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Synthesis and evaluation of tetrahedral intermediate mimic inhibitors of 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase

Aidan N. Harrison, Sebastian Reichau, Emily J. Parker *

Biomolecular Interaction Centre and Department of Chemistry, University of Canterbury, Christchurch 8040, New Zealand

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ABSTRACT

3-Deoxy-D-manno-octulosonate 8-phosphate (KDO8P) synthase catalyses the first committed step in the biosynthesis of 3-deoxy-D-*manno*-octulosonate (KDO), an important component of the lipopolysaccharide of Gram-negative bacteria. The pathway for KDO biosynthesis has been identified as a potential target of antibacterial drug design. The reaction catalysed by KDO8P synthase is an aldol-like condensation between phosphoenolpyruvate (PEP) and D-arabinose 5-phosphate (A5P) and proceeds through a bisphosphorylated tetrahedral intermediate. In this study a bisphosphate analogue of the tetrahedral intermediate was synthesised and was found to inhibit the metal-dependent KDO8P synthase from *Neisseria meningitidis* and the metal-dependent KDO8P synthase from *Acidithiobacillus ferrooxidans* with inhibition constants in the low micromolar range. Additionally, monophosphorylated inhibitors were synthesised to determine the relative importance of the two phosphate groups of this bisphosphate analogue for enzyme inhibition. The removal of either of these two phosphate groups gave less potent inhibitors for both enzymes.

3-Deoxy-D-*manno*-octulosonate phosphate (KDO8P) synthase (Fig. 1) catalyses the aldol condensation between phosphoenolpyruvate (PEP, **1**) and arabinose 5-phosphate (A5P, **2**) to give the eight carbon phosphorylated sugar, 3-deoxy-D-*manno*-octulosonate (KDO8P, **3**). This reaction is the first committed step in the biosynthesis of 3-deoxy-D-*manno*-octulosonate (KDO),[1] an eight carbon sugar that is an important component of lipopolysaccharide (LPS) which is found in the outer membrane of Gram-negative bacteria. Previous studies have shown that disruption of the KDO pathway leads to cell wall disruption and eventual death of the bacterium, and therefore KDO8P synthase has been identified as a potential target for antibacterial drug design.[2]

There are two groups of KDO8P synthases that differ in their requirement for a divalent metal ion for catalytic activity. The KDO8P synthases from *Escherichia coli*[3] and *Neisseria meningitidis*[4] do not require a metal ion for activity, whereas a divalent metal ion is necessary for catalysis by the KDO8P synthases from *Aquifex aeolicus*, *Aquifex pyrophilus*, *Helicobacter pylori*, *Acidithiobacillus ferrooxidans* and *Chlamydia psittaci*.[5–8]

The structures of both metal-independent[9,10] and metal-dependent[11] KDO8P synthases have been determined by X-ray crystallography. The functional forms of these enzymes are homotetramers of $(\beta\alpha)_8$ barrel subunits. The active sites are almost completely conserved between the two groups of the enzyme, apart from a single amino acid substitution in the metal binding site, where a metal binding Cys in the metal-dependent enzyme is substituted for a conserved Asn in the metal-independent form.[10]

Many of the mechanistic aspects of the KDO8P synthase reaction have been determined. It is known that the *si* face of PEP approaches the *re* face of A5P to form the new carbon–carbon bond between the C3 carbon of PEP and the C1 carbon of A5P.[12] The phosphate elimination is known to occur via breakage of the carbon–oxygen bond of the C2 carbon from PEP and the bridging oxygen of the phosphate ester.[13] Computational studies have suggested that this gives an oxocarbenium ion **4** (Fig. 1), of which the electrophilic C2 carbon is attacked by a nucleophilic water molecule.[14] This gives the tetrahedral intermediate **5** which has been directly observed by mass spectrometry (Fig. 1).[15] The same computational studies have suggested that the divalent metal ion is not directly involved in the reaction mechanism, as is thought to occur in the related aldolase 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase,[16] but plays a structural role in orientating the substrates to lower the activation energy of the enzyme reaction, a role that is easily mimicked by amino acid side chains in the metal-independent KDO8P synthases, consistent with relatively facile interconversion between the metal-dependent and metal-independent groups.[5,10,17,18]

