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Brief Article

Synthesis and Evaluation of α-Halogenated Analogues of 3-(Acetylhydroxyamino)propylphosphonic Acid (FR900098) as Antimalarials

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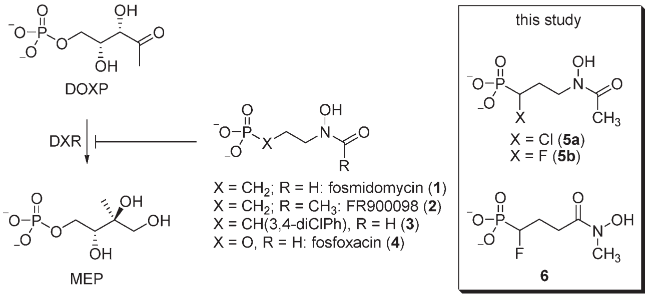
Three α-halogenated analogues of 3-(acetylhydroxyamino)propylphosphonic acid (FR900098) have been synthesized from diethyl but-3-enylphosphonate using a previously described method for the α-halogenation of alkylphosphonates. These analogues were evaluated for antimalarial potential in vitro against *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[a] 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 catalyzes 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 to explore the SAR 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 SAR in this series is that electron withdrawing substituents in α-position decrease the second pK_a of the phosphonate group, which for that reason appears in its double-ionized form. Indeed, earlier SAR 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] Consonant 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

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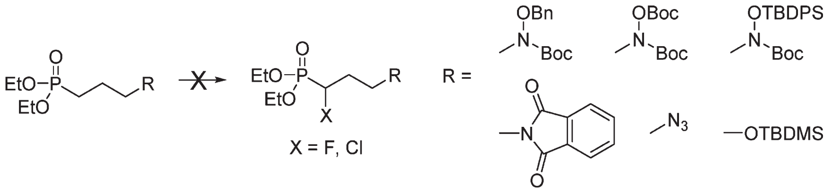
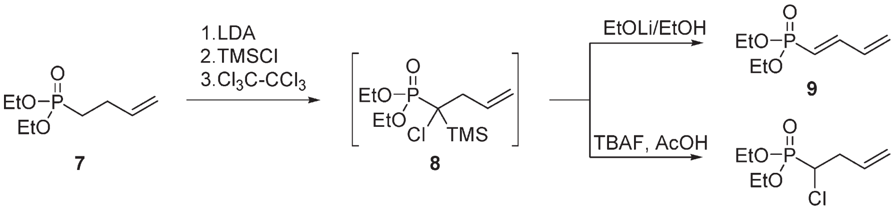
[a] Abbreviations: BAIB, iodobenzene *I,I*-diacetate; CDI, 1,1′-carbonyldiimidazole; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HMG-CoA, 3-hydroxy-3-methylglutarylcoenzyme A; MEP, 2-*C*-methyl-D-erythritol 3-phosphate; MST, mean survival time; NFBS, *N*-fluorobenzenesulfonimide;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

Scheme 1. Substrates Unsuccessfully Tested in the Halogenation Reaction



Scheme 2. Halogenation Strategy with Standard Desilylation Conditions (EtOLi/EtOH) Resulting in Elimination Product **9** and Alternative Acidic Procedure (TBAF/AcOH) towards **10a**



10a

more popular difluoromethylenephosphonates often used for this goal, as the former one has a second pK_a 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 **5b** 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 co-workers.[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-fluorohydroxamate **6**.

Results

For the introduction of the respective halogens into **2**, we adopted the strategy of Iorga et al.,[16] based on the attack of a deprotonated α-monosilylated alkylphosphonate on an electrophilic halogenation reagent, 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 toward 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).

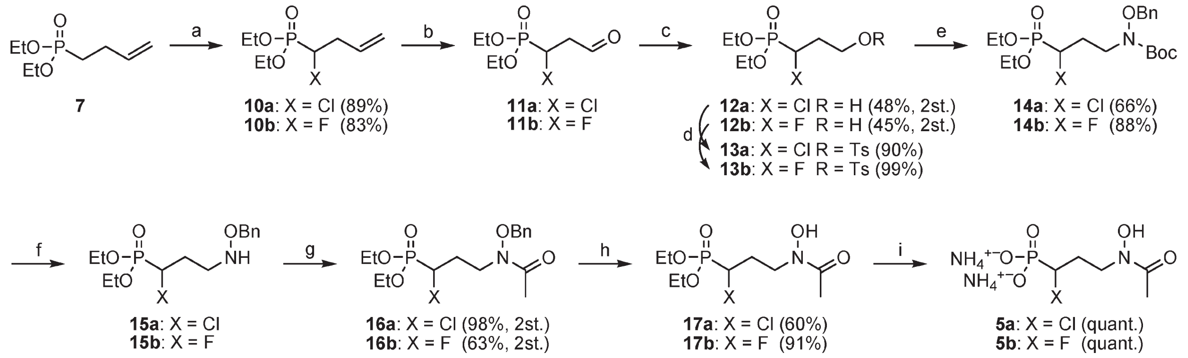
First, three differently protected hydroxyamines, synthesized from diethyl 3-bromopropylphosphonate were tested. Unfortunately all reactions started to turn black after addition of the halogenation reagent, resulting in complex reaction mixtures from which the desired compounds could not or only in very 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** 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).

With the α-halogenated precursors **10a**,**b** in hand, we then assembled the hydroxamate headgroup (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**.

Subsequently, the alcohols were converted into tosylates **13a**, **b**, which were substituted with *N*-Boc-*O*-benzylhydroxylamine. 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-fluorohydroxamate **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).

