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Synthesis and Evaluation of α-Halogenated Analogues of 3-(Acetylhydroxyamino)propyl phosphonic acid (FR900098) as Antimalarials

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[a] Abbreviations: BAIB, iodobenzene *I,I*-diacetate; CDI, 1,1'-carbonyldiimidazole; DOXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HMG-CoA, 3-hydroxy-3-methylglutarylcoenzyme A; MEP, 2-*C*-methyl-D-erythritol-3-phosphate; MST, mean survival time; NFBS, *N*-fluorobenzenesulfonimide; NMO, 4-methylmorpholine *N*-oxide; S.A.R., structure-activity relation; TBAF, tetra n-butylammonium fluoride; TBDMS, *tert*-butyl dimethylsilyl; TEMPO, 2,2,6,6-Tetramethylpiperidine 1-oxyl; TMS, trimethylsilyl; TMSBr, bromotrimethylsilane

ABSTRACT Three α-halogenated analogues of 3-(acetylhydroxyamino)propyl phosphonic acid (FR900098) have been synthesized from diethyl but-3-enylphosphonate using a previously described method for the α-halogenation of alkylphosphonates. These analogues were evaluated for antimalarial potential *in vitro* against *Plasmodium falciparum* and *in vivo* in the *P. berghei* mouse model. All three analogues showed higher *in vitro* and/or *in vivo* potency than the reference compounds.

Introduction

Despite huge efforts already taken, malaria still poses a major threat to public health and the economy of affected countries.[1] With resistance emerging to virtually all available therapeutics, new antimalarials directed against new targets are highly awaited.[2] In this respect, the non-mevalonate pathway for isoprenoid biosynthesis (also known as the MEP pathway) and considered essential in all malaria-causing *Plasmodium* species constitutes a promising target.[3] This pathway is unrelated to the classical mevalonate pathway (HMG-CoA reductase pathway) present in all higher eukaryotes and starts with the condensation of pyruvate and glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose-5-phosphate (DOXP). Thus far, the second enzyme DOXP reductoisomerase (DXR), which catalyses the conversion of DOXP into 2-*C*-methyl-D-erythritol-3-phosphate (MEP), is the best investigated target in the search for new antibiotics, antimalarials and herbicides tackling the non-mevalonate pathway (Chart 1).