Several inhibitors for KDO8PS have been synthesized and tested.[19–22] The most effective of these inhibitors is aminophosphonate **7** (Fig. 2) which was designed to mimic the oxocarbenium ion intermediate (**4**, Fig. 1),[19] with an inhibition constant, K_i, of 0.37 μM for the metal-independent KDO8PS from *E. coli*.[23] Compounds resembling the tetrahedral intermediate or substrate

* Corresponding author. Tel.: +64 3 364 2871; fax: +64 3 364 2110.
E-mail address: Emily.parker@canterbury.ac.nz (E.J. Parker).

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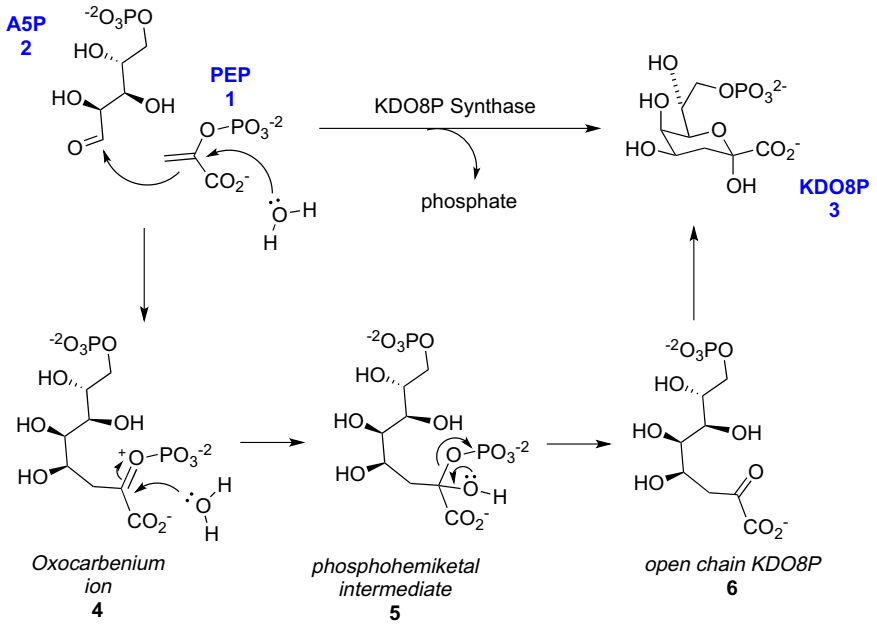


Figure 1. The reaction catalysed by KDO8P synthase. The transformation of PEP (**1**) and A5P (**2**) to KDO8P (**3**) is shown as well as the proposed oxocarbenium ion (**4**) and known tetrahedral intermediate (**5**) as intermediates of the reaction mechanism.

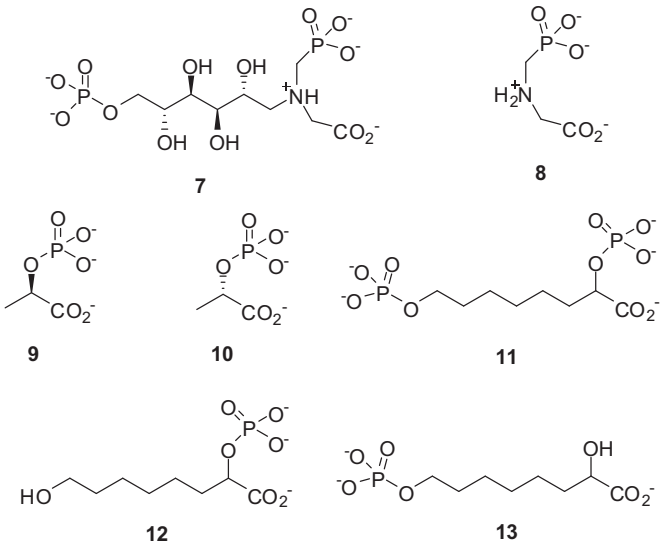


Figure 2. Aminophosphonate **7** and molecules tested as inhibitors of KDO8PS in this study (**8**–**13**).

