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Studies on 3-Deoxy-D-*manno*-octulosonic Acid 8-Phosphate Synthase Using Chorismate Mutase Inhibitors

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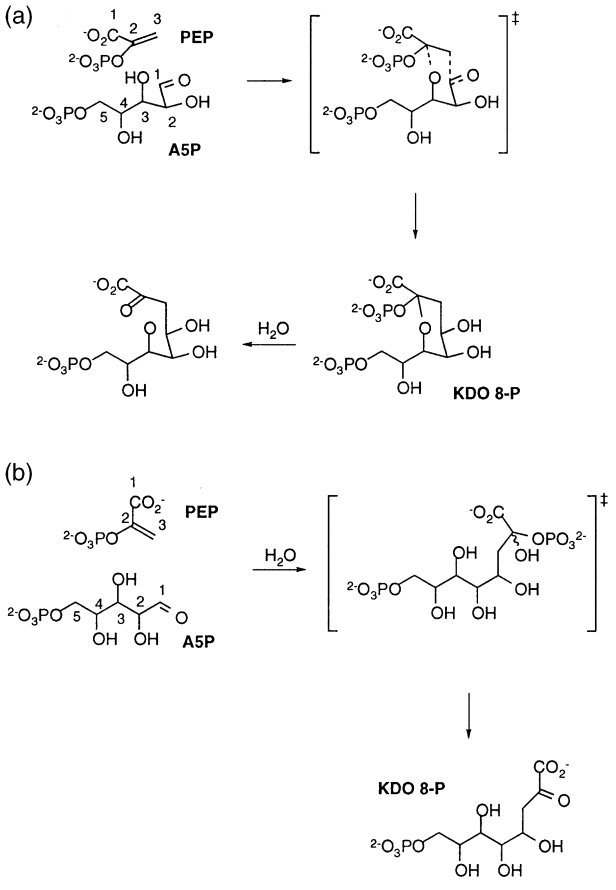
Abstract—The proposed cyclic mechanism of 3-deoxy-D-*manno*-octulosonic acid 8-phosphate synthase and the mechanism of chorismate mutase share certain structural and electronic similarities. In this report, we examine several inhibitors of chorismate mutase for their efficacy against KDO 8-P synthase.

Phospho*enol*pyruvate (PEP) participates in a wide variety of biological reactions that can be divided into two distinct classes. The first class includes reactions wherein the phosphate moiety is cleaved by way of attack of a nucleophile, usually hydroxide, on the phosphate and subsequent dissolution of the P–O bond. The second class of reactions, catalyzed by only four known enzymes, involves breakage of the C–O bond following attack of a nucleophilic oxygen on C2 of PEP.[1] The reactions of both *enol*pyruvyl shikimate 3-phosphate synthase[2] and uridine-5′-diphospho-*N*-acetylglucoseamine *enol*pyruvyl transferase[3] are known to be initiated by the attack of a substrate hydroxyl onto $C2^{PEP}$ while that of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase is proposed to initiated by a water molecule.[4] In the case of 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P; EC 4.1.2.16) synthase, both acyclic[5] and cyclic[6] mechanisms have been proposed. In the cyclic mechanism (Scheme 1a), the C3-OH of arabinose 5-phosphate (A5P) initiates the reaction by attacking $C2^{PEP}$, with subsequent breakdown of the tetrahedral intermediate. The acyclic mechanism (Scheme 1b) is initiated by an active site water attacking $C2^{PEP}$, followed by the $C3^{PEP}$ attack at the carbonyl of A5P. The degree of negative charge build-up at $C3^{PEP}$ has not yet been investigated, leaving open the question of reaction timing (i.e., concerted or stepwise). In a



Scheme 1.

