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Synthesis of Monofluoro- and Difluoro- methylenephosphonate Analogues of *sn*-Glycerol-3-phosphate as Substrates for Glycerol-3-Phosphate Dehydrogenase and the X-Ray Structure of the Fluoromethylenephosphonate Moiety

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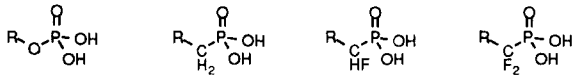
Abstract: The synthesis of the cyclohexylammonium salts of (1*RS*, 3S)-3,4-dihydroxy-1-fluorobutylphosphonic acid **3** and (S)-difluoro-3,4-dihydroxybutylphosphonic acid **4** is reported. These compounds are fluorinated phosphonate analogues of *sn*-glycerol-3-phosphate where the bridging phosphate ester oxygen is replaced by CHF and CF_2 respectively. Kinetic studies are presented for oxidation with NADH linked glycerol-3-phosphate dehydrogenase, which reveal that the CHF-phosphonate **3** performs similarly to the natural substrate *sn*-glycerol-3-phosphate, and is a better substrate than the CF_2-phosphonate **4**. The study also reveals that the diastereoisomers of **3** (**3a** and **3b**) are processed at different rates suggesting that the enzyme can discriminate the CHF stereogenic centres. A synthesis and X-ray crystal structure of 2-amino-1-fluoroethylphosphonic acid **7** is described which allows comparison of the geometry and conformation of CHF-phosphonate with that of analogous CH_2- and CF_2-phosphonates.

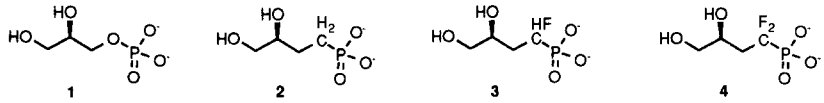
INTRODUCTION

The potential of phosphonates as hydrolytically stable phosphate mimics in bioorganic chemistry has been recognised for many years[1]. The CH_2-moiety of the phosphonate replaces the bridging oxygen of the phosphate group rendering it resistant to phosphatase hydrolysis. The replacement of oxygen for CH_2 maintains the same spatial distribution of functionality as the substrate and allows similar conformations to be accessed, clearly important features for protein binding.

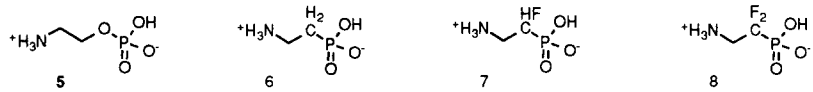


However the high electronegativity of oxygen is not matched by CH_2 and therefore the electronic consequences of such a replacement may prove detrimental. For example the second deprotonation of the phosphonate has a pKa of ~ 7.6 whereas that of the phosphate group is ~ 6.4[2]. The introduction of fluorine atoms onto the methylene group increases the acidity of the phosphonates due to the electron widthdrawing effect of the fluorine atom[3]. In recent years this modification has proved popular and many reports have emerged in the pharmaceutical literature where this substitution is explored in mono-, di- and triphosphate

systems[4]. In view of this it is surprising that the monofluoromethylenephosphonates (CHF-phosphonates) have received little attention despite some obvious advantages. The pKa of the second deprotonation of a CHF-phosphonate is 6.5 [5] and is essentially identical to that of the phosphate group that it is designed to mimic. In a recent theoretical analysis[6] the CHF-phosphonate is predicted to have a close electrostatic profile to the phosphate group.



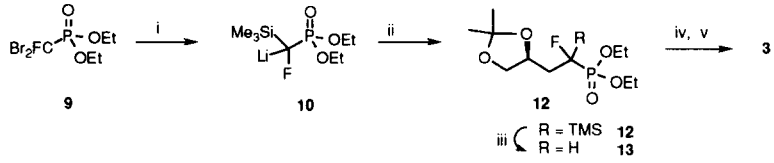
We have had a particular interest in the synthesis of fluorinated phosphonates as mimics for monophosphates of the glycolytic pathway[7]. Previously we prepared in racemic form the CF_2-phosphonate **4**, an analogue of *sn*-glycerol-3-phosphate **1** and demonstrated that it was a substrate for NADH linked glycerol-3-phosphate dehydrogenase[7a]. However our preliminary studies suggested that this CF_2-phosphonate analogue was a poorer substrate than *sn*-glycerol-3-phosphate **1** and also the corresponding CH_2-phosphonate analogue **2**. Interestingly two studies[2,8] have shown that the CH_2-phosphonate analogue is as good a substrate for the dehydrogenase as the natural substrate **1** itself. Clearly it is the addition of the fluorine atoms which is compromising the performance of the CF_2-phosphonate. It became appropriate therefore to evaluate the corresponding CHF-phosphonate analogue **3** as a substrate for the dehydrogenase to assess the intermediate situation with one fluorine atom on the phosphonate α-carbon. In order to draw a direct comparison between the CF_2-phosphonate **4** and the natural substrate **1** we considered it necessary to evaluate the substrate properties of the homochiral CF_2-phophonate rather than the previously prepared racemate of **4**, in order to eliminate the possibility that the unnatural enantiomer is an enzyme inhibitor, and thus contributing to the poorer performance of the racemate.



It is also noteworthy that there is no structural data available for the CHF-phosphonate moiety and therefore we felt it appropriate to address this deficiency. It is clearly of interest to compare the geometry of the CHF-phosphonate with the phosphate and the CH_2- and CF_2- phosphonate analogues. Since the crystallographic data of 2-aminoethanolphosphate **5** [9], 2-aminoethylphosphonic acid **6** [10] and 2-amino-1,1-difluoroethylphosphonic acid **8** [11] are available, we decided to prepare the corresponding monofluorinated phosphonate **7** such that a direct comparison with these structures could be made. In the present paper therefore, we describe full details[12] of our syntheses of the CHF-phosphonate **3** and CF_2-phosphonate **4** and evaluate them as substrates for glycerol-3-phosphate dehydrogenase. The synthesis of the amino CHF-phosphonate **7** is also described and its X-ray crystal structure reported.