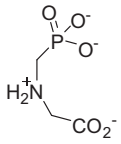
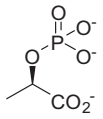
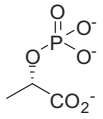
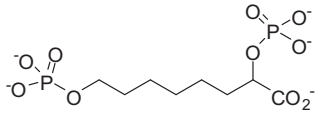
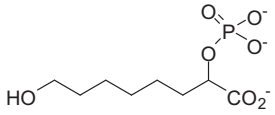
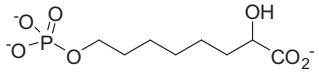
complex, but lacking the A5P-like phosphate moiety have also been tested as inhibitors against KDO8P synthase from *E. coli* and *H. pylori* showing complex, but generally poor inhibition. The best inhibitor of this series has an IC_{50} value of 132 μM against the KDO8PS from *H. pylori*.[21,24]

Recently we described a tetrahedral intermediate mimic as the first potent inhibitor of 3-deoxy-D-*arabinose*-heptulosonate 7-phosphate synthase from *Mycobacterium tuberculosis*,[25] an enzyme which shares many elements of its structure and reaction mechanism with KDO8P synthase. Here we show that this strategy is transferable to the synthesis of simple tetrahedral intermediate mimics for the inhibition of KDO8PS. These compounds incorporate, with appropriate spacing, the key charged carboxylate and phosphate functionalities derived from PEP and the phosphate group of A5P. These findings highlight the potential for compounds mimicking this tetrahedral intermediate to act as inhibitors for both metal-dependent and metal-independent forms of this enzyme.

An initial examination of the potential for mimics of the tetrahedral intermediate to act as inhibitors for this enzyme was carried out by testing the phospholactates **9** and **10** (Fig. 2).[26] Both enantiomers were found to show relatively poor, but competitive inhibition with respect to PEP, of both the metal-dependent KDO8P synthase from *A. ferrooxidans* and the metal-independent KDO8P synthase from *N. meningitidis* (Table 1). There was little discrimination between the enantiomeric phospholactates **9** and **10,** with (2*R*)-2-phospholactate **9** giving K_i values of 870 ± 90 μM and 390 ± 70 μM for the KDO8P synthase from *N. meningitidis* and *A. ferrooxidans* respectively, whereas the (2*S*)-2-phospholactate **10** gave slightly poorer inhibition of both enzymes. The configuration of the stereogenic centre at C2 of the tetrahedral intermediate **5** (Fig. 1) is unclear[27,28] and it is possible that the hydroxyl group is required to be present to create a preference for a specific configuration. Intriguingly the 'PEP head' of the aminophosphate **7**, glyphosate **8** (Fig. 2), was also examined and found to be a relatively poor inhibitor of KDO8PS with K_i values of 260 ± 40 μM and 330 ± 40 μM for the enzymes from *N. meningitidis* and *A. ferrooxidans* respectively. This finding indicates the importance of the A5P-mimicking component of animophosphonate **7**. Based on these inhibition results three inhibitors were proposed. The bisphosphate tetrahedral intermediate mimic **11** (Fig. 2) contains both charged PEP functional groups and a primary phosphate group linked through a hydrocarbon tail. A chain length of eight was selected in order to allow appropriate spacing of the primary phosphate group to mimic the phosphate of the tetrahedral intermediate derived from substrate A5P. The other two compounds **12** and **13** (Fig. 2) lack either the primary or secondary phosphate group and were designed to investigate the relative importance of the two phosphate groups for the inhibition of KDO8P synthase.