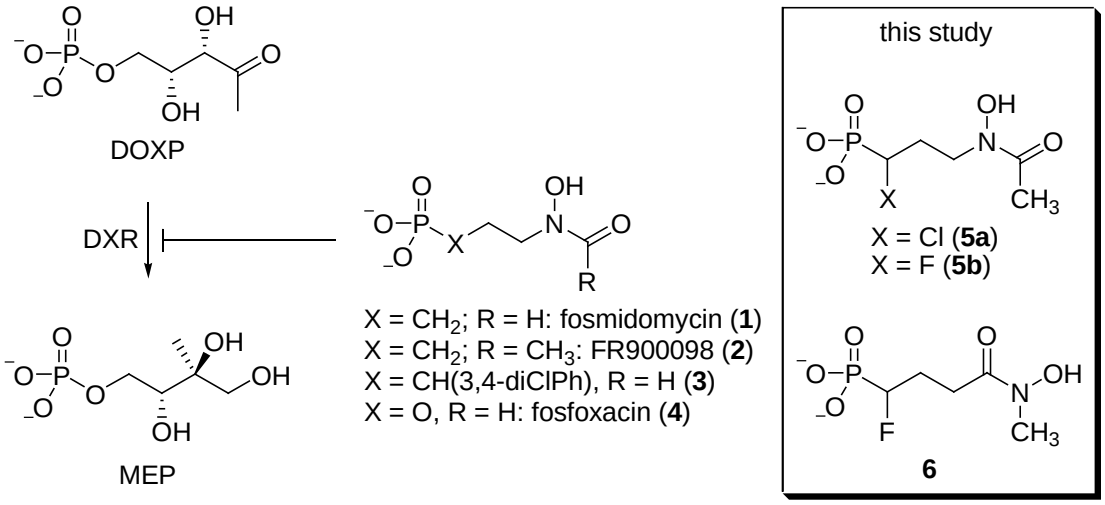


Chart 1

Fosmidomycin (**1**) and its acetyl congener **2** (FR900098)[4], structurally simple antibiotics isolated from *Streptomyces* cultures, are selective inhibitors of DXR[5] and the former has been advanced to clinical trials in Gabon and Cameroon.[6] Since the discovery of their antimalarial activity, different analogues of fosmidomycin / **2** have been synthesized in order to explore the S.A.R. and to develop more potent inhibitors. From these studies, it has become clear that neither the phosphonate[4, 7, 8] nor the (retro)hydroxamate moiety[9] can be replaced without drastic loss of activity. On the other hand, modification of the three-carbon spacer has resulted in DXR inhibitors that surpass fosmidomycin's potency. For example, some α-aryl substituted fosmidomycin analogues proved to be more potent than fosmidomycin in inhibiting the growth of *P. falciparum*.[10, 11] This advantage was especially apparent with electron withdrawing substituents, such as the 3,4-dichlorophenyl group in **3**. One possible explanation for the observed S.A.R. in this series is that electron withdrawing substituents in α-position decrease the second pKa of the phosphonate group, which for that reason appears in its double-ionized form. Indeed, earlier S.A.R. studies at the enzyme level indicate that the presence of two ionizable groups on the phosphonate or phosphate probably plays a key role in the highly potent inhibition of DXR.[7, 12] Consonantly with the hypothesis that increasing the acidity of the phosphonate moiety might confer improved activity, fosfoxacin (**4**), the phosphate analogue of fosmidomycin, and its acetyl congener were found to be more potent inhibitors of *Synechocystis sp.* PCC6803 DXR than fosmidomycin.[7]

Since the metabolic liability of the phosphate precludes its *in vivo* use as a DXR inhibitor, we decided to investigate if the phosphonate group of fosmidomycin could be manipulated to more accurately mimic the electronic nature of the phosphate moiety present in fosfoxacin and the DOXP substrate. Nieschalk et al.[13] showed that a monofluoromethylenephosphonate moiety can be a better phosphate mimic than the more popular difluoromethylenephosphonates often used for this goal, as the former one has a second pKa essentially equal to that of an organophosphate, whereas a difluoromethylenephosphonate is more acidic. Hence, we undertook the synthesis of α-halogenated fosmidomycin analogues **5a** and **b** in which the required electron withdrawing effect comes from the

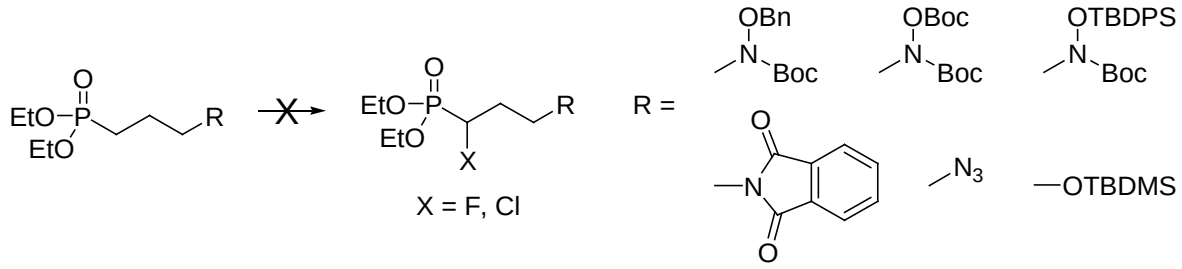
halogen instead of the sterically more demanding aromatic group as in **3**, possibly resulting in a better fit into the active pocket of DXR.

