Substrate and reaction intermediate mimics as inhibitors of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase†

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Received 11th May 2009, Accepted 12th June 2009
First published as an Advance Article on the web 17th June 2009
DOI: 10.1039/b909241b

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase catalyses the aldol-like addition of phosphoenolpyruvate (PEP) to D-erythrose 4-phosphate in the first step of the shikimate pathway to aromatic amino acids. A series of compounds, designed to mimic intermediates in the enzyme-catalysed reaction, have been synthesised and tested as inhibitors for the DAH7P synthase from *Escherichia coli*. The most potent inhibitor was the vinyl phosphonate, (*E*)-2-methyl-3-phosphonoacrylic acid, with a K_i of 4.7 μM.

The shikimate pathway is an essential metabolic pathway, ultimately responsible for the biosynthesis of not only the aromatic amino acids phenylalanine, tyrosine and tryptophan, but also a number of other essential compounds such as iron-scavenging siderophores and quinones for electron transport.[1] This pathway is present in plants and microorganisms, but absent in mammals.[2–4]

The first enzyme of the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase catalyses the aldol-like addition of phosphoenolpyruvate (PEP) to D-erythrose 4-phosphate (E4P), resulting in the formation of the seven-carbon compound DAH7P and inorganic phosphate.

The key mechanistic features of this reaction have been elucidated by the work of a number of groups.[5–16] The phosphate cleavage proceeds *via* breaking of the C–O bond of PEP, and the reaction is stereospecific with respect to both substrates, with the *si* face of PEP C3 attacking the *re* face of E4P.[17–20] The reaction requires the presence of a divalent metal ion, and the identity of the optimum metal ion for catalysis varies depending on the origin of the DAH7P synthase.

While the mechanism of DAH7P synthase continues to be studied, comparatively little is known about the inhibition of this enzyme.[21] Due to the central role of DAH7P synthase in microbial metabolism, inhibitors of DAH7P synthase may be of use as antimicrobial agents. In order to gain an understanding of the inhibition of this enzyme, the interaction of a series of PEP-like compounds with DAH7P synthase was investigated.

A consideration of the mechanism of DAH7P synthase reveals several features of the reaction that could be potentially targeted by inhibitors. The initial attack of PEP on E4P is believed to proceed *via* Lewis acid activation of the E4P aldehyde group, followed by attack of PEP giving an oxocarbenium intermediate **1** (Fig. 1).[11,14] A bisubstrate DAH7P synthase inhibitor **3** ($IC_{50} = 6.6$ μM) with a

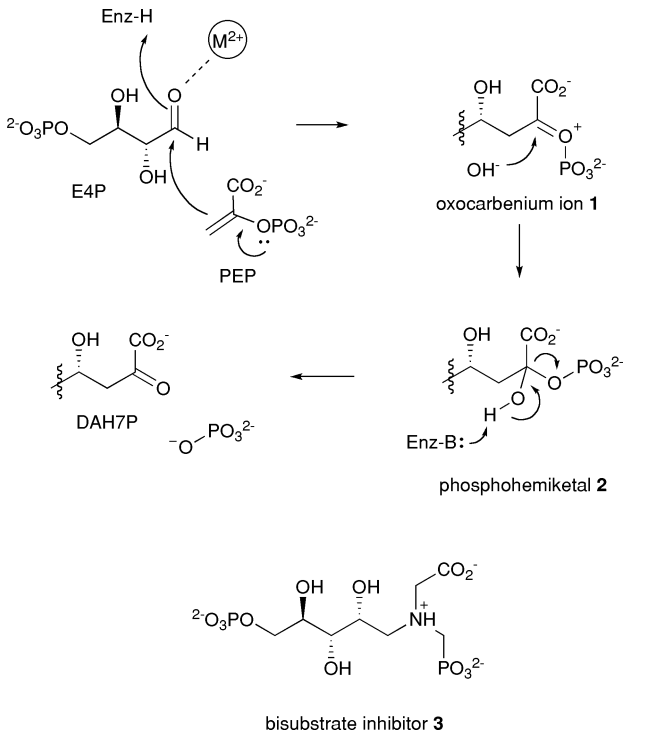

Fig. 1 The key features of the proposed Lewis acid mechanism of DAH7P synthase catalysis, *via* putative oxocarbenium ion species **1** and putative phosphohemiketal intermediate **2**.

