USING SUBSTRATE ANALOGUES TO PROBE THE MECHANISMS OF TWO BIOSYNTHETIC ENZYMES

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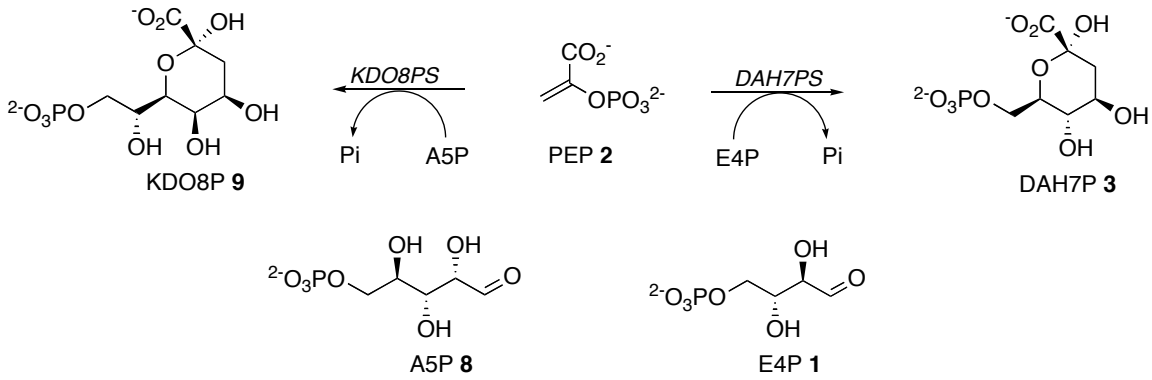
ABSTRACT

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) synthase and 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase (KDO8P) synthase are two enzymes that catalyse very similar reactions. DAH7P synthase is the first enzyme of the shikimate pathway and catalyses the condensation reaction between the four-carbon sugar erythrose 4-phosphate (E4P) **1** and the three-carbon sugar phosphoenolpyruvate (PEP) **2** to give the seven-carbon sugar DAH7P **3**. KDO8P synthase catalyses a similar condensation reaction between the five-carbon sugar arabinose 5-phosphate (A5P) **8** and PEP **2** to give the eight-carbon sugar KDO8P **9**. Early mechanistic studies have shown the reaction mechanisms of these two enzymes to be very similar and structural and phylogenic analysis has suggested that the two enzymes share a common ancestor.



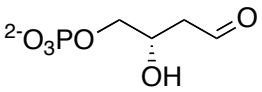
However, there are differences between the two enzymes that have not been explained by the current literature. Whereas all DAH7P synthases require a divalent metal ion for activity, there exists both metallo and non-metallo KDO8P synthases. As well as this, there is the difference in substrate specificity. The natural substrate of KDO8P synthase, A5P, is one carbon longer and has the opposite C2 stereochemistry to E4P, the natural substrate of DAH7P synthase.

This study investigates the role of the C2 and C3 hydroxyl groups of E4P and A5P in the enzyme catalysed reactions. The E4P analogues 2-deoxyE4P **38** and 3-deoxyE4P **39** have been synthesised from β-hydroxy-γ-butyrolactone and malic acid respectively. The two analogues were tested as substrates for DAH7P synthase from a variety of organisms, including *N. meningitidis*, the purification and characterisation of which was

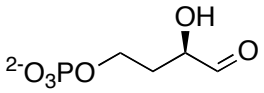
carried out during the course of these studies. It was found that both analogues were substrates for DAH7P synthase. 2-DeoxyE4P was found to be the best alternative substrate for DAH7P synthase to date.

The analogous study was carried out on KDO8P synthase from *N. meningitidis* with 2-deoxyR5P **34** and 3-deoxyA5P **40**. It was found that removal of the C2 and C3 hydroxyl groups of A5P was much more catastrophic for the KDO8P synthase catalysed reaction. Commercially available 2-deoxyR5P was found to be a very poor substrate, whereas 3-deoxyA5P, which was prepared according to a literature procedure was not a substrate.

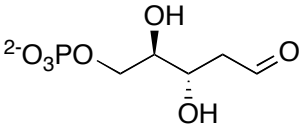
The difference in substrate specificities of DAH7P synthase and KDO8P synthase is consistent with the hypothesis that despite their similarities, these two related enzymes have different mechanisms. The key step for DAH7P synthase appears to be coordination of the E4P carbonyl to the divalent metal. The metal appears to play a less important role in the KDO8P synthase reaction and the key step is the correct orientation of A5P in the active site.



