AN INVESTIGATION OF THE MECHANISM OF
SIALIC ACID SYNTHASE

Dave Simard
B.Sc. (Honours), Dalhousie University, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
Department of Chemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
October, 2004

© Dave Simard, 2004
Library Authorization

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Dave Simard
Name of Author (please print)

An Investigation of the Mechanism of Sialic Acid Synthase

Master of Science 2004

Department of Chemistry

The University of British Columbia

Vancouver, BC Canada
ABSTRACT

Sialic acid synthase (SAS) catalyzes the aldol-like condensation of phosphoenolpyruvate (PEP) and N-acetylmannosamine (ManNAc) resulting in the formation of N-acetylneuraminic acid (NeuNAc). The *neuB* gene, coding for SAS, was cloned out of the *Neisseria meningitidis* genome and inserted into the pET-30 Xa/LIC vector, thus adding a His-tag onto the gene product. The His-tagged protein (SASHIS) was purified and kinetically characterized using a novel continuous coupled UV assay. SAS is a slow enzyme as can be seen by its $k_{cat}$ of $0.8 \pm 0.1 \text{ s}^{-1}$. Furthermore, it binds to PEP more tightly than it does to ManNAc, $K_M = 0.25 \pm 0.07 \text{ mM}$ and $9.4 \pm 1.4 \text{ mM}$, respectively. These kinetic values for SASHIS are in reasonable agreement with literature values obtained with the wild type enzyme. The mechanism employed by SAS to catalyze the condensation reaction was unknown. Through NMR and $^{18}$O-labelled substrate experiments, it was shown that the synthase releases the phosphate of PEP through C-O bond cleavage during the condensation with ManNAc. Thus SAS uses a mechanism analogous to DAHP and KDO-8P synthases, other enzymes forming higher order sugars.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract .................................................. ii</td>
</tr>
<tr>
<td>Table of Contents ......................... iii</td>
</tr>
<tr>
<td>List of Figures................................. v</td>
</tr>
<tr>
<td>List of Tables ...................................... vii</td>
</tr>
<tr>
<td>List of Abbreviations ...................... viii</td>
</tr>
<tr>
<td>Acknowledgements ............................... xi</td>
</tr>
<tr>
<td>Chapter 1. An Introduction to Sialic Acid Synthase .............. 1</td>
</tr>
<tr>
<td>1.1. Higher Order Sugars ...................... 2</td>
</tr>
<tr>
<td>1.2. Sialic Acid and Its Roles in Nature .......... 4</td>
</tr>
<tr>
<td>1.3. Biosynthesis of Sialic Acid ............... 8</td>
</tr>
<tr>
<td>1.4. Characteristics of the Synthase ............ 10</td>
</tr>
<tr>
<td>1.5. Mechanisms of Related Enzymes ............. 12</td>
</tr>
<tr>
<td>1.5.1. Mechanism 1: C-O Bond Cleavage ........... 12</td>
</tr>
<tr>
<td>1.5.2. Mechanism 2: P-O Bond Cleavage .......... 14</td>
</tr>
<tr>
<td>1.6. Aims of this Thesis .......................... 17</td>
</tr>
<tr>
<td>Chapter 2. Expression, Purification, and Kinetic Characterization of the Enzyme ............. 19</td>
</tr>
<tr>
<td>2.1. Introduction ................................. 20</td>
</tr>
<tr>
<td>2.2. Expression of Wild Type Sialic Acid Synthase .......... 22</td>
</tr>
<tr>
<td>2.3. Purification ................................. 24</td>
</tr>
<tr>
<td>2.4. SASHIS ........................................ 28</td>
</tr>
<tr>
<td>2.5. UV Assays .................................... 35</td>
</tr>
<tr>
<td>2.6. Discussion ................................... 39</td>
</tr>
<tr>
<td>2.7. Experimental Methods .................... 40</td>
</tr>
<tr>
<td>2.7.1. Over Expression and Purification of SASHIS .... 40</td>
</tr>
<tr>
<td>2.7.2. NMR Experiments ......................... 43</td>
</tr>
<tr>
<td>2.7.3. Coupled UV Assay .......................... 44</td>
</tr>
</tbody>
</table>
Chapter 3. Synthesis of the Labelled Substrates

3.1. [2-\(^{18}\)O]-Phosphoenolpyruvate
   3.1.1. Ethyl [2-\(^{18}\)O]-3-bromopyruvate
   3.1.2. Ethyl 2-[(dimethoxyphosphinyl)^{18}O-oxy]propenoate
   3.1.3. Deprotection and Anion Exchange

3.2. Overall Synthesis

3.3. [3-\(^{2}\)H]-Phosphoenolpyruvate
   3.3.1. Dibromination Methods
   3.3.2. Ethyl 3,3-dibromopyruvate
   3.3.3. Synthesis of (E,Z)-3-bromo-2-[(dimethoxyphosphinyl)oxy]propenoate
   3.3.4. Additional Peaks Downfield

3.4. Experimental Methods
   3.4.1. Materials
   3.4.2. Ethyl [2-\(^{18}\)O]-3-bromopyruvate
   3.4.3. Ethyl 2-[(dimethoxyphosphinyl)^{18}O-oxy]-propenoate
   3.4.4. Deprotection and Anion Exchange
   3.4.5. 3,3-dibromopyruvate
   3.6.3. Ethyl 3,3-dibromopyruvate
   3.6.4. Ethyl (E,Z)-3-Bromo-2-[(dimethoxyphosphinyl)-oxy]propenoate

Chapter 4. Testing the Mechanism Using the Labelled Substrate

4.1. Results and Discussion

4.2. Experimental Methods
   4.2.1. Materials
   4.2.2. NMR Assay

References
LIST OF FIGURES

Figure 1. Higher order sugars formation .................................................. 3
Figure 2. Sugar linkages in capsules ......................................................... 6
Figure 3. Biosynthesis of NeuNAc ............................................................. 9
Figure 4. Cps gene complex ................................................................. 12
Figure 5. Mechanism 1: C-O bond cleavage ............................................. 13
Figure 6. Mechanism 2: P-O bond cleavage ............................................ 15
Figure 7. PEPCK-catalyzed reaction .................................................... 16
Figure 8. Sialic acid lyase-catalyzed formation of NeuNAc ...................... 17
Figure 9. Michaelis-Menten plot ......................................................... 22
Figure 10. SDS-PAGE gel of partially purified SAS .............................. 24
Figure 11. $^{31}$P NMR spectrum of SAS-catalyzed reaction with
2-phosphoglycerate ............................................................................. 25
Figure 12. Enolase-catalyzed reaction .................................................. 26
Figure 13. pET-30 Xa/LIC cloning protocol .......................................... 30
Figure 14. SDS-PAGE gel of SASHIS purification .............................. 32
Figure 15. $^{31}$P NMR spectra of SASHIS-catalyzed reaction ............... 33
Figure 16. $^1$H NMR spectra of SASHIS-catalyzed reaction ............. 34
Figure 17. $^{31}$P NMR spectra of modified SASHIS-catalyzed reactions 35
Figure 18. Representation of the coupled UV assay .......................... 36
Figure 19. Michaelis-Menten plots for the coupled assay results ........ 37
Figure 20. Lineweaver Burke plots for the coupled assay results ....... 38
Figure 21. Total synthesis of [2- $^{18}$O]-phosphoenolpyruvate ............ 47
Figure 22. (+) LSIMS of ethyl 2-[(dimethoxyphosphinyl)$^{18}$oxy]-
propenoate ......................................................................................... 49
Figure 23. (-) LSIMS of [2- $^{18}$O]-phosphoenolpyruvate
(cyclohexylammonium form) ............................................................... 50
Figure 24. $^1$H NMR spectrum of [2- $^{18}$O]-phosphoenolpyruvate
(sodium form) ................................................................................... 50
Figure 25. $^{31}$P NMR spectrum of [2-$^{18}$O]-phosphoenolpyruvate (cyclohexylammonium form) ........................................ 51

Figure 26. Total synthesis of [2-$^{18}$O]-phosphoenolpyruvate ................................................................. 52

Figure 27. Scheme of the attacks from the faces of PEP .............................................................................. 53

Figure 28. Total Synthesis of [3-$^2$H]-phosphoenolpyruvate ................................................................. 53

Figure 29. $^1$H NMR spectrum of 3,3-dibromopyruvate ................................................................................. 55

Figure 30. $^1$H NMR spectrum of ethyl 3,3-dibromopyruvate ........................................................................ 55

Figure 31. $^1$H NMR spectrum of ethyl ($E,Z$)-3-bromo-2-[(dimethoxyphosphinyl)oxy]propenoate ..................... 56

Figure 32. $^1$H NMR spectrum revealing unknown peaks .............................................................................. 58

Figure 33. $^{31}$P NMR spectra of SASHIS-catalyzed reaction in the presence of [2-$^{18}$O]-phosphoenolpyruvate ................................................................. 64

Figure 34. Zoom region of Figure 33 showing the shift in the free phosphate peak ........................................ 65

Figure 35. Mechanism employed by SAS ................................................................................................. 66
LIST OF TABLES

