0.5 mM respectively. The reaction medium contained inhibitors at various concentrations, depending on the inhibitor and the conditions. When the reaction

was initiated by H-DXR, they were varied from 0.4 to 2 µM for (7), from 0.2 to 0.5 µM for (8) and from 0.1 to 0.5 µM for fosmidomycin. When the enzyme was pre-incubated with the inhibitor, concentrations from 0.04 to 0.15 µM for (7) and from 0.01 to 0.15 µM for (8) and fosmidomycin (4) were used.

Enzyme assays were run under the same conditions as those described above. The concentration of inhibitors were varied from 0.5 to 1.5 µM for (7), from 0.015 to 0.5 µM for (8) and from 0.05 to 0.5 µM for fosmidomycin (4). A control assay in the absence of inhibitor was performed to check the linearity of the rate. The progress of the reaction was monitored at 340 nm over 5 min. Data were fitted using the KaleidaGraph 3.6 software.

Reversibility of the inhibition

The enzyme (10 µM subunit) was incubated with an excess of inhibitor (20 µM) in 50 mM Tris/HCl buffer, pH 7.5, containing 0.2 mM NADPH, 3 mM MgCl₂ and 2 mM DTT (total volume 50 µl) for 5 min at 37°C. An aliquot (5 µl) of the latter solution was added to the same buffer (995 µl) containing 0.2 mM NADPH and 0.5 mM DXP (final concentration). The absorbance decrease at 340 nm was monitored over 10 min. A control experiment without inhibitor was run under the same conditions.

Antimicrobial activity of (7) and (8)

The antimicrobial activity of hydroxamic acids (7) and (8) against *E. coli* XL1 Blue and a fosmidomycin-resistant strain of *E. coli*, selected in our laboratory for resistance against up to 200 µM fosmidomycin concentrations, was determined using the paper disc diffusion method. The efficiency of the synthetic compounds, in comparison to fosmidomycin (4) and fosfomycin (6), was tested as follows (see Figure 5). LB agar plates (9 cm-diameter) were inoculated with bacteria (200 µl, mid-exponential phase). Paper discs (Durieux no. 268, diameter 6 mm) impregnated with (7) (5 or 50 µg), (8) (5 or 50 µg), fosmidomycin (4) (5 or 50 µg) and fosfomycin (6) (5 µg) were placed on Petri dishes. Growth inhibition was examined after 24 h incubation at 37°C.

To test the antimicrobial activity of the inhibitors against *E. coli* XL1 Blue in liquid medium, aliquots of an overnight culture were inoculated into fresh LB medium (50 ml volume, starting *D*₆₀₀ = 0.1) containing (7) (10 µM, 50 µM or 200 µM) or (8) (10 µM or 50 µM). The flasks were incubated at 37°C under constant agitation (125 rev./min). Bacterial growth was monitored by measuring the *D*₆₀₀ and compared with that of the bacteria growing in the absence of inhibitors and in the presence of fosmidomycin (4) (10 or 50 µM). The culture medium was diluted once with water when *D*₆₀₀ exceeded a value of 1.

RESULTS

Synthesis of hydroxamic acids (7) and (8)

The syntheses of the hydroxamic acid (7) and of its methylated analogue (8) are shown in Scheme 2. The first step of the synthesis was the deprotonation of dibenzyl phosphite with sodium hydride in THF to afford the corresponding anion, which upon reaction with commercially available ethyl 4-bromobutyrate (9) provided the expected phosphonate (10) in 74 % yield [24]. Hydrolysis of the ester (10) was accomplished overnight at room temperature in THF with LiOH dissolved in a minimum amount of water to provide the corresponding carboxylic acid (11) in 86 % yield [24]. Treatment of the carboxylic acid (11) with *O*-benzyl-hydroxylamine in the presence of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodi-imide hydrochloride in THF gave the hydroxamic

