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Synthesis of β- and γ-oxa isosteres of fosmidomycin and FR900098 as antimalarial candidates

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Abstract—To expand the structure–activity relationships of fosmidomycin and FR900098, two potent antimalarials interfering with the MEP-pathway, we decided to replace a methylene group in β-position of the phosphonate moiety of these leads by an oxygen atom. β-oxa-FR900098 (**11**) proved equally active as the parent compound. When applied to 4-[hydroxyl(methyl)amino]-4-oxobutyl phosphonic acid, featuring a hydroxamate instead of the retrohydroxamate moiety, a β-oxa modification yielded a derivative (**13**) with superior activity against the *Plasmodium falciparum* 3D7 strain than fosmidomycin, while a γ-oxa modification resulted in less active derivatives. A bis(pivaloyloxymethyl)ester of phosphonate **13** proved twice as active in inhibiting cultured parasites as a similar prodrug of FR900098.

1. Introduction

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate (DOXP, **1**) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **3**). This conversion involves two consecutive steps (Fig. 1). The enzyme first catalyzes an intramolecular rearrangement of DOXP into the intermediate 2-*C*-methyl-D-erythrose 4-phosphate (**2**). This step requires a divalent metal ion to induce partial positive charge on the C-2 carbon, allowing the C-4 atom to attack it. Several studies have been reported on the mechanism of this isomerisation step.[1] A second step involves an NADPH-dependent reduction of the aldehyde intermediate.

DXR is one of the enzymes involved in the MEP pathway for the biosynthesis of isoprenoids. As it is present in most eubacteria, green algae and the plastids of higher plants, but not in humans,[2] this pathway has attracted considerable interest for the development of new and safe antibiotics as well as herbicides. Furthermore, the MEP pathway has been found in *Plasmodium* spp. that harbour this pathway in their apicoplast compartments.[3] The discovery that fosmidomycin (**4**), a metabolite isolated from *Streptomyces lavendulae* in the late 1970s,[4] efficiently inhibits *P. falciparum* growth by targeting DXR,[3] catalyzed research on the synthesis of analogues of this phosphonate and its acetyl congener FR900098 (**5**). Most reported efforts towards the synthesis of fosmidomycin analogues involve modifications of the retrohydroxamate[5] and phosphonate moieties.[6] Modifications of the phosphonate group are generally directed towards improving the relatively poor oral availability of fosmidomycin.

Only recently, some studies were reported on analogues modified at the three-carbon spacer that connects the retrohydroxamate and phosphonate functionalities.[7]

Keywords: Isoprenoid biosynthesis; Fosmidomycin; DOXP reductoisomerase; Methylerythritol phosphate pathway.
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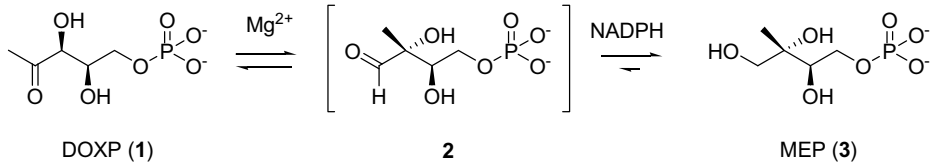


Figure 1. The conversion of 1-deoxy-D-xylulose 5-phosphate (DOXP, **1**) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **3**) by deoxyxylulose 5-phosphate reductoisomerase (DXR).

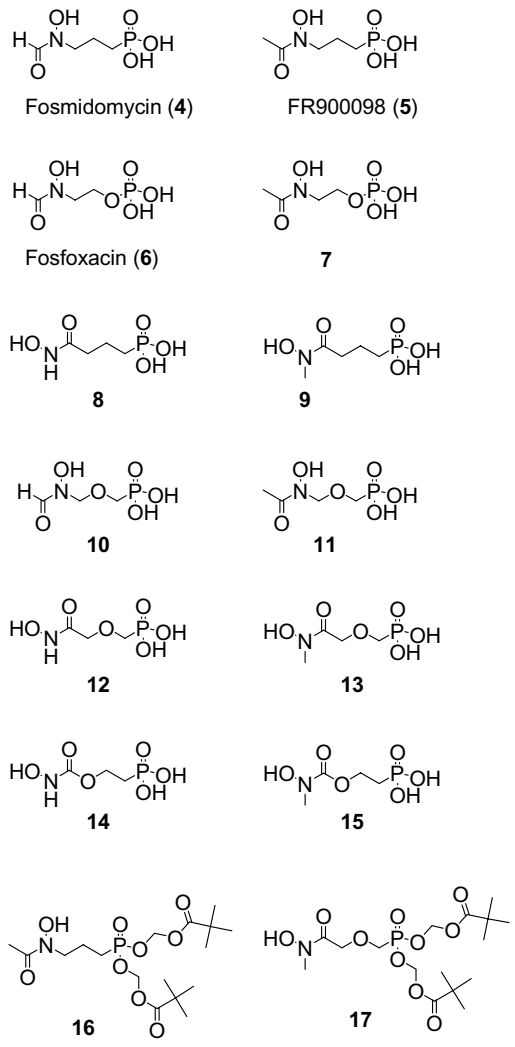


Chart 1. Structures of fosmidomycin, FR900098, reported and synthesized analogues.

The X-ray structural data that are available today should aid in the defining of the structural requirements for the design of potent DXR inhibitors. In a recent ternary complex of DXR with fosmidomycin and NADPH with a tight-binding conformation, hydrophobic contacts were observed between the three-carbon spacer and the rigid part of the binding site or with the partially ordered catalytic loop.[8]

Recently, Proteau and coworkers described the synthesis of the phosphate analogues of fosmidomycin (**6**) and FR900098 (**7**) (Chart 1).[9] The former had previously been isolated from *Pseudomonas fluorescens* PK-52 and is known as fosfoxacin.[10] The latter proved to be a very potent inhibitor of *Synechocystis* DXR. These results prompted us to report our investigations on related series of β- and γ-oxa analogues **10**–**15**.

Compounds **10** and **11** are analogues of fosmidomycin and FR900098, respectively, in which the β-methylene function was replaced by an oxygen atom. Since a phosphonomethoxy group has been successfully employed as a stable iso-electronic alternative of a methyl phosphate moiety to produce antiviral nucleosides,[11] compounds **10** and **11** were selected as possible bioisosteres of **6** and **7**.

On the one hand, the bridging oxygen of the phosphate group of DOXP may be involved in interactions with amino acids in the binding site.[12] Since only hydrophobic contacts with the methylene linker were reported, the replacement of the β- or γ-methylene groups by an oxygen atom in search for hydrogen bonding seems less compelling. On the other hand, the introduction of the electronegative oxygen atom could cause a perturbation of the electronic properties, in casu the deprotonation of the phosphonate group[12] or hydroxamate group, which becomes slightly more acidic. As a consequence of the logical decrease of the second pK_a, the phosphonate could appear rather in its doubly ionised form, which is more favourable for an ideal binding. It can be concluded that differences in ionisation may contribute to a better binding, if the introduction of an oxygen in β-position gives better activity instead of a methylene moiety. Analogues **12** and **13** combine the β-oxa modification with a hydroxamic acid moiety, as generally found as metal-binding group in matrix metalloproteinase inhibitors. Rohmer and coworkers reported that such metal chelating group, when replacing the *N*-hydroxyformamido moiety of fosmidomycin, as in compound **8** or its *N*-methyl analogue **9**, yielded potent inhibitors of DXR which were also capable to inhibit *Escherichia coli* growth.[5b] Poulter and Walker reported an analogue of DOXP in which the acetyl was replaced by a hydroxamic acid, but it did not inhibit the enzyme,[13] probably because the molecule no longer fits in the confined volume of the active site, as a consequence of its one-atom longer chain. This is consistent with the recently reported X-ray structure.[8]