design based on this intermediate has been previously prepared.[21] This inhibitor exploited the similarity of an aminophosphonate to the proposed phosphoxonium substructure, a concept that has been previously used in the inhibition of the related enzyme 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8P synthase) and 5-enolpyruvyl shikimate 3-phosphate synthase.[22–25]

An additional aspect of oxocarbenium species **1** that could be exploited in inhibitor design is the change in bond order around PEP C2. The oxocarbenium ion possesses partial double bond character between PEP C2 and the bridging phosphate oxygen; this bonding could be mimicked by the planarity of vinyl phosphonate **4** (Fig. 2). The (*E*)-configuration of this compound bears the most similarity to the conformation PEP adopts on enzyme binding. Trifluorinated vinyl phosphonate **5** might display improved binding properties compared to vinyl phosphonate **4**, as observed with other phosphonate-based phosphate mimics.[26]

The initial stages of the enzyme reaction could also be interrupted by interfering with the coordination of the E4P carbonyl group, thus blocking the Lewis acid activation of E4P. While no similar approaches to the inhibition of DAH7P synthase have

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† Electronic supplementary information (ESI) available: Synthetic procedures for inhibitors, NMR spectra and inhibition data. See DOI: 10.1039/b909241b

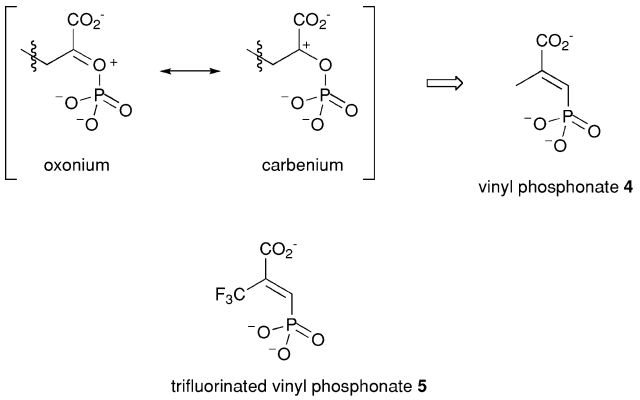

Fig. 2 A comparison between oxonium and carbenium representations of the oxocarbenium species **1** and vinyl phosphonates **4** and **5**.

been reported, we were struck by the similarity in metal activation between DAH7P synthase and the unrelated metalloenzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (Fig. 3). DXR is known to be potently inhibited by the natural product fosmidomycin,[27,28] with a K_i* of 21 nM against the enzyme from *E. coli*.[29] The inhibition of DXR by fosmidomycin is thought to be due to the inhibitor binding in the active site of DXR through interactions with both the phosphate binding pocket and the enzyme's divalent metal ion cofactor.[29,30]

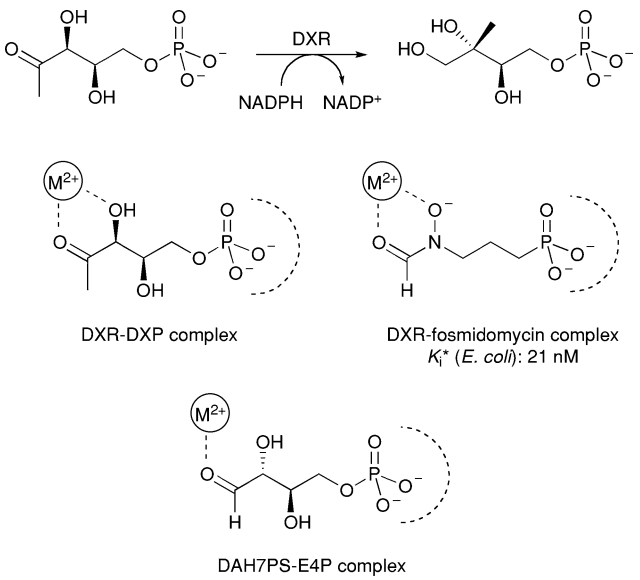

Fig. 3 A comparison of the reaction catalysed by DXR, the known DXR inhibitor fosmidomycin and DXR-DXP and DAH7PS-E4P enzyme–substrate complexes.