As stated above, modification of the *N*-formyl or *N*-acetyl retrohydroxamate in these structures usually results in a total loss of inhibitory activity.[9] A notable exception to this 'rule' is the inversion of the retrohydroxamate into a hydroxamate as proven by Rohmer and coworkers.[12, 14] A β-oxa analogue bearing an *N*-methylhydroxamate functionality showed even better DXR inhibiting properties,[15] hence we also envisaged the synthesis of α-fluoro hydroxamate **6**.

Results

For the introduction of the respective halogens into **2**, we adopted the strategy of Iorga et al.,[16] based upon the attack of a deprotonated α-monosilylated alkylphosphonate on an electrophilic halogenation reagent, *in casu* hexachloroethane or *N*-fluorobenzenesulfonimide (NFBS).[17] This straightforward one-pot strategy allowed for the synthesis of both envisaged precursors **10a** and **10b** with just a minor modification of the desilylation conditions (*vide infra*) and has several advantages such as high yield, high speed, ready availability of electrophilic halogenating reagents and easy elimination of byproducts. Unfortunately, this strategy proved to be very sensitive towards functionalities in the starting alkyl phosphonate, implying that all attempts to introduce a halogen onto a suitably protected fosmidomycin precursor using Iorga's conditions remained unsuccessful (Scheme 1).

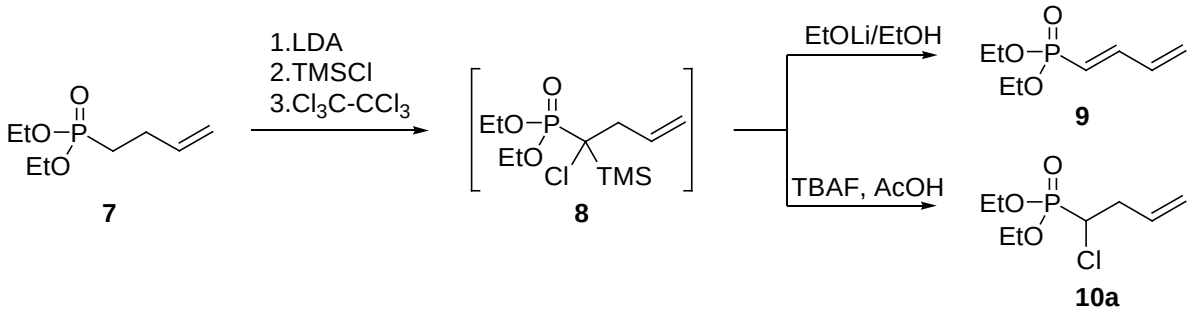
Scheme 1. Substrates unsuccessfully tested in the halogenation reaction.



First, three differently protected hydroxyamines, synthesized from diethyl 3-bromopropylphosphonate were tested. Unfortunately all reactions started to turn black after addition of the halogenation reagent,

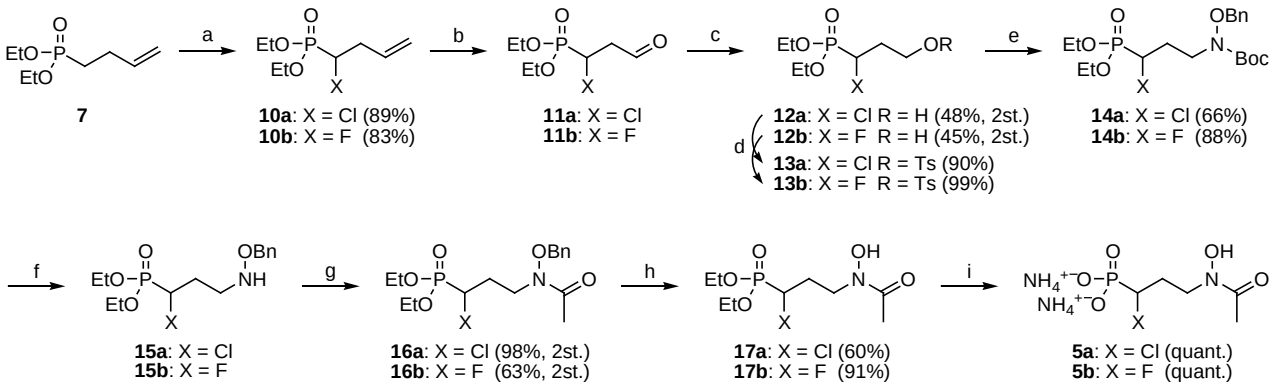
resulting in complex reaction mixtures from which the desired compounds could not or only in very low yield be isolated. Assuming that it was the protected hydroxylamine functionality that did not withstand the reaction conditions, we turned to chemically more resistant (hydroxyl)amine precursors such as a phthalimide or azide group. Here again, the halogenation conditions caused decomposition of the starting materials and were also unsuccessful when applied on TBDMS-protected 3-hydroxypropylphosphonate. These failures led us to explore a simpler phosphonate precursor, i.e. commercially available diethyl but-3-enyl phosphonate (**7**)[18], which could be halogenated in satisfying yields without noticeable breakdown to afford **10b**. One side reaction observed in the synthesis of the α-chloro-derivative **10a**, however, was the formation of conjugated diene **9** caused by elimination of chlorine when using the standard lithium ethoxide-ethanol deprotection for the TMS-group of intermediate **8**. Hence, we decided to remove the installed TMS group by means of an acidic procedure (TBAF in acetic acid), resulting in the desired product **10** (Scheme 2).