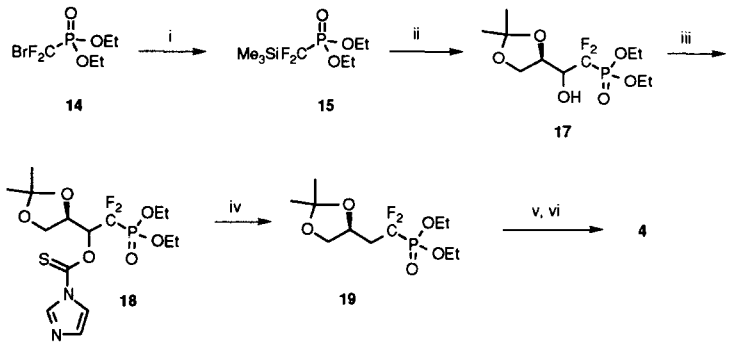
RESULTS AND DISCUSSION

Synthesis of the phosphonate analogues. Several synthetic approaches to α-monofluorophosphonates have been reported. The direct fluorination of phosphonate anions with electrophilic fluorinating reagents has emerged as a recent strategy[13] and the treatment of α-hydroxyphosphonates with reagents such as DAST has also been explored[3,15]. Alternatively, methodology using Michaelis-Becker[14] or Michaelis-Arbuzov[15] reactions, utilising chlorofluoromethane as a starting material has allowed access to a variety of CHF-phosphonate systems, although chlorofluoromethane is toxic and is no longer readily available. For our approach to (1*RS*, 3S)-3,4-dihydroxy-1-fluorobutylphosphonate **3** we exploited a recently reported method[16] for introducing the CHF-phosphonate by employing the α-lithiated-α-fluorotrimethylsilyl-methylphosphonate carbanion **10**. This reagent is readily accessible from dibromofluoromethylphosphonate **9** [17] by double halogen exchange with *n*-butyllithium in the presence of chlorotrimethylsilane (Scheme 1). The α-fluoro(trimethylsilyl)-butylphosphonate **12** was efficiently prepared by alkylation of **10** with (*R*)-2,2-dimethyl-4-methyl-1,3-dioxolane triflate **11**[18]. Although **12** can be isolated, a one pot synthesis proved more expedient, and lithium ethoxide was added directly to the reaction mixture followed by an aqueous workup and recovery of **13**. Treatment of **13** with bromotrimethylsilane and subsequent addition of water provided the desired monofluorophosphonic acid, as a 1:1 mixture of epimers at the CHF stereogenic centre. This compound was isolated after neutralisation as the biscyclohexylammonium salt of **3**.



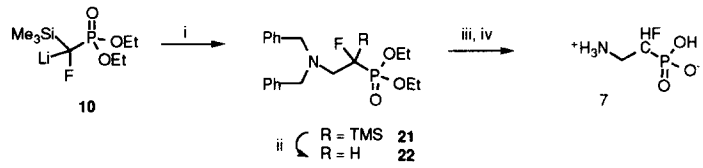
Scheme 1 i, *n*-BuLi (2.2 equiv), Me_3SiCl, THF, -78 °C, 10 min; ii, **11**, -78° C, 40 min; iii, LiOEt/EtOH, 0°C, 1 h then aq. NH_4Cl-ether, 83%; iv, Me_3SiBr, room temp., 3 h then H_2O, room temp., 18 h; v, $C_6H_{11}NH_2$, 63%.

Our route to the CF_2-phosphonate analogue **4** exploited previous methodology for the formation of 1,1-difluoro-2-hydroxyalkylphosphonates, by addition of difluoro(trimethylsilyl)-methylphosphonate **15** to carbonyl compounds under fluoride catalysis[19] (Scheme 2). The silylated phosphonate **15** was readily prepared by direct silylation of the bromodifluoromethylphosphonate **14**[16] using *n*-butyllithium and chlorotrimethylsilane[20]. Thus, reaction of **15** and (*S*)-2,3-O-isopropylideneglyceraldehyde **16**[21] in the presence of tetrabutylammonium fluoride afforded after hydrolysis, the 1,1-difluoro-2-hydroxybutylphosphonate **17** in moderate yield as a mixture of epimers at the newly formed carbinol stereocentre. In order to carry out a Barton deoxygenation[22], **17** was treated with thiocarbonylbismidazole in refluxing THF to give the thiomidazolide **18**.



Scheme 2 i, n-BuLi, Me_3SiCl, THF, -78° C, 20 min, 92%; ii, **16**, TBAF, THF, room temp., 24 h then sat. aq.$NaHCO_3$, 2h, 36%; iii, $Im_2C{=}S$, THF, reflux, 3 h, 81%; iv, Bu_3SnH, AIBN, toluene, reflux, 2 h, 66%; v, Me_3SiBr, room temp., 3 h, then H_2O, 15 h; vi, $C_6H_{11}NH_2$, 30%.

Reduction of **18** with tri-*n*-butyltin hydride in the presence of AIBN in refluxing toluene generated the desired CF_2-phosphonate **19** which was deprotected in the usual manner and isolated as its biscyclohexylammonium salt **4**.



Scheme 3 i, **20**, -78° C, 40 min; ii, LiOEt/EtOH, 0 °C, 1 h then aq. NH_4Cl-ether, 83%; iii, Me_3SiBr, room temp., 3 h then H_2O-$CHCl_3$; iv, $Pd(OH)_2/C$, H_2, room temp., 20h, 68%.

Our route to the amino CHF-phosphonate **7** utilised the facile addition of carbanion **10** to *N,N*-dibenzyl(methylene)imminium chloride **20**[23] to afford adduct **21**. Although **21** could be isolated it was more convenient to desilylate directly to generate **22** (Scheme 3). Hydrolysis of the phosphonate ester moiety of **22** with bromotrimethylsilane and water was followed by hydrogenolysis using palladium hydroxide on charcoal to give the amino CHF-phosphonate **7**. The zwitterionic nature of this compound rendered it crystalline as expected, and the sample was recrystallised from aqueous acetone to afford nice colourless plates which were suitable for X-ray single crystal structure determination.

X-ray crystal structure. Compound **7**, like its phosphonic acid analogues **6** and **8** adopts a *trans*-configuration around the C(1)-C(2) bond as shown in Figure 1. The C(2)-C(1)-P bond angle of 113.3° is close to C-CH_2-P angle in **6** of 112.1°. It is narrower than the corresponding C-O-P angle in **5** of 118.7° and the C-CF_2-P angle of 116.5° in **8** and in this respect deviates from the geometry of the parent phosphate group. Two different conformers (a and b) with respect to the P-C(1) bond, co-exist in the crystal.

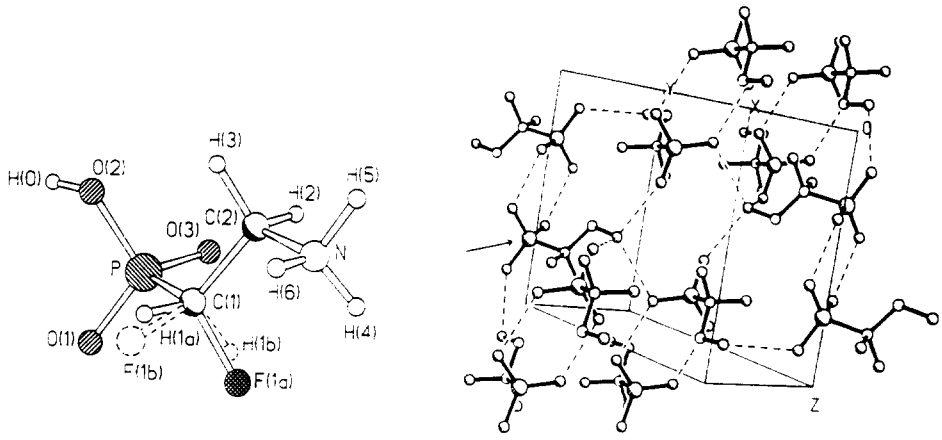


Figure 1 Perspective view of structure **7**, showing the disorder of F(1a/b) and H(1a/b) atoms and a packing plot (hydrogens omitted) highlighting the intermolecular hydrogen bonding contacts (dotted lines). The arrow is pointing to a phosphorous atom.