Whereas DAH7P synthase and DXR catalyse dissimilar reactions, both enzymes utilise a divalent metal ion to activate a phosphorylated carbohydrate-like substrate. The similarity between the two substrates is such that E4P is a poor substrate of some types of DXR.[29] With this in mind, we tested the fosmidomycin as an inhibitor of DAH7P synthase.

The initial attack of PEP on E4P is followed by the addition of water to the oxocarbenium intermediate **1**, to give phosphohemiketal **2** (Fig. 1). This transformation is accompanied by a change in geometry around C2 of PEP, from the trigonal planar geometry present in PEP to the tetrahedral geometry present in the phosphohemiketal **2**. This change in geometry could potentially be exploited to inhibit DAH7P synthase. The phosphohemiketal group is likely to be unstable, limiting its suitability for inclusion in complex inhibitors,[31] however the corresponding phosphate esters **6** incorporate a similar geometry, and thus may be able inhibit DAH7P synthase (Fig. 4). In addition, formation of the phosphohemiketal intermediate **2** is associated with generation of a new stereogenic centre at the ketal carbon, with its configuration dependent on whether attack of water occurs from the *re* or *si* face of the oxocarbenium intermediate.

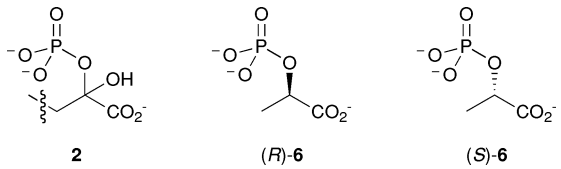

Fig. 4 Phosphohemiketal intermediate **2**, and phospholactate enantiomers (*R*) and (*S*)-**6**.

The configuration of the new stereogenic centre at the ketal carbon of the phosphohemiketal intermediate is yet to be elucidated, despite the intriguing results of Kona *et al*. on the related enzyme KDO8P synthase.[32] Differences in inhibition by phosphates (*R*)-**6** and (*S*)-**6** may provide evidence toward the configuration of the phosphohemiketal intermediate.

In addition to molecules based on the proposed mechanism of DAH7P synthase, it should also be possible to base inhibitors on the structures of the substrates. Accordingly, sulfate **7** and allylic phosphonate **8** were designed to mimic the structure of PEP (Fig. 5). By the proposed DAH7P synthase mechanism allylic phosphonate **8** should be inert due to the lack of a electron-donating oxygen substituent on the alkene. The effect of substituting a phosphate ester for its analogous sulfate is more difficult to predict. Sulfate ions have been previously observed by X-ray crystallography to adventitiously occupy the phosphate binding sites of DAH7P synthase, and sulfate **7** is a known substrate of pyruvate kinase.[7,10,33]

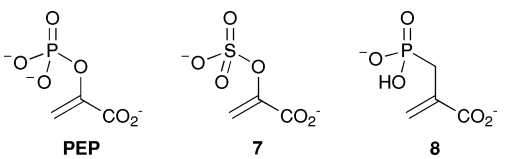

Fig. 5 DAH7P synthase substrate PEP, sulfoenolpyruvate **7** and allylic phosphonate **8**.

The vinyl phosphonates **4** and **5** were prepared by a Horner–Wadsworth–Emmons reaction of the corresponding α-ketoester with tetraethyl methylene bisphosphonate, followed by deprotection with trimethylsilyl bromide and potassium hydroxide (Fig. 6).[34,35] Analysis of these compounds showed that they were competitive inhibitors of the phenylalanine inhibited *E. coli* DAH7P synthase with respect to PEP. The vinyl phosphonate **4** had an inhibition constant of 4.7 μM (Fig. 7). No significant improvement in inhibition was noted with trifluorinated phosphonate **5**, which gave an inhibition constant of 8.8 μM.