Table 1. Kinetic Properties of SASHIS.................................38
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-PG</td>
<td>2-phosphoglycerate</td>
</tr>
<tr>
<td>ACS</td>
<td>American chemical society</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2'-azobisisobutyronitrile</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine 5'-monophosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAHP</td>
<td>3-deoxy-D-arabino-heptulonate 7-phosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanidine 5'-triphosphate</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Deionized distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide 5'-triphosphate</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H₂¹⁸O</td>
<td>¹⁸Oxygen enriched water</td>
</tr>
<tr>
<td>HOD</td>
<td>Singly deuterated water</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kbp</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kₗₐₜ</td>
<td>Rate constant of catalytic step (turnover number)</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>KDN</td>
<td>2-keto-3-deoxy-D-glycero-D-galacto-nononic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>KDO</td>
<td>2-keto-3-deoxy-D-manno-D-octulosonic acid</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSIMS</td>
<td>Liquid Secondary Ion Mass Spectrometry</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Micro ($10^{-6}$)</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetylmannosamine</td>
</tr>
<tr>
<td>MHz</td>
<td>MegaHertz</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Nicotinamide adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPCK</td>
<td>PEP carboxykinase</td>
</tr>
<tr>
<td>PET</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAL</td>
<td>Sialic acid lyase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SAS</td>
<td>Sialic acid synthase</td>
</tr>
<tr>
<td>SASHIS</td>
<td>His-tagged SAS</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine 5'-diphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximal velocity</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Tanner, and my fellow labmates for their help and support during my research. I also want to thank Dr. Elena Polishchuk and Candice Martin for their encouragements. I wish to express my gratitude to Dr. Tanner for allowing me to pursue an intense cycling training and racing schedule while working in his lab.
CHAPTER 1:

AN INTRODUCTION TO
SIALIC ACID SYNTHASE
1. INTRODUCTION

1.1. HIGHER ORDER SUGARS

The common sugars, pentoses and hexoses, have five or six carbon skeletons, respectively. These sugars are mainly used as energy sources for the various metabolic processes occurring in most organisms (1). These simple sugars can bind together and form long chain carbohydrates, which usually serve as energy reserves. For instance, glucose molecules are combined during the synthesis of glycogen and stored in muscle tissue or in the liver (1). These long chain carbohydrates are referred to as complex sugars because their catabolism requires more time, thus they provide energy over a longer period of time.

Carbohydrates are also widely present in the structures which compose the outer layer of cells. While many of these structures, such as bacterial and viral capsules, are formed from the common sugars, some contain carbohydrates with more than six carbon atoms that are collectively known as the higher order sugars. The most recognized family of higher order sugars is the sialic acid family (2). The members of this family are nine carbon α-keto acids which are generated by the condensation of a six member ring sugar and phosphoenolpyruvate (PEP), thus forming neuraminic acid and its derivatives (2). N-acetyleneuraminic acid (NeuNAc) is the most common representative of this class of sugar which, when modified with various substituents, gives rise to most of the other members of the sialic acid family (Figure 1). It was recently shown that 2-keto-3-deoxy-D-glycero-D-
galacto-nononic acid (KDN), which differs from NeuNAc by the presence of a hydroxyl group at C5, also generates some members of this family (2). Higher order sugars are not exclusively nine carbon sugars. 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) is a

![Chemical Structures]

Figure 1. The reactions leading to the formation of four major higher order sugars. A) The condensation of ManNAc and PEP to form NeuNAc as catalyzed by sialic acid synthase (SAS) (5). B) The condensation of mannose-6-phosphate with PEP to yield KDN-9P as catalyzed by KDN-9P synthase (6). C) The reaction catalyzed by DAHP synthase (3). D) The condensation of arabinose-5-phosphate with PEP to form KDO-8P as catalyzed by KDO-8P synthase (4).
seven carbon sugar formed from the condensation of PEP with erythrose-4-phosphate (E4P) (3). On the other hand, 3-deoxy-d-manno-octulosonate 8-phosphate (KDO-8P), an eight carbon sugar, is generated by the condensation of arabinose-5-phosphate (A5P) and PEP (4).

These higher order carbohydrates share a lot of common features. All are α-keto acids and all arise from the condensation of the high energy molecule, PEP, with a simple sugar (Figure 1). It is known that DAHP synthase and KDO-8P synthase both utilize a similar mechanism, explained in Chapter 1.5, to catalyze these reactions (4, 7). Both NeuNAc and KDN are found at the distal ends of glycan chains anchored to the cell wall (2). DAHP is a precursor in the shikimate pathway from which originate the aromatic amino acids (8). KDO is found in the inner core of bacterial lipopolysaccharides (LPS); thus all these sugars serve a higher purpose than as an energy source (9).

1.2. SIALIC ACID AND ITS ROLES IN NATURE

The location of a molecule in an organism can usually give some indication of its function. The importance of a molecule can also be defined by the variety of derivatives obtained from it. Sialic acids are such a type of molecule. Unlike glucose, sialic acids are not ubiquitous in nature, they are mainly found in vertebrates and in certain types of bacteria (2). In the vast majority of cases, sialic acids occupy the distal end of the glycan chains on the surface of the organism. Hence sialic acids are present in the LPS and on lipooligosaccharides (LOS) in bacteria and on the glycolipids and glycoproteins attached to
the cell membranes of vertebrates (9). Furthermore, sialic acids are a class of sugar which contain close to 50 different members all of which are derivatives of NeuNAc or KDN (2).

From their location at the very end of the cells and the number of members in this class of sugar, it could be thought that they are involved in certain recognition processes. This is indeed the case. The recognition role played by these sugars depends on which organism they are found in, thus the function of sialic acids will be discussed in relation to specific classes of organisms.

Bacteria are interesting in terms of their sialic acid molecules since they have opposite functions depending on their location. The sialic acid molecules present in the LPS surrounding the bacterium and in the bacterial capsules show a fair number of modifications from the regular NeuNAc (10). These modifications could be differences in the linkage between two molecules in a chain of sugars or chemical additions onto the sugar hydroxyl groups. This allows these bacteria to evade infection by bacteriophages which identify and bind to a specific set of sugars (2). Sialic acid molecules found on the distal part of LOS try to mimic the glycolipids found on the cell wall of vertebrate cells (10). The function of these terminal sugars is to evade the primary immune response of the invaded organism. The most pathogenic bacteria are those which avoid triggering a host immune response. Hence, sialic acids in bacteria serve to protect them against invaders and to mask them while they are invading other organisms.
The capsule of *Neisseria meningitidis* (*N. meningitidis*) group B, the major cause of meningitis in humans, is composed of sugar molecules linked via α(2-8) or α(2-9) bonds with occasional O-acetylated NeuNAc sugars (Figure 2) (2). With such an external structure, this bacterium can avoid attacks by bacteriophages expressing the Endo-N enzyme which hydrolyzes α(2-8) linked polysialic acid chains, yet are unable to cleave α(2-9) linkages (11). On the other hand, having unmodified sugar molecules allows the bacterium to invade a vertebrate cell without triggering the immune system of invaded cells (2).

Bacterial infections of vertebrates are not the only infections utilizing sialic acid recognition. It has been shown that viruses infect cells by attaching viral lectins to the sialic acids on the cell membranes (12).

In vertebrates the role of sialic acids is also related to recognition. It is unclear how many pathways or processes are regulated by sialic acid recognition, but embryos lacking the
ability to express sialic acids die before the end of their development (2). Several immune responses, in mammals, are controlled by sialic acid recognition mechanisms. Complements attach and pierce any cell that is devoid of Factor H (13). This molecule binds to the terminal sialic acids of cells, thus labelling them as self molecules and preventing their destruction by complement. This is the process avoided by pathogenic bacteria whose LOS sialic acids mimic the glycolipids of vertebrate cells. The recycling of certain cells by macrophages occurs in an analogous fashion. The asialoglycoprotein receptor of the macrophage binds to cells having lost their terminal sialic acid residue during their aging process and engulfs them by phagocytosis (14). Selectins are another class of sialic acid recognizing molecules which are involved in the control of leucocytes and various other immune responses. Certain selectins direct leucocytes to inflammation sites while others direct them back to the lymph nodes (15). Siglecs (sialic acid-binding immunoglobulin superfamily lectins) are the largest class of sialic acid recognizing molecules in mammals. This class of molecules is directly involved in the immune response, since it controls such components as the monocytes, macrophages, NK cells and granulocytes (16).

Though all these molecules serve similar purposes, they act on different cells. This selectivity occurs because of their different lectins (2). Some lectins in the immune system show great specificity in their interactions. Interleukin-4 molecules, for instance, only interact with NeuNAC 1,7-lactone which is a bicyclic derivative of NeuNAc (17). Other lectins interact with broader specificities, such as those of Factor H, which may inadvertently recognize bacterial cells as self.
A faulty regulation of the production and delivery of sialic acids may lead to the development of serious illnesses. Sialidosis and sialic acid storage disorder, both are neurodegenerative disorders, occur if there is absence of lysosomal sialidases or deficiency in the sialic acid transporter (from the nuclei to the Golgi apparatus in vertebrates), respectively (18, 19). Sialuria, on the other hand, occurs when cytidine 5'-monophosphate-NeuNAc (CMP-NeuNAc) does not feedback-inhibit uridine 5'-diphosphate N-acetylg glucosamine (UDP-GlcNAc) 2-epimerase. This leads to the accumulation of NeuNAc in the cells causing acidosis and ultimately mental retardation (20). Recently, cancer cells were observed to have over abundant sialic acids on their surface. There appears to be a link between the sialic acid count on tumour cells and metastasis (21, 22).