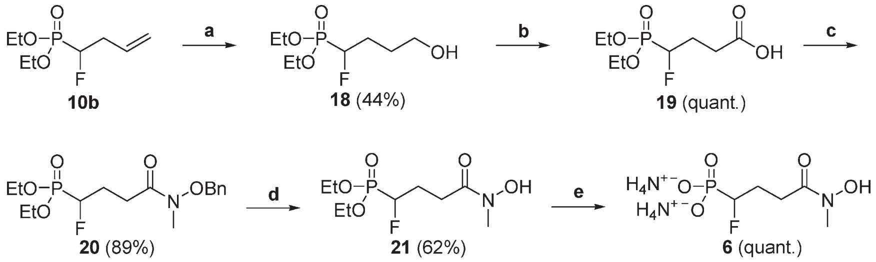
To assess the influence of the introduced halogens on the ionization of these phosphonates, the pK_a values of **5a** and **5b** and reference **2** were estimated from the pH dependence of their ^{31}P chemical shifts (Figure 1). Therefore, the ^{31}P chemical shift of each compound was measured at 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

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) TMSBr, CH_2Cl_2, (ii) NH_4OH_{aq}, THF

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



[a] Reagents and conditions: (a) (i) $BH_3 \cdot THF$, (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.

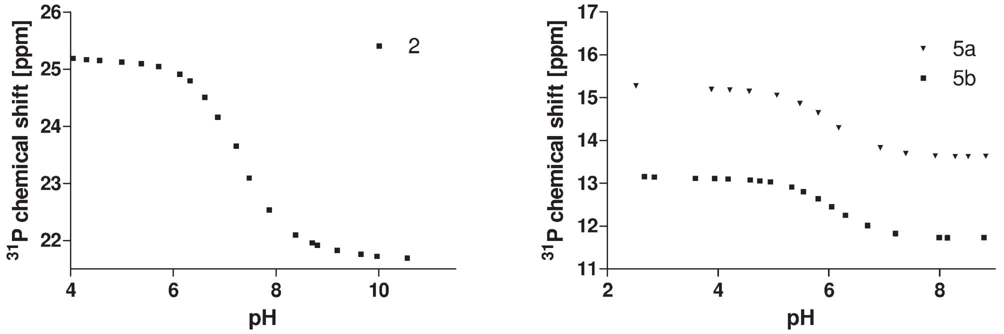


Figure 1. Titration curves of compounds **2**, **5a**, and **5b** around their second equivalence point.

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).

From these figures, a pK_{a2} of 7.35 can be estimated for reference **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 5- to 6-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

	IC$_{50}$ (μM)	
compd	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.

Table 2. Survivors and Mean Survival Time (MST in Days) in the *P. berghei* (GFP ANKA Strain) Acute Mouse Model

		survivors				
treatment (ip for 5 consecutive days)	parasitemia suppression (day 4)	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

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. Parasitemia was determined on days 4, 7, and 14 on surviving animals using flow cytometry (10 μL of blood in 2000 μL of PBS). Percentage reduction of parasitemia 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, **6** resulted in 85% suppression of parasitemia at 4 dpi, which dropped to 42% at 7 dpi and 41% at 14 dpi. The mean survival time was 11.7 days. Compound **5b** resulted in 88% suppression of parasitaemia at 4 dpi, which after ending the treatment also dropped to 62% at 7 dpi and 32% at 14 dpi. The overall MST was 15.8 days. These data clearly demonstrate that all three synthesized compounds have promising in vitro activity and that **5b** and **6** surpass the antimalarial activity of **2** in vivo.

In summary, three α-halogenated analogues of **2** were synthesized and surpass or equal fosmidomycin or **2** in 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}P NMR 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 and 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 mm × 2.00 mm) column or a Phenomenex Luna HILIC 200A 3 μm particle (100 mm × 2.00 mm) column in a Waters Alliance 2695 XE HPLC system spectrometer with quaternary pump and DAD detector, 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 min the ice bath was removed and stirring was continued at room temperature. After 3 days, ^{31}P NMR revealed the presence of incompletely deprotected material, so another 0.2 mL of TMSBr was added. After another 3 days of stirring at room temperature, the volatiles were removed in vacuo and 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, $^3J_{PC}$ = 13.0 Hz), 54.8 (CClH, d, $^1J_{PC}$ = 139.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, DMSO-d_6) 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 min the ice bath was removed and stirring was continued at room temperature. After 3 days, ^{31}P NMR revealed the presence of incompletely deprotected material, so another 0.2 mL of TMSBr was added. After another 4 days of stirring at room temperature, the volatiles were removed in vacuo and 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, $^2J_{CF}$ = 20.2 Hz), 45.0 (CH_2, d, $^3J_{PC}$ = 3.6 Hz), 90.7 (CHF, dd, $^1J_{CF}$ = 171.0 Hz, $^1J_{PC}$ = 154.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.80 (d, $^2J_{PF}$ = 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 ice bath was removed, and the mixture was stirred at room temperature. After 24 h another 0.3 mL of TMSBr was added and the mixture 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. Lyophilization 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, $^2J_{CF}$ = 19.6 Hz), 28.5 (CH_2, d, $^3J_{PC}$ = 1.0 Hz), 36.1 (CH_3), 92.9 (CHF, dd, $^1J_{CF}$ = 171.1 Hz, $^1J_{PC}$ = 153.7 Hz), 175.5 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.74 (d, $^2J_{PF}$ = 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 for **2**, **5a**, and **5b**; ^{1}H, ^{31}P, and ^{13}C spectra for **5a**, **5b**, and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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