Analogues **14** and **15**, which are constitutional isomers of **12** and **13**, were also synthesized. On paper they combine a hydroxamic acid moiety with an oxygen in γ-position that could possibly mimic the C4 hydroxyl group present in the substrate. The C4 hydroxyl group is known to be involved in hydrogen bonding with Glu152, Asn227 and Lys228.[8]

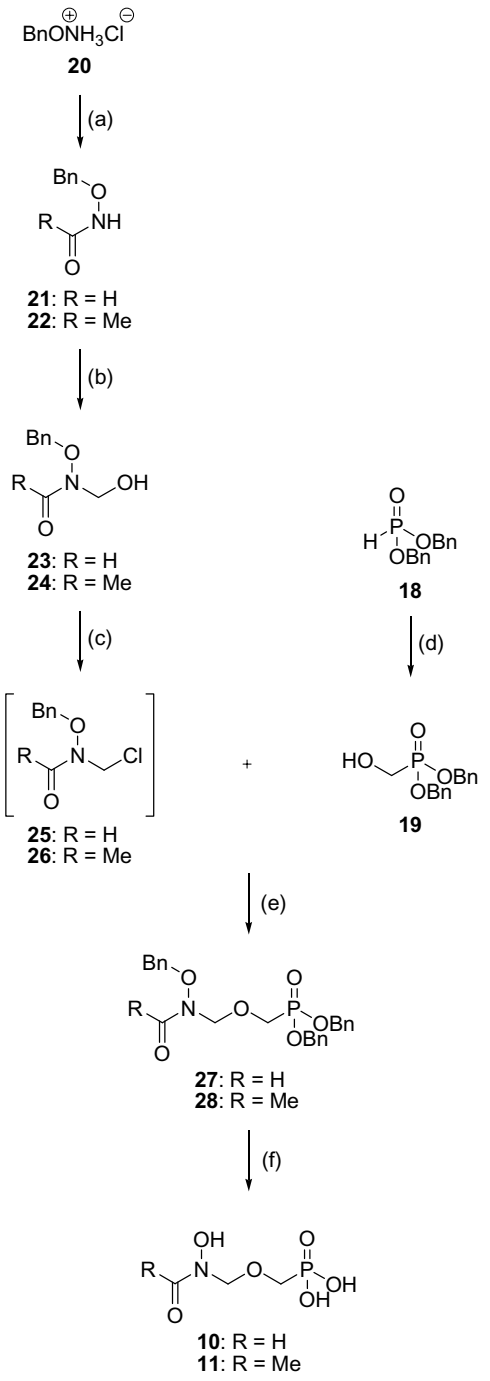
Prompted by the instability of phosphonic acid **13**, we set out on the synthesis of its bis(pivaloyloxymethyl)ester analogue **17**.

2. Results and discussion

Scheme 1 depicts the synthesis of analogues **10** and **11**. Retrohydroxamic acids **21** and **22**, obtained by acylation of *O*-benzylhydroxylamine **20**, were treated with paraformaldehyde and potassium *tert*-butoxide to give the hydroxymethylated products **23** and **24**, which were subsequently converted to the chlorides **25** and **26** as reported by Zhu et al.[14] Since attempts to crystallize the chlorides led to hydrolysis to alcohols **23** and **24**, crude **25** and **26** were directly used in the Williamson synthesis with **19**. Addition of one equivalent of NaH did not prove effective to increase the moderate yield. Compound **19** was prepared by a neat reaction with dibenzyl phosphite, paraformaldehyde and triethylamine. The perbenzylated compounds **27** and **28** were finally deprotected by catalytic hydrogenation to yield the phosphonic acids **10** and **11**, which were purified by reversed phase HPLC. Since the *N*,*O*-acetal in **27** and **28** is acid sensitive, it was very important to use neutral Pd/C catalyst to prevent cleavage. Sajiki et al. reported a remarkable supplier dependent disparity in properties of Pd/C catalysts. Pd/C catalysts of some suppliers appeared to be acidic enough to cleave triethylsilylethers without H_2.[15] β-Oxa analogue **11** proved to be stable in H_2O for more than 2 weeks, as revealed by ^1H NMR.

The synthesis of the hydroxamate **12** and its *N*-methyl analogue **13** is shown in Scheme 2. Diethylhydroxymethyl phosphonate (**29**) was reacted with ethylbromoacetate in the presence of two equivalents of NaH in THF to give **30** in 80% yield. Hydrolysis of **30** with one equivalent of sodium hydroxide followed by neutralisation gave acid **31** in 94% yield. An attempt to react diethylhydroxymethyl phosphonate directly with chloroacetic acid in the presence of two equivalents of NaH resulted in a poor yield. Treatment of carboxylic acid **31** with carbonyldiimidazole and *O*-benzylhydroxylamine afforded the *N*-benzyloxyamide **32**. A possible simplification consists of the direct conversion of the ester **30** to the *N*-benzyloxyamide **32** by a recently published procedure.[16] A methyl group was introduced by deprotonation of **32** with NaH in THF followed by addition of methyl iodide to give **34**. Finally, hydroxamic acid **12** and its N-methylated analogue **13** were obtained after catalytic debenzylation (Pd/C) and deprotection of the phosphonate moieties with two equivalents of bromotrimethylsilane in dichloromethane.

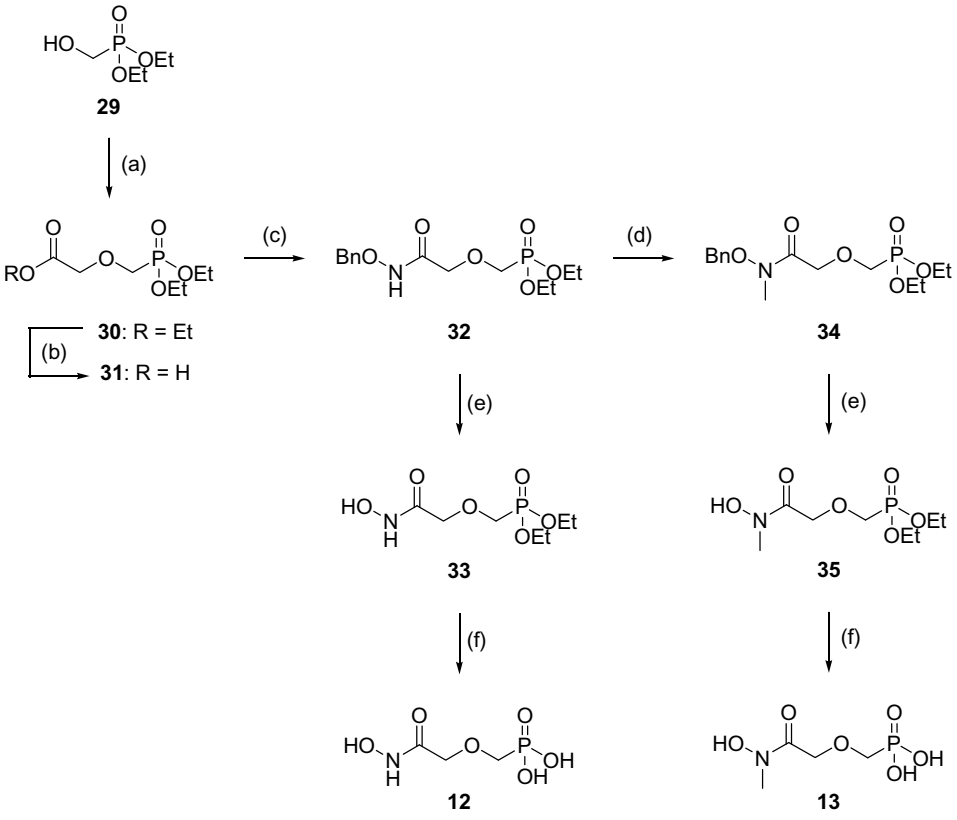
The synthesis of the *N*-hydroxy carbamates **14** and **15** is depicted in Scheme 3. Treatment of dimethylhydroxyethyl phosphonate (**34**) with carbonyldiimidazole and benzylhydroxyl amine afforded **37**. *N*-methylation, as described for **34**, yielded **39**. The benzyl protecting groups of **37** and **39** were removed by catalytic hydrogenolysis (Pd/C) to give **38** and **40**. Finally, the phosphonate esters were deprotected as outlined above (Scheme 4).