Following our recently published procedure,[25] the syntheses for all three compounds proceeded via a common precursor, the α-hydroxy ester **18**, which was synthesised in four steps from 1,6-hexanediol (Scheme 1). 1,6-Hexanediol **14**, was monoprotected with *tert*-butyldimethylsilyl chloride, and this alcohol **15** was then oxidised using Dess–Martin periodinane[29] to give the aldehyde **16**.

Table 1
Inhibition constants of the inhibitors tested in this study[a]

Inhibitor	K_i *Nme* (μM)	K_i *Afe* (μM)
 **8**	260 ± 40	330 ± 40
 **9**	870 ± 90	390 ± 70
 **10**	1200 ± 50	1000 ± 220
 **11**	7.9 ± 1.6	20 ± 3
 **12**	1000 ± 80	540 ± 50
 **13**	270 ± 40	190 ± 40

[a]Inhibitors were assayed on KDO8P synthase from *N. meningitidis* (*Nme*) and *A. ferrooxidans* (*Afe*) KDO8P synthases using a direct assay system following the loss of PEP at 232 nm. All compounds were found to be competitive inhibitors with respect to PEP.

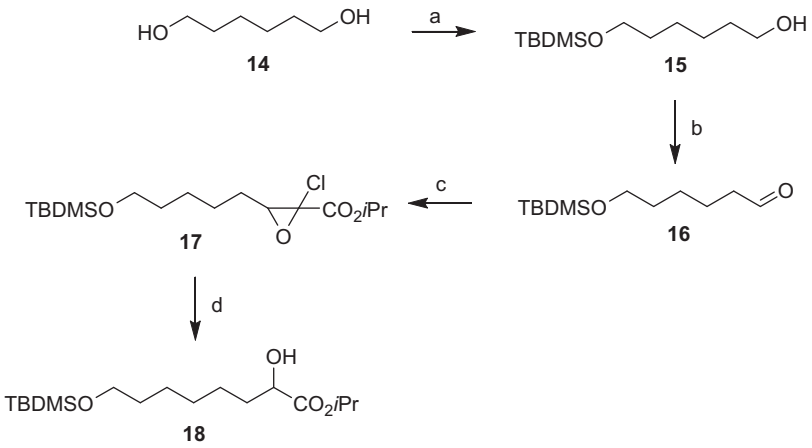
Aldehyde **16** was then used to produce the chloroepoxide **17** using a modified Darzen's condensation.[30,31] The crude epoxide **17** was then reduced using sodium cyanoborohydride[32] to give the α-hydroxy ester **18** which was purified by flash chromatography. The reaction sequence from **15** to **18** did not require purification of the intermediates and could be conveniently carried out in one day.

The synthesis of bisphosphate **11** proceeded by deprotection of the silyl ether of α-hydroxy ester **18** with *tert*-butylammonium fluoride[33] to give the diol **19**. This diol was then diphosphorylated in two steps via the phosphite, which was subsequently oxidised with *meta*-chloroperbenzoic acid to give the protected bisphosphate **20** (Scheme 2).[34]Bisphosphate **20** was then subjected to final deprotection by removal of the phosphate benzyl ether groups by hydrogenolysis, followed by the hydrolysis of the isopropyl ester group under basic conditions to give the bisphosphate **11**.