1.3. BIOSYNTHESIS OF SIALIC ACID

The biosynthesis of NeuNAc is a fairly long process involving many enzymes (Figure 3). Mammals and bacteria do not produce this molecule in exactly the same way, in fact, the mammalian pathway requires more steps and more energy. The first committed step to the biosynthesis of NeuNAc in both bacteria and mammals is the conversion of UDP-GlcNAc to N-acetylmannosamine (ManNAc) by the enzyme UDP-GlcNAc 2-epimerase (23). In bacteria, the enzyme NeuNAc synthase (Sialic acid synthase, SAS) [E.C. 4.1.3.19] condenses ManNAc with PEP to form NeuNAc (5). However, mammals must phosphorylate ManNAc using ManNAc kinase to form ManNAc 6-phosphate which is then condensed with PEP by NeuNAc 9-phosphate synthase. The product, NeuNAc 9-phosphate, is then dephosphorylated
Figure 3. Biosynthesis of NeuNAc in both bacteria and mammals. ManNAc is produced by UDP-GlcNAc 2-epimerase (1) in both bacteria and mammals; mammalian ManNAc kinase (2) phosphorylates ManNAc; NeuNAc 9-phosphate synthase (3) condenses PEP and ManNAc-6P; NeuNAc phosphatase (4) cleaves the phosphate to yield NeuNAc; bacterial NeuNAc synthase (SAS, 5) condenses ManNAc and PEP directly; CMP-NeuNAc synthetase (6) activates the sugar by condensing it with a molecule of CTP.

by NeuNAc phosphatase to yield NeuNAc (24). The sugar, which was synthesized in the cytoplasm, now enters the nucleus where it is condensed with a CTP molecule by CMP-NeuNAc synthetase to be exported onto the outer portions of the cell wall. In mammals, the CMP-NeuNAc exits the nucleus and penetrates the Golgi via a specific transporter. The
activated sugar is then placed onto a sialyltransferase which delivers NeuNAc onto an elongating glycolipid or glycoprotein. This completed structure would then migrate to the cell surface (25).

1.4. CHARACTERISTICS OF THE SYNTHASE

Sialic acid synthase has been found in bacteria, mammals and viruses. Though they do not use the same substrates, the human enzyme, nonetheless, shares 36.1% sequence identity and 56.1% sequence similarity with the Escherichia coli (E. coli) enzyme (24). The Campylobacter jejuni (C. jejuni) SAS shares 58.7% sequence identity and 82.2% sequence similarity with that of N. meningitidis (26).

The mammalian enzyme is interesting because of its loose substrate selectivity. In addition to the regular substrates, ManNAc-6P and PEP, this enzyme can accept mannose-6-phosphate (Man-6P) and PEP, and generate NeuNAc-9P and KDN-9P, respectively (24, 27). This latter activity is not shared by all mammals, since studies on rat SAS did not reveal any KDN-9P formation (28). The rat SAS was, however, shown to be a dimeric enzyme with a divalent cation requirement (28). This cation requirement is shared with bacterial enzymes; SAS from N. meningitidis has a preference for manganese (5).

There may be differences in the structures of SAS from various bacteria since Hwang et al (29) claim to have identified, by LC-MS, a tetrameric structure for the E. coli enzyme.
On the other hand, Suryanti et al (30) used gel filtration and assigned a dimeric structure to the SAS of *Streptococcus agalactiae* (*S. agalactiae*) which shares 72 % sequence similarity with the *E. coli* enzyme.

The enzyme forming NeuNAc in *E. coli* was not fully identified until the late 1990s. It was thought that NeuNAc was generated by condensation of pyruvate with ManNAc as catalyzed by sialic acid lyase (31). Even though SAS had been isolated from many bacterial sources, it was only in 1997 that Vann et al (32) isolated it from *E. coli* K1 and gave evidence for a lyase-independent synthesis of NeuNAc. Blacklow and Warren were the only group to determine kinetic values for any bacterial SAS. They found the $K_M$ for PEP and ManNAc to be 0.042 mM and 6.5 mM, respectively, using a thiobarbituric assay on the enzyme from *N. meningitidis* (5).

The gene coding for SAS is designated by many different names in the literature. In this thesis, this gene will be referred to as *neuB*. This gene has slightly over 1000 base pairs (bp) in both bacteria and mammals: 1029 bp for *C. jejuni* (26), 1051 bp for *N. meningitidis* (33), and 1073 bp for humans (24). The *neuB* gene in *N. meningitidis* is found in region A of the 24-kbp cps gene cluster (Figure 4) (34). The three other genes located in this region code for capsule-producing proteins: *neuC* is the UDP-GlcNAc 2-epimerase (35), *neuA* is the CMP-NeuNAc synthetase (36), and *neuD* is the α(2-8)polysialyltransferase (37).
Figure 4. The cps gene complex and its five regions. The genes involved with the production of the capsular component are located in region A (34). The gene that is studied in this research is in bold, neuB.

From the neuB gene of *N. meningitidis*, the amino acid sequence for the protein can be identified. Hence SAS is 349 amino acids long and has a theoretical molecular weight of 38347 Da (33).

1.5. MECHANISM OF RELATED ENZYMES

1.5.1. MECHANISM 1: C-O BOND CLEAVAGE

The first mechanism that will be discussed is the mechanism that seems counter intuitive to the bioorganic chemist. In most reactions involving PEP, nature cleaves the phosphate ester bond to liberate about 14 kcal/mol of energy and thus drive the reaction to completion (9). However the enzymes, DAHP synthase and KDO-8P synthase, catalyze their respective reactions without cleaving the phosphate ester bond, but rather the C-O bond...
adjacent to it (4, 7). Based on the mechanisms proposed for the synthesis of DAHP and KDO-8P, a type 1 mechanism for the synthesis of NeuNAc would resemble the scheme illustrated in Figure 5. The first step is a nucleophilic attack by the alkene onto the aldehyde of the open chain ManNAc. This is immediately followed by addition of a water molecule to the oxocarbenium ion intermediate. The resulting tetrahedral intermediate will readily eliminate phosphate and spontaneous ring closure would give the product NeuNAc.

Figure 5. Inspired from the reactions of DAHP synthase and KDO-8P synthase, this is the illustration of the formation of NeuNAc if it happened via a C-O bond cleavage.
The evidence for this mechanism, in the case of KDO-8P (4) and DAHP (7) synthases, is based on a simple, yet interesting labelling experiment. Researchers synthesized PEP molecules with an $^{18}$O label at the bridging position (Figure 5). The labelled substrate was incubated with the enzyme and the phosphate produced was isolated. It was either derivatized to trimethylphosphate (4) or analyzed directly as potassium dihydrogen phosphate (7) by mass spectrometry. An increase of two atomic mass units confirmed the presence of one labelled oxygen on the free phosphate, thus supporting C-O bond cleavage. A modern method that could also be used is to monitor the reaction by $^{31}$P NMR spectroscopy. It is known that $^{16}$O- and $^{18}$O-isotopes in a phosphate group cause different chemical shifts in the phosphorus signal. A signal due to a molecule in which the phosphorus is attached to an $^{18}$O-isotope will be 0.02 ppm upfield from the signal caused by a molecule containing only $^{16}$O-isotopes on the phosphorus (38). As can be deduced from this reaction scheme (Figure 5), the labelled oxygen would remain on the phosphate, thus the free phosphate peak would be shifted upfield by 0.02 ppm in the $^{31}$P NMR spectrum. The use of this technique to investigate the mechanism of SAS will be described in Chapter 4.

1.5.2. MECHANISM 2: P-O BOND CLEAVAGE

The enzymes catalyzing the second mechanism utilize the cleavage of the high energy bond between phosphorus and oxygen in PEP to release a reactive enolate species. A hypothetical type 2 mechanism for the reaction catalyzed by SAS is represented in Figure 6. The first step is a nucleophilic attack by water onto the phosphorus atom of PEP. This results in the release of a pyruvate enolate ion that can attack the aldehyde of the open chain
ManNAc. The resulting open chain NeuNAc will spontaneously cyclize to the pyranose form. In this mechanism an $^{18}$O-label in the bridging position of PEP would be expected to end up in the sugar as opposed to the phosphate.

Figure 6. A hypothetical mechanism for the sialic acid synthase reaction that employs P-O bond cleavage.

Many enzymes utilize mechanisms involving attack at the phosphate of PEP and the release of pyruvate. Phosphoenolpyruvate carboxykinase (PEPCK) will be presented as an example. It is a vital regulatory enzyme and catalyzes the first committed step in the gluconeogenesis (39). In this pathway, PEPCK catalyzes the reversible decarboxylation and the phosphorylation of oxaloacetate to PEP in the presence of nucleotide triphosphate and divalent metals (39, 40) (Figure 7).
An enzyme that uses an aldol-addition strategy that is similar to the second step of mechanism 2 is sialic acid lyase (sialate-pyruvate lyase, E.C. 4.1.3.3). It catalyzes the reversible conversion of NeuNAc to ManNAc and pyruvate (31). This enzyme is found in both bacteria and mammals where it plays a role in free sialic acid regulation (41). Some bacteria, like *Clostridium perfringens* (*C. perfringens*), utilize sialidases to cleave terminal sialic acid on the surface of cells from invaded organisms. The free sialic acid is then transported through the bacterial cell wall, where sialic acid lyase cleaves it, and ManNAc can then be used as an energy source (42).

The sialic acid lyase isolated from *E. coli* K1 strain has a monomeric weight of 33 kDa and is thought to be a tetramer in solution (31). The affinity ($K_M$) of this enzyme for its substrates has been reported to be 7.7 mM for ManNAc, 8.3 mM for PEP and 4.8 mM for NeuNAc (31). The crystal structure of the sialic acid lyase from *Haemophilus influenzae* (*H. influenzae*) has been reported (43) and the mechanism of the enzyme has been identified (41). The first step is the formation of a Schiff base between the ketone group of pyruvate and lysine 161 (Figure 8). Tyrosine 133 deprotonates the methyl group of pyruvate to give a nucleophilic enamine. This undergoes an aldol-like attack onto the carbonyl of the open chain ManNAc. Hydrolysis of the imine linkage releases the open chain form of sialic acid.
which can spontaneously close to the pyranose form (41). This type of mechanism is typical of class 1 aldolases.