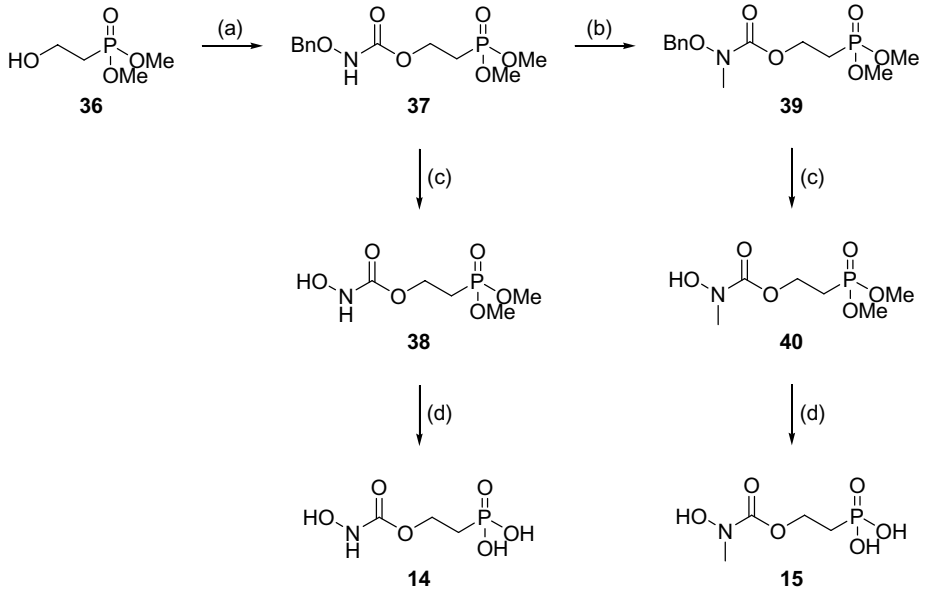
Scheme 1. Reagents and conditions: (a) acetyl chloride, CH_2Cl_2, Et_3N, 0 °C or carbonyldiimidazole, HCOOH, rt; (b) paraformaldehyde, *t*-BuOK, 60 °C; (c) $SOCl_2$, CH_2Cl_2, 10 °C; (d) paraformaldehyde, Et_3N, 90 °C; (e) CH_2Cl_2, rt; (f) H_2, Pd/C, MeOH, rt.

The synthesis of the bis(pivaloyloxymethyl)ester of **13** from its diethyl ester **35** was realized under standard conditions.

All target compounds were tested for inhibition of recombinant *E. coli* DXR (Table 1). The assay was based on the photometric measurement of the NADPH turnover associated with the DXR catalyzed reaction.[17] Fosmidomycin and FR900098 were included as reference compounds.

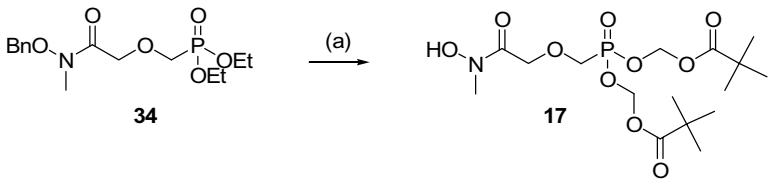


Scheme 2. Reagents and conditions: (a) ethylbromoacetate, NaH, DMF, 60 °C; (b) NaOH, H_2O, rt; (c) carbonyldiimidazole, $BnONH_2{\cdot}HCl$, Et_3N, rt; (d) MeI, NaH, rt; (e) H_2, Pd/C, MeOH, rt; (f) TMSBr, CH_2Cl_2, rt.



Scheme 3. Reagents and conditions: (a) carbonyldiimidazole, $NH_2OBn{\cdot}HCl$, Et_3N, rt; (b) MeI, NaH, rt; (c) H_2, Pd/C, MeOH, rt; (d) TMSBr, CH_2Cl_2, rt.

In the series with the inversed hydroxamates and carbamates the *N*-methyl substituted analogues proved superior to their non-methylated analogues. Compound **9**, made by Rohmer et al., was marginally less active than fosmidomycin and FR900098 in the *E. coli* DXR inhibition assay. The results of **13** and **15** showed that introduction of an oxygen atom in the propyl chain is more favourable in the β-position of the phosphonate moiety than in γ-position of the phosphonate moiety. Introduction of an oxygen atom in the γ-position of the phosphonate moiety generates carbamates **14** and **15** which failed to inhibit *E. coli* DXR at a concentration of 3 μM. In con-



Scheme 4. Reagents and conditions: (a) i—TMSBr in CH_2Cl_2 6 h, 0 °C to rt, then H_2O in THF, rt; ii—$(CH_3)_3CC(O)OCH_2Cl$, Et_3N, DMF, 70 °C to rt, 2d; iii—H_2, Pd/C, EtOAc, rt.

Table 1. IC_{50} values against recombinant *E. coli* DXR and in vitro growth inhibition of the *P. falciparum* strain 3D7

Compound	IC_{50} (μM) *E. coli* DXR[a]	IC_{50} (μM) 3D7[a]
9	0.097 ± 0.012	0.98 ± 0.06
10	1.11 ± 0.14	4.9 ± 0.4
11	0.087 ± 0.015	0.36 ± 0.02
12	>3	
13	0.072 ± 0.012	0.24 ± 0.09
14	>3	
15	>3	
16	>30	0.099 ± 0.012
17	>30	0.051 ± 0.010
Fosmidomycin	0.054 ± 0.012	1.20 ± 0.18
FR900098	0.062 ± 0.012	0.43 ± 0.06

[a] Mean values ± standard deviation of 3–5 independent measurements.

trast, compound **13** proved to be almost equipotent to FR900098 and at least as active as compound **9**. Comparison of analogues **11** and **13** demonstrated that, when combined with a β-oxa modification, the hydroxamate seems favourable to the *N*-hydroxyacetamido group as metal chelating moiety. Inhibitor **13**, when assayed as a fresh solution, is slightly more potent than **11**. The introduction of an oxygen atom in the linker does not contribute favourably to the hydrophobic interactions around the linker which are present according to the crystal structure of DXR from Sweeney et al., although in this *E. coli* DXR inhibition assay analogue **13** was comparably potent to analogue **9** with the methylene linker. This points out that an oxygen atom in β-position slightly influences the ionisation and as such the binding, even if hydrophobic interactions are playing a part. Likewise, it is also possible that the ether linkage is still hydrophobic enough to ensure a tight bonding of the inhibitor. In order to confirm that there is no hydrogen bonding involved, docking experiments would be useful.