Table 1 Influence of inhibitor pre-incubation with DXR on the IC₅₀ values

Inhibitor	IC ₅₀ (nM) without pre-incubation	IC ₅₀ (nM) with pre-incubation (2 min)
Compound (7)	1000	170
Compound (8)	500	48
Fosmidomycin (4)	250	32

acid derivative (**12**) in 66% yield [25,26]. The methyl group was introduced by reaction of (**12**) with sodium hydride in THF, followed by addition of methyl iodide to give (**13**) in 90% yield. Removal of the protective benzyl groups of (**12**) and (**13**) was achieved by catalytic hydrogenolysis with palladium over charcoal at atmospheric pressure and room temperature. Hydroxamic acids (**7**) and (**8**) were obtained in quantitative yields and required no further purification.

Inhibition studies

Hydroxamic acids (**7**) and (**8**) were revealed as potent inhibitors of *E. coli* DXR. Pre-incubation of H-DXR with (**7**) or (**8**) and initiation of the enzymic reaction by the substrate led to a significant decrease in the IC₅₀ value (Table 1). A similar behaviour was also observed with fosmidomycin (**4**), considered as a slow-binding inhibitor of DXR [9]. These preliminary results suggested that both (**7**) and (**8**) were slow-binding inhibitors. Thus we performed specific studies, as detailed in the literature previously [27–29], to confirm this observation. Progress curves for NADP⁺ formation in the presence of (**7**), (**8**) or fosmidomycin (**4**) were non-linear. Such a non-linearity is indicative of slow binding, and was not due to substrate depletion, since the control assay showed steady-state kinetics. Both (**7**) and (**8**), like fosmidomycin (**4**), were revealed as time-dependent inhibitors of H-DXR, with their binding apparently tightening over time (Figure 1). As shown in Scheme 3, three different mechanisms would result in slow-binding inhibition kinetics [28,29]. In mechanism (A), the binding of the inhibitor to the enzyme and its dissociation are slow. Mechanism (B) assumes a rapid formation of an EI complex, which undergoes slow equilibrium to form a tight EI* complex. In mechanism (C), the enzyme exists in two states, undergoing a slow reversible interconversion between E and E*, of which only E* is capable of binding the inhibitor. The analysis of the reaction progress curves in the presence of the inhibitor at various concentrations should help to distinguish between the inhibition modes. Therefore data points from the progress curves were fitted to integrated Michaelis–Menten eqn (1) [27,28]:

$$[P] = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (1)$$

where [P] is the product concentration at any *t*, *v*₀ and *v*_s are the initial and final steady-state rates respectively, and *k* is the apparent first-order rate constant for the establishment of the final steady-state equilibrium. The parameters *v*₀, *v*_s and *k* were calculated for each concentration of inhibitor used to determine the kind of inhibition. For mechanism (A), the initial velocity *v*₀ is independent of the concentration of inhibitor [I], and the rate of inhibition *k* would increase linearly with [I]. If inhibition occurs via mechanism (B), 1/*v*₀ and 1/*v*_s are linear functions of [I], and *k* would increase with inhibitor concentration, but tend towards a saturation point as the concentration becomes higher than the *K*_i value. A plot of *k* versus [I] would be a hyperbola. Difficulties in distinguishing between the two mechanisms would occur, however, when the initial interaction between the inhibitor and the enzyme is not fast, so that the free enzyme and the EI and EI* complexes are in

Figure 1 Time-dependent inhibition of DXR by hydroxamic acids (**7**) (A) and (**8**) (B), and fosmidomycin (**4**) (C)

The reaction was performed in a 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM MgCl₂ and 2 mM DTT at 37 °C. The concentrations of NADPH and DXR were 0.2 and 0.5 mM respectively. The upper lines (traces **a** in panels **A–C**) were control runs without inhibitor. The concentrations of inhibitor were: compound (**7**), 500 nM (trace **b**), 1000 nM (trace **c**) and 1500 nM (trace **d**); compound (**8**), 150 nM (trace **b**), 250 nM (trace **c**) and 500 nM (trace **d**); and fosmidomycin (**4**), 50 nM (trace **b**), 150 nM (trace **c**), 250 nM (trace **d**) and 500 nM (trace **e**). The enzymic reaction was initiated by addition of DXR (2.25 μg), and followed by monitoring the decrease of absorbance at 340 nm due to the oxidation of NADPH to NADP⁺.