Selected bond lengths (Å) and angles (°). P-O(1) 1.508 (2); P-O(2) 1.555 (2); P-O(3) 1.503 (2); P-C(1) 1.820 (2); F(1a)-C(1) 1.380 (3); F(1b)-C(1) 1.310 (5); N-C(2) 1.492 (3); C(1)-C(2) 1.500 (3); C(2)-C(1)-P 113.28 (14); F(1a)-C(1)-C(2) 110.3 (2); F(1b)-C(1)-C(2) 117.6 (3); N-C(2)-C(1) 111.9 (2); P-C(1)-C(2)-N -175.1(1); O(1)-P-C(1)-C(2) -179.2(1); O(2)-P-C(1)-F(1A) 175.2(2).

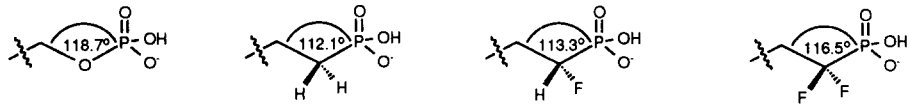
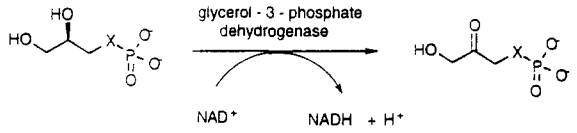
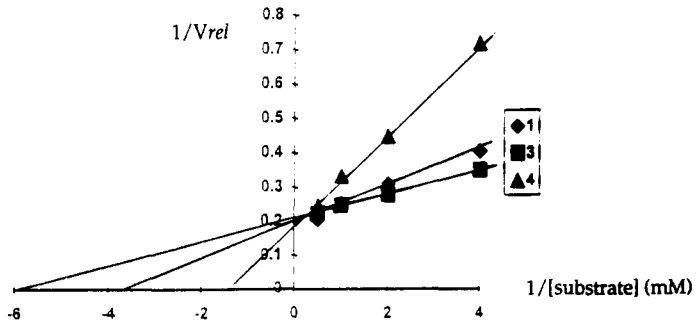


Figure 2 The C-X-P angles of the phosphate and phosphonates as determined from X-ray structure data.

They occupy the same crystallographic site so that the F(1a) and H(1a) atoms essentially change places with the H(1b) and F(1b) respectively, due to lack of significant steric discrimination between the fluorine and hydrogen atoms in the crystal packing. In each confomer the C-F bond is antiperiplanar to one of the P-O bonds. A similar conformation was observed in the structure of **8**[11] and can be explained by donation of electron density from the electron rich oxygen atom into the σ* antibonding orbital of the C-F bond. A three dimensional net-work of hydrogen bonds exists in the crystal of **7** between the phosphate oxygen atoms and amino hydrogens (see packing plot) and like the structure of **8**[11] it is noteworthy that there are no hydrogen bonds which involve the fluorine atom as an acceptor.

Assessment of the fluoromethylenephosphonates 3 and 4 as substrates for G-3-P dehydrogenase. With the CHF- and CF_2- phosphonates **3** and **4** in hand they were evaluated as substrates for glycerol-3-phosphate dehydrogenase (Sigma Type I from rabbit muscle). The results are summarised in Figure 3.





Substrate	Km	V*rel*
1 X = O	0.2 mM[2]	1.0
2 X = CH_2	0.18 mM[2,8]	0.8
3 X = CHF	0.17 mM	0.9
4 X = CF_2	0.73 mM	0.8

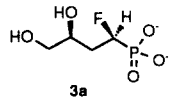
Figure 3

Both of the fluorinated phosphonates **3** and **4** emerged as good substrates showing classic Michaelis-Menten behaviour and the secondary plots of 1/V*rel* (at different NAD^+ concs.) versus 1/[substrate] are shown in Figure 3. The Michaelis-constants (K_m) revealed however that the CHF-phosphonate **3** is a significantly better substrate for the dehydrogenase than the CF_2-phosphonate **4** (K_m **3** = 0.17 mM, K_m **4** = 0.73 mM). In fact **3** shows the same K_m value as the CH_2-phosphonate **2** and both **2** and **3** have a *lower* K_m values than glycerol-3-phosphate itself[2]. We anticipated that the CHF-phosphonate substrate **3** would have an intermediate Km value between that of the CH_2- and CF_2- phosphonates **2** and **4**. In the event this was not the case. Since **3** was submitted to the enzyme assay as a mixture of diastereoisomers (**3a and 3b**), epimeric at the CHF stereogenic centre, the experimental Km value necessarily represents an average of the values for the two diastereoisomers. These values may of course be very similar. All of the substrates were turned over at similiar maximal rates (V*rel*) relative to **1** at substrate saturation levels, however the CF_2-phosphonate emerged uniquely, as a poorer substrate by Km. The enzyme can presumably feel the influence of the second fluorine atom, perhaps due to an adverse steric or more likely an adverse electrostatic interaction with the surface of the protein. It is anticipated for example that the magnitude of the negative electrostatic potential of a CF_2-P group will be significantly larger than that of CHF-P or O-P[6]. Adverse electrostatic interactions will depend on a lack of complementarity with the enzyme surface, and will vary from one enzyme to

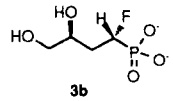
another, thus the CF_2-phosphonate may, and indeed does[24,25], emerge as a good phosphate mimic in other enzyme systems. Presumably in this particular system however the magnitude of the electrostatic potential is detrimental.