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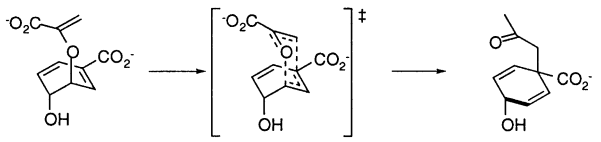
series of communications by Baasov and co-workers,[7–9] it was concluded that the cyclic mechanism was not operative, due to the observation that the proposed cyclic intermediate was not converted to KDO 8-P by the enzyme. These data were obtained before it was known that the active site of KDO 8-P synthase is sealed from bulk solvent by the closure of two loop regions. These closures are caused by the binding of the two substrates.[10] This finding allows for the possibility that an intermediate may not bind to the correct form of the enzyme for catalysis (the correct form for intermediate binding having the active site closed to solvent) and thus may not be converted to product. Furthermore, the two compounds synthesized by Baasov as mimics of the cyclic[7] and acyclic[8] transition states both failed to inhibit KDO 8-P synthase strongly enough (35 and 3 μM, respectively) to draw meaningful mechanistic conclusions. Therefore, while evidence exists to support both the cyclic and acyclic hypotheses,[10–13] no experiment has conclusively established the true catalytic mechanism. Structures obtained by X-ray diffraction of the *Escherichia coli*[14] and *Aquifex aeolicus*[10] KDO 8-P synthases further compound this problem. In the structure of the *E. coli* enzyme, the location of the phosphate groups of PEP and A5P (inferred by the location of two bound sulphates) are 13 Å apart, precluding the cyclic mechanism, while in the *A. aeolicus* structure they are only 10 Å apart, allowing for the possibility that $C2^{PEP}$ and $C3\text{-}OH^{A5P}$ are within the requisite distance for bond formation and hence the cyclic mechanism.

Chorismate mutase (CM; EC 5.4.99.5) catalyzes the pericyclic rearrangement of chorismate to prephenate (Scheme 2), and represents a key enzyme in the biosynthesis of phenylalanine and tyrosine along the shikimate pathway. The rearrangement proceeds via a cyclic transition state resembling that shown in Scheme 1 for KDO 8-P synthase.

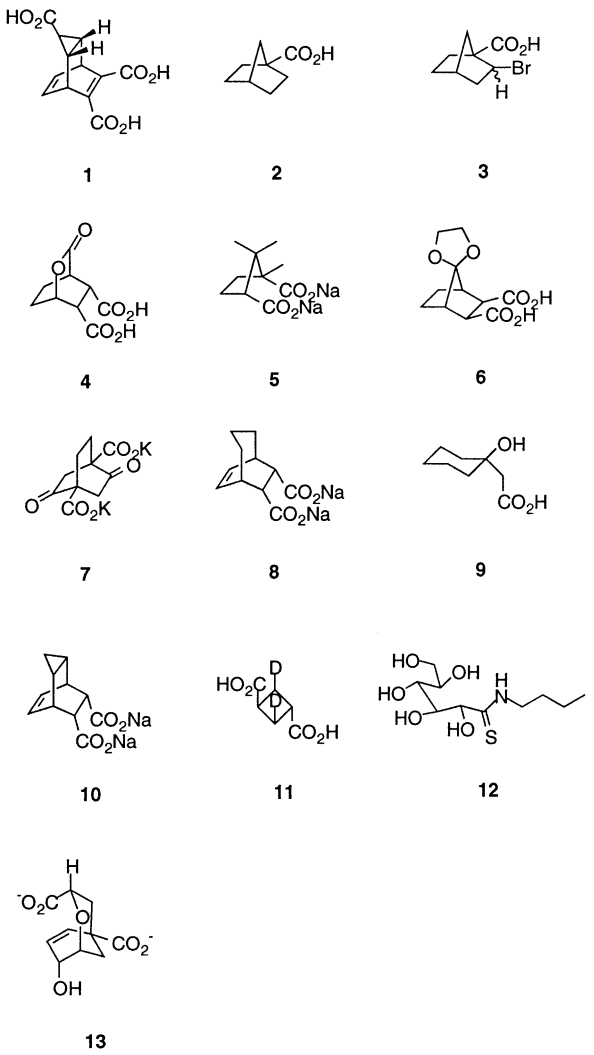
Interestingly, a search of available genetic information using the Basic Local Alignment Search Tool[15] with *E. coli* KDO 8-P synthase as the query sequence returned, among others, a sequence identified as the chorismate mutase from *Staphylococcus xylosus* (55% similar). Both CM and the cyclic mechanism of KDO 8-P synthase catalyze the attack of $C3^{PEP}$ (or the PEP-like moiety in the case of CM) onto an electrophilic sp^2 carbon in a six-membered transition state. Such a mechanistic similarity between the two enzymes suggested that CM inhibitors might serve as promising leads for KDO 8-P synthase inhibitors. Neither enzyme is expressed in mammalian cells and are thus both attractive targets for the design of new antibiotic agents. Therefore, a series of compounds (Scheme 3, **1**–**11**) that had earlier been screened as CM inhibitors[16] was tested against KDO 8-P synthase. Compound **12**[17] was included in this screen empirically, based on its similarity to the structure of A5P.