Because of the varying IC_{50} values obtained with **13**, we assessed its stability and inferred that in aqueous solution, slow but significant degradation of this compound took place. Hence, we evaluated its bis(pivaloyloxymethyl)ester **17**, which was compared to the same ester prodrug of FR900098. While both compounds were unable to inhibit *E. coli* DXR, **17** proved approximately twice more potent in inhibiting the growth of a 3D7 *P. falciparum* strain, further warranting in vivo evaluation of this compound.

Furthermore, compound **11** outperformed its formyl analogue **10**. Nevertheless, in this series of compounds fosmidomycin stays the best inhibitor in this *E. coli* DXR inhibition assay.

In summary, we established an efficient and reliable synthesis for β- and γ-oxa fosmidomycin analogues. Analogue **13**, which combines a β-oxa modification with a hydroxamate moiety, was almost as potent in inhibiting *E. coli* DXR as FR900098.

3. Experimental

3.1. Chemicals and solvents

Most chemicals were obtained from Sigma–Aldrich or Acros Organics and were used without further purification. Compound **16** was obtained as described earlier.[5b] All reactions were carried out under inert (N_2) atmosphere.

3.2. Chromatography

Precoated Merck silica gel F_{254} plates and precoated Macherey–Nagel (Düren, Germany) silica gel F_{254} plates were used for TLC and spots were examined under UV light at 254 nm and revealed by a phosphomolybdic-cerium sulfate solution, iodine vapour or bromocresol green solution. Column chromatography was performed on ICN silica gel (63–200 μM).

3.3. Instruments and analyses

NMR spectra were obtained with a Varian Mercury 300 spectrometer. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak, in the case of $CDCl_3$ 7.26 ppm for ^1H and 77.4 ppm for ^{13}C). Coupling constants are expressed in Hz. Abbreviations used are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad signal. All signals assigned to hydroxyl and to amino groups were exchangeable with D_2O. Structural assignment was confirmed with COSY, DEPT and/or HMQC if necessary. Mass spectra and exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2, Micromass, Manchester, UK) equipped with a standard electrospray ionisation (ESI) interface. Samples were infused in an acetonitrile/water (1:1) mixture at 3 μL/min.

3.4. Dibenzyl hydroxymethylphosphonate (19)

Dibenzyl phosphite (20 mL, 76.27 mmol), triethylamine (10.60 mL, 76.27 mmol) and paraformaldehyde (2.45 g, 130 mmol) were mixed in a reaction vessel. The vessel

was tightly sealed and the mixture was mechanically stirred for 12 h at 90 °C. All volatile components were subsequently removed under reduced pressure, and the remaining semisolid was dissolved in 25 mL of CH_2Cl_2. The solution was successively washed with saturated aqueous solutions of NH_4Cl, K_2CO_3 and again NH_4Cl, dried with $MgSO_4$ and the solvent removed under reduced pressure. The residue, a pale-yellow oil, was purified by column chromatography (CH_2Cl_2/ethyl acetate, 2:1 to CH_2Cl_2/acetone 1:1, after all benzyl alcohol had eluted from the column) to yield compound **19** (12.48 g, 42.7 mmol, 56%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.92 (d, J = 5.6 Hz, 2 H, CH_2P), 5.06 (m, 4 H, CH_2Ph), 7.34 (s, 10H, arom. H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 57.66 (d, J = 160.7 Hz, CH_2P), 68.27 (d, J=6.6 Hz, OCH_2Ar), 128.26 (=CH), 128.78 (=CH), 128.86 (=CH), 136.29 (=C) ppm.

Exact mass (ESI–MS): calculated for $C_{15}H_{16}O_4P$ $[M-H]^-$: 291.0785; found: 291.0772.

3.5. *N*-(Benzyloxy)formamide (21)

Formic acid (1.18 mL, 31.3 mmol) was dissolved in dry CH_2Cl_2 (60 mL). To this solution carbonyldiimidazole (5.08 g, 31.3 mmol) was added. After stirring for 30 min, a suspension of benzylhydroxylamine hydrochloride (5 g, 31.3 mmol) in 31 mL of CH_2Cl_2 containing Et_3N (4.35 ml, 31.3 mmol) was added. When TLC revealed disappearance of all the starting material, the reaction mixture was diluted with 150 mL CH_2Cl_2 and washed six times with 1 N HCl (100 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and the residue purified by chromatography (CH_2Cl_2/MeOH, 95:5) to yield 4.13 g of *N*-(benzyloxy)formamide **21** (yield 87%) as a pale-yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): mixture of amide rotamers δ = 4.74 and 4.80 (2× s, 2H, CH_2Ph, minor and major), 7.30 (m, 5H, arom. H), 7.67 and 8.01 (2× s, 1H, C(O)H, major and minor), 9.60 and 10.32 (2× s, 1H, NH, minor and major) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): mixture of amide rotamers δ = 78.56 and 81.05 (2× s, OCH_2Ar, major and minor), 128.79 (=CH), 129.07 (=CH), 129.41 (=CH), 135.09 (=C), 158.35 and 165.08 (2× s, C(O)H, major and minor) ppm.

Exact mass (ESI–MS): calculated for $C_8H_{10}NO_2$ $[M+H]^+$: 152.0712; found: 152.0726.

3.6. *N*-(Benzyloxy)acetamide (22)

O-benzylhydroxylamine hydrochloride (1 g, 6.2 mmol) was dissolved in CH_2Cl_2 (20 mL) and triethylamine (1 mL, 6.8 mmol) and stirred for 1 h. This suspension was treated with acetyl chloride (0.5 mL, 6.8 mmol) dropwise and stirred for 1.5 h at room temperature. The reaction mixture was diluted with 60 mL of CH_2Cl_2 and washed with 100 mL of water. The organic solvent was removed under reduced pressure and the residue was purified (CH_2Cl_2/MeOH 95:5) to yield 0.43 g of *N*-(benzyloxy)acetamide **22** (yield 86%) as white crystals.

^{1}H NMR (300 MHz, $CDCl_3$): mixture of amide rotamers δ = 1.80 and 1.96 (2× s, 3 H, $C(O)CH_3$, major and minor), 4.77 and 4.84 (2× s, 2 H, CH_2Ph, minor and major), 7.30 (m, 5H, arom. H), 8.57 and 9.59 (2× s, 1H, NH, minor and major) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): mixture of amide rotamers δ = 19.95 and 19.96 (2× s, CH_3, minor and major); 78.24 and 79.53 (2× s, OCH_2Ar, major and minor), 128.74 (=CH), 128.82 (=CH), 129.32 (=CH), 135.67 (=C), 168.45 and 175.23 (2× s, $C(O)CH_3$, major and minor) ppm.

Exact mass (ESI–MS): calculated for $C_9H_{12}NO_2$ $[M+H]^+$: 166.0868; found: 166.0875.

3.7. General method for hydroxymethylation of 21 and 22

A mixture of the *N*-benzyloxyamide (5.71 mmol), paraformaldehyde (0.26 g, 8.56 mmol) and *t*-BuOK (0.064 g, 0.57 mmol) was heated, in the absence of solvent, at 60 °C during 4 h. The reaction mixture was cooled and diluted with water (80 mL). After extraction with CH_2Cl_2 (3 × 80 mL) the organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was purified by chromatography (hexane/ethyl acetate, 1:1 for **24** or pentane/acetone/Et_3N, 6:4:0.1 for **23**) to give **23** and **24** in 79 and 48% yield as white solids.