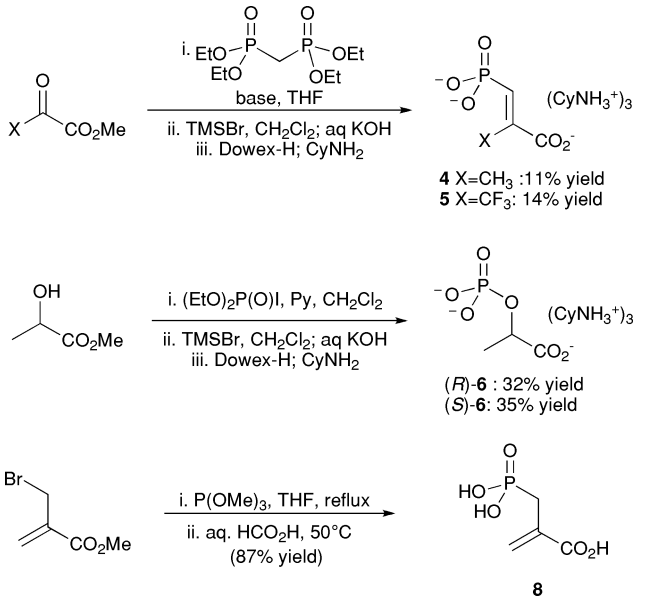

Fig. 6 A summary of the synthesis of compounds in this study. Details are presented in the ESI.†

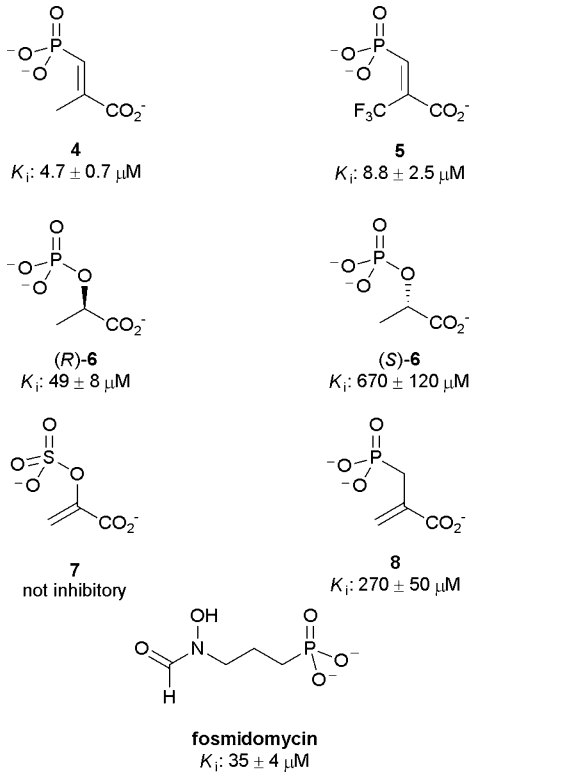

Fig. 7 Inhibitory behaviour of compounds in this study. All of the compounds shown (bar sulfoenolpyruvate **7**) were inhibitors of *E. coli* DAH7P synthase, competitive with respect to PEP. In each case a mixture of inhibitor, PEP, E4P and manganese(II) sulfate in buffer (bis-tris-propane, 50 mM, pH 6.8) was monitored by UV–visible spectroscopy at 232 nm, and the reaction was initiated by the addition of recombinant *E. coli* DAH7P synthase (~2 μg). Initial rates were determined over varied PEP and inhibitor concentrations, and the initial rates ($n = 12$) were fitted by global non-linear regression to the Michaelis–Menten model using GraFit.[38]

Fosmidomycin was analysed as an inhibitor of DAH7P synthase, based on the hypothesis that fosmidomycin would be able to bind to the monosaccharide-phosphate binding pocket of DAH7P synthase with its phosphonate group, while the formylhydroxamate group interacted with the active site metal ion. Investigation showed that fosmidomycin was indeed an inhibitor of DAH7P synthase. However, surprisingly fosmidomycin was found to be an uncompetitive inhibitor with respect to E4P, as evidenced by analysis with a Lineweaver–Burk plot (K_i(app): 282 ± 14 μM). A time dependence in the onset of inhibition by fosmidomycin was also noted, with a maximum level of inhibition attained after fosmidomycin and DAH7P synthase were incubated together for ten minutes. The extent of inhibition by fosmidomycin was also dependent on the metal cofactor used to activate the *E. coli* DAH7P synthase. Inhibition was approximately 2-fold greater with both manganese(II) and iron(II) than with cobalt(II).