All compounds (Scheme 3, **1**–**12**) were initially assayed at 1 mM [90 μM ($\sim 10\times K_M$) PEP, 270 μM ($\sim 10\times K_M$) arabinose 5-phosphate (A5P), 200 nM KDO 8-P synthase, and 100 μM Tris–OAc (pH 7.4) in a total of 1 mL at 37 °C]. The absorbance due to the α,β-unsaturated carboxylate of PEP was monitored at 232 nm ($\varepsilon = 2840\ M^{-1}\ cm^{-1}$) as described previously.[18] Compound **1** absorbed strongly at 232 nm, therefore a purine nucleoside phosphorylase (PNPase) based phosphate release assay was utilized [90 μM ($\sim 10\times K_M$) PEP, 270 μM ($\sim 10\times K_M$) arabinose 5-phosphate (A5P), 100 μM 7-methylinosine, ~200 nM KDO 8-P synthase, ~200 nM PNPase, and 100 μM Tris-OAc (pH 7.4) in a total of 1 mL at 37 °C]. The PNPase coupled assay was monitored at 280 nm as described previously.[18,19]

Of the 12 compounds tested as inhibitors of KDO 8-P synthase, only one exhibits an IC_{50} below 500 μM



Scheme 2.



Scheme 3.

(Table 1). Compound **12**, which displays saccharide like structural characteristics, but lacked a phosphate group at C6, is a surprisingly potent inhibitor. Phosphate groups are required for binding of other monosaccharides tested (arabinose and ribose are neither substrates nor inhibitors of KDO 8-P synthase).[20] The progress curve of **12** (not shown) indicates that this compound binds very slowly to KDO 8-P synthase and appears to be an irreversible inactivator of the enzyme. This inactivation, which follows apparent first order kinetics, proceeds with a K_i of 2.3 mM and a k_{inact} of 0.01 min^{-1} (Fig. 1). A low millimolar K_i places **12** in a range similar to ribose 5-phosphate (1 mM),[20] which differs from A5P only in the stereochemistry at C2. The absence of the terminal phosphate, while detrimental to binding, is no longer requisite for inhibition. At all concentrations of **12** tested, complete inactivation of the enzyme is achieved (compared to inhibitor-free control). The inactivation of KDO 8-P synthase appears to be kinetically irreversible, but is completely reversed by the addition of a reductant [either 1 mM DTT or 100 μM *tris*-(carboxyethyl)phosphine (TCEP)] (data not shown).

Although the reactions of chorismate mutase and KDO 8-P synthase may have certain similarities, there appears to be little cross-reactivity between inhibitors. The only compound that inhibited KDO 8-P synthase to any great extent (**12**) is obviously similar to the linear form of the substrate, A5P, and the product, KDO 8-P. Conversely, the best CM inhibitor (**9**, $IC_{50} = 350$ μM[16]) had little effect on KDO 8-P synthase. This result is consistent with the recent conclusion of Duewel et al.[10] that the cyclic mechanism is less likely than the acyclic mechanism wherein the reaction is initiated by attack of an activated water molecule on $C2^{PEP}$. Although the activities of compounds **1**–**11** against CM are uninspiring,[16] the general steric and electronic properties of these compounds are similar to those of true CM transition state analogues, such as **13**.

