Inactivation of *Micromonospora viridifaciens* Sialidase by Fluorinated Sialic Acids; Binding Specificities of the Hydrophobic Pocket

by

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B.Sc., Karaj Islamic Azad University, 1993

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

in the Department of Chemistry Faculty of Science

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SIMON FRASER UNIVERSITY

Fall 2013

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Abstract

The sialidase from *Micromonospora viridifaciens* (MvS) is inactivated by the sialic acid analogue 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride (DiFSA), and by the Kdn analogue 2,3-dideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride (DiFKdn). The second-order rate constant for inhibition of MvS by DiFSA at 25 °C at a pH of 7.00 is $3.60 \times 10^6$ M$^{-1}$ s$^{-1}$. Whereas, the corresponding rate constant for inhibition by the Kdn inactivator is approximately one thousand-fold smaller ($2.92 \times 10^3$ M$^{-1}$ s$^{-1}$ at 25 °C and with a pH of 5.25). This decrease in activity on substitution of an acetamido group (DiFSA) for a hydroxyl functionality (DiFKdn) is remarkably similar to that for MvS-catalyzed hydrolysis of 4-nitrophenyl glycosides of sialic acid and Kdn. These observations are consistent with the difluoro containing inhibitors being 'mechanism-based' inactivators and that the hydrophobic pocket of MvS providing approximately 22 kJ/mol transition state stabilization by virtue of its hydrophobic interaction with the 5-acetamido group.

**Keywords:** *Micromonospora viridifaciens* sialidase; DiFSA; DiFKdn; hydrophobic pocket; transition state stabilization
I dedicate this thesis to my family, especially to my parents Tahereh and Manouchehr Khazaei who have always supported me in my endeavours.
Acknowledgements

I would like to thank my senior supervisor Dr. Andrew Bennet for giving me the opportunity to work in his laboratory. He supported me with kindness, passion and encouragement.

I would like to thank to my committee members Dr. Margo Moore and Dr. Robert Britton for their great support and advice. A special thanks to Dr. Robert Young for being my internal examiner.

I am grateful to Dr. Jacqueline Watson for providing the enzyme that I used in this research. I would like to thank to my friend and colleague Dr. Fahimeh Shidmoossavee for her great support and encouragement. I also offer my gratitude to the Bennet laboratory members both past and present as well as my close friends whose good humor made this journey enjoyable.

I would like to thank the technical assistance of Dr. Andrew Lewis and Mr. Colin Zhang for their help with NMR spectroscopy. A special thanks to Mr. Frank Haftbaradaran (Instrumental technician) for his support and encouragement.

I would like to express my gratitude to Simon Fraser University and NSERC for financially supporting this work.
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<td>Neu5Ac</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>N-glycolyneuraminic acid</td>
</tr>
<tr>
<td>RDE</td>
<td>Receptor-destroying enzyme</td>
</tr>
<tr>
<td>Neu5,9Ac₂₈Me</td>
<td>9-O-acetyl-8-O-methyl-N-acetylneuraminic acid</td>
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<td>Neu5Gc₇,₈,₉-Ac₃</td>
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</tr>
<tr>
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<td>Neu5Ac-9-phosphate</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Uridine diphosphate N-acetylg glucosamine</td>
</tr>
<tr>
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<td>Glucosamine-6-phosphate</td>
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<tr>
<td>Kdn</td>
<td>3-deoxy-D-glycero-D-galacto-non-2-ulosonic acid</td>
</tr>
<tr>
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<td>Polysialoglycoprotein</td>
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<tr>
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<tr>
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<td>4-methylumbelliferyl α-sialoside</td>
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<td>Adenosine 5'-diphosphates</td>
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</tr>
<tr>
<td>pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
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1. Introduction

1.1. Sialic Acid

Sialic acids are a family of monosaccharides that possess a nine-carbon backbone. These sugars are often found at the terminal position of oligosaccharide chains. Sialic acids occur naturally throughout all kingdoms of life.1