![Reaction mechanism](image)

**Figure 8.** Reaction mechanism for the formation of NeuNAc catalyzed by sialic acid lyase.

1.6. **AIMS OF THIS THESIS**

This research project was aimed at identifying the mechanism utilized by the enzyme, sialic acid synthase (SAS), for the condensation of phosphoenolpyruvate (PEP) with N-acetylmannosamine (ManNac). The project also aimed at kinetically characterizing the His-tagged version of the wild-type enzyme (SASHIS). Both goals required the synthesis of
labelled substrates, \(^{18}\)O-PEP and \([3,^{2}\text{H}]\)-PEP, and the development of a continuous UV spectrophotometric assay.
CHAPTER 2:

EXPRESSION, PURIFICATION, AND KINETIC CHARACTERIZATION OF THE ENZYME
2. EXPRESSION, PURIFICATION, AND KINETIC CHARACTERIZATION OF THE ENZYME

2.1. INTRODUCTION

Valuable mechanistic information can be obtained through good kinetic data. For instance, the binding and release order for substrates and products can be determined with specific types of kinetic reactions. These useful details are, however, often difficult to obtain. Basic information can nonetheless be obtained by plotting the rate of an enzymatic reaction versus the substrate concentration. This type of figure is called a Michaelis-Menten plot, which provides the maximal velocity of the reaction ($V_{\text{max}}$) and the enzyme's affinity for a substrate ($K_M$) (44). Equation 1 depicts a one substrate, one product reaction system,

$$
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_2} E + P
$$

(1)

where $E$ is the amount of free enzyme and $S$ is the substrate concentration. ES is the enzyme-substrate complex and $P$ is the concentration of products formed. There is one rate constant for each direction of the reversible reaction between free enzyme and substrate and the enzyme complex. On the other hand, the reaction between ES and the formation of free product is essentially irreversible. For such a system, the Michaelis-Menten equation
(Equation 2) relates the reaction velocity to the substrate and enzyme concentrations and to the Michaelis constant ($K_M$),

$$V = \frac{k_2[E][S]}{K_M + [S]}$$  \hspace{1cm} (2)

To comprehend this equation, the Michaelis constant has to be defined first. This constant is the sum of the rate constants for the disappearance of ES, $k_1$ and $k_2$, divided by the rate constant for the rate of formation of ES, $k_1$. Plotting the reaction velocity versus substrate concentration will generate the graph shown in Figure 9. The rate approaches $V_{\text{max}}$ at very high substrate concentrations, since $K_M$ becomes insignificant with respect to $[S]$ and the substrate concentrations in the numerator and denominator of Equation 2 cancel. $V_{\text{max}}$ is thus related to the rate of product formation and the total concentration of enzyme. $K_M$ is the substrate concentration required to reach half $V_{\text{max}}$ (44).

This chapter describes our work on the expression of the wild type *N. meningitidis* SAS expressed in *E. coli* cells and our attempts to purify the protein. The construction and purification of SASHIS, a modified enzyme onto which a His-tag was added is also discussed in this chapter. Finally, the development of a continuous, coupled assay for sialic acid synthase activity is presented.
2.2. EXPRESSION OF WILD TYPE SIALIC ACID SYNTHASE

Only one article had been published on the isolation of SAS from *N. meningitidis* (5). The article dated back to 1962, hence the expression and purification protocols mentioned in it were designed for exogenous proteins. To isolate small amounts of this enzyme, large amounts of cell cultures had to be grown and then purified. Ultimately, only 0.75 mg of pure SAS was obtained from eight litres of culture (5). Thus a recombinant SAS was created to allow for greater expression of the protein.

Over the past few decades, methodologies that enable one to over express any target protein have been developed (45). The gene coding for the desired protein is cloned into a
vector next to an inducible promoter region, then that plasmid is transformed into expression cells. Cell cultures containing the recombinant plasmid will continuously express the target protein upon addition of an inducer molecule. Over a short period of time, the target protein will accumulate in large quantities.

To obtain over expression of SAS, the \textit{neuB} gene was cloned out of the genome of \textit{N. meningitidis} and inserted into the pCWori+ vector by Dr. Wakarchuk of the NRC in Ottawa. This plasmid has a T4 promoter region slightly upstream of the inserted gene and a \textit{lacI} gene (46, 47). Transcription of mRNA is regulated by the binding of a repressor molecule onto the promoter region, thus preventing the assembly of RNA polymerase onto DNA. When the repressor is absent, RNA polymerase can bind to DNA and start transcribing all the genes that come downstream of the promoter region. The repressor binds to the promoter when it has its regular conformation. However, an inducer molecule can bind to the repressor and modify its conformation, thus hindering its attachment to the promoter region. The product of the \textit{lacI} gene is \(\beta\)-galactosidase which leads to the production of allolactose. This latter product is the inducer molecule in vivo (1). In vitro, isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) is the preferred inducing agent since it is not cleaved by \(\beta\)-galactosidase hence its concentration remains constant (1). Upon addition of IPTG, the \textit{neuB} gene is transcribed continuously and SAS accumulates as seen in lane 1 of the SDS-PAGE gel (Figure 10).
2.3. PURIFICATION

The enzyme obtained by this process did not have any tag for facile purification, thus a purification procedure had to be developed. The wild-type enzyme obtained from the pCWori+ plasmid was partially purified by running the crude lysate through a HiTrap Q HP (1 cm) anion exchange column and eluted using a step gradient of NaCl (0 to 0.4 M) in triethanolamine-HCl buffer pH 7.5. The column is a strong anion binder, thus negatively charged proteins stick to its sepharose resin (48). Based on SDS-PAGE gels, an over expressed protein of approximately 39 kDa and a less prominent 45 kDa protein remained after purification (Figure 10). The over expressed protein was SAS, but the 45 kDa band was unknown, thus only partial purification was obtained after running this column. Linear gradients and smaller fraction sizes were used in an attempt to remove this other protein without changing the purification method. This was not successful, yet NMR experiments were conducted with partially purified enzyme.

These preliminary $^{31}$P NMR experiments confirmed the activity of the recombinant SAS and revealed the identity of the 45 kDa band. When PEP and ManNAc were incubated...
in the presence of SAS, the PEP peak decreased with time and a peak corresponding to free phosphate increased with time (Figure 11). This activity was completely dependent on the presence of ManNAc, indicating it was due to SAS as opposed to a phosphatase impurity. However, when the partially purified protein was used in this reaction, a third peak was observed at 5.05 ppm (Figure 11).

Figure 11. $^{31}$P NMR spectrum of a SAS-catalyzed reaction using partially purified enzyme. The peak at 5.05 ppm was unexpected and was identified as 2-PG, a product of enolase activity.

This peak was similar to an unexpected peak observed during a KDO-8P synthase reaction which was assigned to 2-phosphoglycerate (2-PG) (49). Addition of commercial 2-PG to the reaction mixture led to an increase in the size of the peak at 5.05 ppm, thus
confirming the assignment. This unexpected peak could be caused by an enolase impurity, as previously observed (5), or by an ‘enolase-like’ activity as reported for KDO-8P synthase (49). Since, the 2-PG peak was not generated by every enzyme fraction, this suggested the presence of an impurity rather than an unexpected side reaction from SAS.

The *E. coli* enolase, or phosphoenolpyruvate hydratase, converts PEP to 2-PG in gluconeogenesis and catalyzes the reverse reaction in glycolysis (50) (Figure 12). The

![Figure 12. Reaction mechanism for the reversible reaction catalyzed by the enolase impurity in the partially purified enzyme extracts.](image)

formation of 2-PG by the enolase is problematic since it depletes the PEP stocks in our experiments. Therefore, the enzymes had to be completely purified.

SAS and the enolase could easily be separated from the other cytoplasmic proteins in one simple step using a short anion exchange column. Charge differences between SAS and the enolase may not have been perceived because of the short length of the HiTrap Q HP column, thus a longer column (56 cm) with a weak anion exchange resin (DE52) was used. Unfortunately, the proteins behaved similarly as on the smaller column and coeluted in the same 0.1 M NaCl fractions.
The next option was to attempt running the ion exchange column at different pH values. The crude lysate was loaded onto the HiTrap Q HP column at pH 6. At that pH, some residues are still protonated, thus some proteins will stick less on the anion exchange column. Based on the amino acid composition of SAS, the pH change should protonate the enzyme sufficiently for it to elute in the flow through or at very low salt concentrations. SAS and the enolase were both found in the flow through of the pH 6 column, so the enzymes still behaved similarly. The flow through was, nonetheless, exchanged into a pH 8.0 triethanolamine-HCl buffer, prior to being loaded onto a new HiTrap column. At pH 8.0, most residues are deprotonated and the proteins were found to adhere very tightly to the column. A linear salt gradient was used to elute the proteins, however, the proteins were found to co-elute under these conditions as well.

It was unclear, at the start of this experiment, what form SAS adopted in solution. The monomeric weight of the enolase was 45 kDa (50) and that of SAS was 39 kDa. The crystal structure of the \textit{E. coli} enolase and gel filtration experiments confirmed that the protein was a homodimer of about 90 kDa (50, 51). Size exclusion chromatography would easily separate the enolase from SAS if the latter was a monomer in solution. Sephadex G-75 Superfine was the resin used in an attempt to separate the two enzymes. This resin has the particularity of trapping molecules under 75 kDa, but anything larger would elute in the void volume. Thus, a monomeric SAS would be retained, while the enolase would rapidly elute from the column. When the column was run, however, it was found that both enzymes coeluted in the void volume, presumably because SAS is not monomeric.
When all the modern purification techniques fail to purify a protein, older methods may be of some help. Ammonium sulfate precipitation was a technique routinely used to selectively precipitate out proteins from solution. Salts have a greater charge ratio than proteins. Hence, salts attract water thus desolvating the proteins which aggregate and precipitate out of solution. Based on previous purification results, it was uncertain if this method would be successful. In fact, both enzymes were still present together in the highest salt concentration (60%) fraction. This step was, from then on, used on the crude lysate as a preliminary purification step before trying all the previous purification steps again. Unfortunately, this addition did not change the outcome of any purification method.

Having been unable to completely separate SAS from the enolase using traditional purification techniques, the addition of a His-tag was the next option utilized.