An alternative hypothesis for the poorer performance of the CF_2-phosphonate is that it is a less good phosphate mimic on ionisation and/or geometric grounds. With a pKa for the second deprotonation of 5.6 [7b], more acidic than a phosphate group, it can be assumed that the CF_2-phosphonate will be processed by the enzyme in its diionic form, the form generally considered to be important for phosphate binding to an enzyme. However the CH_2-phosphonate **2**, the least acidic of the series (pKa = 7.6) emerged as an excellent substrate, so acidity does not appear to underly the poorer performance of the CF_2-phosphonate. The X-ray structure data gives some geometric insights. From the combined values of the C-CX_2-P angles of the CH_2-, CHF- and CF_2- phosphonates, it is the CF_2-phosphonate angle of 116.5°, which most closely resembles that of the phosphate C-O-P angle of 118.7°. The angle closes to ~112° and ~113° for CH_2- and CHF-phosphonates respectively. So on geometric grounds the CF_2-phosphonate would appear again to be a good phosphate mimic. Thus the performance of the phosphonate analogues (CH_2- ~ CHF- > CF_2-) cannot easily be attributed to ionisation or geometry and is most likely due to an adverse electrostatic interaction associated with the CF_2 group in this enzyme system.

*A comparison of the rates of enzymatic oxidation of diastereoisomers **3a** and **3b**.* It became pertinent to investigate if the enzyme could discriminate kinetically between the two diastereoisomers of **3** (**3a** and **3b**).



3a



3b

The 19-fluorine signals for **3a** and **3b** are resolved in the ^{19}F-NMR spectrum of the mixture of diastereoisomers, although we are unable to assign the signals unambigiously to each diastereoisomer. The signals, which are complex multiplets, can be simplified and enhanced in intensity, by applying proton-fluorine decoupling to give two sets of doublets (J_{PF} =63 Hz) with excellent signal to noise sensitivity. Accordingly an enzyme incubation was carried out in a NMR tube in order to monitor the reaction directly by $^{19}F\{^1H\}$ NMR spectroscopy. Analysis of the change in ratio of the integrals of the signals associated with each diastereoisomer, over time, revealed that one diastereoisomer (arbitrarily assigned **3a**) decreased relative to the other by 20% within 65 min. The data are shown below in Table 1.

t (min)	0	3	10	21	40	65
decrease of **3a**	100%	98.2%	92.7%	87.8%	81.7%	79.6%

Table 1 A solution of **3a** and **3b** (2.7 mg, 7 mmol) in water (0.33ml) was introduced into an NMR tube and then sequentially a 12.7 mM solution of NAD (0.33 ml, 4 mmol) in water and 0.1 M glycine/hydrazine buffer (0.33 ml) was added. The first $^{19}F\{^1H\}$ spectrum was recorded without addition of the enzyme in order to determine at zero time, the ratio of integrals (1 : 0.97) for the signals at -201.8 ppm and -205.7 ppm which correspond in an arbirary assignment to diastereoisomers **3a** and **3b** respectively. After addition of 80 ml of a glycerol-3-phosphate dehydrogenase solution (1Unit) spectra were recorded at various time intervals within 65 min and the integral ratio was determined.

We were unable to accurately assess the level of conversion as the oxidised product of the reaction, which should be a hydrazone under the assay conditions (hydrazine, pH 9.5), did not accumulate and was not apparent by ^{19}F-NMR. It is possible that the product is decomposed under the basic conditions. However if it is assumed that all of the NAD^+ is consumed then the level of conversion would reach a maximum of 60%. On this basis the enzyme processed 1.47 molecules of **3a** for every molecule of **3b** over the 65 minute reaction. Clearly if the level of conversion is less than 60% then the selectivity is higher, so this is a minimum estimate. The experiment clearly demonstrates that the dehydrogenase can distinguish the diastereoisomers to a significant extent. The origin of this distinction does not appear to be due to binding, as the average Km for **3a** and **3b** is lower than that of the natural substrate (0.17 mM versus 2.0mM) and forces the conclusion that a kinetic preference underlies this discrimination. The origin of this kinetic preference is not clear. We are currently investigating this reaction without hydrazine and under neutral conditions using co-factor recycling protocols. This should allow us to observe product and monitor the consumption of the diastereomers with conversion, through a complete reaction course.

Acknowledgements. We thank the EC (HCM Programme) *European Network on the Synthesis and Molecular Recognition of Fluorinated Molecules* for a grant (JN). We also thank Mr Ian H. McKeag, University of Durham, for recording ^{19}F{^{1}H}-NMR spectra.

EXPERIMENTAL

General:.IR spectra were recorded on a Perkin-Elmer 257 Spectrometer and mass spectra were recorded on a VG-7070E instrument. NMR spectra were obtained on Bruker AC-250 and Varian XL200 instruments in $CDCl_3$ or D_2O. Chemical shifts are quoted relative to TMS for ^{1}H- and ^{13}C- NMR spectra, ^{19}F chemical shifts are quoted as negative values relative to fluorotrichloromethane and ^{31}P chemical shifts are quoted relative to phosphoric acid. Solvents were dried and distilled prior to use. Reactions requiring anhydrous conditions were conducted under an atmosphere of nitrogen and column chromatography was carried out over silica gel (Merck, Kieselgel 60, 230 - 400 mesh).

Diethyl (1RS, 3S)-1-fluoro-3(S),4-dihydroxy-3,4-O-isopropylidenebutylphosphonate (**13**). 1.6 M *n*-Butyllithium in hexane (6.6 ml, 10.6 mmol) was added to a stirred solution of ethyl dibromofluoromethylphosphonate **9** (1.57 g, 4.8 mmol) and chlorotrimethylsilane (0.52 g, 4.8 mmol) in tetrahydrofuran (25 ml) at -78° C. After 10 min a solution of (*R*)-2,2-dimethyl-4-methyl-1,3-dioxolane triflate **11**[18] (1.27 g, 4.8 mmol in 5 ml THF) was added dropwise to the reaction mixture and the temperature (-78°C) was maintained for an additional 40 min. Subsequently a solution of lithium ethoxide (prepared by addition of 6.6 ml 1.6 M butyllithium to 2 ml ethanol (2 ml) and THF (10 ml)) was added and the mixture stirred for 1h at 0°C. Saturated aqueous NH_4Cl was added and the product was extracted into diethyl ether. The combined organic extracts were washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$ and the solvent evaporated to give the crude product. Purification by column chromatography (ethanol/petrol ether 1:7) afforded **13** as a 1:1 mixture of diastereoisomers (1.12 g, 83%). ^{1}H NMR ($CDCl_3$): 1.29 - 1.38 (12H, m, $(CH_3)_2C$, $(CH_3CH_2O)_2P$), 1.88 - 2.29 (2H, m, CH_2CHF), 3.54 - 3.65 (1H, m, CH_aH_bO), 4.02 - 4.36 (6H, m, $(CH_3CH_2O)_2P$, CH_aH_bO, OCH_2CH), 4.71 - 5.05 (1H, m, CHF). ^{13}C NMR ($CDCl_3$): 16.4 (d, $J_{C\text{-}P}$ = 6.9 Hz, CH_3CH_2O), 25.5, 26.7, 26.9, (s, $(CH_3)_2C$), 33.8, 34.6 (d, $J_{C\text{-}F}$ = 19.5 Hz, CH_2CHF), 62.7, 63.1 (d, $J_{C\text{-}P}$ = 6.5 Hz, CH_3CH_2O), 63.2 (m, CH_3CH_2O), 68.7 (d, J.= 1.9 Hz, OCH_2CH), 69.3 (s, OCH_2CH), 71.6 (dd, $J_{C\text{-}P}$ = 15.2 Hz, $J_{C\text{-}F}$ = 1.8 Hz, OCH_2CH), 72.3 (dd, $J_{C\text{-}P}$ = 11.8 Hz, $J_{C\text{-}F}$ = 3.2 Hz, OCH_2CH), 85.9 (dd, J = 178.3 Hz, J = 171.8 Hz, CHF), 86.1 (dd, J = 178.7 Hz, J = 170.5 Hz, CHF), 109.1 (s, $(CH_3)_2C$). ^{19}F NMR ($CDCl_3$): -207.7 (1F, dddd, $J_{F\text{-}P}$ = 75.7 Hz, $J_{F\text{-}H}$ = 46.8 Hz, $J_{F\text{-}H}$ = 31.7 Hz, $J_{F\text{-}H}$= 20.7 Hz), -212.5 (1F, m). ^{31}P NMR ($CDCl_3$): 17.5 (1F, d, $J_{P\text{-}F}$ = 74.0 Hz), 17.8 (1F, d $J_{P\text{-}F}$ = 75.3 Hz). IR (neat): 2986, 2935 (CH), 1372, 1260 (P=O), 1160, 1054, 1025, 973. $[\alpha]^{20}_D$ = -3.0° (CH_2Cl_2, c = 3.4). (Anal. calcd. for $C_{11}H_{17}FO_5P$: C, 46.48; H, 7.80. Found: C, 46.56; H, 8.10%).

