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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

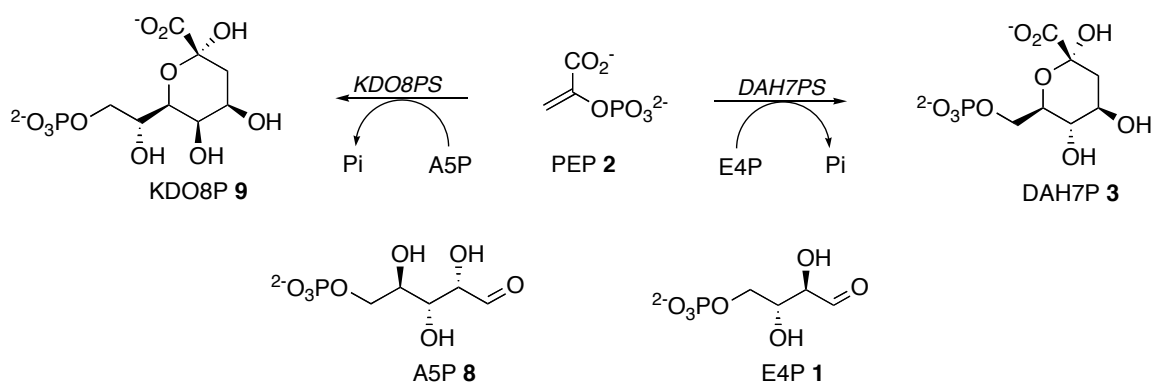
**Doctor of Philosophy
In
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ABSTRACT

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.



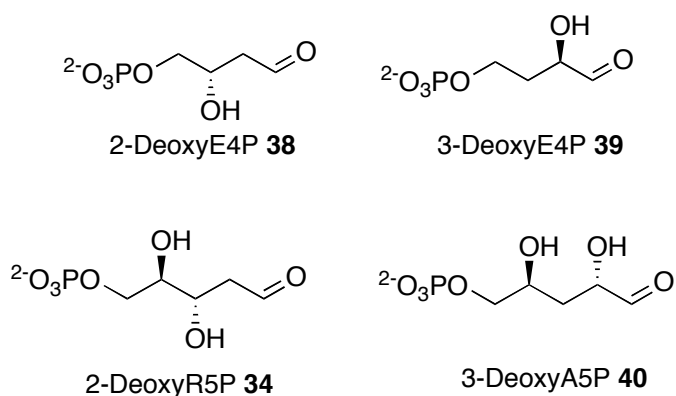
However, there are differences between the two enzymes that have not been explained by the current literature. Whereas all DAH7P synthases require a divalent metal ion for activity, there exists both metallo and non-metallo KDO8P synthases. As well as this, there is the difference in substrate specificity. 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.



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TABLE OF CONTENTS

ABBREVIATIONS.....	viii
INDEX OF FIGURES	xi
INDEX OF TABLES	xvi

CHAPTER ONE: INTRODUCTION

1.1	The shikimate pathway.....	1
1.2	3-Deoxy-D- <i>arabino</i> -heptulosonate 7-phosphate synthase.....	2
1.3	3-Deoxy-D- <i>manno</i> -octulosonate 8-phosphate synthase.....	2
1.4	Classification	3
1.5	Mechanism.....	5
1.6	Metal activation	
	1.6.1 DAH7P synthase.....	9
	1.6.2 KDO8P synthase.....	11
1.7	Structural analysis	
	1.7.1 Type I α	12
	1.7.2 Type I β_D	15
	1.7.3 Type I β_K (non metallo)	17
	1.7.4 Type I β_K (metallo)	17
	1.7.5 Type II.....	19
1.8	Structural analysis and the implications for the catalytic mechanism	20
1.9	Regulation	22
1.10	Inhibition	23
1.11	Substrate specificity	
	1.11.1 DAH7P synthase.....	26
	1.11.2 KDO8P synthase.....	29
1.12	Outline of thesis.....	30

CHAPTER TWO: PURIFICATION AND CHARACTERISATION OF A TYPE I α DAH7P SYNTHASE FROM *NEISSERIA MENINGITIDIS*

2.1	Introduction.....	31
2.2	Cloning and expression.....	32
2.3	Purification	
2.3.1	Purification by Ion Exchange Chromatography (IEC)	33
2.3.2	Purification by Hydrophobic Interaction Chromatography	34
2.3.3	Summary	37
2.4	Molecular mass determination	38
2.5	Initial kinetic parameters	40
2.6	Metal Dependency.....	41
2.7	Temperature studies	43
2.8	Feedback inhibition studies.....	44
2.9	Substrate specificity	45
2.10	Summary.....	46