2.4. SASHIS

The progression of molecular biology has seen the development of tags with metal affinity which help in the purification of gene products. The most common tag is the His-tag, which is six consecutive histidine residues placed on the C or N termini of the protein. The tag is incorporated at the genetic level, where the gene is inserted into a specific vector which contains a set of base pairs coding for six adjacent histidines. This region is located
downstream of the start codon, thus upon mRNA translation, the novel protein would have an additional short peptide tail terminating in six histidine amino acids (52).

Cloning the neuB gene into the pET-30 Xa/LIC vector was completed by following the instruction manual provided by Novagen (52). The first step, as illustrated in Figure 13, is to adapt the neuB gene to be inserted into the vector. Two primers were designed to link the end of the vector and the beginning of the gene in both the forward (5' to 3' - SASFOR) and in the reverse (3' to 5' - SASREV) directions. These primers have to be complementary to both the vector and gene strands which they will bind to. The primers were constructed by the Nucleic Acids Protein Services (NAPS) Unit of UBC.

The primers were used to amplify the SAS gene in a polymerase chain reaction (PCR) using a procedure developed by Dr. Andrew Murkin that was based on a Novagen protocol. This resulted in an extended gene with double stranded portions that were complementary to a cut vector bearing single stranded ends (purchased from Novagen). This modified gene was then submitted to T4 DNA polymerase treatment in the presence of dGTP. In this step the exonuclease activity of the T4 polymerase cleaved off the residues from one of the strands of the overhanging portions of the gene, until it discovered a guanine base whence it came to a halt. At this point, the gene contained single stranded overhangs which were complementary to the ends of the vector and was thus ready to be annealed to it. The T4-treated PCR product and the cut vector were annealed and transformed directly into NovaBlue competent cells without a ligation step. The resulting plasmid was then extracted from these cells using the QIAprep plasmid extraction kit (Qiagen) because this cell line is
not optimal for protein expression. The pET-30: SAS plasmid was transformed into *E. coli* strain JM109 (DE3) competent cells which are recognized for their protein expression capacity.

![Diagram](image-url)

**Figure 13. Illustration of the protocol used for the insertion of the *neuB* gene into the pET-30 Xa/LIC vector.** Representation copied from 52.

The expression of the His-tagged protein (SASHIS) was conducted in *E. coli* strain JM109 (DE3) expression cells. The antibiotic, kanamycin, was added to these cultures to ensure that only the cells containing the pET-30: SAS plasmid would grow. It was found that 0.5 mM IPTG was sufficient to obtain over expression of the synthase within a 4 h incubation period, as seen in lane 1 of Figure 14.
The addition of the His-tag renders protein purification much simpler. The crude lysate is loaded onto a nickel affinity chromatography column. The nickel atoms bound to the iminodiacetate groups on the sepharose fast flow resin are chelated by the histidines of the tag, which is sufficient to immobilize the protein onto the column (53). Various ways may be used to elute the protein such as changing the pH of the buffer to protonate the histidines or the more typical method, addition of an imidazole gradient to compete for the chelation sites. Low imidazole concentrations eliminate all the non-specific interactions such as proteins with a few external histidine, cysteine or tyrosine residues (53). Higher imidazole concentrations are used to elute anything left on the column which, at this point, should only be the His-tagged protein. This technique is excellent and the purity of the enzyme, as seen on SDS-PAGE gels, is over 95% homogeneity. The imidazole is removed during overnight dialysis.

This tag is widely used, because it normally does not affect the folding or the activity of the enzyme, thus it is acceptable to leave the tag on during the study of the protein. However, if the tag had to be removed, various methods are possible such as cleavage with thrombin or Factor Xa depending on how the vector was designed. In the case of the pET-30 Xa/LIC vector, the tag could be removed by thrombin which would leave 31 amino acids at the N-terminus of the new protein. Alternatively it could be cleaved by Factor Xa which would leave no residual amino acids (52).
As can be seen in Figure 14, SASHIS elutes with the addition of 500 mM imidazole. The 500 mM imidazole fractions were pooled and then dialyzed for 24 h at 4 °C with at least one change of buffer. The concentration of the purified enzyme was assayed using the Bradford method before being concentrated or frozen (54).

Lane 4) The 500 mM imidazole fraction which contains the active SASHIS.

The first $^{31}$P NMR experiment consisted of 5 mM PEP (neutral pH), 2.5 mM ManNAc, 1 mM dithiothreitol (DTT) and MgCl$_2$ in 30 mM triethanolamine-HCl pH 7.5 buffer. At time “0,” the spectrum showed only a peak around 0.1 ppm (PEP; spectrum was not calibrated against an external standard) (Figure 15). Less than 60 minutes after the addition of 0.3 mg SASHIS, a second peak developed 3.1 ppm downfield of the PEP peak. This second peak was thought to be free phosphate. To test this, phosphate buffer (pH 7.5) was added to the NMR tube at the end of the reaction. The new spectrum showed an increased peak at 3.2 ppm, thus confirming the assignment. No 2-PG was observed indicating that enolase was not present. In the absence of ManNAc, no phosphate was formed; showing that there was no phosphatase impurity.
Figure 15. $^{31}$P NMR spectra, at times 0, 60 min and 24 h, showing the emergence of the inorganic phosphate peak and the diminishing signal for the PEP peak during the SASHIS-catalyzed reaction.

An important experiment to confirm the $^{31}$P NMR results was the repetition of the above experiment, but followed by $^1$H NMR. The spectrum was fairly complex because of the presence of all the substrates. However, when focusing on a region between 1.6 ppm and 2.4 ppm, two doublets of doublets emerged at 1.69 ppm and 2.08 ppm (Figure 16). These peaks had previously been assigned to the axial and equatorial C-3 methylene protons of NeuNAc (55), thus SAS was active and producing the correct product.

The next experiment was identical to the first, except that MgCl$_2$ was omitted. After 24 hours of incubation at room temperature the PEP peak (0.1 ppm) had slightly decreased, while the free phosphate peak (3.2 ppm) was still very weak (Figure 17). Hence, in the
absence of exogenous metals, the reaction still takes place, but at a reduced rate compared to the rate observed in Figure 15. Another reaction was conducted, in the presence of EDTA (5 mM) in the initial (time 0) mixture. After 24 hours at room temperature, only the PEP signal (0.1 ppm) was present. This experiment emphasizes the need for the enzyme to have access to a divalent metal. In the previous experiment, small concentrations of metal must have been present in the enzyme solution.

Figure 16. $^1$H NMR spectra of the SASHIS-catalyzed condensation of ManNAc and PEP. The times are in minutes. The emergence of the signals for the two C-3 methylene protons of NeuNAc are illustrated.
Figure 17. $^{31}$P NMR spectra of various SASHIS-catalyzed reactions at times 0 and 24 hours. The second spectrum was the result of an experiment which did not contain additional magnesium salt. The third spectrum was obtained from a reaction conducted in the presence of 5 mM EDTA.

2.5. UV ASSAYS

Direct UV assays are an ideal method of detecting enzymatic activity because they don't require any additives that may interfere with enzyme function. In this project, the direct UV assay would monitor the disappearance of the double bond of PEP at 232-nm on a UV spectrophotometer. This assay was attempted at pH 7.5. Unfortunately at this pH value, the absorbance of PEP at 232 nm is not a maximum. This caused the absorption values to
fluctuate depending on how accurately the buffer pH was set. The lack of reproducibility made this technique unusable, thus an alternate method of monitoring the enzymatic activity was developed.

Coupled assays are a complex method of determining the activity of a target enzyme. They often employ UV active cofactors and coupling enzymes that catalyze irreversible reactions of the product to be analyzed. For such an assay to work, the other enzymes must be in great excess relative to the target enzyme. If not, the results will reflect all the enzymatic activities and not only that of the target enzyme. A triple coupled assay involving: SAS (actually SASHIS), sialic acid lyase (SAL), and lactic dehydrogenase (LDH) was developed for this project. As is illustrated in Figure 18, PEP and ManNAc are condensed together by SASHIS to produce NeuNAc. This product is then cleaved to ManNAc and pyruvate by SAL (see page 16 for a description of SAL). LDH reduces pyruvate to lactic acid while oxidizing the cofactor, reduced nicotinamide adenine dinucleotide (NADH). The assay monitors the disappearance of NADH at 340 nm.

![Diagram of coupled assay](image)

**Figure 18.** Representation of the coupled assay to monitor the activity of SASHIS.

Prior to using the coupled assay in kinetic experiments, it had to be shown that the assay was fully coupled. When a reaction is coupled, the target enzyme will solely control
the rate of the reaction, thus doubling the amount of target enzyme will double the rate and halving the amount of that enzyme will reduce the rate to half its original value. The coupled assay was tested with double the original amount of target enzyme and the rate increased by 100%.

The kinetic data was initially plotted as a Michaelis-Menten plot to verify that all the data were within experimental error (Figure 19). The kinetic values, $K_M$ and $k_{cat}$, were obtained from Lineweaver-Burke, double reciprocal, plots (Figure 20). The $K_M$ for ManNAc, $9.4 \pm 1.4$ mM, was obtained from the average of the two trials presented in Table 1; while the affinity for PEP was $0.25 \pm 0.07$ mM. The turnover number, $k_{cat}$, was found to be $0.8 \pm 0.1$ s$^{-1}$.

![Figure 19. Michaelis-Menten plots for the coupled assay. The left plot represents the experiments where ManNAc was maintained at 30 mM (saturating) and PEP concentrations were varied from 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mM. The right plot represents the experiments where PEP was maintained at 1 mM (saturating) and ManNAc concentrations were varied from 2.5, 6.25, 10, 15 and 30 mM. The lines are for the different experiments conducted (trials 1 (●), 2 (○)).](image)
Figure 20. Lineweaver-Burke plots for the coupled assay. The left plot represents the experiments where ManNAc was maintained at 30 mM (saturating) and PEP concentrations were varied from 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mM. The right plot represents the experiments where PEP was maintained at 1 mM (saturating) and ManNAc concentrations were varied from 2.5, 6.25, 10, 15 and 30 mM. The lines are for the different experiments conducted (trials 1 (•), 2 (○)).