Given that none of these compounds inhibits either enzyme well, the cross-reactivity of CM and KDO 8-P synthase was examined. First, *Bacillus subtilis* CM[21] was incubated with PEP and A5P, and the production of KDO 8-P was monitored using the deoxy-monosaccharide assay described by Aminoff[22] as modified by Ray.[20] Given the sensitivity of the Aminoff assay, as little as 500 nM KDO 8-P would have been readily detectable. Second, chorismic acid was incubated with KDO 8-P synthase while monitoring both the disappearance of chorismate and the production of prephenate.[23,24] Finally, a CM catalytic antibody (DMH6 1F7, raised against **13**, a transition state analogue)[25] was incubated with PEP and A5P and KDO 8-P production was monitored as above. It was hoped that the active site flexibility of this antibody, while not a particularly efficient catalyst of the CM reaction (10^4 times slower than CM itself), would allow it to bind A5P and PEP in the appropriate conformation for the cyclic reaction. No cross-reactivity was observed in any of these experiments, suggesting that the intermediates in these two reactions do not closely resemble one another, and that the cyclic mechanism may not be operative in KDO 8-P synthase.

Although **12** was not intentionally designed as a CM inhibitor, the unexpected results with this compound are interesting, especially its ability to bind KDO 8-P synthase in the absence of a phosphate ester on the primary carbon. However, the reversal of inhibition by **12** can be explained. It has been known for some time that the active site of *E. coli* KDO 8-P synthase contains a mechanistically important cysteine residue.[26] This Cys

Table 1. Inhibition of KDO 8-P synthase

Compd	IC_{50} (μM) KDO 8-P synthase
1	> 500
2	n.a.[a]
3	n.a.
4	n.a.
5	> 1000
6	n.a.
7	> 1000
8	> 1000
9	> 1000
10	> 1000
11	n.a.
12	400[b]

[a]n.a. = not active.
[b]Due to spectroscopic interference by **12**, data were only obtainable below 500 μM. Therefore, the IC_{50} for **12** was determined using only data between 50 and 500 μM.

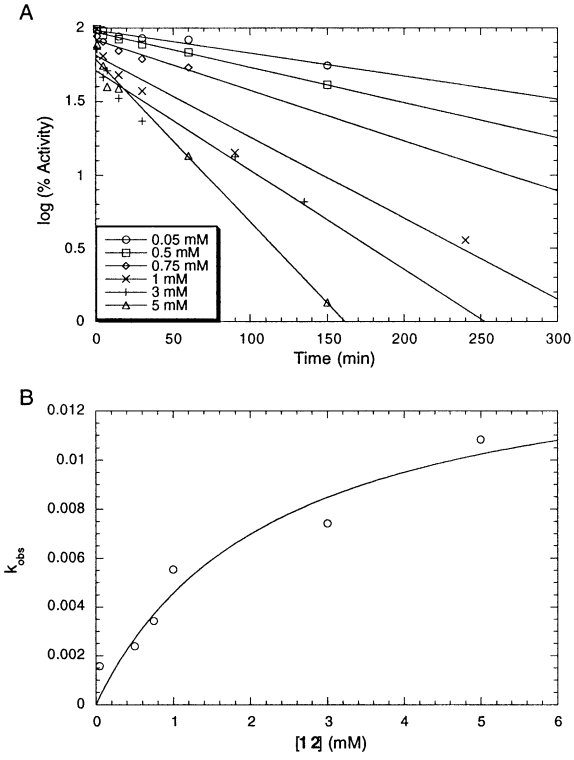


Figure 1. Inactivation of KDO 8-P synthase by **12**. (A) Time and concentration dependence of inactivation. Homogenous KDO 8-P synthase (~5 μM) was incubated with **12** for the indicated time and then assayed by diluting 5 μL of the enzyme–inhibitor solution into 995 μL of 100 mM Tris–HCl (pH 7.5), 150 μM PEP, and 150 μM A5P at 37 °C. The change in absorbance (λ = 232 nm) was monitored; (B) Hyperbolic plot of observed inactivation rates (k_{obs}) versus concentration of **12**. From the fit of the hyperbolic plot: $K_i = 2.3$ nM and $k_{inact} = 0.01$ min^{-1}.

could form a disulfide bond with an appropriately bound thiolate. The formation of this disulphide could be accomplished though a resonance form of the thioamide. Any disulphide would then be easily reduced by the addition of DTT or TCEP.