Mechanism A

Mechanism B

Mechanism C

Scheme 3 Mechanisms for competitive slow-binding enzyme inhibition

See the text for further details.

Figure 2 Determination of K_i and K_i^*

Progress curves in Figure 1 were fitted to eqn (1) to determine the initial and final steady-state rates (v_0 and v_s) respectively. The constants were estimated from the plots of $1/v_0$ (A) and $1/v_s$ (B) against the concentration of inhibitor, according to eqns (2) and (3). Symbols used: compound (7), ■; compound (8), □; and fosmidomycin (4), ▲.

equilibrium. In that case, the function of $1/v_0$, $1/v_s$ and k versus $[I]$ could be either linear or hyperbolic, depending on the (k_4/k_5) ratio [29]. In the case of mechanism (C), k would decrease with increasing concentration of inhibitor.

The fitting of the progress curves obtained during the inhibition of H-DXR by the three tested compounds revealed the clear dependency of the initial rate v_0 on inhibitor concentration (Figure 2A). Accordingly, inhibition via mechanism (B), a fast equilibrium preceding the formation of a slow dissociating enzyme–inhibitor complex, was favoured. A clear-cut answer could not be given, since the plot of k against the concentration of inhibitor yielded straight lines (Figure 3). Experimental data suggested that fosmidomycin inhibits DXR in a competitive inhibition manner with respect to DXP via mechanism (B) [9]. The two related hydroxamic acids (7) and (8) were assumed to follow a similar inhibition pattern, and the inhibition constants K_i (k_4/k_3) and K_i^* ($K_i[k_6/(k_5 + k_6)]$) were estimated by fitting our data to the equations for competitive inhibition (Figure 2):

$$\frac{1}{v_0} = \frac{K_m}{V_{\max}[S] \cdot K_i} + \frac{1(1 + K_m)}{V_{\max}[S]} \quad (2)$$

$$\frac{1}{v_s} = \frac{K_m[I]}{V_{\max}[S]K_i^*} + \frac{1(1 + K_m)}{V_{\max}[S]} \quad (3)$$

where $[S]$ and $[I]$ are the substrate and the inhibitor concentrations, K_m is the Michaelis constant and $V_{\max}$ the maximum reaction rate of the uninhibited reaction. Under our assay conditions, a K_m of 100 μM for DXP and a $V_{\max}$ of 18 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein were found with the H-DXR preparation. As envisaged for slow binding inhibition, K_i^* values were lower than K_i values. The highest K_i/K_i^* ratio was observed for (8).

Figure 3 Determination of slow-binding mechanism by plotting k against the inhibitor concentration

Progress curves in Figure 1 were fitted to eqn (1) to determine k , the apparent first-order rate constant, for the establishment of the final steady-state equilibrium. Symbols used: compound (7), ◆; compound (8), □; and fosmidomycin (4), ▲.

Figure 4 Dissociation of the inhibitor–H-DXR complex

The enzyme (10 μM) was incubated for 5 min with the inhibitor (20 μM) in the presence of 0.15 mM NADPH at 37 °C in a 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM MgCl_2 and 2 mM DTT. An aliquot (5 μl) was taken off and diluted into the same buffer (995 μl) containing 0.2 mM NADPH and 0.5 mM DXP. The activity regaining was monitored at 340 nm by following the oxidation of NADPH to NADP^+ . (trace a) compound (8); (trace b) fosmidomycin (4); (trace c) compound (7); (trace d) control; DXR was incubated under the same conditions without inhibitor.