Table 1. Kinetic properties of SASHIS from *N. meningitidis* identified by the coupled assay.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat} (s^{-1})$</th>
<th>$K_M$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ManNAc</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.79 ± 0.04</td>
<td>9.2 ± 1.3</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.73 ± 0.03</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>Average</td>
<td>0.76 ± 0.05</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td><strong>PEP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.92 ± 0.02</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.87 ± 0.08</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>Average</td>
<td>0.90 ± 0.08</td>
<td>0.25 ± 0.07</td>
</tr>
</tbody>
</table>
2.6. DISCUSSION

The purification of the wild type SAS revealed itself to be a daunting task. Various traditional purification techniques were attempted, but none succeeded in the separation of SAS from the enolase impurity. Thus, SASHIS was constructed and its purification using nickel affinity chromatography was readily achieved.

$^1$H and $^{31}$P NMR experiments confirmed that SASHIS was free of enolase and phosphatase impurities and that it catalyzed the correct reaction. These NMR experiments also confirmed the enzyme's requirement for a divalent metal cation. The UV coupled assay afforded kinetic values for the SASHIS-catalyzed reaction. The hyperbolic curve obtained on the Michaelis-Menten plot indicated that SAS did not exhibit cooperativity. The $K_M$ values shown in Table 1 reveal that the enzyme's affinity for PEP is much greater than that of ManNAc and that the enzyme is not that efficient in catalyzing this condensation. These values are not surprising and correlate reasonably well with the only published values of $K_M$ for the SAS of *N. meningitidis* (5). The $K_M$ for ManNAc, 9.41 mM, was 1.5 times greater than that of the literature; while the affinity for PEP, 0.25 mM, was six times greater. It is possible that the tag interferes with the PEP binding site and therefore the affinity of the enzyme for this substrate is reduced. Differences in the conditions used in the two assays could also lead to slight kinetic incongruities. For example, the literature experiment utilizes 5 mM manganese, however, only 1 mM manganese could be used in the coupled assay,
because SASHIS activity was non-linear otherwise. Hence the assay could not be optimized with the same conditions as in the literature.

2.7. EXPERIMENTAL METHODS

Pyruvate, ManNAc, DTT, NADH, MgCl₂, Tris base, triethanolamine, and LDH (Type II from rabbit muscle) were purchased from Sigma-Aldrich Chemical Co. SAL was purchased from Jülich Fine Chemicals (Germany). The Xia/LIC ligation kit and the SpinPrep PCR Clean-up Kit were purchased from Novagen. The oligonucleotide primers were synthesized by NAPS at UBC. The plasmids were extracted from cell cultures using the QIAgen plasmid extraction kit (Qiagen). Nucleotides and Taq polymerase were purchased from Invitrogen. Chelating Sepharose® Fast Flow was purchased from Pharmacia Biotech. HiTrap Q HP columns were purchased from Amersham Biosciences.

2.7.1. OVER EXPRESSION AND PURIFICATION OF SASHIS

All the PCR reactions were conducted in the Biorad iCycler apparatus in the Biological Services laboratory. A forward (SASFOR) and a reverse (SASREV) direction primers were constructed by NAPS. Part of the sequence of these oligonucleotides was complementary to the end of the vector, while the other portion was complementary to the beginning of the gene. SASFOR 5' GGT ATT GAG GGT CGC ATG CAA ACC AAC 3' and SASREV 5' AGA GGA GAG TTA GAG CCT TAT TCA ATA TCA G 3'. The
primers and the genes were combined together using a PCR protocol developed by Dr Andrew Murkin. 5 μL 10X PCR buffer, 1 μL 10 mM dNTP, 1.5 μL 50 mM MgCl₂, 2.5 μL SASFOR (10 μM), 2.5 μL SASREV (10 μM), 1 μL dsDNA (20 ng), and 36.25 μL ddH₂O were mixed into a PCR tube. This mixture was subjected to three minutes of heating at 94 °C. After this, 0.25 μL Taq polymerase was added to the tube and 30 PCR cycles were run as follows: 45 seconds at 94 °C (denaturing), 30 seconds at 55 °C (annealing) and 90 seconds at 72 °C (elongation). Once the 30 cycles were complete, the mixture would remain at 72 °C for another 10 minutes. The PCR product was purified using the SpinPrep PCR Clean-up Kit (Novagen). The purified products were observed on a 1 % agarose gel and visualized with ethidium bromide under UV light. A single band slightly above the 1 kDa marker was observed in the lanes containing the gene products. These purified gene products were then inserted into the pET-30 Xa/LIC vector following the Xia/LIC cloning kit protocol (Novagen). Once the plasmid and the gene were annealed, the vector was transformed into Novagen NovaBlue competent cells.

These cells were grown overnight at 37 °C with shaking (225 rpm) in LB medium in the presence of 30 μg/mL kanamycin. The plasmid from these cell cultures was extracted and then transformed into E. coli strain JM109 (DE3) expression cells and plated onto agar plates containing 30 μg/mL kanamycin. A 10 mL culture of LB medium was inoculated with colonies from the previously plated cells. This culture was incubated at 37 °C in a shaker (225 rpm) overnight. The next morning this culture was used to inoculate a larger volume of LB medium (500 mL) with kanamycin (30 ng/μL). This new culture was incubated in the same conditions until it reached an OD₆₀₀ 0.6. At that point, the expression of the SASHIS
protein would commence with the addition of IPTG (0.5 mM). The culture flask was returned to the shaking incubator for an additional 4 hours. After this period, the culture was spun at 5000 rpm in a cool (4 °C) centrifuge for 15 min. The supernatant was discarded, while the cell pellet could either be flash frozen in liquid nitrogen and stored in the deep freezer (-80 °C) or dissolved in less than 10 mL of 30 mM triethanolamine-HCl buffer pH 7.5 (10 % glycerol, 1 μg/L aprotinin and pepstatin, 1 mM PMSF) and subjected to two runs on the French Press. The double treatment is solely to ensure that all the cells have been lysed. The lysate was spun at 5000 rpm for 60 min. The supernatant was then filtered through a 0.45 μ filter.

The crude lysate of SASHIS was then loaded onto a nickel affinity chromatography column (10 mL resin) which had been prepared according to the instruction manual (53) and equilibrated with the same buffer, but at pH 8.0. Once the crude lysate was loaded, the column was washed with the buffer and 10 mM imidazole. The concentration of imidazole in the buffer was increased in a step gradient fashion. The imidazole concentration was increased after two column volumes had been used and no change had occurred or once the recorder had returned to baseline. After the wash buffer, a peak was observed with 50 mM imidazole, but this was not the protein since it had no activity. SASHIS eluted from the column with 500 mM imidazole as confirmed by SDS-PAGE gels. All the fractions containing the enzyme were pooled into dialysis tubing and dialysed overnight in 30 mM triethanolamine-HCl buffer pH 7.5 with 1 mM DTT and 10 % glycerol. SASHIS concentration was established with the Bradford method using bovine serum albumin as the standard (54).
2.7.2. **NMR EXPERIMENTS**

All the NMR experiments were conducted on the Bruker Avant 300 MHz NMR spectrometer. All the $^{31}$P NMR experiments were conducted in dH$_2$O, but 200 µL of D$_2$O was added to the NMR tube to allow locking of the signal. In the case of $^1$H NMR studies, the substrates were dissolved in 30 mM triethanolamine-HCl pH 8.0 buffer prepared with D$_2$O, while the enzyme was exchanged into this buffer using spin dialysis.

All the $^1$H and $^{31}$P NMR experiments conducted in this section contained 5 mM PEP (neutral pH), 2.5 mM ManNAc, 1 mM DTT and MgCl$_2$ in 12 mM triethanolamine-HCl pH 7.5 buffer (final concentrations). All the experiments were initiated with the addition of 0.3 mg SASHIS. The exceptions are the experiments where the absence of MgCl$_2$ was being tested or when 5 mM EDTA was added as an extra component. 2.5 mM phosphate buffer pH 7.5 and 2 mM 2-PG were added to different reaction tubes, after the end of the study, to confirm the assignment of the various peaks. All the $^{31}$P NMR substrates were dissolved in dH$_2$O, except for PEP which was dissolved in buffer and had to be adjusted to pH 7.5 with the addition of 1 M NaOH. All these experiments were conducted at room temperature, including the incubation periods.
2.7.3. COUPLED UV ASSAY

All the solutions used were made fresh and stored on ice prior to their use in the assay. All the enzyme (SASHIS and SAL) and PEP stocks were diluted in Tris-acetic acid buffer pH 8.3, while the other substrates and cofactors were dissolved in dH2O. The stock solutions were made in such a way that the smallest volume used was 25 µL, except LDH which was purchased at a set concentration. The reactions were conducted in a 1 mL disposable cuvette, but the total reaction volume was kept at 800 µL. The assay was conducted in a UV spectrophotometer which included a heating device, maintaining the cuvette’s content at 37 °C. The oxidation of NADH was followed at 340 nm.