CHAPTER THREE: EVALUATION OF 2-DEOXYE4P AND A5P ANALOGUES WITH DAH7P AND KDO8P SYNTHASES

3.1	Introduction.....	48
3.2	Use of γ-butyrolactones to synthesise E4P analogues.....	49
3.3	Synthesis of (S)-2-deoxyE4P from β-hydroxy-γ-butyrolactone.....	50
3.4	Enzymatic reaction of (S)-2-deoxyE4P with DAH7P synthase.....	54
3.5	Analysis of the product formed by the reaction of 2-deoxyE4P and PEP	56
3.6	Determination of the utilisation of racemic 2-deoxyE4P by <i>E. coli</i> DAH7P synthase	59
3.7	Initial kinetic parameters of 2-deoxyE4P with DAH7P synthase from various organisms	
3.7.1	<i>E. coli</i> DAH7P synthase	63
3.7.2	<i>N. meningitidis</i> DAH7P synthase	65
3.7.3	<i>P. furiosus</i> DAH7P synthase	66

3.7.4	<i>M. tuberculosis</i> DAH7P synthase.....	67
3.8	Stereospecific deuteration by DAH7P synthase.....	68
3.9	Initial kinetic parameters of 2-deoxyR5P with KDO8P synthase.....	69
3.10	Summary.....	71

CHAPTER FOUR: SYNTHESIS AND EVALUATION OF 3-DEOXYE4P AND 3-DEOXYA5P WITH DAH7P AND KDO8P SYNTHASES

4.1	Introduction.....	73
4.2	Synthesis of 3-deoxyE4P	
4.2.1	Previous investigations into the synthesis of 3-deoxyE4P	74
4.2.2	Synthesis of 3-deoxyE4P from α -hydroxy- γ -butyrolactone.....	75
4.2.3	Synthesis of 3-deoxyE4P from malic acid.....	78
4.3	Initial kinetic parameters of 3-deoxyE4P with DAH7P synthase from various organisms	
4.3.1	<i>E. coli</i> DAH7P synthase (phe).....	83
4.3.2	<i>N. meningitidis</i> DAH7P synthase	85
4.3.3	<i>P. furiosus</i> DAH7P synthase	86
4.3.4	<i>M. tuberculosis</i> DAH7P synthase.....	87
4.4	Proof of product formation.....	88
4.5	Use of erythronic lactone to synthesise fluorinated E4P analogues	89
4.6	Synthesis of 3-deoxyA5P.....	95
4.7	Investigation of methyl 2,3-anhydro-D-<i>lyxo</i>-furanoside as a precursor to C3-fluorinated A5P analogues	100
4.8	Summary.....	104

CHAPTER FIVE: MECHANISTIC INSIGHT INTO DAH7P AND KDO8P SYNTHASES

5.1	Introduction.....	105
5.2	The role of the E4P hydroxyl groups in DAH7P synthase	
5.2.1	Role of the C2-hydroxyl of E4P	106
5.2.2	Role of the C3-hydroxyl of E4P	112
5.3	The role of the A5P hydroxyl groups in KDO8P synthase	
5.3.1	Role of the C2-hydroxyl of A5P.....	115
5.3.2	Role of the C3-hydroxyl of A5P.....	116
5.4	Mechanism of DAH7P and KDO8P synthases.....	117
5.5	Summary and future directions.....	121

CHAPTER SIX: EXPERIMENTAL METHODS

6.1	General biochemical methods.....	123
6.2	General chemical methods	126
6.3	Experimental methods for chapter two	128
6.4	Experimental methods for chapter three.....	132
6.5	Experimental methods for chapter four	139

REFERENCES	157
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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- <i>arabino</i> -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- <i>manno</i> -octulosonic acid
KDO8P	3-deoxy-D- <i>manno</i> -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	<i>tert</i> -butyldimethylsilyl
TBDPS	<i>tert</i> -butyldiphenylsilyl
THF	tetrahydrofuran
TLC	thin layer chromatography
Trp	tryptophan
Tyr	tyrosine
UV	ultra-violet