Biscyclohexylammonium 3,4-dihydroxy-1-fluorobutylphosphonate (**3**). Bromotrimethylsilane (0.35 g, 2.3 mmol) was added to **13** (100 mg, 0.35 mmol) and the reaction mixture stirred for 3 h at room temperature. Volatiles were removed under reduced pressure and water (3 ml) was added to give a turbid solution which was stirred for an additional 18 h. The acidic solution became clear and was neutralised by dropwise addition of cyclohexylamine. Removal of the solvent under reduced pressure gave a cream coloured solid which was recrystallised from methanol/acetone to afford the cyclohexylammonium salt **3** (86 mg, 63%) as a white amorphous solid. ^{1}H NMR (D_2O): 1.03-1.26 (8H, m, $C_6H_{11}N$), 1.40-1.86 (14H, m, $C_6H_{11}N$, CH_2CHF), 2.84-3.00 (2H, m, $C_6H_{11}N$), 3.25-3.50 (2H, m, CH_2OH), 3.62-3.81 (1H, m, $CHOH$), 4.26-4.41, 4.44-4.61 (1H, dm, $J_{H\text{-}F}$ = 47.5 Hz, CHF). ^{13}C NMR ($CDCl_3$): 26.7 (s, C-3', C-5'), 27.2 (s, C-4'), 33.2 (s, C-2', C-6'), 37.4 (d, $J_{C\text{-}F}$ = 19.5 Hz, CH_2CHF), 53.1 (s, C-1'), 67.7, 68.6 (s, CH_2OH), 71.2 (dd, $J_{C\text{-}P}$ = 11.2 Hz, $J_{C\text{-}F}$ = 3.2 Hz, CHOH), 72.8 (dd, $J_{C\text{-}P}$ = 11.2 Hz, $J_{C\text{-}F}$ = 2.5 Hz, CHOH), 92.5 (dd, J = 169.7 Hz, J = 154.0 Hz, CHF), 93.9 (dd, J = 171.0 Hz, J = 153.0 Hz, CHF). ^{19}F NMR (D_2O): -201.58 (dddd, $J_{F\text{-}P}$ = 63 Hz, $J_{F\text{-}H}$ = 47.5 Hz, $J_{F\text{-}H}$ = 35.7 Hz, $J_{F\text{-}H}$ = 23 Hz), -204.48 (dddd, $J_{F\text{-}H}$ = 63 Hz, $J_{F\text{-}H}$ = 47.9 Hz, $J_{F\text{-}H}$ = 42.3 Hz, $J_{F\text{-}H}$ = 14.8 Hz). ^{31}P NMR (D_2O): 12.09, 12.41 (d, $J_{P\text{-}F}$ = 63 Hz). IR (KBr): 3417 (NH), 2936, 2565, 2215, 1634, 1564, 1076 (P=O), 1051 (P=O). M.p. = 172 - 174° C. $[\alpha]^{20}_D$ = -6.7° (CH_3OH, c = 0.9). Negative FAB : 187 (80%) M-2 $C_6H_{11}NH_2$-H$^+$. Positive FAB : 288 (7%) M-$C_6H_{11}NH_2$+H$^+$. (Anal. calcd. for $C_{16}H_{36}FN_2O_5P$: C, 49.73; H, 9.39; N, 7.25. Found: C, 49.52; H, 9.46; N, 7.13%).

Diethyl difluoro(trimethylsilyl)-methylphosphonate (**15**). 2.5 M *n*-Butyllithium in hexane (6.5 ml, 16.3 mmol) was added to a solution of diethyl bromodifluoromethylphosphonate **14** (3 g, 11.2 mmol) and chlorotrimethylsilane (1.7 g, 15.8 mmol) in THF (60 ml) at -78° C and the mixture was stirred for 20 min at the same temperature. The reaction was allowed to warm to 0° C and then saturated aqueous NH_4Cl was added. The product was extracted into diethyl ether, dried over $MgSO_4$ and the solvent removed under reduced pressure to give **15** (2.7 g, 92%) as a clear oil. Since the crude material was essentially pure as judged by ^{1}H NMR, **15** was used without further purification for the following reaction. ^{1}H NMR ($CDCl_3$): 0.26 (9H, s, $(CH_3)_3Si$), 1.36 (6H, t, $J_{H\text{-}H}$ = 7 Hz, $(CH_3CH_2O)_2P$), 4.25 (4H, m, $(CH_3CH_2O)_2P$). ^{19}F NMR ($CDCl_3$): -131.2 (d, $J_{F\text{-}P}$ = 92.2 Hz).