The assay was composed of three phases. The first phase consisted of incubating ManNAc (variable), NADH (160 µM), LDH (250 U), Tris-acetic acid pH 8.3 (150 mM), SAL (20 U), MnCl2 (1 mM) DTT (1 mM), and dH2O for 10 minutes at 37 °C: This incubation was done to allow all the components to reach thermal equilibrium. In the second phase, PEP (variable) was added. Hydrolysis of PEP caused by non-SAS activity did occur, but after a few minutes this background activity became linear. The background rate was calculated with the least square method on the last two minutes of data of the 10 minutes phase. The last phase marked the beginning of the assay, since this was when SASHIS (1 µg) was added. The assay was run for 15 minutes after the addition of enzyme. The rate calculated for phase 3 was the value obtained after submitting all the data for the final five minutes of the assay, as long as they were linear, to the least square method. The final rate of the reaction was obtained by subtracting the background rate in phase 2 from the rate
observed in phase 3. Two types of experiments were conducted in this section: 1) saturating ManNAc concentration (30 mM) and 0.05, 0.1, 0.25, 0.5, 0.75, and 1 mM PEP; and 2) saturating PEP concentration (1 mM) and 2.5, 6.25, 10, 15, and 30 mM ManNAc. The results generated hyperbolic Michaelis-Menten plots, which were replotted as Lineweaver-Burke plots using GraFit 4 (Erithacus Software, Horley, UK).
CHAPTER 3:

SYNTHESIS OF THE

LABELLED SUBSTRATES
3. SYNTHESIS OF LABELLED SUBSTRATES

3.1. [2-\(^{18}\)O]-PHOSPHOENOLPYRUVATE

As was explained in Chapter 1.5, the result from the \(^{31}\)P NMR using this compound will yield evidence strongly favouring one of the two possible mechanisms used by SAS. If the \(^{18}\)O-label ends up in the phosphate, the C-O bond cleavage mechanism is supported, whereas if it ends up in the NeuNAc, the P-O bond cleavage mechanism is supported. The synthesis of [2-\(^{18}\)O]-PEP was modified from procedures reported in the literature (56, 57) and is shown in Figure 21.

![Diagram of the synthesis of [2-\(^{18}\)O]-PEP](image-url)

Figure 21. Total synthesis of [2-\(^{18}\)O]-PEP
3.1.1. ETHYL [2-\(^{18}\text{O}\)]-3-BROMOPYRUVATE

The incorporation of the 18-oxygen label into PEP is a facile process. Mixing the starting material, ethyl 3-bromopyruvate, in methanol/\(\text{H}_2^{18}\text{O}\) containing acetic acid as a proton catalyst, and stirring in the dark for 24 hours, will lead to the incorporation of the label. After lyophilization, the compound was carried to the next step, where the introduction of a phosphate group will lock the label in its bridging position. The product was not characterized following lyophilization to avoid any washout of label with adventitious water.

3.1.2. ETHYL 2-[(DIMETHOXYPHOSPHINYL)^{18}\text{OXY}]PROPENOATE

This step is crucial to ensure that the 18-oxygen label stays at the bridging position. Once the phosphate group has been added, the label should not wash out and scrambling will not happen. The phosphate group was added by treating ethyl [2-\(^{18}\text{O}\)]-3-bromopyruvate with trimethyl phosphite in refluxing dioxane. The solvent was then removed under vacuum. Previous trials conducted without the label indicated that this reaction did not generate impurities, hence the compound was not purified to maximize the reaction yield.

The product was characterized by positive liquid secondary ion mass spectrometry (LSIMS) to confirm the incorporation of the label. The peak at 227 m/z was the labelled product, whereas the smaller peak at 225 m/z was the unlabelled product (Figure 22). There appeared to be 66% incorporation of the label into this intermediate product. This was ideal
since the NMR experiments will contain two peaks; one for each of the labelled and unlabelled products.

Figure 22. (+) LSIMS spectrum for ethyl 2-[(dimethoxyphosphinyl)\(^{18}\text{oxy}\)]propenoate. The labelled product is the 227 m/z peak, while the unlabelled product is the 225 m/z peak.

3.1.3. DEPROTECTION AND ANION EXCHANGE

This is the final step in this synthesis since all the protecting groups were removed from the carboxyl and phosphoryl groups with bromotrimethylsilane. The deprotected product was then extracted into an aqueous layer and protonated. Quickly it was mixed with cyclohexylamine to facilitate its recrystallization as a cyclohexylammonium salt. After this purification step, the labelled PEP was run through a Dowex (\(\text{Na}^+\) form) column to exchange the cyclohexylammonium cation for sodium. It was noticed, however, that the sodium form of this product was slightly hygroscopic, thus the labelled product was stored in its cyclohexylammonium form.
Figure 23. (−) LSIMS spectrum for $^{18}$O-PEP (cyclohexylammonium form). The labelled product is the 169 m/z peak, while the unlabelled product is the 167 m/z peak. The peaks at 275 and 277 are the products complexed with a thioglycerol molecule.

Figure 24. 200 MHz $^1$H NMR spectrum of the sodium form of $^{18}$O-PEP. The peaks identifying the product are those slightly downfield from the HOD peak.
Figure 25. 300 MHz $^{31}$P NMR spectrum of $^{18}$O-PEP (cyclohexylammonium form). The tall peak arises from phosphorus atoms attached to 18-oxygen, while the small one is caused by phosphates containing $^{16}$O-isotopes. The shift is approximately 0.02 ppm between the two peaks.

The product was characterized by negative LSIMS and the 167/ 169 m/z peaks represent the unlabelled and labelled products in their unprotected state (Figure 23). The ratio seems to be closer to 2:1 in this spectrum. The other interesting peaks are the pair at 275/ 277 m/z. These appear to be the product complexed with thioglycerol which was a component of the matrix used. A proton NMR of the sodium form of [2- $^{18}$O]-PEP in D$_2$O was obtained (Figure 24). The two peaks revealing the identity of the product were the peaks at 5.15 and 5.30 ppm. These peaks agree with values published for the vinyl protons of PEP (58). The peak around 1.8 ppm was an unknown impurity occurring after the conversion of the product to its sodium form. On the other hand, Figure 25 revealed the 0.02 ppm shift between the two PEP peaks in the $^{31}$P NMR spectrum (D$_2$O).

3.2. OVERALL SYNTHESIS

The total synthesis used in this research was slightly different than that mentioned in the literature (Figure 26) (56, 57, 59). The overall yield of this reaction was poor (17%). The least efficient step was the recrystallization.
3.3. [3-²H]-PHOSPHOENOLPYRUVATE

The face of PEP leading the attack onto ManNAc could be determined with this compound (Figure 27). If (E) [3-²H]-PEP was incubated with ManNAc in the presence of SAS and that the reaction occurred by a si face attack, then the NeuNAc would have a deuterated label in the axial position at C3. The ¹H NMR spectrum of such a compound would only have a signal for the equatorial proton at C3, but it would not be coupled to its axial counterpart. The deuterated PEP is referenced in the literature (57), thus its synthesis should have been facile (Figure 28). However, various problems were encountered so different methods had to be used in its synthesis.
Figure 27. Scheme showing the attack by one of the faces of PEP and their respective products.

Figure 28. Total synthesis of [3-^3H]-PEP
3.3.1. DIBROMINATION METHODS

The first step, in the article that was initially followed, was the addition of a second bromine atom onto position 3 of ethyl 3-bromopyruvate using NBS, AIBN and UV light while refluxing in carbon tetrachloride (57). Both photo and thermal initiated reactions were attempted, but were unsuccessful. This may have been due to the poor quality of the AIBN which is now unavailable in Canada. Ultimately, the radical approach was discarded.

An alternate article discussed the bromination of pyruvate using bromine in refluxing chloroform (60). After several trials at identifying the correct conditions, 3,3-dibromopyruvate was synthesized. Esterification with an ethyl group would yield the original target compound, ethyl dibromopyruvate. The purification of this compound was attempted by benzene recrystallization. This step however did not improve the purity of the product, since the $^1$H NMR spectrum showed that peaks for 3-bromopyruvate (3.66 ppm) and pyruvate (1.70 ppm) remained (Figure 29). The dibrominated product was formed because a peak at 6.03 ppm was present.

3.3.2. ETHYL 3,3-DIBROMOPYRUVATE

Dibromopyruvate was refluxed in ethanol with catalytic amounts of acetyl chloride. The Fischer esterification was conducted for six hours, prior to having the solvent removed by reduced pressure. The purification of this product was done by flash chromatography. This method did not remove all the impurities as can be seen in Figure 30.
Figure 29. The 200 MHz $^1$H NMR spectrum for 3,3-dibromopyruvate in D$_2$O.

Figure 30. The 200 MHz $^1$H NMR spectrum for ethyl 3,3-dibromopyruvate in D$_2$O.
3.3.3. SYNTHESIS OF ETHYL \((E,Z)\) 3-BROMO-2-\([(DIMETHOXYPHOSPHINYL)OXY]\)PROPENOATE

This reaction was identical to the one used in producing the \(^{18}\)O-labelled PEP and involved refluxing the ethyl 3,3-dibromopyruvate with trimethyl phosphite in dioxane. The reaction was successful, but separating the \(E\) and \(Z\) stereoisomers presented a great challenge. The literature recommendations for the column size and elution mixtures were followed (57), but all failed or minute amounts of pure material were obtained. In the crude mixture there was a 2:1 ratio in favour of the \(Z\) stereoisomer. This difference was obvious in the NMR spectra and therefore the mixture of \(E\) and \(Z\) stereoisomers could still be used in the enzymatic experiments. However, a pure mixture was required and this was not a trivial task.

![Figure 31. The 200 MHz \(^1\)H NMR of ethyl \((E, Z)\) 3-bromo-2-\([(dimethoxyphosphinyl)oxy]\)propenoate after flash chromatography purification.](image)
A spectrum of the quality observed in Figure 31 was obtained only once and very little material was recovered after the two flash chromatography steps which were required to arrive to this stage. A stereoisomeric mixture purified as in Figure 31 would be the ideal and could be brought to the next step.

3.3.4. ADDITIONAL PEAKS DOWNFIELD

When the synthetic experiments were repeated, they usually afforded an impurity just downfield of the hydrogen peak in the $^1$H NMR spectrum of dibromopyruvate. This impurity was generated during the dibromination reaction or during the esterification. In either case, its cause was unclear and none of the purification steps, previously used, worked to remove it. The compound causing this peak would also react with the phosphate group, hence four peaks would be obtained around 7 ppm (Figure 32) instead of two as seen in Figure 31. Since fresh reagents did not alleviate this problem nor did any of the purification steps tried, this project was halted.