1.1.1. Background

Early research on sialic acid was mainly pioneered by two groups, one in the United States (Landsteiner and Levene 1927)2 and Walz’s group in Germany.3 These two groups independently discovered a sugar-like animal lipid that showed different properties to those of the simpler sugar hexoses.2,4 In 1935, Klenk and his group isolated a brain glycolipid fraction, and called these complex sugar structures "gangliosides" from which they purified a crystalline compound after treating the ganglioside with Bial's reagent⁵ (a solution of orcinol, HCl and ferric chloride).5 This crystalline compound was called neuraminic acid (Neu) (1) (Neuro + amine + acid).⁶ In the following year, Blix also isolated a sugar from submaxillary mucin using a mild procedure with Bial's reagent and it was purified to an unmodified crystalline form that was called sialic acid (2).⁴ The name sialic acid (2) came from the Greek word sialos for saliva. The chemical structure of this molecule was determined by Blix and his group to be that depicted for sialic acid (2).⁷ Two years later, sialic acid (2) was synthesized both biochemically and chemically by Comb and Roseman as well as by Brug and Paerels.⁸-¹⁰ After the discovery of sialic acid (2), Gottschalk and Lind showed that sialic acid can be removed from complex glycoproteins by the action of the receptor-destroying enzyme (RDE) from Vibrio cholera.¹¹,¹² During the early 1960s, the structural chemistry, diversity and biological roles of sialic acid (2)

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¹ Bial's reagent consists of a solution of orcinol (0.4 g), concentrated hydrochloric acid (200 ml) and a 10% of ferric chloride solution (0.5 ml).
received a great deal of attention. Today it has been shown that in nature the sialic acid family of sugars contains more than 50 neuraminic acid (1) derivatives and analogues.\(^6\) (Figure 1.1)

1.1.2. **Diversity in Structure and Linkage**

Sialic acid (2) is the generic name for N-acetylneuraminic acid (Neu5Ac, 2). Members of the sialic acid family have a remarkable diversity with more than 50 representatives.\(^{16}\) It was shown that the parent amino sugar of sialic acids is neuraminic acid (Neu, 1). The derivatization of the amine group at the C5 position of neuraminic acid by an acetyl or a glycolyl group gives the two most common derivatives of sialic acids N-acetylneuraminic acid (Neu5Ac, 2) and N-glycolylneuraminic acid (Neu5Gc 3), respectively.\(^6\) (Figure 1.1) The replacement of the amino group by a hydroxyl group gives Kdn 4, another member of the sialic acid family.\(^{17}\) Many other sialic acid family members are biosynthetic derivatives of Neu5Ac (2), Neu5Gc (3), and Kdn (4). That is, modification of the hydroxyl groups at C9, C8, C7, and C4 by various groups such as lactate, methyl, acetate, sulfate and phosphate on 2, 3, and 4 result in further structural diversity. Other unusual modifications on sialic acids, which provide more diversity within this 2-keto-3-deoxynononic acid family, include intra and intermolecular lactonization between C1 and N5.

![Figure 1.1](Figure 1.1. Structure of four naturally occurring sialic acids.)
1.1.3. **Nomenclature and Abbreviation**

In 1982, Schauer proposed a simple systematic nomenclature for the naming of sialic acids. In this system, Neu, and Kdn are the abbreviations for the core structures neuraminic acid (1) and 2-keto-3-deoxy-non-2-ulosonic acid (4), respectively. The following detail substitutions (Ac) stands for acetyl, (Gc) stands for glycolyl, (Me) stands for methyl, and (S) stands for sulfate. The numbers beside these codes are used to indicate the position of substitution. For instance, 9-O-acetyl-8-O-methyl-N-acetyleneuraminic acid is abbreviated to Neu5,9Ac28Me and 7,8,9-tri-O-acetyl-N-glycolylnearaminic acid is abbreviated to Neu5Ge7,8,9-Ac3. In this system, when it is not certain about the exact structure of the sialic acid that is attached to the specific position of oligosaccharide, the generic abbreviation of Sia is used. If partial information is available, it can be applied to such an abbreviation. For example, an O-acetylated group with an uncertain location abbreviates as SiaOAc. Lastly, if there is a substitution present but the type of substitution is not known, this is abbreviated as SiaX.