To test the reversibility of the inhibition, samples of H-DXR were incubated with a 2-fold excess of each inhibitor in the presence of NADPH and MgCl_2 , the cofactors required for substrate binding [8,9]. They were then assayed for enzyme activity after a 200-fold dilution. Curves clearly showed that the enzyme regained activity with all three tested compounds (Figure 4). Total recovery could not be obtained, since a 0.1 μM concentration of inhibitor remained in the reaction medium. The regaining of enzyme activity was faster with hydroxamic acid (7) and fosmidomycin (4) than with (8). The release of the latter from the enzyme is very slow, indicating that hydroxamic acid (8) may be considered as a slow tight-binding inhibitor.

Antimicrobial activity

The paper disc diffusion method showed that fosmidomycin (4) and fosfomycin (6) have nearly the same efficiency against *E. coli* XL1 Blue (Figure 5a). At a 5 μg dose, hydroxamic acid (7) presented no activity, whereas (8) showed already significant growth inhibition activity, which was exemplified for both compounds (7) and (8) at a dose of 50 μg (Figure 5b). With the fosmidomycin-resistant strain, cross-resistance with fosfomycin was observed (Figure 5c). Interestingly, this fosmidomycin/fosfomycin-resistant strain remained sensitive to (8) (Figures 5c and 5d).

The antimicrobial activity of (7) and (8) was also tested in liquid medium and compared with the activity of fosmidomycin. The latter was more effective than (7) and (8): a 10 μM fosmidomycin concentration was sufficient to stop the division of *E. coli*, whereas

Figure 5 Antimicrobial activity of (7) and (8) in comparison with fosmidomycin (4) and fosfomycin (6)

(a) Wild-type *E. coli* strain: discs impregnated with a 5 μ g dose. (b) Wild-type *E. coli* strain: discs impregnated with a 50 μ g dose. (c) Fosmidomycin-resistant *E. coli* strain: discs impregnated with a 5 μ g dose. (d) Fosmidomycin-resistant *E. coli* strain: discs impregnated with a 50 μ g dose.

Figure 6 Antimicrobial activity of (7) and (8) on an *E. coli* XL1 Blue culture

The bacteria were grown in an LB medium in the presence of the DXR inhibitors at different concentrations at 37 °C. The influence of the compounds on the cell proliferation was studied by monitoring the increase in D_{600} as a function of time. In both (A) and (B), the growth was compared with a control performed without inhibitors (■), and an assay carried out in the presence of 10 μ M fosmidomycin (◆). (A) Data for compound (7) are shown, at the following concentrations: 10 μ M (□), 50 μ M (▲) and 200 μ M (△); in (B), data for compound (8) are shown, at the following concentrations: 10 μ M (□) and 50 μ M (▲).

a 50 μ M concentration was required for (8) (Figure 6B), and a 200 μ M concentration for (7) was not sufficient to stop cell proliferation (Figure 6A). Compound (8) retained some anti-microbial activity against the fosmidomycin-resistant *E. coli* strain. A

Figure 7 Antimicrobial activity of (8) on a fosmidomycin-resistant *E. coli* XL1 Blue strain

The bacteria were grown in an LB medium containing 100 μ M fosmidomycin in the presence of (8) at different concentration: 50 μ M (▲), 200 μ M (△) at 37 °C. The influence of the compounds on the cell proliferation was followed by monitoring the increase in D_{600} nm as a function of time. The growth was compared with a control (■).

200 μ M concentration was capable of significantly reducing cell proliferation (Figure 7).