3.4. EXPERIMENTAL METHODS

3.4.1. MATERIALS

All the reagents used were purchased from Sigma-Aldrich Chemical Co., except the $^{18}$O-enriched water (95%) which was purchased from Icon Stable Isotopes. These reagents
were ACS grade or better. The solvents used in the following experiments were purified and/or dried by standard methods (61).

Figure 32. The 200 MHz $^1$H NMR of ethyl (E, Z) 3-bromo-2-[(dimethoxyphosphinyl)oxy]propenoate with the two unknown peaks in the vicinity of the alkenyl protons.

3.4.2. ETHYL [2- $^{18}$O]-3-BROMOPYRVATE

A solution of ethyl 3-bromopyruvate (1.11 g, 5.5 mmol), $\text{H}_2^{18}$O (400 $\mu$L, 0.02 mmol), acetic acid (300 $\mu$L, 5.2 mmol) and methanol (400 $\mu$L) was stirred in the dark for 24 hours. The solution was then freeze-dried. A white powder (0.85 g, 77 %) was obtained. It was not characterized, since the label could exchange with water.
3.4.3. **ETHYL 2-[(DIMETHOXYPHOSPHINYL)\(^{18}\)OXY]- PROPENOATE**

Ethyl [2-\(^{18}\)O]-3-bromopyruvate (0.85 g, 6.9 mmol) was brought to reflux in dioxane (25 mL) prior to the addition of trimethyl phosphite (0.73 g, 5.9 mmol). The reflux was maintained for an additional 30 minutes. The solvent was then removed by reduced pressure with slight heating (35 °C). The resulting oil (0.63 g, 41 %) was not purified to maximize the yield of the labelled product and because this reaction generates few impurities. MS (+LSIMS) 225 (33 %), 227 (100 %)

3.4.4. **DEPROTECTION AND ANION EXCHANGE**

Ethyl 2-[(dimethoxyphosphinyl)\(^{18}\)oxy]propenoate (0.63 g, 2.7 mmol) was dissolved in dichloromethane (25 mL) in a flask which was flushed with argon for 10 minutes. Bromotrimethylsilane (1.22 g, 8.0 mmol) was added to the solution which was stirred at room temperature for two hours. The solvent was removed under reduced pressure. The residual solid was dissolved in KOH (16 mL, 1 M). This solution was washed with dichloromethane (2 X 15 mL) and the aqueous layer was run through a Dowex 50WX8-200 (H\(^+\)) column. Once the product started eluting from the column, it was mixed with cyclohexylamine (0.29 g, 2.9 mmol). This solution was freeze-dried and 0.74 g of white powder was obtained. This was recrystallized (1:1 MeOH/ ether) to yield 0.18 g of white powder. Most of the material was conserved as the cyclohexylammonium form of the labelled PEP, but some was dissolved in water and run through a Dowex (Na\(^+\)) column. Cyclohexylammonium form: \(^1\)H NMR (200 MHz, D\(_2\)O) \(\delta\) 5.30 (s, 1 H, E), 5.18 (s, 1 H, Z)
(spectrum not shown); MS (-LSIMS) 167 (45 %), 169 (100 %). Sodium form: $^1$H NMR (200 MHz, D$_2$O) δ 5.30 (s, 1 H, E), 5.15 (s, 1 H, Z).

3.4.5. **3,3-DIBROMOPYRUVATE**

Pyruvate (7.1 g, 81 mmol) was refluxed with bromine (28 g, 175 mmol) in dichloromethane (100 mL) for 30 hours. The solvent was then removed under reduced pressure. The dark orange oil was recrystallized in benzene. The overall yield for this reaction after recrystallization is under 40 %. $^1$H NMR (200 MHz, D$_2$O) δ 6.03 (s, 1H).

3.4.6. **ETHYL 3,3-DIBROMOPYRUVATE**

Ethanol (7.9 g, 170 mmol) was cooled in a dry ice/acetone bath prior to the addition of acetyl chloride (200 μL). After warming to RT, it was added to dibromopyruvate (4.23 g, 17 mmol) and the mixture was stirred until everything was dissolved. The solution was refluxed for six hours. The solvent was removed by reduced pressure. The yellow oil was purified by flash chromatography (silica gel, 35% ethyl acetate (EtOAc)/petroleum ether (PET)) to yield about 10 % of pure product. $^1$H NMR (200 MHz, D$_2$O) δ 6.03 (s, 1 H), 4.24 (q, 2 H), 1.22 (t, 3 H).
3.4.7. ETHYL (E,Z)-3-BROMO-2-[(DIMETHOXYPHOSPHINYL)OXY]-PROPENOATE

Trimethyl phosphite (0.20 g, 1.8 mmol) was added to a refluxing solution of ethyl 3,3-dibromopyruvate (0.488 g, 1.8 mmol) in dioxane (8.5 mL). The reflux was maintained for an additional 30 minutes. The solvent was removed by reduced pressure with slight heating (35 °C). The mixture of E/Z should be separated by flash chromatography and a step gradient of EtOAc in PET. A first column was run with 70 % EtOAc/ PET as the eluent. The product was quickly removed from the column and concentrated by rotary evaporation. The second column was run with 20 % EtOAC/ PET as the eluent, but the two enantiomers and the impurities were still found in the same fractions.

Mixture: $^1$H NMR (200 MHz, CDCl$_3$) δ 7.21 (d, 1 H), 6.90 (d, 1 H), 4.30 (m, 4 H), 3.85 (d, 3 H), 3.75 (d, 3 H), 1.35 (t, 3 H), 1.25 (t, 3 H).
CHAPTER 4:

TESTING THE MECHANISM USING
THE LABELLED SUBSTRATE
4. TESTING THE MECHANISM WITH THE $^{18}\text{O}$-LABELLED SUBSTRATE

The mechanism employed by SAS to condense ManNAc and PEP was probed using the $^{18}\text{O}$-PEP which was successfully synthesized in this project. This will reveal which bond of the phosphate group was cleaved during the catalysis, hence it will allow the classification of SAS into a certain category of enzyme.

4.1. RESULTS AND DISCUSSION

This experiment relies on there being a difference in the $^{31}\text{P}$ NMR spectrum between a phosphorus atom attached to a $^{16}\text{O}$-isotope and an $^{18}\text{O}$-isotope. There is literature precedence for a $0.02$ ppm shift between such two peaks (38). This chemical shift is fairly small and observing it is not trivial since components of the assay may broaden the signals. The shift was observed when a sufficient number of scans were taken and the sweeping bandwidth was limited to a 20 ppm window (10 ppm to -10 ppm). Using this parameter, the shift was clearly observed without the addition of chelex or EDTA which would have terminated the reaction, but increased the sharpness of the signals. The addition of 10 mg labelled substrate was also essential to having a signal which had a satisfactory intensity.

A time “0” $^{31}\text{P}$ NMR spectrum was taken in the presence of all the assay components except SASHIS. Once a clear spectrum was obtained, SASHIS was added to the NMR tube and the reaction was monitored at room temperature. Scans would be recorded continuously
up to a set time point when another spectrum was taken. After each spectrum was taken, the experiment was halted and a new set of scans was immediately initiated. This permits the analysis of the spectrum for each time point instead of having only one large file containing all the scans leading to the last spectrum.

Figure 33. The 300 MHz $^{31}$P NMR spectrum of the reaction catalyzed by SASHIS in the presence of $^{18}$O-PEP as a substrate. The free phosphate peak is shifted by 0.02 ppm, thus revealing the attachment of an 18-oxygen to the phosphorus.
Figure 34. A zoom view of the free phosphate peak observed at t= 60 min in Figure 33. The 0.02 ppm shift is clearly noticeable.

The time “0,” spectrum shows a main peak at 0.08 ppm and a second smaller peak emerging from the base of the taller one at 0.1 ppm (Figure 33). The upfield signal is due to the molecules in which phosphorus is attached to an 18-oxygen, while the downfield signal is due to molecules containing 16-oxygen. The following spectrum (t= 30 min) showed the emergence of a second peak at 2.90 ppm (Figure 33). This peak appears in the same area as the free phosphate in the preliminary experiments, except that it is broader and is slower in developing. After 60 minutes, the two peaks at 2.90 ppm are clearly distinct and they are separated by 0.02 ppm (Figures 33 and 34). This observation means that the 18-oxygen is still attached to the phosphorus atom in the product of the reaction. Hence the enzyme employs a C-O bond cleavage mechanism. This experiment reveals that SAS behaves similarly to KDO-8P synthase and DAHP synthase for the condensation of PEP with their respective sugars (Figure 35).
4.2. EXPERIMENTAL METHODS

4.2.1. MATERIALS

Pyruvate, ManNAc, DTT, MgCl₂, and triethanolamine were purchased from Sigma-Aldrich Chemical Co. Deuterated water was obtained from Cambridge isotopes. The $^{18}$O-PEP was synthesized as described in Chapter 3. The concentration of the labelled substrate
was not determined, thus a significant portion of its weight could be caused by the presence of salts.

4.2.2. NMR ASSAY

The experiment was conducted on a 300 MHz Bruker Avant NMR spectrometer with the sweep bandwidth limited to 10 and -10 ppm. Time “0” reaction mixture contained $^{18}\text{O}$-PEP (10 mg), ManNAc (2.5 mM), DTT (1 mM), MgCl$_2$ (1 mM), triethanolamine-HCl pH 7.5 (12 mM) (final concentration), and D$_2$O (200 μL for locking the NMR signal). Once a clean spectrum was obtained, the synthase (0.3 mg) was added and the reaction was monitored for two hours. Spectra were taken every 30 minutes during the reaction, hence four distinct NMR experiments were run. Each time a spectrum was obtained, a new experiment was commenced with the collection of a new set of scans.
REFERENCES