Further investigation revealed fosmidomycin is a competitive inhibitor of DAH7P synthase with respect to PEP, with an inhibition constant of 35 μM with manganese(II) as the enzyme's metal cofactor, without any preincubation of fosmidomycin and DAH7P synthase.

The (*R*)- and (*S*)-phospholactates (*R*)-**6** and (*S*)-**6** were selected for study based on their similarity to the proposed phosphohemiketal intermediate **2** of the DAH7P synthase reaction. Both compounds have been previously reported as inhibitors of a number of PEP-utilising enzymes,[36] but not as inhibitors of DAH7P synthase or related enzymes. The (*R*)- and (*S*)-enantiomers of phospholactate were prepared by phosphorylation of the corresponding enantiomer of methyl lactate with diethylphosphoryl iodide,[37] followed by deprotection with trimethylsilyl bromide and potassium hydroxide.

Both phospholactates were found to be inhibitors of DAH7P synthase, competitive with respect to PEP. The stereochemistry of these compounds has a strong influence on their affinity for the enzyme, as evidenced by the greater than ten-fold weaker enzyme-inhibitor interaction of (*S*)-phospholactate (670 μM) compared to (*R*)-phospholactate (49 μM).

Sulfoenolpyruvate (SEP) **7** was selected for study based on its structural similarity to PEP. SEP was prepared by the route of Peliska and O'Leary,[33] and the interaction of SEP with DAH7P synthase was investigated. Remarkably, despite the similarity between PEP and SEP, SEP did not inhibit DAH7P synthase, with no significant inhibition observed up to a concentration of 10 mM. No consumption of SEP was observed spectrophotometrically with *E. coli* DAH7P synthase in the presence of manganese(II), copper(II), zinc(II) or cobalt(II) as metal cofactors, and analysis by ^{1}H NMR spectroscopy showed no decrease in SEP concentration after incubation with E4P, manganese(II) sulfate and *E. coli* DAH7P synthase overnight.

Allylic phosphonate **8** shares a close similarity in structure with PEP, and has also been previously reported to inhibit several PEP-utilising enzymes, in addition to being a substrate for enolase.[39] Allylic phosphonate **8** was prepared by the route of Stubbe and Kenyon,[40] and its interaction with DAH7P synthase was investigated by enzyme assay. Allylic phosphonate **8** was not a substrate for DAH7P synthase, instead it acted as a competitive inhibitor with respect to PEP, with an inhibition constant of 270 μM. This result reinforces the importance of the

enol functionality in contributing to the nucleophilicity of the double bond of PEP in the enzyme-catalysed reaction.

By examining the proposed mechanism of DAH7P synthase, and focussing on the enzyme's PEP subsite, this study has discovered several molecules that inhibit DAH7P synthase.

The most potent of these molecules, vinyl phosphonate **4** (K_i: 4.7 μM) was designed to mimic the geometry and bond order of the PEP-portion of the proposed oxocarbenium intermediate. The success of this mechanism-based approach can be verified by comparison with the isomeric allylic phosphonate **8**, a substrate-based mimic of PEP with a much weaker ability to inhibit DAH7P synthase (K_i: 270 μM). While this difference in binding affinity between these two compounds has been ascribed here to the mimicry of the oxocarbenium ion intermediate **1**, the potency of vinyl phosphonate **4** may also have an entropic contribution. Vinyl phosphonate **4**, by virtue of its (*E*)-configured alkene, is locked into a similar conformation to that of PEP in several DAH7P synthase X-ray crystal structures. Due to this conformational locking, the binding of vinyl phosphonate **4** to DAH7P synthase perhaps incurs a smaller entropic disadvantage than the binding of more flexible phosphonates such as allylic phosphonate **8**.[41]

The enantiomeric phospholactates (*R*)-**6** and (*S*)-**6** were chosen as potential mimics of the proposed phosphohemiketal intermediate **2**. The stereochemistry of the lactates had a strong effect on inhibition, with the (*S*)-isomer displaying a greater than 10-fold larger inhibition constant than its (*R*)-counterpart. The origin of this significant difference in affinity of the two stereoisomers for DAH7P synthase is intriguing, and is expected to be related to configuration at C2 of the phosphohemiketal intermediate **2**. This effect is under further investigation in our laboratory.