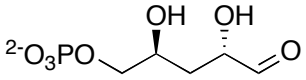
2-DeoxyE4P **38**



3-DeoxyE4P **39**



2-DeoxyR5P **34**



3-DeoxyA5P **40**

ACKNOWLEDGEMENTS

I owe the greatest thanks to my supervisor, Emily Parker, whose stubborn refusal to give up on me saw me follow this project through to the end. Her enthusiasm for her work and dedication to her students is inspirational.

Thanks to Linley Schofield for teaching me about protein purification and of course, for the chocolate cake! Many thanks also to the various members of the 'Shikimate Group' who have come and gone over the years and who have contributed to this project in various different ways. I owe particular thanks to the biochemists, who supplied the purified enzymes that were used in these studies, Dr Linley Schofield, Dr Fiona Cochrane and Dr Celia Webby.

Finally, thanks to my husband Mark, who has been incredibly patient and supportive through all my years of studying and has always been so proud of me.

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ABBREVIATIONS

A5P	arabinose 5-phosphate
AEC	anion exchange chromatography
ATP	adenosine triphosphate
BSA	bovine serum albumin
BTCA	benzyltrichloroacetimidate
BTP	1,3-(tris(hydroxymethyl)-methylamino)propane
CSA	camphor sulfonic acid
Conc.	concentrated
Da	dalton
DAH7P	3-deoxy-D-*arabino*-heptulosonate-7-phosphate
DAST	diethylaminosulfurtrifluoride
DIBAL	diisobutylaluminium hydride
DMF	N, N-dimethylformamide
E4P	erythrose-4-phosphate
EDTA	ethylenediaminetetraacetic acid
EPSP	5-enolpyruvyl shikimate 3-phosphate
EtOAc	ethyl acetate
EtOH	ethanol
FPLC	fast protein liquid chromatography
G3P	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
Hex	hexane
HIC	hydrophobic interaction chromatography
IPTG	isopropyl-1-thio-β-D-galactopyranoside
K_{cat}	turnover number
KDO	3-deoxy-D-*manno*-octulosonic acid
KDO8P	3-deoxy-D-*manno*-octulosonate 8-phosphate
K_i	inhibition constant
K_M	Michaelis constant
L5P	lyxose 5-phosphate
LAH	lithium aluminum hydride

LB	luria broth
MWCO	molecular weight cut-off
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
2-PGA	2-phosphoglyceric acids
Phe	phenylalanine
P_i	inorganic phosphate
pI	isoelectric point
ppm	parts per million
R5P	ribose 5-phosphate
R_F	retention factor
Rpm	revolutions per minute
Sat.	saturated
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
T4P	threose 4-phosphate
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
THF	tetrahydrofuran
TLC	thin layer chromatography
Trp	tryptophan
Tyr	tyrosine
UV	ultra-violet

DAH7P synthase	3-deoxy-D-*arabino*-heptulosonate-7-phosphate
EPSP synthase	5-enolpyruvyl shikimate 3-phosphate synthase
G6P dehydrogenase	glucose 6-phosphate dehydrogenase
KDO8P synthase	3-deoxy-D-*manno*-octulosonate 8-phosphate synthase

A. aeolicus	*Aquifex aeolicus*
A. pyrophilus	*Aquifex pyrophilus*
B. subtilis	*Bacillus subtilis*
C. psittaci	*Chlamydia psittaci*
E. coli	*Escherichia coli*
H. pylori	*Helicobacter pylori*
M. tuberculosis	*Mycobacterium tuberculosis*
N. crassa	*Neurospora crassa*
N. gonorrhoeae	*Neisseria gonorrhoeae*
P. furiosus	*Pyrococcus furiosus*
S. typhimurium	*Salmonella typhimurium*
S. cerevisiae	*Saccharomyces cerevisiae*
T. maritima	*Thermotoga maritima*
N. meningitidis	*Neisseria meningitidis*

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PUBLICATIONS

Parts of this thesis have been published in the following publications:

Ahn, M.; Pietersma, A. L.; Schofield, L. R.; Parker, E. J., Mechanistic divergence of two closely related aldol-like enzyme-catalysed reactions. *Organic & Biomolecular Chemistry* **2005,** 3, (22), 4046-4049.

Williamson, R. M.; Pietersma, A. L.; Jameson, G. B.; Parker, E. J., Stereospecific deuteration of 2-deoxyerythrose 4-phosphate using 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. *Bioorganic & Medicinal Chemistry Letters* **2005,** 15, (9), 2339-2342.