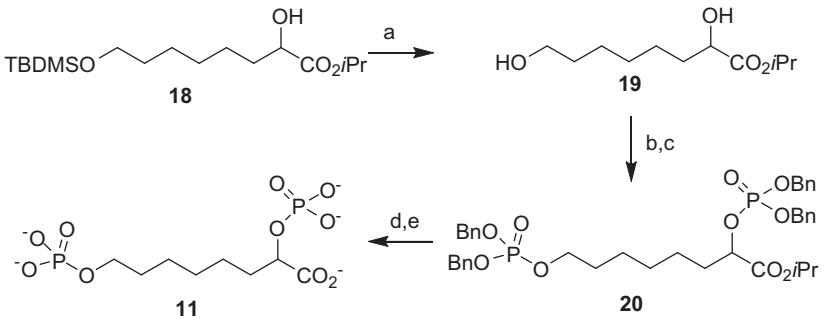
The synthesis of monophosphate inhibitor **12** proceeded from the α-hydroxy ester **18** by monophosphorylation of the C2 hydroxyl group, prior to deprotection of the silyl ether group of ester **21** to give the benzyl protected monophosphate **22** (Scheme 3). The phosphate benzyl ether groups were deprotected by hydrogenolysis, however purification by anion exchange chromatography proved to be difficult due to the poor resolution of the compound from the column. Passage of the crude product through a DOWEX-H^+ column proved to be a more efficient alternative purification method, with ^1H, ^{13}C and ^{31}P NMR analysis indicating no impurities. The monophosphate inhibitor **12** dissolved in an aqueous solution was lyophilised and the compound obtained as a white powder.

A similar route was followed for the synthesis of monophosphate inhibitor **13** (Scheme 4). The C2 hydroxyl group of α-hydroxy ester **18** was benzylated before removal of the silyl ether. Alcohol **24** was then phosphorylated to give the protected monophosphate **25**. Deprotection and purification by anion exchange chromatography gave the product **13** as a white powder.

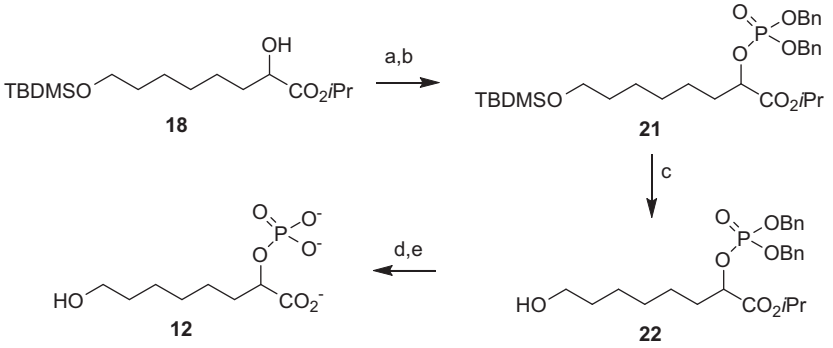
The ability of the three compounds (**11**, **12**, **13**) to inhibit the metal-independent KDO8P synthase from *N. meningitidis* and the metal-dependent KDO8P synthase from *A. ferrooxidans* was determined by kinetic assay (Table 1). The bisphosphate inhibitor **11** was found to give competitive inhibition with respect to PEP with an inhibition constant of 7.9 ± 1.6 μM for the KDO8P synthase from *N. meningitidis* and 20 ± 3 μM against KDO8P synthase from *A. ferroxidans*. These K_i values are approximately 2–3 fold higher than the K_m values for PEP for each enzyme, making the tetrahedral intermediate mimic **11** the second most potent inhibitor for KDO8P synthase, behind the aminophosphonate **7**. The importance of both the A5P and PEP phosphate moieties for the inhibition of KDO8P synthase is highlighted by the results for the inhibition of both KDO8P synthase enzymes by the monophosphate inhibitors.