Based on the superficial similarities between DXR substrate DXP and the DAH7P synthase substrate E4P, we trialled fosmidomycin as an inhibitor of DAH7P synthase. Fosmidomycin was successful as an inhibitor of DAH7P synthase; however, surprisingly fosmidomycin was a competitive inhibitor of DAH7P synthase with respect to PEP, with a relatively potent binding constant of 35 μM. Given the approximately 1000-fold poorer inhibition of DAH7P synthase by fosmidomycin compared with DXR, it seems likely that the inhibition of DAH7P synthase by fosmidomycin is of little consequence to the anti-microbial effects of fosmidomycin *in vivo*. The competitive inhibition of DAH7P synthase by fosmidomycin with relation to PEP suggests fosmidomycin occupies the PEP binding region of the active site, rather than the E4P binding region as initially hypothesised. The observed metal dependence of this inhibition may suggest that fosmidomycin is also capable of interacting with the metal cofactor of the DAH7P synthase active site.

The failure of sulfoenolpyruvate **7** to act as either a substrate or inhibitor of DAH7P synthase is a surprising finding. One major difference between PEP and SEP is the charge state of each compound; whereas PEP can be expected to be in both di- or tri-anionic forms at pH 6.8, sulfoenolpyruvate can only be a dianion. The failure of SEP to inhibit or be processed by DAH7P synthase may suggest that PEP and analogues bind to DAH7P synthase exclusively as trianionic species.

In conclusion, the ability of the PEP site of DAH7P synthase to bind a series of inhibitors of varying structure has been investigated. Inhibitors bearing both a carboxylate group and a phosphonate or phosphate separated by a similar distance to those found in PEP were found to be effective inhibitors, whilst the sulfate-containing SEP was ineffective. Interestingly, fosmidomycin, bearing both a formylhydroxamate and phosphonate group, was also found to inhibit DAH7PS. The inhibitory potency of compounds against DAH7PS was found to be sensitive to minor variations in compound structure, with changes such as stereochemical inversion and alkene isomerisation providing large differences in binding affinity. The further investigation of these compounds against DAH7P synthase and KDO8P synthase continues in our laboratory, in addition these results are guiding the design of more potent DAH7P synthase inhibitors.

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34 Phosphonate **4**: Treatment of tetraethyl methylene bisphosphonate (1.1 eq) with sodium bis(trimethylsilylamide) (1 eq) in THF, followed by the addition of methyl pyruvate (1 eq) gave a mixture of *E*/*Z* vinyl phosphonates which were separated by chromatography. The *E*-isomer was deprotected by trimethylsilylbromide and potassium hydroxide to give **4** ^{1}H NMR (500 MHz, D_2O) δ ppm 6.19 (dd, J = 14.6, 1.2 Hz, 1H, H3′), 3.10–2.88 ($CyNH_3^+$), 1.87 (dd, J = 2.9, 1.2 Hz, 3H, H3), 1.85–1.78 (m, $CyNH_3^+$), 1.67–1.59 (m, $CyNH_3^+$), 1.48 (dm, J = 12.8 Hz, $CyNH_3^+$), 1.24–1.09 (m, $CyNH_3^+$), 1.01 (ddm, J = 22.2, 10.1 Hz, $CyNH_3^+$). HRMS (electrospray negative ion, 20% AcOH matrix) required for $C_4H_6O_5P^-$: 164.9953; found: 164.9948. Similarly, treatment of methyl trifluoropyruvate (1 eq) with tetraethyl methylene bisphosphonate (1.25 eq) and LDA (1.1 eq) gave a vinyl phosphonate which was deprotected to give **5**: ^{1}H NMR (300 MHz, D_2O) δ ppm 6.75 (d, J = 6.2 Hz, H3′), 3.21–2.89 (m, $CyNH_3^+$), 2.02–1.75 (m, $CyNH_3^+$), 1.75–1.59 (m, $CyNH_3^+$), 1.52 (dm, J = 12.2 Hz, $CyNH_3^+$), 1.21 (m, $CyNH_3^+$). HRMS (electrospray negative ion, 20% AcOH matrix): required for $C_4H_3O_5F_3P$: 218.9670, found 218.9670. Full details are given in the ESI†.
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