DISCUSSION

DXR is an essential enzyme for the viability of bacteria and represents an attractive target for the development of antibacterial compounds. Fosmidomycin (4) and the related FR-900098 (5) are the most efficient inhibitors of this enzyme known today. The two molecules bind to DXR via their phosphonate and hydroxamic acid moieties. The phosphonate group fits into the phosphate-recognizing site, whereas the hydroxamic acid group, known to be a metal chelator, tightly anchors the inhibitors to the enzyme by co-ordination to the metal cation required for enzyme activity [21]. The two novel related inhibitors (7) and (8) that we synthesized are characterized by the same features, with a negatively charged phosphonate moiety and a chelating hydroxamate group linked by a chain of the same length as that found between the functional groups of fosmidomycin, but in a different arrangement (Scheme 1). Many compounds bearing a hydroxamic acid group have been reported to be efficient enzyme inhibitors. Among them, the (*R*)-enantiomer of trichostatin A, a natural product isolated from *Streptomyces* exerting antifungal antibiotic activities [30], and the structurally related suberoylanilide hydroxamic acid are potent inhibitors of histone deacetylases [31,32]. The hydroxamic moiety binds to the Zn^{2+} -containing pocket in the catalytic site, and thus causes their reversible inhibition [33]. *N*-Hydroxy-*N*-isopropylloxamate tightly inhibits acetohydroxy acid isomeroreductase from bacteria and plants, a key enzyme in the biosynthetic pathway of the branched-chain amino acids [34,35]. Like DXR, this enzyme catalyses first an alkyl migration, followed by a concomitant NADPH-dependent reduction step. In contrast with DXR, which accepts several bivalent ions for enzymic activity, acetohydroxy acid isomeroreductase has an absolute requirement for Mg^{2+} . *N*-Hydroxy-*N*-isopropylloxamate is a competitive time-dependent inhibitor. Anchoring to the active site takes place through the binding of the oxygen atoms of the hydroxamic acid moiety to the bivalent cations present in the active site [36].

Like fosmidomycin, the hydroxamic acids (7) and (8) are slow-binding inhibitors. The K_i and K_i^* values of both compounds against DXR were, respectively, 169 and 68 nM for (7) and 54 and 3 nM for (8) when determined for initial (v_0) and final (v_s) velocities (Table 2). Under the same assay conditions and with the same preparation of H-DXR, 40 and 10 nM values were obtained for fosmidomycin. With non-tagged DXR, Koppisch et al. [9]

Table 2 Inhibition constants (K_i), assuming a competitive slow-binding inhibition via mechanism (B) (cf. Scheme 3)Fitting errors for K_i values are indicated.

Inhibitor	Kinetic parameter		
	K_i (nM)	K_i^* (nM)	K_i/K_i^*
Compound (7)	169 $\pm$ 4	68 $\pm$ 1	2.5
Compound (8)	54 $\pm$ 1	3 $\pm$ 1	18
Fosmidomycin 4	40 $\pm$ 1	10 $\pm$ 1	4

found values of 215 nM and 21 nM for the inhibition constants with fosmidomycin. Hydroxamic acid (8), in which the hydrogen atom of the hydroxamic acid (7) is replaced by a methyl group, inhibited H-DXR more efficiently. The same phenomenon was reported for FR-900098 (5), bearing a methyl group in the place of the formyl hydrogen of fosmidomycin (4) and also showing an increased affinity towards DXR [3]. This observation is in favour of a similar binding of the inhibitors (7) and (8) with the normal substrate DXP (1). Such a substrate-like binding was also suggested for fosmidomycin (4) and FR-900098 (5) [21].

However, as far as antimicrobial activity is concerned, DXR inhibitors (7) and (8) are less efficient against *E. coli* than fosmidomycin. Differences in the herbicidal potency of two inhibitors of plant acetohydroxy acid isomeroreductase are apparently correlated with their rates of association with the enzyme: the slower the binding to the active site, the lower the herbicidal activity [35]. Concerning hydroxamic acid (8), the estimated values of k obtained by fitting the progress curves to eqn (1) seemed to indicate that fosmidomycin (4) binds faster to H-DXR than (8) does. At the same concentrations of fosmidomycin (4) and (8), the values of k for the former were significantly higher than those of (8), suggesting that the slower binding may contribute to a lower efficiency of (8) as an antimicrobial agent.