Scheme 2. Halogenation strategy with standard desilylation conditions (EtOLi/EtOH) resulting in elimination product **9** and alternative acidic procedure (TBAF/AcOH) towards **10a**.



With the α-halogenated precursors **10a-b** in hand, we then assembled the hydroxamate head group (Scheme 3). First the double bond was oxidized with NMO and osmium tetroxide as a catalyst. The resulting vicinal diol was then cleaved oxidatively with sodium periodate and the resulting aldehyde reduced with sodium borohydride to give alcohols **12a-b**.

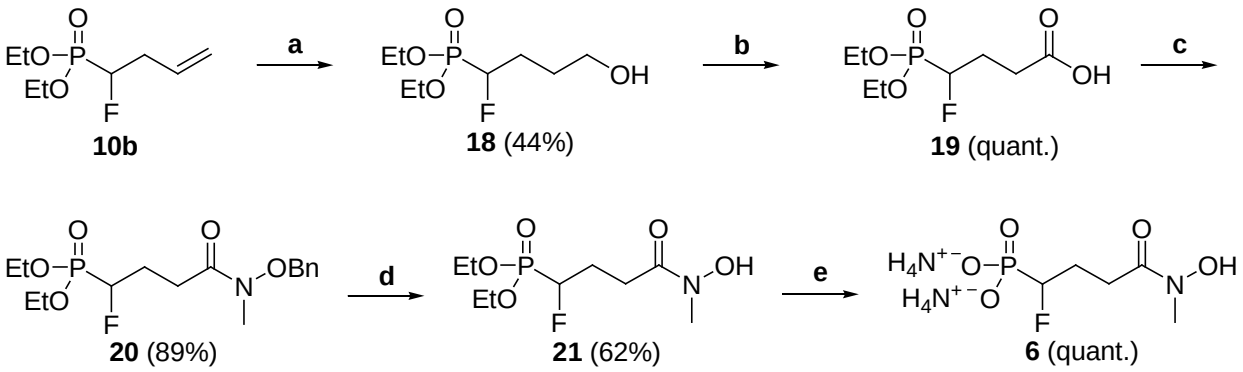
Scheme 3. Synthesis of α-chloro and α-fluoro analogues of FR900098[a]



[a]Reagents and conditions: (a) (i) LDA, TMSCl, C_2Cl_6 or $(PhSO_2)_2NF$, THF, -78 °C, (ii) TBAF, AcOH, THF; (b) (i) OsO_4, dioxane, (ii) $NaIO_4$; (c) $NaBH_4$, MeOH; (d) TsCl, Et_3N, CH_2Cl_2; (e) BocNH(OBn), NaH, DMF; (f) TFA, CH_2Cl_2; (g) Ac_2O, Et_3N, DMAP, CH_2Cl_2; (h) H_2, Pd/C, EtOAc; (i) (i) TMSBr, CH_2Cl_2, (ii) $NH_4OH_{aq.}$, THF