The inactivation of KDO 8-P synthase by **12** is interesting for several reasons: its resemblance to the linear (acyclic) intermediate, the absence of any phosphate moieties, and the potential covalent nature of the inhibition. The extended linear nature of **12** once again suggests that the mechanism of KDO 8-P synthase is acyclic. In all of the substrate-like inhibitors thus far, a phosphate or phosphate isostere has been mandatory. The fact that **12** is able to bind to KDO 8-P synthase in the absence of this phosphate suggests that other non-phosphorylated structures may bind to and inhibit KDO 8-P synthase. Elimination of a phosphate requirement would vastly ease design of a bioavailable substrate analogue or transition state analogue.

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References and Notes

1. Walsh, C. T.; Benson, T. E.; Kim, D. H.; Lees, W. J. *Chem. Biol.* **1996**, *3*, 93.
2. Bondinell, W. E.; Vnek, J.; Knowles, P. F.; Sprecher, M.; Sprinson, D. B. *J. Biol. Chem.* **1971**, *246*, 6191.
3. Brown, E. D.; Marquardt, J. L.; Lee, J. P.; Walsh, C. T.; Anderson, K. S. *Biochemistry* **1994**, *33*, 10638.
4. DeLeo, A. B.; Dayan, J.; Sprinson, D. B. *J. Biol. Chem.* **1973**, *248*, 2344.
5. Hedstrom, L.; Abeles, R. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 816.
6. Dotson, G. D.; Dua, R. K.; Clemens, J. C.; Wooten, E. W.; Woodard, R. W. *J. Biol. Chem.* **1995**, *270*, 13698.
7. D'Souza, F. W.; Benenson, Y.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2457.
8. Du, S.; Tsipori, H.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2469.
9. Liang, P.-H.; Kohen, A.; Baasov, T.; Anderson, K. S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2463.
10. Duewel, H. S.; Radaev, S.; Wang, J.; Woodard, R. W.; Gatti, D. L. *J. Biol. Chem.* **2001**, *276*, 8393.
11. Sheffer-Dee-Noor, S.; Belakhov, V.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1583.
12. Kohen, A.; Belakhov, V.; Baasov, T. *Tetrahedron Lett.* **1994**, *35*, 3179.
13. Baasov, T.; Kohen, A. *J. Am. Chem. Soc.* **1995**, *117*, 6165.
14. Radaev, S.; Dastidar, P.; Patel, M.; Woodard, R. W.; Gatti, D. L. *J. Biol. Chem.* **2000**, *275*, 9476.
15. Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J. Mol. Biol.* **1990**, *215*, 403.
16. Husain, A.; Galopin, C. C.; Zhang, S.; Pohnert, G.; Ganem, B. *J. Am. Chem. Soc.* **1999**, *121*, 2647.
17. *N*-Butyl-D-gluco(thio)amide **12** was synthesized from the known *N*-butyl-D-gluconamide by reaction with trimethylsilyl chloride, followed by Lawesson's reagent and hydrolysis in methanolic 1 M HCl.
18. Sheflyan, G. Y.; Howe, D. L.; Wilson, T. L.; Woodard, R. W. *J. Am. Chem. Soc.* **1998**, *120*, 11027.
19. Rieger, C. E.; Lee, J.; Turnbull, J. L. *Anal. Biochem.* **1997**, *246*, 86.
20. Ray, P. H. *J. Bacteriol.* **1980**, *141*, 635.
21. Hilvert, D.; Carpenter, S. H.; Nared, K. D.; Auditor, M. T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4953.
22. Aminoff, D. *Biochem. J.* **1961**, *81*, 384.
23. Christendat, D.; Turnbull, J. *Biochemistry* **1996**, *35*, 4468.
24. Koch, G. L. E.; Shaw, D. C.; Gibson, F. *Biochim. Biophys. Acta* **1970**, *212*, 375.
25. Kast, P.; AsifUllah, M.; Hilvert, D. *Tetrahedron Lett.* **1996**, *37*, 2691.
26. Salleh, H. M.; Patel, M. A.; Woodard, R. W. *Biochemistry* **1996**, *35*, 8942.