1.1.4. **Biosynthesis and Turnover of Sialic Acid**

Since Neu5Ac is a starting compound for biosynthesis of other sialic acid family members, the biosynthetic pathway of this sialic acid is shown in Figure 1.2. In mammalian cystol, Neu5Ac may be made biosynthetically in nine steps from glucose (Figure 1.2). In this synthetic pathway, ManNAc is phosphorylated at the 6-position, a reaction that is catalyzed by ManNAc-6-kinase, to afford ManNAc-6-phosphate (ManNAc-6-P). The condensation of ManNAc-6-P and phosphoenolpyruvate is catalyzed by the enzyme sialic acid-9-phosphate synthase to give Neu5Ac-9-phosphate (Neu5Ac-9-P), which subsequently has the 9-phosphate removed by the action of sialic acid-9-phosphatase to afford free sialic acid. Degradation of sialic acid is catalyzed by the enzyme Neu5Ac aldolase. This enzyme breaks the carbon bond between C3 and C4 to generate pyruvate and ManNAc. In the nucleus, free sialic acid is activated to Neu5Ac-CMP by the action of CMP-Neu5Ac synthase. The activated Neu5Ac-CMP is then transferred to an acceptor oligosaccharide or glycoprotein in the Golgi (Figure 1.2).
Sialic acid can be prepared chemoenzymatically using Neu5Ac aldolase to couple ManNAc and pyruvate. As this coupling reaction has an equilibrium constant of $28.7 \text{ M}^{-1}$, the yield of Neu5Ac is increased by using higher concentrations of either ManNAc/pyruvate or both (Figure 1.3).
1.1.5. **Biological Importance of Sialic Acid**

All sialic acids by virtue of their carboxylic acid group, which has a $\text{pK}_a$ of around 2.2, are negatively charged at physiological pH values.\(^{23}\) As sialic acids are often located at the non-reducing end of glycoconjugates, they have numerous and varied biological roles. That is, sialic acids affect their physicochemical environment due to a combination of their negative charge, size and hydrophilicity.

**Non-specific roles:** By virtue of their negative charge sialic acids bind and help transport positively charged ions such as $\text{Ca}^{2+}$. In addition, negative charge repulsion between sialylated molecules can cause conformational stabilization of enzymes and proteins in cell membranes as well as decreasing the viscosity of mucus substrates.\(^{24,25}\) The negative charged sialic acids that cover host cell surfaces protects the cells by slowing infection because bacterial cell envelopes are also negatively charged and thus limiting bacterial colonization. In addition, glycoproteins can be protected from proteolytic and thermal degradation by masking the protein itself from proteases.\(^{15}\) Furthermore, chemical derivatization of sialic acid residues modifies the glycoprotein’s physicochemical properties.\(^{25}\) For instance, the hydrophobicity of these complex structures is increased by both $O$-methylation and $O$-acetylation whereas the hydrophilicity increases on hydroxylation ($\text{Neu5Ac} \rightarrow \text{Neu5Gc}$) and the acidity is increased by sulfation. Therefore, any of these modifications may result in changes to the specific functions of these carbohydrates.

**Sialic acid as a biological mask:** Another significant biological role for sialic acids is to mask glycoprotein recognition by certain cells. The presence of sialic acids at the termini of blood cell glycoconjugates results in the material remaining in the blood stream. For instance, the glycans of erythrocytes (red blood cells) are terminated by sialic acids and the half life of erythrocytes is about 120 days. In this period of time, sialic acids are removed by serum sialidases.
(hydrolysis). These “unmasked” erythrocytes display the penultimate galactose residues, which bind to a specific receptor (Ashwell) in liver cells where they are degraded.\textsuperscript{26-28} This same mechanism also functions for thrombocytes and leucocytes, which are highly sialylated on their surfaces. After a certain time, these blood cells become desialylated and now bind to lectins exposed on macrophages, which results in degradation of these cells.\textsuperscript{27} The immune system often distinguishes between self and nonself by the presence of sialic acids; however, failure of this process may result in undesired immune system effects.\textsuperscript{24} Highly sialylated molecules or sialylation of foreign tissues may not be detected by the immune system.\textsuperscript{28} For instance, some tumor cells have a higher degree of sialylation on their surface (exogenous) molecules in comparison to normal tissue. As a result, the immune system cannot recognize these tumors and these abnormal cells are not removed by activated lymphocytes.\textsuperscript{29}