Subsequently, the alcohols were converted into tosylates **13a-b**, which were substituted with *N*-Boc *O*-benzyl-hydroxylamine. Treatment of **14a-b** with trifluoroacetic acid in dichloromethane gave hydroxylamines **15a-b**, which were acetylated with acetic anhydride. Finally, debenzylation of the retrohydroxamate by hydrogenation on palladium on carbon, followed by TMSBr-deprotection of the phosphonate esters and basic workup gave **5a-b** as bisammonium salts. The synthesis of α-fluoro hydroxamate **6** could easily be elaborated from the α-fluoro precursor **10b** (Scheme 2). Hydroboration of **10b** gave rise to alcohol **18** which was oxidized with TEMPO-BAIB to carboxylic acid **19**. This acid was activated with CDI followed by coupling with *N*-methyl *O*-benzylhydroxylamine to give **20**, which was then deprotected in the same way as **16a-b** to give **6** as the bisammonium salt.

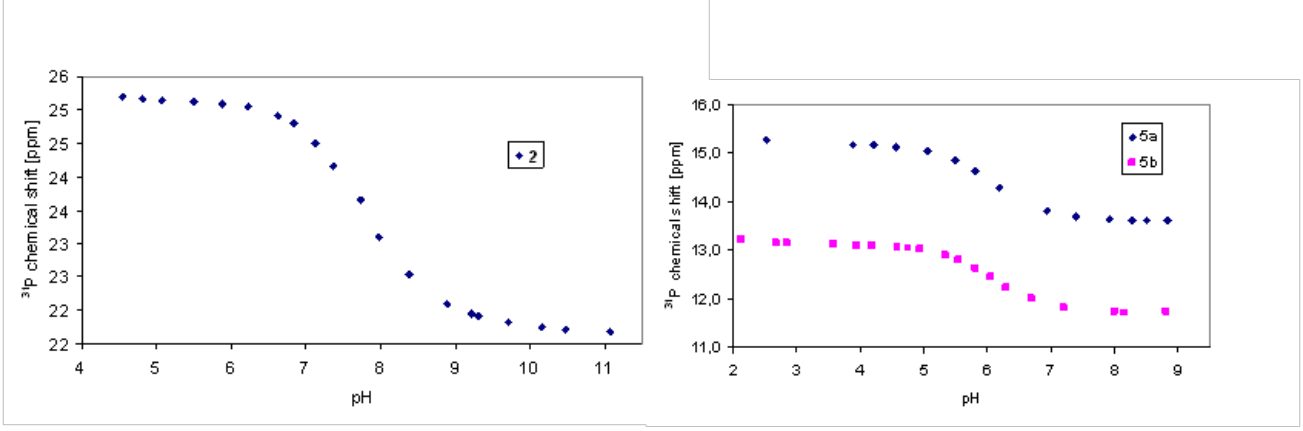
Scheme 4. Synthesis of α-fluorohydroxamate **6**[a]



[a]Reagents and conditions: (a) (i) BH_3.THF (ii) NaOH, H_2O_2; (b) TEMPO, BAIB, CH_3CN, H_2O; (c) 1,1'-carbonyldiimidazole, Me-NH-OBn; (d) H_2, Pd/C, EtOAc; (e) (i) TMSBr, CH_2Cl_2, (ii) NH_4OH, THF

In order to assess the influence of the introduced halogens on the ionization of these phosphonates, the pK_a's of compounds **5a** and **5b** as well as reference compound **2** were estimated from the pH dependence of their ^{31}P chemical shift. Therefore, the ^{31}P chemical shift of each compound was measured at different pH and plotted as a function of these pH values. The pK_a of each compound is estimated to be at the pH of the inflection point of its titration curve. Of special interest here is the second pK_a of each molecule, as the value of this pK_a determines whether the phosphonate will be in its single- or double-ionized form at physiological pH. Therefore a cutout of the titration curves is displayed from which the pK_a2 can be estimated (for the complete curves see Supporting Information).