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

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

INDEX OF FIGURES

Figure		Page
1.1	The seven enzyme-catalysed reactions of the shikimate pathway	1
1.2	The reaction catalysed by DAH7P synthase	2
1.3	The reaction catalysed by KDO8P synthase	3
1.4	The steric course of the DAH7P synthase reaction between E4P and PEP	6
1.5	Two proposed mechanisms for DAH7P synthase catalysis	7
1.6	Proposed cyclic mechanism for the formation of KDO8P	8
1.7	Phosphonate analogue 14 of proposed cyclic intermediate 13	8
1.8	Comparison of the quaternary structures of <i>E. coli</i> DAH7P synthase (phe) and <i>S. cerevisiae</i> DAH7P synthase (tyr)	13
1.9	<i>E. coli</i> DAH7P synthase (phe) monomer structure	14
1.10	PEP binding site of <i>E. coli</i> DAH7P synthase (phe)	15
1.11	Monomer structure of <i>P. furiosus</i> DAH7P synthase	16
1.12	Comparison of the active sites of <i>Aquifex aeolicus</i> KDO8P synthase and <i>P. furiosus</i> DAH7P synthase	18
1.13	Borohydride reduction of KDO8P	23
1.14	Inhibitors for KDO8P synthase	24
1.15	Modified KDO8P synthase inhibitors	25
1.16	Isosteric phosphonate inhibitor for KDO8P synthase	25
1.17	Amino phosphonate inhibitor for DAH7P synthase	26
1.18	PEP analogues tested as substrates for <i>E. coli</i> (phe) DAH7P synthase	27
1.19	Phosphonate and homophosphophonate E4P analogues	27
1.20	E4P analogues tested as substrates for <i>E. coli</i> DAH7P synthase (phe)	28
1.21	Phosphonate analogue of PEP	29
2.1	Chromatogram of AEC using Source 15Q [®] column	34
2.2	Chromatogram trace of HIC, using Source Phe [®] column	35

2.3	SDS-PAGE analysis of the stages of purification of <i>N. meningitidis</i> DAH7P synthase	36
2.4	SDS-PAGE analysis of <i>N. meningitidis</i> DAH7P synthase, before and after size-exclusion chromatography	37
2.5	Standard curve of log molecular mass versus elution time for <i>N.meningitidis</i> DAH7P synthase	39
2.6	Native PAGE analysis of <i>N. meningitidis</i> DAH7P synthase and <i>E. coli</i> DAH7P synthase	40
2.7	Michaelis-Menten plots for determination of K_M values for E4P and PEP with <i>N. meningitidis</i> DAH7P synthase	41
2.8	Effect of temperature on specific activity of purified <i>N. meningitidis</i> DAH7P synthase	43
2.9	Partial sequence alignment of <i>S. cerevisiae</i> (phe), <i>S. cerevisiae</i> (tyr), <i>E. coli</i> (tyr), <i>E. coli</i> (phe) and <i>N. meningitidis</i> DAH7P synthases	44
2.10	Phosphorylated monosaccharides tested as substrates for <i>N. meningitidis</i> DAH7P synthase	46
3.1	Synthesis of racemic 2-deoxyE4P	48
3.2	Potential E4P analogue products from γ -butyrolactones	49
3.3	Synthesis of (<i>S</i>)-2-deoxyE4P	50
3.4	The six membered ring product 47 with the primary alcohol exposed	52
3.5	The two possible five-membered ring products 50 from the protection of the aldehyde of 46	52
3.6	¹ H NMR spectra of DAH7P and 5-deoxyDAH7P	57
3.7	The two ways that 5-deoxyDAH7P could be formed by reaction of 2-deoxyE4P with PEP	58
3.8	Glucose-6-phosphate dehydrogenase assay	60
3.9	Assays of enantiopure and racemic 2-deoxyE4P with <i>P. furiosus</i> DAH7P synthase	61

3.10	Assays following the loss of PEP at 232nm in the presence of enantiopure and racemic 2-deoxyE4P with <i>E. coli</i> DAH7PS (phe)	63
3.11	Michaelis–Menten and Lineweaver-Burk plots for determination of K_M values for racemic 2-deoxyE4P and PEP in the presence of racemic 2-deoxyE4P for <i>E. coli</i> DAH7P synthase (phe)	64
3.12	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M values for (<i>S</i>)-2-deoxyE4P and PEP in the presence of (<i>S</i>)-2-deoxyE4P for <i>E. coli</i> DAH7P synthase (Phe)	65
3.13	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M value for (<i>S</i>)-2-deoxyE4P with <i>N. meningitidis</i> DAH7P synthase	66
3.14	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M values for (<i>S</i>)-2-deoxyE4P and PEP in the presence of (<i>S</i>)-2-deoxyE4P for <i>M. tuberculosis</i> DAH7P synthase	67
3.15	^1H NMR spectra of 5-deoxyDAH7P and (<i>S</i> S)-[5- ^2H]-5-deoxyDAH7P	68
3.16	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M value for 2-deoxyR5P with <i>N. meningitidis</i> KDO8P synthase	71
4.1	Outline of the strategy used by Dr Rost to synthesise 3-deoxyE4P	75
4.2	Synthesis of 3-deoxyE4P from α -hydroxy- γ -butyrolactone	76
4.3	The possible products from the aldehyde protection reaction and their phosphorylated products	77
4.4	Synthesis of 3-deoxyE4P from malic acid	79
4.5	^1H NMR spectra showing the deprotection of the dimethylacetal to 3-deoxyE4P	81
4.6	Potential products from the commercially available isomers of malic acid	82
4.7	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M value for racemic 3-deoxyE4P with <i>E. coli</i> DAH7P synthase (phe)	84
4.8	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M value for (<i>R</i>)-3-deoxyE4P with <i>E. coli</i> DAH7P synthase (phe)	85