Diethyl (2R, 3S)- and (2S, 3S)-3,4-dihydroxy-1,1-difluoro-2-hydroxy-3,4-O-isopropylidenebutylphosphonate (**17**). Compound **15** (2.94 g, 11.3 mmol) and 3(S),4-isopropylideneglyceraldehyde **16** (1.77 g, 13.6 mmol) were dissolved in THF (50 ml) and stirred over 3Å molecular sieves. This mixture was cooled to 0° C and a solution of tetra-n-butylammonium fluoride (0.6 ml, 0.6 mmol) was added. After stirring at room temperature for 24 h the molecular sieves were filtered off and saturated aqueous $NaHCO_3$ was added to the THF-solution. The reaction was stirred for 2 h at room temperature, extracted into diethyl ether, dried over $MgSO_4$ and the solvent evaporated under reduced pressure. Purification by column chromatography (ethyl acetate/petrol ether 1:1) afforded a diastereomeric mixture (1 : 1) of **17** (1.3 g, 36%) as a clear oil. ^{1}H NMR ($CDCl_3$): 1.27 - 1.40 (12H, m, $(CH_3)_2C$,$(CH_3CH_2O)_2P$), 3.75 - 4.55 (9H, m, OCH_2CH, $(CH_3CH_2O)_2P$, $CHOH$, OCH_2CH, OH). ^{13}C NMR ($CDCl_3$): 17.6 (d, $J_{C\text{-}P}$ = 5.5 Hz, CH_3CH_2O), 26.8, 27.6 (s, $(CH_3)_2C$), 66.1 (m, CH_3CH_2O), 67.7, 67.8 (s, OCH_2CH), 71.5 - 73.1 (m, $CHOH$), 74.1, 74.9 (m, OCH_2CH), 110.3, 111.1 (s, $(CH_3)_2C$), 119.8, 120.5 (m, CF_2). ^{19}F NMR ($CDCl_3$): Diastereoisomer 1: -116.62 (2F, m). Diastereoisomer 2 : -124.86 (1F,ddd, $J_{F\text{-}F}$ = 309.6 Hz, $J_{F\text{-}P}$ = 99.8 Hz, $J_{F\text{-}H}$ = 19.4), -125.05 (1F, ddd, $J_{F\text{-}F}$ = 309.6 Hz, $J_{F\text{-}P}$ = 102.6 Hz, $J_{F\text{-}H}$ = 22.8). ^{31}P NMR ($CDCl_3$): Diastereoisomer 1: 6.01 (t, $J_{P\text{-}F}$ = 99.6 Hz). Diastereoisomer 2 : 6.19 (dd, $J_{P\text{-}F}$ = 102.6 Hz, $J_{P\text{-}F}$ = 97.5 Hz). IR (neat) 3374 (OH), 2986, 2964, 2917, 1372, 1261 (P=O), 1025, 800. (Anal. calcd. for $C_{11}H_{21}F_2O_6P$: C, 41.51; H, 6.65; Found: C, 41.95; H, 6.82%).

Diethyl (2R, 3S)- and (2S, 3S)-3,4-dihydroxy-2-(N-1H-imidazolylthiocarbonyl)-oxy-3,4-O-isopropylidenebutylphosphonate (**18**). Thiocarbonylbisimidazole (1.91 g, 10.7 mmol) was added to a solution of **17** (1.7 g, 5.3 mmol) in THF (65 ml) and was heated under reflux for 2 h. After concentration under reduced pressure the residue was diluted with dichloromethane (40ml), washed with water and saturated aqueous $NaHCO_3$ and then dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (ethyl acetate/petrol ether 1:1) to afford **18** (1.85 g, 81%) as a colourless oil. ^{1}H NMR ($CDCl_3$): 1.23- 1.40 (12H, m, $(CH_3CH_2O)_2P$, $(CH_3)_2C$), 3.98 - 4.36 (6H, m, $(CH_3CH_2O)_2P$, OCH_2CH), 4.69 - 4.84 (1H, m, OCH_2CH), 6.15 - 6.33 (1H, m, $OCHCF_2$), 6.51 (1H, t.t, $J_{F\text{-}H}$ = 14.4 Hz, $J_{H\text{-}H,H\text{-}P}$ = 2.8 Hz, $OCHCF_2$), 7.06 (1H, br, H-Im), 7.70 (1H, br, H-Im), 8.38 (1H, br, H-Im). ^{13}C NMR ($CDCl_3$): 16.1 (d, $J_{C\text{-}P}$ = 5.5 Hz, CH_3CH_2O), 24.6, 25.0, 25.7, 25.9 (s, $(CH_3)_2C$), 64.4, 65.0 - 65.7 (m, CH_3CH_2O, OCH_2CH), 72.1 (q, $J_{C\text{-}F,C\text{-}P}$ = 2.9 Hz, OCH_2CH), 72.8 (q, $J_{C\text{-}F,C\text{-}P}$ = 2.5 Hz, OCH_2CH), 76.9 - 79.1 (m, $OCHCF_2$), 109.4, 110.0 (s, $(CH_3)_2C$), 116.4 (dt, $J_{C\text{-}F}$ = 267 Hz, $J_{C\text{-}P}$ = 211 Hz, CF_2), 118.0, 118.3 (s.C-Im), 130.8, 131.0 (s, C-Im), 137.1 (s, C-Im), 182.9, 183.0 (s, C=S). ^{19}F NMR ($CDCl_3$): -115.77 (1F, ddd, $J_{F\text{-}F}$ = 319.9 Hz, $J_{F\text{-}P}$ = 98.6 Hz, $J_{F\text{-}H}$ = 9.0 Hz), -119.18 (1F, ddd, $J_{F\text{-}F}$ = 319.9 Hz, $J_{F\text{-}P}$ = 100.8 Hz, $J_{F\text{-}H}$ = 15.0 Hz). ^{31}P NMR ($CDCl_3$): 3.67 (t, $J_{P\text{-}F}$ = 98.3 Hz), 3.86 (t, $J_{P\text{-}F}$ = 98.7 Hz,

$J_{P\text{-}F}$ = 100.6 Hz). IR (neat): 2987, 2936, 1468, 1395, 1321, 1287 (P=O), 1225, 1182, 1021 (C-O-P), 828, 746. (Anal. calcd. for $C_{15}H_{23}F_2N_2O_6PS$: C, 42.06; H, 5.41; N, 6.54; Found: C, 41.88; H, 5.96; N, 5.88%).