Figure 1 Titration curves of compounds **2**, **5a** and **5b** around their second equivalence point



From these figures, a pK_a2 of 7.35 can be estimated for reference compound **2**, whereas both the α-chlorinated analogue **5a** and the α-fluorinated analogue **5b** show a pK_a2 of around 6. It can thus be concluded that introduction of a halogen in α-position of the phosphonate moiety indeed lowered the pK_a2 of those compounds to a pK_a2 comparable to that of a phosphate[13] and that, at a physiological pH, they will be present in their double-ionized form.

The title compounds were tested in duplicate for their inhibitory effect against intraerythrocytic forms of *P. falciparum* (strains GHA and K1) using a microdilution assay.[19] The results are summarized in Table 1. All three analogues show submicromolar activity on both strains and appear to be five- to six-fold more active than the parent compound fosmidomycin and slightly superior to **2** on the K1 strain.

Table 1. *In vitro* growth inhibition of the *P. falciparum* strains GHA and K1

Compound	IC_{50} (μM)	
	Pf-GHA	Pf-K1
Fosmidomycin (**1**)	-	1.73 ± 0.89
2	-	0.42 ± 0.17
3	0.60 ± 0.01	0.16 ± 0.01
5a	0.82 ± 0.10	0.30 ± 0.06
5b	0.70 ± 0.08	0.29 ± 0.06
6	0.73 ± 0.11	0.31 ± 0.07

The initial α-aryl derivative **3** and the fluorinated analogues **5b** and **6** were subsequently evaluated *in vivo* in the *P. berghei* (GFP ANKA-strain) acute mouse model after intraperitoneal dosing at 50 mg/kg for 5 consecutive days. Chloroquine (10 mg/kg for 5 days) was included as reference treatment. The animals were observed for the occurrence/presence of clinical or adverse effects during the course of the experiment. In case of very severe clinical signs, either due to toxicity or malaria, animals were euthanized for welfare reasons. Parasitaemia was determined on days 4, 7 and 14 on surviving animals using flow cytometry (10 μL blood in 2000 μL PBS). Percentage reduction of parasitaemia compared to vehicle-treated infected controls is used as a measure for drug activity and the mean survival time (MST) was calculated (Table 2). Compound **3** did not show any relevant activity. On the other hand, compound **6** resulted in 85% suppression of parasitaemia at 4dpi, which dropped to 42% at 7dpi and 41% at 14dpi. The mean survival time was 11.7 days. Compound **5b** resulted in 88% suppression of parasitaemia at 4dpi, which after ending the treatment also dropped to 62% at 7dpi and 32% at 14dpi. The overall MST was 15.8 days. These data clearly demonstrate that all three synthesized compounds have promising *in vitro* activity and that compounds **5b** and **6** surpass the antimalarial activity of (**2**) *in vivo*.

Table 2. Survivors and mean survival time (MST in days) in the *P. berghei* (GFP ANKA-strain) acute mouse model.

Treatment (IP for 5 consecutive days)	**Parasitaemia suppression** (day 4)	**Survivors** day 7	day 11	day 14	Day 25	**MST**
Vehicle	**0**	1/6	1/6	1/6	0/6	**8.5**
Chloroquine (10 mg/kg)	**100**	6/6	6/6	6/6	3/6	**20.7**
Fosmidomycin **(1)** (50 mg/kg)	**82**	6/6	4/6	3/6	nd	**11.5**
2 (50 mg/kg)	**93**	6/6	3/6	2/6	nd	**10.8**
3 (50 mg/kg)	**46**	4/5	0/5	0/5	0/5	**7.0**
5b (50 mg/kg)	**88**	4/4	2/4	2/4	0/4	**15.8**
6 (50 mg/kg)	**85**	2/3	2/3	1/3	0/3	**11.7**