3.7.1. *N*-(Benzyloxy)-*N*-(hydroxymethyl)formamide (23).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.91 (br s, 1H, OH), 4.76 (s, 2H, CH_2Ph), 4.98 (br s, 2H, $HOCH_2$), 7.37 (m, 5H, arom. *H*), 8.08 (s, 1H, O=CH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 69.55 (s, $HOCH_2$, major), 73.10 (s, $HOCH_2$, minor), 77.71 (s, CH_2Ph, minor), 79.81 (s, CH_2Ph, major), 128.96 (=CH), 129.37 (=CH), 129.87 (=CH), 134.54 (=C), 159.48 (s, O=CH, minor), 164.27 (s, C(O)H, major) ppm.

Exact mass (ESI–MS): calculated for $C_9H_{12}NO_3$ $[M+H]^+$: 182.0817; found: 182.0832.

3.7.2. *N*-(Benzyloxy)-*N*-(hydroxymethyl)acetamide (24).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.06 (s, 3H, $C(O)CH_3$), 3.96 (br s, 1H, OH), 4.93 (s, 2H, CH_2Ph), 5.03 (br s, 2 H, $HOCH_2$), 7.38 (m, 5 H, arom. H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 20.86 (s, CH_3), 71.17 (s, $HOCH_2$), 78.45 (s, OCH_2Ar), 128.94 (=CH), 128.94 (=CH), 128.94 (=CH), 134.65 (=C), 174.26 (s, $C(O)CH_3$) ppm.

Exact mass (ESI–MS): calculated for $C_{10}H_{14}NO_3$ $[M+H]^+$: 196.0974; found:196.0981.

3.8. General method for synthesis of *N*-(benzyloxy)-*N*-(chloromethyl)formamide (25) and *N*-(benzyloxy)-*N*-(chloromethyl)acetamide (26)

To a cooled solution (10 °C) of **23** or **24** (2.7 mmol) in 5.5 mL of CH_2Cl_2 was added thionyl chloride (0.55 mL, 7.5 mmol). After stirring for 30 min, the mixture was evaporated under vacuum to yield an oily residue that could be solidified by adding a drop of hexane and connecting the flask to high vacuum. In our hands it was not possible to recrystallize **26** from ethyl acetate due to hydrolysis to the alcohol **24**. Therefore, **26** was directly used in the next step without further purification. The same holds for **25**.

3.9. Dibenzyl(*N*-(*N*-benzyloxyformamido)methoxy)methylphosphonic acid (27)

To a solution of crude **25** (~0.37 mmol) in 5 mL of CH_2Cl_2 was added a solution of **19** (0.16 g, 0.54 mmol) in 5 mL of CH_2Cl_2. After stirring for 16 h at room temperature the mixture was evaporated to dryness and purified by column chromatography (CH_2Cl_2/ethyl acetate, 2:1), affording **27** (35 mg, 21%) as a clear oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.75–3.87 (m, 2H, CH_2P), 4.74–5.15 (m, 8H, NCH_2O and PhCH_2O), 7.28–7.35 (m, 15H, arom. H), 8.07 and 8.15 (br s and br s, 1H, C(O)H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 63.60 (d, $^1J_{C,P}$ = 171.0 Hz, CH_2P), 68.24 and 68.32 (2× s, PhCH_2), 80.04 (NCH_2O), 128.33 (=CH), 128.86 (=CH), 129.37 (=CH), 129.80 (=CH), 134.44 (=C), 136.18 (=C), 164.25 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{24}H_{27}NO_6P$ $[M+H]^+$: 456.1576; found: 456.1580.

3.10. Dibenzyl(*N*-(*N*-benzyloxyacetamido)methoxy)methylphosphonic acid (28)

To a solution of crude **26** (~2.5 mmol) in 2 mL of CH_2Cl_2 was added a solution, **19** (1.46 g, 5 mmol) in 3 mL of CH_2Cl_2. After stirring for 16 h at room temperature the mixture was evaporated to dryness and purified by column chromatography (CH_2Cl_2/ethyl acetate, 2:1) to give **28** (0.44 g, 38%) as a clear oil.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.06 (s, 3H, C(O)CH_3), 3.97 (d, J = 8.8 Hz, 2H, CH_2P), 4.92 (s, 2H, NCH_2O), 5.02 (s, 2H, PhCH_2), 5.05 (d, J = 1.5 Hz, 2H, PhCH_2), 5.08 (d, J = 2.0 Hz, 2H, PhCH_2), 7.30–7.40 (m, 15H, arom. H) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 21.16 (CH_3), 62.76 (d, $^1J_{C,P}$ = 219.3 Hz, CH_2P), 67.70 and 67.78 (2× s, PhCH_2), 77.41 (NCH_2O), 128.48 (=CH), 128.93 (=CH), 129.13 (=CH), 129.15 (=CH), 129.40 (=CH), 130.10 (=CH), 135.35 (=C), 137.03 (d, $J_{C,P}$ = 6.1 Hz, =C), 173.18 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{25}H_{29}NO_6P$ $[M+H]^+$: 470.1733; found: 470.1728.

3.11. General procedure for the benzyl deprotection of 27 and 28

A solution of compound **27** or **28** (140 mg) in MeOH (3 mL) was hydrogenated at atmospheric pressure in the presence of Pd 10 wt.% on activated carbon (70 mg). After 3 h stirring the reaction mixture was filtered over a Celite pad. The solvent was removed under vacuum and the crude mixture was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 90:10). Phosphonic acids **10** and **11** were purified according to the purification of compounds **12**–**15** (see further).

3.11.1. (*N*-(*N*-hydroxyformamido)methoxy)methylphosphonic acid (10). Yield: 34% according to HPLC analysis.

^{1}H NMR (300 MHz, D_2O): δ = 3.68 (d, $J_{H,P}$ = 9.4 Hz, 2H, CH_2P), 5.01 (s, 2H, OCH_2), 7.94 (s, 1H, C(O)H, minor), 8.21 (s, 1H, C(O)H, major) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 58.11(d, $^1J_{C,P}$ = 153.88 Hz, CH_2P, major), 63.98 (d, $^1J_{C,P}$ = 153.88 Hz, CH_2P, minor), 81.26 (s, OCH_2, major), 81.40 (s, OCH_2, minor), 160.19 (s, C=O, minor), 161.032 (s, C=O, major) ppm.

^{31}P NMR (121 MHz, D_2O): δ = 15.05 ppm.

Exact mass (ESI–MS): calculated for $C_3H_7NO_6P$ $[M-H]^-$: 184.0010; found: 184.0025.

3.11.2. (*N*-(*N*-hydroxyacetamido)methoxy)methylphosphonic acid (11). Yield: 43% according to HPLC analysis.

^{1}H NMR (300 MHz, D_2O): δ = 2.07 (s, 3H, CH_3, major), 2.08 (s, 3H, CH_3, minor), 3.43 (d, $J_{H,P}$ = 8.9 Hz, 2H, CH_2P, major), 3.49 (d, $J_{H,P}$ = 8.9 Hz, 2H, CH_2P, minor), 4.87 (s, 2H, OCH_2, major), 4.95 (s, 2H, OCH_2, minor) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 19.79 (CH_3), 66.38 (d, $^1J_{C,P}$ = 137.0 Hz, CH_2P, major), 70.87 (d, $^1J_{C,P}$ = 137.0 Hz, CH_2P, minor), 77.78 (s, OCH_2, major), 77.93 (s, OCH_2, minor), 175.74 (C=O) ppm.

^{31}P NMR (121 MHz, D_2O): δ = 15.16 ppm.

Exact mass (ESI–MS): calculated for $C_4H_{11}NO_6P$ $[M+H]^+$: 200.0324; found: 200.00316.