The phosphonohydroxamic acids (7) and (8) are potent inhibitors of the *E. coli* DXR, especially (8), which is nearly as efficient as fosmidomycin in enzyme assays. All other tested substrate analogues are either weak inhibitors [8] or substrate surrogates [37]. Both compounds (7) and (8), and again especially (8), are growth inhibitors of *E. coli*, although they are less efficient than fosmidomycin. Interestingly, hydroxamate (8) is still active against an *E. coli* strain with cross-resistance towards fosmidomycin (4) and fosfomycin (6), which have different targets. Fosmidomycin inhibits DXR, whereas fosfomycin interferes with the formation of the bacterial cell wall by inactivation of UDP-*N*-acetylglucosamine enolpyruvyltransferase [38]. Both antibiotics, however, have been described to enter the cells via the same pathway, i.e. via the *L*- α -glycerophosphate and the glucose-6-phosphate uptake systems [39–42], which are probably also utilized for (7) and (8), although their efficiency in hampering bacterial growth was lower. The inhibitory activity of hydroxamic acid (8) on the growth of the fosmidomycin/fosfomycin-resistant *E. coli* strain suggests differences in the uptake and/or the detoxification [43,44] of the antibiotics, opening new perspectives for overcoming bacterial antibiotic resistance.

We thank Dr R. Graf and Mr J.D. Sauer for NMR experiments and Mr R. Hueber (all at the Université Louis Pasteur, Strasbourg, France) for the MS analyses. This work was supported by a grant to M.R. from the 'Institut Universitaire de France'.

REFERENCES

- Rohmer, M. (1999) The discovery of the mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep.* **16**, 565–573
- Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **6**, 78–84
- Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Türbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K. et al. (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* **285**, 1573–1576
- Zhou, W. X. and Nes, W. D. (2000) Stereochemistry of hydrogen introduction at C-25 in ergosterol synthesized by the mevalonate-independent pathway. *Tetrahedron Lett.* **41**, 2792–2795
- Rohmer, M., Grosdemange-Billiard, C., Seemann, M. and Tritsch, D. (2004) Isoprenoid biosynthesis as a novel target for antibacterial and antiparasitic drugs. *Curr. Opin. Invest. Drugs* **5**, 154–162
- Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H. (1998) A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzes the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9879–9884
- Kuzuyama, T., Takahashi, S., Takagi, M. and Seto, H. (2000) Characterization of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic amino acid residues. *J. Biol. Chem.* **275**, 19928–19932
- Hoeffler, J. F., Tritsch, D., Grosdemange-Billiard, C. and Rohmer, M. (2002) Isoprenoid biosynthesis via the methylerythritol phosphate pathway. Mechanistic investigations of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase. *Eur. J. Biochem.* **269**, 4446–4457