Diethyl (S)-difluoro-3,4-dihydroxy-3,4-O-isopropylidenebutylphosphonate (**19**). Tri-*n*-butyltin hydride (1 g, 3.44 mmol) and azobisisobutyronitrile (60 mg, 0.37 mmol) were added to a solution of **18** (1.6 g, 3.73 mmol) in toluene (20 ml) and the mixture heated under reflux for 2 h. The solvent was removed under reduced pressure and the crude material purified by column chromatography (ethyl acetate/petrol ether 1:2) to give **19** (0.74 g, 66%) as a clear oil. ^{1}H NMR ($CDCl_3$): 1.35 (3H, s, $(CH_3)_2C$), 1.37 (6H, t, $J_{H\text{-}H}$ = 7.0 Hz, $(CH_3CH_2O)_2P$), 1.39 (3H, s, $(CH_3)_2C$), 2.13 - 2.66 (2H, m, CH_2CF_2), 3.62 (1H, dd, $J_{H\text{-}H}$ = 8.2 Hz, $J_{H\text{-}H}$ = 7.2 Hz, OCH_aH_bCH), 4.14 (1H, dd, $J_{H\text{-}H}$ = 8.2 Hz, $J_{H\text{-}H}$ = 6.1 Hz, CH_aH_bCH), 4.26 (4H, m, $(CH_3CH_2O)_2P$), 4.45 (1H, m, CH). ^{13}C NMR ($CDCl_3$): 16.3 (d, $J_{C\text{-}P}$ = 5.4 Hz, CH_3CH_2O), 25.6, 26.7 (s, $(CH_3)_2C$), 38.04 (dt, $J_{C\text{-}F}$ = 20.0 Hz, $J_{C\text{-}P}$ = 14.1 Hz, CH_2CF_2), 64.55, 64.62 (d, $J_{C\text{-}P}$ = 6.7 Hz, CH_3CH_2O), 69.7 (s, OCH_2CH, OCH_2CH),108.8 (s, $(CH_3)_2C$), 119.5 (dt, $J_{C\text{-}F}$ = 260.1 Hz, $J_{C\text{-}P}$ = 216.6 Hz, CF_2). ^{19}F NMR ($CDCl_3$): -110.75 (1F, dddd, $J_{F\text{-}F}$ = 300.3 Hz, $J_{F\text{-}P}$ = 106.1 Hz, $J_{F\text{-}H}$ = 20.9 Hz, $J_{F\text{-}H}$ = 14.1 Hz), -112.26 (1F, dddd, $J_{F\text{-}F}$ 300.3 Hz, $J_{F\text{-}P}$ = 105.7 Hz, $J_{F\text{-}H}$ = 24.7 Hz, $J_{F\text{-}H}$ = 16.9 Hz). ^{31}P NMR ($CDCl_3$): 6.51 (t, $J_{P\text{-}F}$ = 105.8). IR (neat): 2987, 2917, 1371, 1272 (P=O), 1162, 1022. (Anal. calcd. for $C_{11}H_{21}F_2N_2O_5P$: C, 43.71; H, 7.00: Found: C, 43.42; H, 7.13%).

Biscyclohexylammonium (S)-difluoro-3,4-dihydroxybutylphosphonate (**4**). Bromotrimethylsilane (1.68 g, 11 mmol) was added to **19** (500 mg, 1.65 mmol) and the reaction mixture was allowed to stir for 3 h at room temperature. Volatiles were removed under reduced pressure and water (15 ml) was added to generate a turbid solution which was stirred for an additional 18 h. The acidic solution became clear and was neutralised by dropwise addition of cyclohexylamine. Removal of the solvent under reduced pressure gave a cream coloured solid which was recrystallised from methanol/acetone to afford a colourless powder of cyclohexylammonium salt **4** (200 mg, 30%) as an amorphous white solid. ^{1}H NMR (D_2O): 1.03-1.25 (8H, m, $C_6H_{11}N$), 1.40-1.83 (12H, m, $C_6H_{11}N$), 1.89-2.07 (2H, m, CH_2CF_2), 2.84-3.00 (2H, m, $C_6H_{11}N$), 3.33 (1H, dd, $J_{H\text{-}H}$ = 11.7 Hz, $J_{H\text{-}H}$ = 6.4 Hz, $CHaHbOH$), 3.42 (1H, dd, $J_{H\text{-}H}$ = 11.7 Hz, $J_{H\text{-}H}$ = 4.3 Hz, $CHaHbOH$), 3.87-3.94 (1H, m, CHOH). ^{13}C NMR (D_2O): 26.7 (s, C-3', C-5'), 27.1 (s, C-4'), 33.2 (s, C-2', C-6'), 40.8 (dt, $J_{C\text{-}F}$ = 21.1 Hz, $J_{C\text{-}P}$ = 12.9 Hz, CH_2CF_2), 53.1 (s, C-1'), 68.3 (s, CH_2OH), 69.4 (s, CHOH), 126.4 (dt, $J_{C\text{-}F}$ = 259.7 Hz, $J_{C\text{-}P}$ = 183.7 Hz, CF_2). ^{19}F NMR (D_2O): -108.51 (ddt, $J_{F\text{-}F}$ = 282, Hz $J_{F\text{-}P}$ = 85.2 Hz, $J_{F\text{-}H}$ = 21.4 Hz), -111.50 (ddt, $J_{F\text{-}F}$ = 282 Hz, $J_{F\text{-}P}$ = 85.2 Hz, $J_{F\text{-}H}$ = 21.4 Hz). ^{31}P NMR (D_2O): 5.55 (t, $J_{P\text{-}F}$ = 85.9 Hz). IR (KBr): 3420 (NH), 2933, 2861, 2211, 1627, 1559, 1127 (P=O), 1078 (P=O). M.p. = 227° C. $[a]^{20}_D$ = -7.1° (CH_3OH, c = 1.1). (Anal. calcd. for $C_{16}H_{35}F_2N_2O_5P$: C, 47.52; H, 8.72; N, 6.93: Found: C, 47.09; H, 8.81; N, 6.61%).