1.2. Kdn

1.2.1. General Background

3-Deoxy-D-glycero-D-galacto-non-2-ulosonic acid is the IUPAC name for Kdn (often called 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) (Figure 1.4), which was discovered in 1986 by Sadako Inoue and Ken Kitajima. The first reported characterization of Kdn was from the eggs of rainbow trout, and it was located at the terminal positions in the oligosialyl chains from a polysialoglycoprotein (PSGP).\textsuperscript{30} In 1978, Inoue and Iwasaki discovered the PSGPs to be a class of glycoproteins and characterization showed that; (i) more than 60 % of weight of PSGPs is sialic acid, and further (ii) oligo(poly)sialylglycan with a chain length of 6 occur in alveoli.\textsuperscript{31}
The term KDNoology was used in a review article on Kdn that detailed various aspects of its biology, chemistry and molecular biology. Several research groups have shown that Kdn is best categorized as a distinct and new member of the sialic acid family as it shares many properties in common with Neu5Ac (Figure 1.4). Although well documented natural occurrences of Kdn are limited and it is expected that this situation will change as research into the biological function of Kdn continues. Of note, Kdn can be found in all types of sialoglycoconjugates such as glycoproteins, glycolipids and bacterial polysaccharides. In addition, the analysis of all Kdn-linked structures shows that Kdn residues can be substituted for Neu5Ac and Neu5Gc in glycan structures. In theory, it is possible that biosynthesis of Kdn occurs by two steps, deacylation and deamination, from Neu5Ac. However, no evidence exists for the formation of Kdn from Neu5Ac.  

### Figure 1.4. Structures of Kdn.

![Structure of Kdn](image)
Figure 1.5. Proposed De novo pathway for biosynthesis of Kdn in Eukaryotes. 5'-triphosphates; ADP, Adenosine 5'-diphosphates; PEP, phosphoenolpyruvate; p_i, Inorganic phosphate; p_p_i, Inorganic pyrophosphate.

Kdn biosynthesis is similar to that of Neu5Ac and is shown in Figure 1.5, in which mannose (the precursor sugar) is acted upon by several enzymes. After activation of Kdn to Kdn-CMP, it is transferred to an acceptor sugar. Each reaction uses a specific enzyme that catalyzes the corresponding reaction. Both sialic acid (Neu5Ac) and Kdn are precursors for a number of modified sialic acid derivatives.

1.2.2. Biological Importance

It has been shown that Kdn residues can be clearly distinguished from Neu5Ac due to their distinctive properties and biological activities. For instance, Kdn glycoconjugates are protected from the attack of many bacteria and viruses since the ketosidic linkage of Kdn is
resistant to enzyme-catalyzed hydrolysis by the sialidases from these species.\textsuperscript{30,34-37} When Kdn residues are added to the non-reducing termini of the polysialylated groups, further elongation of such polysialylated chains will be terminated. Thus, Kdn capping of polysialyl chains effectively inhibits polysialyltransferases, which catalyze polysialylation.\textsuperscript{30,38,39} Due to its unique properties, a new area of research centred on the biosynthesis of Kdn-glycoconjugates is emerging. Although chemists are able to synthesize Kdn attached to small oligosaccharides, the enzymatic synthesis of Kdn-glycoconjugates is preferable due to the mild physiological-like conditions used in the coupling reactions.\textsuperscript{40,41} These mild conditions allow the glycoproteins, which become adorned with Kdn residues, to retain their biological activity. Examples of such glycoproteins are hormones and growth factors.\textsuperscript{39}

Recently, it has been shown that there is a relationship between malignant cancer tumors and Kdn expression. Thus, an increase in Kdn expression in certain human cancer cells is a marker for the reappearance of the cancer. Due to the importance of Kdn in cancer, research into Kdn and its derivatives will continue to grow.\textsuperscript{42}