In summary, three α-halogenated analogues of **2** were synthesized and surpass or equal fosmidomycin or **2** in both *in vitro* and *in vivo* antimalarial activity. These findings consolidate the assumption that electron withdrawing substituents, causing a decrease in phosphonate pK_a, favor the antimalarial activity of fosmidomycin analogues. Furthermore, we provided a new example of a fosmidomycin analogue in which the (*N*-formyl-*N*-hydroxy)amino moiety, involved in a chelating interaction with a Mn^{2+} cation, can be replaced by a *N*-hydroxy-*N*-methylamide group as found in **6**. This opens new perspectives to combine other favorable α-modifications with a hydroxamate moiety.

An important outcome of the current study is that the promising in vitro activity of the α-fluorinated analogues **5b** and **6** is reflected in the *P. berghei* acute mouse model, while the α-aryl fosmidomycin analogue **3** failed to show significant *in vivo* activity despite its promising *in vitro* activity.

Experimental Section

Synthesis. General. ^{1}H, ^{13}C, ^{19}F and ^{31}PNMR spectra were recorded in $CDCl_3$, acetone-d_6, DMSO-d_6 or D_2O on a Varian Mercury 300 spectrometer. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak for ^{1}H and ^{13}C and to external D_3PO_4 for ^{31}P). Silica gel (60 Å, 0.063 – 0.200 mm) was purchased from Biosolve. All solvents and chemicals were used as purchased unless otherwise stated. Purity of the final compounds was deduced from clean ^{1}H, ^{13}C and ^{31}P NMR spectra, high resolution mass spectra and assessed by LC-DAD-MS. Reversed phase chromatograms were recorded on a Phenomenex Luna C-18 2.5 µm particle (100 x 2.00 mm) column or a Phenomenex Luna HILIC 200A 3µm particle (100 x 2.00 mm) column in a Waters Alliance 2695 XE HPLC system spectrometer with quaternary pump, DAD detector and coupled to a Waters LCT Premier XE orthogonal time-of-flight spectrometer with API-ES source. High resolution mass spectroscopy spectra for all compounds were also recorded on a Waters LCT Premier XE orthogonal time-of-flight spectrometer with API-ES source. Purity of all final compounds was 95% or higher.

(±)-3-(*N*-hydroxyacetamido)-1-chloropropylphosphonic acid, bisammonium salt (5a). To a solution of **17a** (150 mg; 0.52 mmol) in dry dichloromethane (5 mL) was added TMSBr (0.7 mL, 5.20 mmol) while stirring at 0 °C. After 45 minutes the ice bath was removed and stirring was continued at room temperature. After three days, ^{31}P-NMR revealed the presence of uncompletely deprotected material, so another 0.2 mL of TMSBr was added. After another three days of stirring at room temperature, the volatiles were removed *in vacuo*, the residue was redissolved in 5% aqueous ammonia and washed with diethyl ether. The aqueous phase was then lyophilized to give 138 mg of a very hygroscopic, off-white powder. ^{1}H NMR (300.01 MHz, D_2O) δ 1.92-2.57 (2H, m), 2.16 (3H, s), 3.71-3.92 (2H, m), 3.92-4.06 (1H, m); ^{13}C NMR (75.44 MHz, D_2O): δ 19.5 (CH_3), 30.7 (CH_2), 45.9 (CH_2, d, $^{3}J_{PC}$ = 13.0 Hz), 54.8 (CClH, d, $^{1}J_{PC}$ = 139.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, DMSO-d6) δ 11.85; HRMS (ESI) *m/z* 232.0135 [$(M+H)^+$, calcd for $C_5H_{12}ClNO_5P^+$ 232.0136].