- Koppisch, A. T., Fox, D. T., Blagg, B. S. J. and Poulter, C. D. (2002) *E. coli* MEP synthase: steady-state kinetic analysis and substrate binding. *Biochemistry* **41**, 236–243
- Okuhara, M., Kuroda, Y., Goto, T., Okamoto, M., Terano, H., Kohsaka, M., Aoki, H. and Imanaka, H. (1980) Studies on new phosphonic acid antibiotics III. Isolation and characterization of FR-31564, FR-32863 and FR-33289. *J. Antibiot.* **33**, 24–28
- Shigi, Y. (1989) Inhibition of bacterial isoprenoid synthesis by fosmidomycin, a phosphonic acid-containing antibiotic. *J. Antimicrob. Chemother.* **24**, 131–145
- Kuzuyama, T., Shimizu, T., Takahashi, S. and Seto, H. (1998) Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett.* **39**, 7913–7916
- Grolle, S., Bringer-Meyer, S. and Sahm, H. (2000) Isolation of the *dxr* gene of *Zymomonas mobilis* and characterization of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase. *FEMS Microbiol. Lett.* **191**, 131–137
- Zeidler, J. G., Schwender, J., Müller, C., Wiesner, J., Weidemeyer, C., Beck, E., Jomaa, H. and Lichtenthaler, H. K. (1998) Inhibition of the non-mevalonate 1-deoxy-D-xylulose-5-phosphate pathway of plant isoprenoid biosynthesis by fosmidomycin. *Z. Naturforsch.* **53**, 980–986
- Fellermeier, M., Kis, K., Sagner, S., Maier, U., Bacher, A. and Zenk, M. H. (1999) Cell-free conversion of 1-deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate into β -carotene in higher plants and its inhibition by fosmidomycin. *Tetrahedron Lett.* **40**, 2743–2746
- Reichenberg, A., Wiesner, J., Weidemeyer, C., Dreiseidler, E., Sanderbrand, S., Altincicek, B., Beck, E., Schlitzer, M. and Jomaa, H. (2001) Diaryl ester prodrugs of FR900098 with improved *in vivo* antimalarial activity. *Bioorg. Med. Chem. Lett.* **11**, 833–835
- Ortmann, R., Wiesner, J., Reichenberg, A., Henschker, D., Beck, E., Jomaa, H. and Schlitzer, M. (2003) Acyloxyalkyl ester prodrugs of FR900098 with improved *in vivo* anti-malarial activity. *Bioorg. Med. Chem. Lett.* **13**, 2163–2166
- Wiesner, J., Borrmann, S. and Jomaa, H. (2003) Fosmidomycin for the treatment of malaria. *Parasitol. Res.* **90**, S71–S76
- Lell, B., Ruangweeayut, R., Wiesner, J., Anoumou-Missinou, M., Schindler, A., Baranek, T., Hintz, M., Hutchinson, D., Jomaa, H. and Krenschner, P. G. (2003) Fosmidomycin, a novel chemotherapeutic agent for malaria. *Antimicrob. Agents Chemother.* **47**, 735–738
- Wiesner, J., Henschker, D., Hutchinson, D. B., Beck, E. and Jomaa, H. (2002) *In vitro* and *in vivo* synergy of fosmidomycin, a novel antimalarial drug, with clindamycin. *Antimicrob. Agents Chemother.* **46**, 2889–2894
- Steinbacher, S., Kaiser, J., Eisenreich, W., Huber, R., Bacher, A. and Rohdich, F. (2003) Structural basis of fosmidomycin action revealed by the complex with 2-C-methyl-D-erythritol 4-phosphate synthase (IspC). *J. Biol. Chem.* **278**, 18401–18407
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254
- Leloir, L. F. and Cardini, C. E. (1957) Characterisation of phosphorus compounds by acid lability. *Methods Enzymol.* **3**, 840–850