\section*{1.3. Sialidases}

Sialidases (EC 3.2.1.18, \(N\)-acetylneuraminosyl glycohydrolases), which are also called neuraminidases, are a family of glycosidases (glycosyl hydrolases GH), and are classified in family GH33 (viral) and GH34. Exo-sialidases are responsible for catalyzing the hydrolytic cleavage of sialosidic linkages located at the terminal position of glycolipids and glycoproteins.\textsuperscript{43} The most prominent characteristic of this family is that hydrolysis occurs with net retention of configuration at the anomeric centre.\textsuperscript{44} Sialidases are found in bacteria, birds, mammals, fungi, mycoplasma and viruses but not in plants.\textsuperscript{45-49}

\subsection*{1.3.1. Background}

The discovery of sialidases occurred in the early 1940s by George Hirst. He showed that influenza virus can agglutinate red blood cells at low temperature, but after warming to room temperature these red blood cells become non-agglutinable.\textsuperscript{50} This lead to the postulate that an enzyme was present on the surface of the influenza virus. This enzyme was called the 'receptor-destroying enzyme' (RDE). Similar results were shown for red blood cells using enzymes from
*Vibrio cholerae* and *Clostridium*. The phenomenon of agglutination of red blood cells lead to the structural elucidation of sialic acid and of the interaction of this sugar and the sialoglycoconjugates present on cell surfaces with a second protein, hemagglutinin, which is found on the surface of the virus particles.

In 1956, Gottschalk showed that *N*-acetylneuraminic acid was the hydrolysis product of the receptor destroying enzyme (RDE) from viral and bacterial extract on salivary mucin, and thus, he named this enzyme "neuraminidase". During the same year, Heimer and Mayer showed that sialic acid was the cleavage product of receptor destroying enzyme (RDE) from a pneumococcal extract on salivary mucins and other glycoprotein substrates. He called this enzyme "sialidase", which was purified from *Vibrio cholerae*. Therefore, it was demonstrated that both enzymes "neuraminidase" and "sialidase" possess the same function.

The two terms "neuraminidase" and "sialidase" have been applied interchangeably in the literature. Nowadays, the term "sialidase" is preferred as these enzymes function on sialic acid and its derivatives, but not on neuraminic acid itself.

### 1.3.2. **Biological Importance of Sialidase**

Sialidases are widely distributed throughout nature, for example they can be found in mammals, viruses, bacteria and fungi, but not in plants. Although sialidases have extensive variations in their primary structures, substrate specificities and biochemical properties, all sialidases appear to have evolved from a common ancestor. As animals make and display diverse sialic acid residues on their glycoconjugates, they produce sialidases with distinct substrate specificities and subcellular locations. Factors that control the rate of sialidase-catalyzed hydrolysis of sialosides, include; (i) type of substrate (sialic acid derivatives); (ii) type of linkage to the underlying carbohydrate, (iii) identity of the underlying sugar residue. Thus, animals control the rate of sialoglycoconjugate clearance based on these factors. It is known that certain microorganisms produce sialidases which are involved in pathogenesis. These are described in detail below.
1.3.3. **Sialidases in Disease**

The influenza virus particle surface is studded with sialidase, this pathogenic enzyme makes influenza a constant threat to public health.\(^5^7\) As one of the two glycoproteins on the viral surface, influenza sialidase plays a significant role during infection. That is, its position on the virion surface allows it to assist in the release of progeny viral particles from infection sites by removal of sialic acid residues preventing viral recapture by hemagglutinin.\(^4^4,5^8\)

As noted above, sialidases can be found in a wide range of bacteria, including *Clostridium*, *Salmonella*, *Vibrio*, *Micromonaspora* and *Streptococcus*.\(^4,5^7\) Many bacteria use Neu5Ac as an energy and carbon source. For example, enteric bacteria, those that live inside the large intestine have a sialidase that hydrolyzes mucin carbohydrates (oligosaccharide) so that the organism can scavenge the resulting sialic acid.\(^4,5^9\) An example of a non-enteric pathogenic bacterium that possesses a sialidase is *Pseudomonas aeruginosa*, which is involved in respiratory disease. In many cases, pathogenic bacteria have excreted sialidases that are soluble in water, while other organisms have enzymes tethered to their bacterial surface e.g. *Streptococcus pneumoniae*, a major main cause of infection in the lower respiratory tract.\(^6^0\) On the other hand, some bacterial sialidases are not secreted; an example is *Clostridium perfringens*, which produces a small sialidase.\(^4^4,6^1\)