3.12. Diethyl((ethoxycarbonyl)methoxy)methylphosphonate (30)

Sodium hydride (400 mg, 11.9 mmol) was added to a solution of diethylhydroxymethyl phosphonate (1 g, 5.9 mmol) in THF (26 mL) and stirred for 1 h. Subsequently, ethyl bromoacetate (1.29 g, 7.7 mmol) was added and, after 3 h, the reaction was quenched with

sat. NH_4Cl (aq). EtOAc was added, the two phases separated and the aqueous layer further extracted with three portions of EtOAc (100 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 95:5) to give **30** (1.21 g, 80%) as an oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25 (t, J = 7.0 Hz, 6H, C(O)OCH$_2$CH_3), 1.32 (t, J = 7.0 Hz, 6H, POCH$_2$CH_3), 3.91 (d, $J_{H,P}$ = 8.5 Hz, 2H, CH$_2$P), 4.12–4.22 (m, 8H, C(O)CH$_2$O and POCH_2CH$_3$) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.37 (C(O)OCH$_2$*C*H$_3$), 16.64 (d, $^3J_{C,P}$ = 5.8 Hz, POCH$_2$*C*H$_3$), 61.19 (C(O)O*C*H$_2$CH$_3$), 62.79 (d, $^2J_{C,P}$ = 6.3 Hz, PO*C*H$_2$CH$_3$), 65.30 (d, $^1J_{C,P}$ = 165.5 Hz, CH$_2$P), 69.66 (d, $^3J_{C,P}$ = 11.2 Hz, C(O)*C*H$_2$O), 169.79 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_9H_{20}O_6P$ $[M+H]^+$: 255.0998; found: 255.0987.

3.13. 2-((Ethoxyphosphono)methoxy)acetic acid (31)

A solution of **28** (1.21 g, 4.8 mmol) in 4.8 mL of 1 N aq NaOH was stirred for 2 h at 30 °C. When the hydrolysis was finished, as indicated by TLC, the reaction mixture was neutralised with 1 N HCl until pH 6. The desired carboxylic acid was extracted with CH_2Cl_2 (5 × 50 mL) and EtOAc (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Purification by flash chromatography (CH_2Cl_2/MeOH/HCOOH, 95:5:0.1) afforded **31** (1.0 g, 94%). The carboxylic acid was visualized on TLC with bromocresol green.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.36 (t, J = 7.0 Hz, 6H, OCH$_2$CH_3), 3.98 (d, $J_{H,P}$ = 8.2 Hz, 2H, CH$_2$P), 4.16–4.26 (m, 6H, C(O)CH$_2$O and OCH_2CH$_3$), 6.79 (br s, 1H, OH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.61 (d, $^3J_{C,P}$ = 5.8 Hz, OCH$_2$*C*H$_3$), 63.34 (d, $^2J_{C,P}$ = 6.3 Hz, O*C*H$_2$CH$_3$), 65.40 (d, $^1J_{C,P}$ = 165.8 Hz, CH$_2$P), 69.74 (d, $^3J_{C,P}$ = 10.1 Hz, C(O)CH$_2$O), 171.91 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_7H_{16}O_6P$ $[M+H]^+$: 227.0685; found: 227.0683.

3.14. Diethyl((benzyloxycarbamoyl)methoxy)methylphosphonate (32)

Compound **31** (0.80 g, 3.5 mmol) was dissolved in CH_2Cl_2 (4 mL). To this solution 1.1 eq carbonyldiimidazole (0.60 g, 3.7 mmol) was added. After stirring for 0.5 h, a suspension of benzylhydroxylamine hydrochloride (0.59 g, 3.7 mmol) in 4 mL of CH_2Cl_2 containing Et_3N (0.38 g, 3.7 mmol) was added. After disappearance of all starting material as indicated by TLC, the reaction mixture was diluted with 50 mL CH_2Cl_2 and washed with 1 N HCl (60 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification by flash chromatography (CH_2Cl_2/MeOH, 95:5) afforded **32** as an oil in 83% yield (970 mg).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.31 (t, J = 7.0 Hz, 6H, OCH$_2$CH_3), 3.76 (d, $J_{H,P}$ = 8.8 Hz, 2H, CH$_2$P), 4.07–4.17 (m, 6H, C(O)CH$_2$O and OCH_2CH$_3$), 4.96 (s, 2H, PhCH_2O), 7.34–7.44 (m, 5H, arom. H), 9.46 (br s, 1H, NH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.65 (d, $^3J_{C,P}$ = 5.8 Hz, OCH$_2$*C*H$_3$), 63.01 (d, $^2J_{C,P}$ = 6.6 Hz, O*C*H$_2$CH$_3$), 66.19 (d, $^1J_{C,P}$ = 168.4 Hz, CH$_2$P), 72.33 (d, $^3J_{C,P}$ = 10.1 Hz, CH$_2$), 78.59 (Ph*C*H$_2$O), 128.78 (=CH), 128.95 (=CH), 129.39 (=CH), 135.0 (=C), 168.91 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{14}H_{23}NO_6P$ $[M+H]^+$: 332.1263; found: 332.1272.

3.15. Diethyl((hydroxycarbamoyl)methoxy)methylphosphonate (33)

Benzyl deprotection of **32** was essentially performed as described for **10** and **11**, yielding the title compound in 80% yield (92 mg).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.30 (m, 6H, OCH$_2$CH_3), 3.85 (m, 2H, CH$_2$P), 4.14 (m, 6H, C(O)CH$_2$O and OCH_2CH$_3$), 10.02 (br s, 1H, NH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.59 (d, $^3J_{C,P}$ = 5.5 Hz, OCH$_2$*C*H$_3$), 63.23 (d, $^2J_{C,P}$ = 6.3 Hz, O*C*H$_2$CH$_3$), 65.91 (d, $^1J_{C,P}$ = 166.7 Hz, CH$_2$P), 71.57 (d, $^3J_{C,P}$ = 10.0 Hz, C(O)*C*H$_2$O), 166.24 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_8H_{17}NO_6P$ $[M+H]^+$: 254.0794; found: 254.0806.

3.16. Diethyl((*N*-(benzyloxy)-*N*-methylcarbamoyl)methoxy)-methylphosphonate (34)

To a solution of **32** (0.51 g, 1.5 mmol) in 16 mL of THF were added sodium hydride (67 mg, 1.7 mmol) and methyl iodide (0.24 g, 1.7 mmol). The mixture was stirred overnight at room temperature, quenched with a saturated solution of NH_4Cl (40 mL) and extracted with ether (3 × 40 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification by flash chromatography (CH_2Cl_2/MeOH, 95:5) afforded **34** (300 mg, 56%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.30 (t, J = 7.0 Hz, 6H, OCH$_2$CH_3), 3.17 (s, 3H, NCH$_3$), 3.83 (d, $J_{H,P}$ = 8.2 Hz, 2H, CH$_2$P), 4.09–4.19 (m, 4H, OCH_2CH$_3$), 4.25 (s, 2H, C(O)CH$_2$O), 4.77 (s, 2H, PhCH_2O), 7.26–7.37 (m, 5H, arom. H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.63 (d, $^3J_{C,P}$ = 5.8 Hz, OCH$_2$*C*H$_3$), 33.73 (NCH$_3$), 62.71 (d, $^2J_{C,P}$ = 6.6 Hz, O*C*H$_2$CH$_3$), 65.11 (d, $^1J_{C,P}$ = 164.7 Hz, CH$_2$P), 69.91 (d, $^3J_{C,P}$ = 11.5 Hz, C(O) *C*H $_2$O), 76.53 (Ph*C*H$_2$O), 129.00 (=CH), 129.42 (=CH), 129.65 (=CH), 134.28 (=C), 171.13 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{15}H_{25}NO_6P$ $[M+H]^+$: 346.1420; found: 346.1408.