Diethyl 2-(N,N-dibenzylamino)-1-fluoroethylphosphonate (**22**). Acetyl chloride (310 mg, 4 mmol) was to a stirred solution of *N,N,N',N'*-tetrabenzylmethylenediamine (1.65 g, 4 mmol) in diethyl ether under an atmosphere of nitrogen. *N,N*-Dibenzyl(methylene)imminium chloride **20** began to precipitate after 10 min and the stirring was stopped in order not to crush the crystals. The precipitate was left to stand for 1 h and then the supernatant was removed by pipette. The remaining solid was mixed with diethyl ether and the procedure was repeated taking care to exclude moisture as **20** is readily hydrolysed. To a stirred solution of ethyl dibromofluoromethylphosphonate (400 mg, 1.22 mmol) and chlorotrimethylsilane (132 mg, 2.71 mmol) in tetrahydrofuran (6 ml) was added 1.6 M *n*-butyllithium in hexane (1.7 ml, mmol) at -78° C. The imminium salt **20** was suspended in THF (2 ml) and added to the phosphonate solution as a slurry. After 30 min stirring at the same temperature a solution of lithium ethoxide (prepared by addition of 1.7 ml 1.6 M butyllithium to 1 ml ethanol and 3 ml THF) was added and the mixture stirred for 1 h at 0° C. Saturated NH_4Cl solution (10ml) was added and the product extracted into diethyl ether. The combined organic extracts were washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and the solvent evaporated. The product was purified by column chromatography (ethyl acetate/petrol ether 1:2) and **22** (316 mg, 68%) was recovered as a colourless oil. ^{1}H NMR ($CDCl_3$): 1.25 (6H, t, $J_{H\text{-}H}$ = 7.1 Hz, $(CH_3CH_2O)_2P$), 2.73 - 3.15 (2H, m, CH_2CHF), 3.72 (4H, s, $(PhCH_2)_2N$), 4.09 (4H, q, $J_{H\text{-}H}$ = 7.1 Hz, $(CH_3CH_2O)_2P$), 4.98 (1H, dm, $J_{H\text{-}F}$ = 48.0 Hz, CHF), 7.23 - 7.41 (10H, m, $(C_6H_5CH_2)_2N$). ^{13}C NMR ($CDCl_3$): 16.3 (s, CH_3CH_2O), 52.7 (dd $J_{C\text{-}F}$ = 19.1 Hz, $J_{C\text{-}P}$ = 6.4, CH_2CHF), 58.3 (s, CH_2Ph), 62.7, 63.1 (d, $J_{C\text{-}P}$ = 6.7 Hz, CH_3CH_2O), 88.1 (dd, J = 182.2 Hz, J = 164.9 Hz, CHF), 127.0 (s, C-4'), 128.2, 128.8 (s, C-2', C-3', C-5', C-6'), 138.8 (s, C-1'). ^{19}F NMR ($CDCl_3$): -208.4 (dddd, $J_{F\text{-}P}$ = 76.7 Hz, $J_{F\text{-}H}$ = 48.0 Hz, $J_{F\text{-}H}$ = 36.2, $J_{F\text{-}H}$ = 23.3 Hz). ^{31}P NMR ($CDCl_3$): 17.0 (d, $J_{P\text{-}F}$ = 75.8 Hz). IR (neat): 3061, 3027 (Ph), 2981, 2929 (CH), 1494, 1452, 1368, 1260 (P=O), 1027 (P-O-C), 973, 747, 699. (Anal. calcd. for $C_{20}H_{27}FNO_3P$: C, 63.31; H, 7.17; N, 3.69: Found: C, 63.95; H, 7.24; N, 3.63%).

2-Amino-1-fluoroethylphosphonic acid (**7**). Bromotrimethylsilane (0.7 g, 4.6 mmol) was added to **22** (295 mg, 0.78 mmol) and the reaction mixture was allowed to stir for 3 h at room temperature. Volatiles were removed under reduced pressure and then water (10ml) and chloroform (10ml) were added and the mixture stirred until all the material was dissolved. After the solvents were removed under reduced pressure the residue was dissolved in ethyl acetate and evaporated to dryness. Addition of a small amount of chloroform

to the oily residue and a quick evaporation under reduced pressure afforded 2-(*N,N*-dibenzylamino)-1-fluoroethylphosphonic acid. This crude material was dissolved in ethanol (5ml) and 20% palladium hydroxide on charcoal (100 mg) was added. The reaction mixture was then stirred under an atmosphere of hydrogen at ambient pressure for 20 h. The catalyst was filtered off and evaporation of the solvent gave **7** as a solid which was recrystallised from water/acetone to afford colourless crystals (76 mg, 68%). ^{1}H NMR (D_2O): 3.05-3.48 (2H, m, CH_2CHF), 4.52-4.65 + 4.75-4.90 (1H, dm, $^{2}J_{HF}$ = 47.9 Hz, CH_2CHF). ^{13}C NMR (D_2O): 43.1 (dd, $J_{C\text{-}F}$ = 19.5 Hz, $J_{C\text{-}P}$ = 7.1 Hz, CH_2CHF), 89.9 (dd, J = 177.3 Hz, J = 156.4 Hz, CH_2CHF). ^{19}F NMR (D_2O): -211.07 (dddd, J_{FP} = 63.1 Hz, J_{FH} = 47.9 Hz, J_{FH} = 33.3 Hz, J_{FP} = 18.2 Hz). ^{31}P NMR (D_2O): 8.68 (d, J_{PF} = 63.1 Hz). IR (KBr): 3040 - 2571 (NH_3^+), 2282, 1646, 1543, 1136, 976, 568, 511, 449. M.p. = 283 -284° C. (Anal. calcd. for $C_2H_7FNO_3P$: C, 16.79; H, 4.93; N, 9.79: Found: C, 16.79; H, 5.01; N, 9.44%).

Enzyme assay: β-Nicotinamide adenine dinucleotide (98%, Sigma Grade II from yeast), L-glycerol-3-phosphate di(monocyclohexylammonium) salt (synthetic, 95%) and *sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase (EC 1.1.1.8, type I from rabbit muscle) were purchased from the Sigma Chemical Co. Ltd. The rabbit muscle glycerol-3-phosphate dehydrogenase was diluted (1:50) with 0.1 M phosphate buffer (pH 6.5) and stored at 0° C. Each assay was carried out in a 3 ml cuvette containing 1ml 0.1 M glycine/hydrazine buffer (pH 9.5), 1 ml aqueous NAD^+ solution, 1ml aqueous substrate solution and 50 ml of the diluted enzyme (0.5 U) at 26° C. Initial rates of the enzymatic reactions were determined by monitoring the formation of NADH from NAD^+. The increase in the extinction at 340 nm was recorded using a Pye Unicam SP 8 - 100 ultraviolet spectrometer and the reaction rates were evaluated during the first 40 s of the reaction. The initial rates during this time period were linear in all cases and the Km and relative V_{max} values were determined by a graphical method.[26]

X-Ray structure determination Crystal data for **7**: $C_2H_7FNO_3P$, M = 143.06, orthorhombic, space group *Pbca*(No. 63), at 150 K a = 7.533 (4), b = 12.052 (3), c = 12.263 (6) Å, U = 1113.3 (8) Å^3 (from 25 reflections with 12.5 < q < 15.0°), Z = 8, D_c = 1.71 g cm^{-3}, F(000) = 592, graphite-monochromated Mo-K_α radiation, l = 0.71073 Å, μ = 4.3 cm^{-1}; data collection on a Siemens P4 diffractometer with an Oxford Cryosystems open-flow N_2 gas cryostat[27], Lehman-Larssen ω scan, 2q ≤ 50°, 1360 data total, 979 unique, R_{int} = 0.010. The structure was solved by direct methods (SHELXS-86)[28] and refined by full-matrix least squares (SHELXL-93)[29] against F^2 of all data with Chebyshev weights (non-H atoms anisotropic, H atoms refined isotropic, 106 variables, $wR(F^2)$ = 0.081 for all data, R(F) = 0.041 for 821 observed data with I > 2σ(I), goodness-of-fit 1.064, Δr_{max} = 0.26, Δr_{min} = -0.24 eÅ^{-3}). The F(1) atom (and correspondingly, H(1)) is disordered over two positions, a and b, with occupancies refined to 72 and 28%. Atomic co-ordinates and temperature factors, bond distances and angles have been deposited at the Cambridge Crystallographic Data Centre.

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