- 24 Cushman, M., Yang, D., Mihalic, J. T., Chen, J., Gerhardt, S., Huber, R., Fischer, M., Kis, K. and Bacher, A. (2002) Incorporation of an amide into 5-phosphonoalkyl-6-D-ribitylamino-pyrimidinone lumazine synthase inhibitors results in an unexpected reversal of selectivity for riboflavin synthase vs lumazine synthase. *J. Org. Chem.* **67**, 6871–6877
- 25 Hanessian, S., Moitessier, N. and Viau, M. (2001) *N*-Arylsulfonyl homocysteine hydroxamate inhibitors of matrix metalloproteinases: further probing of the S₁, S₁' and S₂' pockets. *J. Med. Chem.* **44**, 3066–3073
- 26 Stoermer, D., Liu, Q., Hall, M. R., Flanary, J. M., Thomas, A. G., Rojas, C., Slusher, B. S. and Tsukamoto, T. (2003) Synthesis and biological evaluation of hydroxamate-based inhibitors of glutamate carboxypeptidase II. *Bioorg. Med. Chem. Lett.* **13**, 2097–2100
- 27 Morrison, J. F. and Walsh, C. T. (1988) The behavior and significance of slow-binding enzyme inhibitors. *Adv. Enzyme Relat. Areas Mol. Biol.* **61**, 201–301
- 28 Copeland, R. A. (2000) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2nd edn, pp. 318–349, Wiley-VCH, New York
- 29 Sculley, M. J., Morrison, J. F. and Cleland, W. W. (1996) Slow-binding inhibition: the general case. *Biochim. Biophys. Acta* **1298**, 78–86
- 30 Tsuji, N., Kobayashi, M., Nagashima, K. and Koizumi, K. (1976) A new antifungal antibiotic, trichostatin. *J. Antibiot.* **29**, 1–6
- 31 Yoshida, M., Kijima, M., Akita, M. and Beppu, T. (1990) Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J. Biol. Chem.* **265**, 17174–17179
- 32 Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A. and Marks, P. A. (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3003–3007
- 33 Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. and Pavletich, N. P. (1999) Structures of a histone deacetylase homologue bound to TSA and SAHA inhibitors. *Science* **401**, 188–193
- 34 Aulabaugh, A. and Schloss, J. V. (1990) Oxalyl hydroxamates as reaction intermediate analogues for ketol-acid reductoisomerase. *Biochemistry* **29**, 2824–2830
- 35 Dumas, R., Cornillon-Bertrand, C., Guigue-Talet, P., Genix, P., Douce, R. and Job, D. (1994) Interactions of plant acetohydroxy acid isomeroreductase with reaction intermediate analogues: correlation of the slow, competitive, inhibition kinetics of enzyme activity and herbicidal effects. *Biochem. J.* **301**, 813–820
- 36 Biou, V., Dumas, R., Cohen-Addad, C., Douce, R., Job, D. and Pebay-Peyroula, E. (1997) The crystal structure of plant acetohydroxy acid isomeroreductase complexed with NADPH, two magnesium ions and a herbicidal transition state analog determined at 1.65 Å resolution. *EMBO J.* **16**, 3405–3415
- 37 Meyer, O., Grosdemange-Billiard, C., Tritsch, D. and Rohmer, M. (2003) Isoprenoid biosynthesis via the MEP pathway. Synthesis of (3*R*,4*S*)-dihydroxy-5-oxohexylphosphonic acid, an isosteric analogue of 1-deoxy-D-xylulose 5-phosphate, the substrate of the 1-deoxy-D-xylulose 5-phosphate reducto-isomerase. *Org. Biomol. Chem.* **1**, 4367–4372
- 38 Kahan, F. M., Kahan, J. S., Cassidy, P. J. and Kroop, H. (1974) The mechanism of action of fosfomycin (phosphonomycin). *Ann. N.Y. Acad. Sci.* **235**, 364–385
- 39 Sakamoto, Y., Furukawa, S., Ogihara, H. and Yamasaki, M. (2003) Fosmidomycin resistance in adenylate cyclase deficient (*cya*) mutants of *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **67**, 2030–2033
- 40 O'Hara, K. and Hashimoto, H. (1996) Mechanism of fosfomycin resistance in clinical isolates. *J. Antibiot.* **49**, 533–544
- 41 Winkler, H. H. (1973) Distribution of an inducible hexose phosphate transport system among various bacteria. *J. Bacteriol.* **116**, 1079–1081
- 42 Wong, K. K. and Kwan, H. S. (1992) Transcription of *glpT* of *Escherichia coli* K12 is regulated by anaerobiosis and *fnr*. *FEMS Microbiol. Lett.* **94**, 15–18
- 43 Shimizu, M., Nonomiya, T., Shigenobu, F., O'Hara, K. and Sawai, T. (1998) Fosfomycin resistance in *Escherichia coli* in Japan. *J. Antibiot.* **51**, 889–892
- 44 Fujisaki, S., Ohnuma, S., Horiuchi, T., Takahashi, I., Tsukui, S., Nishimura, Y., Nishino, T., Kitabatake, M. and Inokuchi, H. (1996) Cloning of a gene from *Escherichia coli* that confers resistance to fosmidomycin as a consequence of amplification. *Gene* **175**, 83–87

Received 13 August 2004/7 October 2004; accepted 11 October 2004

Published as BJ Immediate Publication 11 October 2004, DOI 10.1042/BJ20041378