3.17. Diethyl((*N*-hydroxy-*N*-methylcarbamoyl)methoxy)-methylphosphonate (35)

Benzyl deprotection of **34** was essentially performed as described for **10** and **11**, yielding the title compound in 89% yield (140 mg).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.31 (t, J = 7.0 Hz, 6H, OCH_2CH_3), 3.19 (s, 3H, NCH_3), 3.91 (d, $J_{H,P}$ = 8.2 Hz, 2H, CH_2P), 4.09–4.19 (m, 4H, OCH_2CH_3), 4.41 (s, 2H, $C(O)CH_2O$), 9.38 (br s, 1H, OH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.57 (d, $^3J_{C,P}$ = 5.8 Hz, OCH_2CH_3), 36.03 (NCH_3), 63.22 (d, $^2J_{C,P}$ = 6.6 Hz, OCH_2CH_3), 65.02 (d, $^1J_{C,P}$ = 166.1 Hz, CH_2P), 70.01 (d, $^3J_{C,P}$ = 11.8 Hz, C(O) CH_2O), 169.83 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_8H_{19}NO_6P$ $[M+H]^+$: 256.0950; found: 256.0952.

3.18. Dimethyl-2-(*N*-benzyloxycarbonyloxy)ethylphosphonate (37)

The title compound was prepared in 73% yield from **36** using a similar procedure as described for **32**.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.13 (dt, $J_{H,H}$ = 7.3 Hz and $J_{H,P}$ = 18.8 Hz, 2H, CH_2P), 3.68 (d, J = 11.1 Hz, 6H, OCH_3), 4.30 (dt, $J_{H,H}$ = 7.6 Hz and $J_{H,P}$ = 12.9 Hz, 2H, CH_2CH_2P), 4.81 (s, 2H, $PhCH_2O$), 7.29–7.34 (m, 5H, arom. H), 8.15 (br s, 1H, NH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.42 (d, $^1J_{C,P}$ = 140.5 Hz, CH_2P), 52.73 (d, $^2J_{C,P}$ = 6.6 Hz, OCH_3), 59.79 (CH_2CH_2P), 78.78 ($PhCH_2O$), 128.69 (=CH), 128.78 (=CH), 129.32 (=CH), 135.68 (=C), 157.16 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{12}H_{19}NO_6P$ $[M+H]^+$: 304.0950; found: 304.0947.

3.19. Dimethyl-2-(*N*-hydroxycarbonyloxy)ethylphosphonate (38)

Benzyl deprotection of **37** was essentially performed as described for **10** and **11**, yielding the title compound in 98% yield (350 mg).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.21 (dt, $J_{H,H}$ = 7.0 Hz and $J_{H,P}$ = 18.8 Hz, 2H, CH_2P), 3.77 (d, J = 11.1 Hz, 6H, OCH_3), 4.40 (dt, $J_{H,H}$ = 7.0 Hz and$J_{H,P}$ = 15.3 Hz, 2H, CH_2CH_2P), 7.40 (br s, 1H, NH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.45 (d, $^1J_{C,P}$ = 141.4 Hz, CH_2P), 52.96 (d, $^2J_{C,P}$ = 6.6 Hz, OCH_3), 59.82 (CH_2CH_2P), 158.38 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_5H_{13}NO_6P$ $[M+H]^+$: 214.0481; found: 214.0479.

3.20. Dimethyl-2-(*N*-benzyloxy-*N*-methylcarbonyloxy)-ethylphosphonate (39)

The title compound was prepared in 68% yield from **37** using a similar procedure as described for **34**.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.03 (dt, $J_{H,H}$ = 7.0 Hz and $J_{H,P}$ = 18.8 Hz, 2H, CH_2P), 2.91 (s, 3H, NCH_3), 3.56 (d, J = 10.8 Hz, 6H, OCH_3), 4.20 (dt, $J_{H,H}$ = 7.3 Hz and $J_{H,P}$ = 13.2 Hz, 2H, CH_2CH_2P), 4.69 (s, 2H, $PhCH_2O$), 7.15–7.25 (m, 5H, arom. H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.33 (d, $^1J_{C,P}$ = 140.5 Hz, CH_2P), 36.89 (NCH_3), 52.52 (d, $^2J_{C,P}$ = 6.3 Hz, OCH_3), 60.08 (CH_2CH_2P), 76.65 ($PhCH_2O$), 128.52 (=CH), 128.70 (=CH), 129.48 (=CH), 135.47 (=C), 157.06 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_{13}H_{21}NO_6P$ $[M+H]^+$: 318.1107; found: 318.1111.

3.21. Dimethyl-2-(*N*-hydroxy-*N*-methylcarbonyloxy)ethylphosphonate (40)

Benzyl deprotection of **39** was essentially performed as described for **10** and **11**, yielding the title compound in 97% yield (300 mg).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.09 (dt, $J_{H,H}$ = 6.2 Hz and $J_{H,P}$ = 18.8 Hz, 2H, CH_2P), 3.08 (s, 3H, NCH_3), 3.64 (d, J = 10.8 Hz, 6H, OCH_3), 4.40 (dt, $J_{H,H}$ = 6.5 Hz and $J_{H,P}$ = 13.8 Hz, 2H, CH_2CH_2P) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.27 (d, $^1J_{C,P}$ = 140.5 Hz, CH_2P), 38.34 (NCH_3), 52.82 (d, $^2J_{C,P}$ = 6.3 Hz, OCH_3), 59.76 (CH_2CH_2P), 157.22 (C=O) ppm.

Exact mass (ESI–MS): calculated for $C_6H_{15}NO_6P$ $[M+H]^+$: 228.0637; found: 228.0624.

3.22. General method for the phosphonate deprotection towards compounds 12, 13, 14 and 15

Esters **33**, **35**, **38** or **40** (0.39 mmol) were dissolved in CH_2Cl_2 (1.1 mL) and treated dropwise with 2 eq of TMSBr (0.77 mmol, 83 mg) under N_2. The reaction mixture was stirred for 2 h at room temperature. After completion of the reaction the volatile compounds were removed in vacuo to give the corresponding phosphonic acids. All final compounds were purified using a preparative HPLC system on a C18 column (5μm; Phenomenex; Luna; 250 × 21.2 mm) with a linear gradient of acetonitrile in 5 mM NH_4OAc solution over 20 min at a flow rate of 17.5 mL/min. The purity of all target compounds was assessed by analytical HPLC (5 μm; Phenomenex; C18(2); 250 × 4.6 mm) using the same gradient at a flow rate of 1 mL/min. All final compounds were obtained as hygroscopic foams or slight-yellow oils after lyophilisation. For compounds **12** and **13**, this procedure was not very efficient since the target compounds showed poor retention (elution time: 3.6 min). Nevertheless it permitted to produce a pure fraction.

3.22.1. ((Hydroxycarbamoyl)methoxy)methylphosphonic acid (12). Yield: 52% according to HPLC analysis.

^{1}H NMR (300 MHz, D_2O): δ = 3.49 (d, $J_{H,P}$ = 9.1 Hz, 2H, CH_2P), 3.92 (s, 2H, $C(O)CH_2O$, minor), 4.07 (s, 2H, $C(O)CH_2O$, major) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 69.47 (d, $^1J_{C,P}$ = 152.3 Hz, CH_2P), 70.76 (d, $^3J_{C,P}$ = 13.3 Hz, C(O)*C*H_2OO), 169.31 (C=O) ppm.