4.9	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M value for (<i>R</i>)-3-deoxyE4P with <i>N. meningitidis</i> DAH7P synthase	86
4.10	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M values for (<i>R</i>)-3-deoxyE4P and PEP in the presence of (<i>R</i>)-3-deoxyE4P with <i>P. furiosus</i> DAH7P synthase	87
4.11	Michaelis–Menten and Lineweaver-Burk plots for determination of the K_M values for (<i>R</i>)-3-deoxyE4P and PEP in the presence of (<i>R</i>)-3-deoxyE4P with <i>M. tuberculosis</i> DAH7P synthase	88
4.12	Thiobarbituric acid test	89
4.13	Potential route to fluorinated E4P analogues	90
4.14	Elimination product 86 from the benzylation of erythronic lactone in DMF	92
4.15	Fluorination of α -hydroxy- γ -butyrolactone with DAST	92
4.16	^{19}F NMR spectra of 82 and 83	93
4.17	Protection of erythronic lactone with TBDMSCl	94
4.18	Synthesis of 2,3-anhydro-D- <i>lyxo</i> -furanoside from D-xylose	95
4.19	Potential products from the epoxide opening of 94 by the hydride ion	97
4.20	^1H NMR spectrum of the α -anomer of 95	98
4.21	Synthesis of 3-deoxyA5P	99
4.22	^1H NMR spectra of 99 and 3-deoxyA5P 40	100
4.23	Methyl 2,3-anhydro-5- <i>O</i> -benzyl-D- <i>lyxo</i> -furanoside	101
4.24	Fluorination of epoxide 94 to give 101	102
4.25	^{19}F NMR of 101	102
4.26	Benzylation of 94 to give 100	103
5.1	The reactions catalysed by DAH7P and KDO8P synthases	105
5.2	Phosphorylated monosaccharides shown to be substrates for DAH7P synthase	106
5.3	Active site of <i>P. furiosus</i> DAH7P synthase with E4P modeled in, showing the two different conformations of Pro61	109
5.4	L-T4P	112
5.5	Proposed cyclic mechanism of DAH7P synthase	113
5.6	Active site of <i>P. furiosus</i> DAH7P synthase with E4P modeled in	114

5.7	Active site of <i>A. aeolicus</i> KDO8P synthase showing the C2-hydroxyl of A5P interacting with the metal ion <i>via</i> a Water molecule	117
5.8	Comparison of active sites and proposed (partial) reaction mechanisms for <i>A. aeolicus</i> KDO8P synthase and <i>P. furiosus</i> DAH7P synthase	121

INDEX OF TABLES

Table		Page
2.1	Two step purification procedure of <i>N. meningitidis</i> DAH7P synthase	32
2.2	Protein standard molecular masses and elution times from the Superdex S200 column	39
2.3	Kinetic parameters of characterised type I α DAH7P synthases	41
2.4	Activation of purified <i>N. meningitidis</i> DAHPS by various divalent metal ions	42
2.5	The effect of aromatic amino acids on the activity of <i>N. meningitidis</i> DAH7P synthase	45
3.1	Kinetic parameters for E4P and PEP with enzymes from various organisms	62
3.2	Kinetic parameters of racemic 2-deoxyE4P with <i>E. coli</i> DAH7P synthase (phe)	62
3.3	Kinetic parameters of (<i>S</i>)-2-deoxyE4P with DAH7P synthase from various organisms	62
3.4	Kinetic parameters of A5P and 2-deoxyR5P with KDO8P synthase from <i>E. coli</i> and <i>N. meningitidis</i>	70
4.1	Kinetic analysis of 3-deoxyE4P with DAH7P synthase from various organisms	83
5.1	Kinetic parameters of DAH7P synthase with four-carbon analogues of E4P	110
5.2	Kinetic parameters of DAH7P synthase with five-carbon analogues of E4P	111
5.3	Kinetic parameters of KDO8P synthase with five-carbon analogues of A5P	115

PUBLICATIONS

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

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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.