(±)-3-(*N*-hydroxyacetamido)-1-fluoropropylphosphonic acid, bisammonium salt (5b). To a solution of **17b** (223 mg, 0.82 mmol) in dry dichloromethane (8 mL) was added TMSBr (1.1 mL, 8.2 mmol) while stirring at 0 °C. After 45 minutes the icebath was removed and stirring was continued at room temperature. After 3 days, ^{31}P NMR revealed the presence of uncompletely deprotected material, so another 0.2mL of TMSBr was added. After another 4 days of stirring at room temperature, the volatiles were removed *in vacuo*, the residue was redissolved in 5% aqueous ammonia and washed with diethyl ether. The aqueous phase was then lyophilized to give 207 mg of **5b** as a very hygroscopic, off-white powder. ^{1}H NMR (300.01 MHz, D_2O) δ 1.96-2.22 (2H, m), 2.11 (3H, s), 3.58-4.00 (2H, m), 4.33-4.64 (1H, m); ^{13}C NMR (75.44 MHz, D_2O): δ 19.5 (CH_3), 28.4 (CH_2, d, $^{2}J_{CF}$ = 20.2 Hz), 45.0 (CH_2, d, $^{3}J_{PC}$ = 3.6 Hz), 90.7 (CHF, dd, $^{1}J_{CF}$ = 171.0 Hz, $^{1}J_{PC}$ = 154.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.80 (d, $^{2}J_{PF}$ = 62.3 Hz); HRMS (ESI) m/z 216.0455 [$(M+H)^+$, calcd for $C_5H_{12}FNO_5P^+$ 216.0432].

(±)-3-(*N*-hydroxy-*N*-methylcarbamoyl)-1-fluoropropylphosphonic acid, bisammonium salt (6). **21** (119 mg, 0.44 mmol) was dissolved in dry dichloromethane under inert atmosphere and cooled to 0 °C. TMSBr (0.6 mL, 4.4 mmol) was added dropwise while stirring. The icebath was removed and the reaction was stirred at room temperature. After 24 hours another 0.3 mL of TMSBr were added and the reaction was stirred for another 4 days. The volatiles were removed *in vacuo*, the crude material was dissolved in 5% aqueous ammonia and washed with diethyl ether. Lyophilisation of the ammonia solution yielded the product as a brown solid in quantitative yield. ^{1}H NMR (300.01 MHz, D_2O) δ 1.82-2.00 (2H, m), 2.30-2.62 (2H, m), 3.07 (3H, s), 4.18-4.45 (1H, m); ^{13}C NMR (75.44 MHz, D_2O) δ 27.0 (CH_2, d, $^{2}J_{CF}$ = 19.6 Hz), 28.5 (CH_2, d, $^{3}J_{PC}$ = 1.0 Hz), 36.1 (CH_3), 92.9 (CHF, dd, $^{1}J_{CF}$ = 171.1 Hz, $^{1}J_{PC}$ = 153.7 Hz), 175.5 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.74 (d, $^{2}J_{PF}$ = 63.27 Hz); HRMS (ESI) m/z 216.0437 [$(M+H)^+$, calcd for $C_5H_{12}FNO_5P^+$ 216.0432].

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Supporting Information Available: Experimental details and spectral information for intermediates **11a/b-17a/b**, **18**, **19**, **20**, **21;** pK_a determination of compounds **2, 5a** and **5b**; ^{1}H, ^{31}P and ^{13}C spectra for compounds **5a**, **5b** and **6**. This material is available free of charge via Internet at http://pubs.acs.org.

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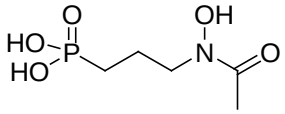
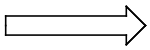
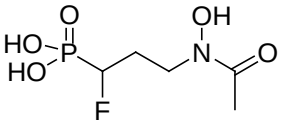
Table of Contents graphic



FR 900098
IC_{50} for K1 strain = 0.42µM





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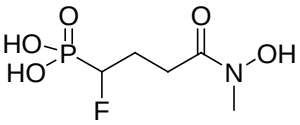

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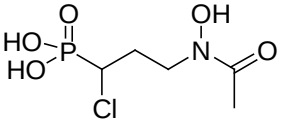

IC_{50} = 0.30 µM