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USING SUBSTRATE ANALOGUES TO PROBE THE MECHANISMS OF TWO BIOSYNTHETIC ENZYMES

A thesis presented in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Chemistry

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New Zealand

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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 phylogenetic 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.

![Chemical structures](image-url)
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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<td>arabinose 5-phosphate</td>
</tr>
<tr>
<td>AEC</td>
<td>anion exchange chromatography</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BTCA</td>
<td>benzyltrichloroacetimidate</td>
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<td>BTP</td>
<td>1,3-(tris(hydroxymethyl)-methylamino)propane</td>
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<td>CSA</td>
<td>camphor sulfonic acid</td>
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<td>concentrated</td>
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<td>DAH7P</td>
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<td>DAST</td>
<td>diethylaminosulfurtrifluoride</td>
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<td>DIBAL</td>
<td>diisobutylaluminium hydride</td>
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<tr>
<td>DMF</td>
<td>N, N-dimethylformamide</td>
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<tr>
<td>E4P</td>
<td>erythrose-4-phosphate</td>
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<td>L5P</td>
<td>lyxose 5-phosphate</td>
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<td>LAH</td>
<td>lithium aluminum hydride</td>
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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
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PUBLICATIONS

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