^{31}P NMR (121 MHz, D_2O): δ = 15.58 ppm.

Exact mass (ESI–MS): calculated for $C_3H_7NO_6P$ $[M-H]^-$: 184.0010; found: 184.0021.

3.22.2. ((*N*-Hydroxy-*N*-methylcarbamoyl)methoxy)methylphosphonic acid (13). Yield: 45% according to HPLC analysis.

^{1}H NMR (300 MHz, D_2O): δ = 3.16 (s, 3H, NCH_3, major), 3.22 (s, 3H, NCH_3, minor), 3.51 (d, $J_{H,P}$ = 8.5 Hz, 2H, CH_2P), 4.25 (s, 2H, C(O)CH_2O, minor), 4.38 (s, 2H, C(O)CH_2O, major) ppm.

^{13}C NMR (75 MHz, D_2O): δ = 36.09 (NCH_3), 68.40 (d, $^1J_{C,P}$ = 157.5 Hz, CH_2P), 69.37 (d, $^3J_{C,P}$ = 11.5 Hz, C(O)*C*H_2O), 171.73 (C=O) ppm.

^{31}P NMR (121 MHz, D_2O): δ = 15.42 ppm.

Exact mass (ESI–MS): calculated for $C_4H_9NO_6P$ $[M-H]^-$: 198.0166; found: 198.0160.

3.22.3. 2-(*N*-Hydroxycarbonyloxy)ethylphosphonic acid (14). Yield: 71 mg (quantitative).

^{1}H NMR (300 MHz, MeOD): δ = 2.14 (dt, $J_{H,H}$ = 7.0 Hz and $J_{H,P}$ = 19.1 Hz, 2H, CH_2P), 4.31 (dt, $J_{H,H}$ = 7.6 Hz and $J_{H,P}$ = 10.6 Hz, 2H, C*H*$_2$$CH_2P$) ppm.

^{13}C NMR (75 MHz, MeOD): δ = 27.51 (d, $^1J_{C,P}$ = 137.0 Hz, CH_2P), 59.97 (*C*$H_2$$CH_2P$), 159.15 (C=O) ppm.

^{31}P NMR (121 MHz, MeOD): δ = 25.63 ppm.

Exact mass (ESI–MS): calculated for $C_3H_8NO_6P$ $[M-H]^-$: 184.0010; found: 184.0007.

3.22.4. 2-(*N*-Hydroxy-*N*-methylcarbonyloxy)ethylphosphonic acid (15). Yield: 153 mg (quantitative).

^{1}H NMR (300 MHz, MeOD): δ = 2.16 (m, 2H, CH_2P), 3.16 (s, 3H, NCH_3), 4.32 (m, 2H, C*H*$_2$$CH_2P$) ppm.

^{13}C NMR (75 MHz, MeOD): δ = 27.43 (d, $^1J_{C,P}$ = 138.2 Hz, CH_2P), 37.68 (NCH_3), 60.68 (*C*$H_2$$CH_2P$), 157.74 (C=O) ppm.

^{31}P NMR (121 MHz, MeOD): δ = 25.92 ppm.

Exact mass (ESI–MS): calculated for $C_4H_9NO_6P$ $[M-H]^-$: 198.0166; found: 198.0173.

3.23. Bis(pivaloyloxymethyl)ester of 13 (17)

To a solution of **34** in CH_2Cl_2 was added TMSBr (4 eq) dropwise at 0 °C. After one hour the icebath was removed and the mixture was stirred at rt for another 5 h. All volatiles were removed under vacuum, the residual oil was redissolved in THF and treated with water. After 5 min the solvent was evaporated and the residue was dried in vacuo overnight. For the alkylation step, the resulting oil was dissolved in anhydrous DMF, treated with triethylamine and stirred for 10 min. Chloromethyl pivalate was added and the mixture was stirred at 70 °C for 6.5 h, then at room temperature during the weekend. DMF was removed under reduced pressure and the residue was taken up in diethyl ether, filtered and extracted with water and saturated $NaHCO_3$ solution. The organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was purified by flash chromatography on silica gel with diethyl ether as an eluent. Benzyl deprotection was performed in ethyl acetate with H_2 at atmospheric pressure and Pd/C 10% as a catalyst. After hydrogenating for 4 h at room temperature the mixture was filtered over a Celite pad, the solvent was evaporated and the resulting oil was purified by column chromatography on silica gel (Et_2O to Et_2O:EtOAc 1:1) to give **17** as a slightly purple oil in 56% yield.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (s, 18H, $(CH_3)_3$C(O)O), 3.26 (br s, 3H, NCH_3), 4.00 (d, $J_{C,P}$ = 6.6 Hz, 2H, CH_2P), 4.41 (s, 2H, C(O)CH_2O), 5.70 (m, 4H, $(CH_2O)_2P$), 8.23 (br s, 1H, NOH) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ = 27.02 ($(CH_3)_3$), 36.11 (NCH_3), 38.95 ($C(CH_3)_3$), 65.20 (d, J_{CP} = 165.59 Hz, CH_2P), 70.07 (d, J_{CP} = 9.96 Hz, C(O) *C*H_2O), 82.08 (d, J_{CP} = 6.64 Hz, OCH_2O), 169.66 (C(O)N), 177.13 (C(O)O) ppm.

^{31}P NMR (121 MHz, $CDCl_3$): δ = 22.91 (s) ppm.

Exact mass (ESI–MS): calculated for $C_{16}H_{29}NO_{10}P$ $[M-H]^-$: 428.16799; found: 428.16729.

3.24. Biological assays

3.24.1. DOXP reductoisomerase inhibition assay. The assay was performed in a reaction mixture containing 100 mM Tris–HCl (pH 7.5), 0.2% bovine serum albumin, 1 mM $MnCl_2$, 1 mM DOXP, 0.3 mM NADPH, and 1 μg/ml recombinant DOXP reductoisomerase of *E. coli*. The mixture without substrate was pre-incubated with a serial dilution of the test compounds on a 96-well plate at 30 °C for 5 min. The compounds had been dissolved in 100 mM Tris HCl (pH 7.5) and pre-diluted to the tenfold final concentrations on a separate plate. The reaction was started by the addition of DOXP. The decrease of absorption was monitored at 340 nm using a SpectraMax 340PC microplate reader (Molecular Devices).

3.24.2. In vitro culture of *P. falciparum*. *Plasmodium falciparum* parasites were cultured in RPMI 1640 medium supplemented with 10% human serum and 25 mM Hepes buffer.[18] Human erythrocytes served as host cells. For maintenance of the culture, Petri dishes with 10 cm diameter were used. Each Petri dish contained 10 mL medium with a haematocrit of 5%. The cultures were

incubated at 37 °C under an atmosphere of 5% O_2, 3% CO_2 and 92% N_2. The medium was changed daily, and the cultures diluted at a maximal parasitaemia of 5%. The parasitaemia was checked microscopically using Giemsa-stained smears.

3.24.3. In vitro determination of antimalarial activity. For the in vitro determination of antimalarial activity a 96-well plate assay was used.[19] To each well, 0.2 mL of a suspension of *P. falciparum* infected erythrocytes with 2% haematocrit and 0.4% parasitaemia was added. Then, a serial dilution of the test compounds was prepared on the plate. After incubation for 48 h, 0.8 μCi [^{3}H]hypoxanthine in 50 μL medium was added to each well. The plates were further incubated for 24 h. The parasites were collected on glass fibre filters using a cell harvester (Micromate 196, Packard). The incorporated radioactivity was measured with a β-counter (Matrix 9600, Packard).

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