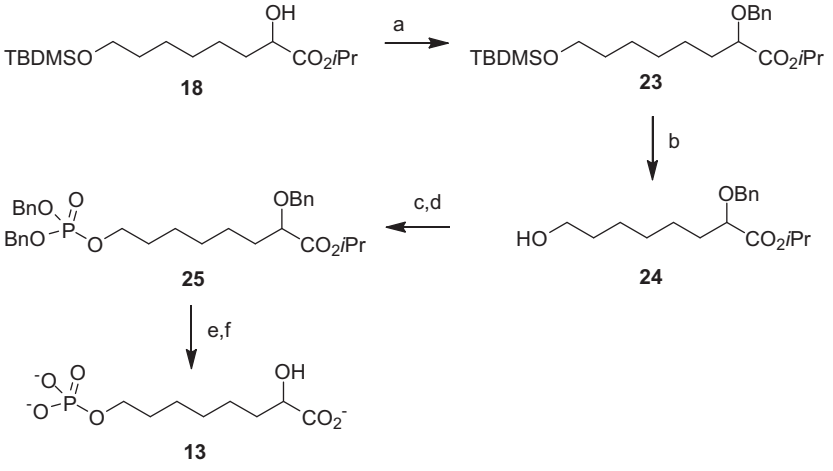
Scheme 1. Synthesis of hydroxy ester **18**. (a) TBDMSCl, imidazole, THF (72%), (b) Dess–Martin periodinane, CH_2Cl_2, (c) Cl_2CHCO_2iPr, *i*PrOK, *i*PrOH, Et_2O, (d) $NaCNBH_3$, *i*PrOH (26% from **15**).



Scheme 2. Synthesis of bisphosphate inhibitor **11.** (a) TBAF, THF (83%), (b) 1*H*-tetrazole, $(i\text{Pr})_2\text{NP}(\text{OBn})_2$, CH_2Cl_2, (c) *m*-CPBA (65% from **19**), (d) H_2, Pd/C, EtOAc, (e) KOH/H_2O, DOWEX-H^+ (2% from **20**).



Scheme 3. Synthesis of monophosphate inhibitor **12**. (a) 1*H*-tetrazole, $(i\text{Pr})_2\text{NP}(\text{OBn})_2$, CH_2Cl_2, (b) *m*-CPBA (80% from **18**), (c) TBAF, THF (56%), (d) H_2, Pd/C, EtOAc, (e) KOH/H_2O, DOWEX-H^+ (41% from **22**).



Scheme 4. Synthesis of monophosphate inhibitor **13**. (a) Ag_2O, KI, BnBr, CH_2Cl_2 (66%), (b) TBAF, THF (83%), (c) 1*H*-tetrazole, $(i\text{Pr})_2\text{NP}(\text{OBn})_2$, CH_2Cl_2, (d) *m*-CPBA (84% from **24**), (e) H_2, Pd/C, EtOAc, (f) KOH/H_2O, DOWEX-H^+ (12% from **25**).

Monophosphate inhibitor **12**, lacking the A5P-like phosphate moiety inhibited the KDO8P synthase from *N. meningitidis* with an inhibition constant of 1000 ± 80 μM and the KDO8P synthase from *A. ferrooxidans* with an inhibition constant of 540 ± 50 μM. These values are similar to the K_i values found for the average of the K_i values for phospholactates **9** and **10**, which is unsurprising since the hydrocarbon tail of **12** is unlikely to form contacts with KDO8P synthase active site residues, and therefore the presence of this tail does not incur any inhibitory advantage. Interestingly monophosphate inhibitor **13**, lacking the PEP phosphate moiety, inhibited both KDO8P synthase enzymes more effectively than monophosphate inhibitor **12**. This result suggests that the ability of the inhibitor to bind in both substrate-binding sites at either end of the enzyme active site through charged groups provides more effective inhibition of KDO8P synthase.

In conclusion an inhibitor designed as a tetrahedral intermediate mimic, incorporating only key charged moieties but without the stereochemically and synthetically complex hydroxyl functionality of A5P was able to effectively inhibit both the metal-independent KDO8P synthase from *N. meningitidis* and the metal-dependent KDO8P synthase from *A. ferrooxidans*. This compound was considerably more effective than inhibitors that lacked either of the phosphate groups, indicating that these charged groups both play an important role in the inhibition of KDO8P

synthases from both bacterial sources. These findings suggest that the charged functionalities of both substrates are important for inhibitor binding, and that further elaboration of the tetrahedral bisphosphate inhibitor **11** to include the A5P-derived hydroxyl moieties may show increased inhibition of the enzyme.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.12.025.

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