1.3.4. **Conserved Active Site Residues**

In 1983, Varghese and Laver solved the crystal structure of a sialidase from influenza type A virus.\(^6^2,6^3\) Based on protein sequence analysis and solved crystal structures, it was proposed that all sialidases possess several conserved active site residues.\(^6^4\) Figure 1.6 shows the important conserved active site residues in the crystal structure of the sialidase from influenza A/N2 complexed with Neu5Ac. The residues involving in the catalytic mechanism are a tyrosine (Tyr406), which acts as nucleophile, glutamic acid and aspartic acid residues (Glu277 & Asp151), that general acid/bases, and three arginine residues (Arg371, Arg118, Arg292) that bind the carboxylate group in Neu5Ac (Figure 1.6, 1.7). Figure 1.7 shows the important interactions corresponding to the strong cation-anion interactions between the positively charged arginine residues (Arg118, Arg371, Arg292) and the carboxylate group of Neu5Ac. Another ion-dipole interaction exists between an arginine residue (Arg152) and the oxygen atom of the 5-acetamido group. Hydrophobic interactions also occur between tryptophan and isoleucine residues with the
amidic methyl group. Other interactions involve hydrogen bonding between a glutamate residue (Glu276) and the hydroxyl groups on C8 and C9 and a similar interaction between glutamate 119 (Glu119) and the C4 hydroxyl group.44,65

Figure 1.6. X-ray crystal structure of the N2 influenza sialidase active site with α-Neu5Ac (blue backbone) bound (PDB Code 2BAT).
1.3.5. **Catalytic Mechanism of Sialidases**

As sialidases are retaining glycosidases, they hydrolyze α-linked Neu5Ac glycosides with net retention of anomeric configuration. The product of this enzymatic reaction is α-Neu5Ac, which has been shown by X-ray crystallography to bind to the active site of the influenza virus enzyme in a boat conformation. Guo and Sinnott performed a kinetic isotope effect (KIE) study on the *Vibrio cholera* sialidase-catalyzed hydrolysis of 4-nitrophenyl α-sialoside. These authors postulated that an oxacarbenium ion-like transition state was involved. The nucleophilic role of the active site tyrosine was shown by Watson et al. when they performed mechanistic studies on three different tyrosine mutant *Micromonospora viridifaciens* sialidas (MvS). They showed that for the serine, aspartic acid and glycine mutants, the reaction product was β-Neu5Ac; specifically, the reaction mechanism now involved an inversion of
configuration at the anomeric centre. These authors proposed that water, which could fit into the hole created by mutagenesis, was now the nucleophile.\textsuperscript{71} Watts \textit{et al} reported the first three-dimensional crystal structure of a sialosyl-intermediate using the \textit{T. rangeli} sialidase and two different fluorinated inactivators. Kinetic studies on these two inactivators allowed these authors to show that structural modifications in these inactivators altered the half-life of the covalent intermediate. Characterization of the glycosyl-intermediate complex was carried out using techniques such as mass spectroscopy and X-ray crystallography.\textsuperscript{72} These results supported the hypothesis that all sialidase mechanisms involve formation of a covalent sialosyl-intermediate between the tyrosine residue and the anomeric centre of the substrate.\textsuperscript{72}

![Diagram of proposed sialidase mechanism](image)

**Figure 1.8.** The proposed mechanism for retaining sialidase-catalyzed hydrolysis of α-sialosides.\textsuperscript{73}

Figure 1.8 shows the currently accepted catalytic mechanism for wild-type sialidases.\textsuperscript{73} Acid catalysis is provided by the aspartic acid residue, which assists cleavage of the glycosidic bond by protonation of the aglycone oxygen as it departs from the active site. Deprotonation of the nucleophilic tyrosine by the proximal glutamate residue makes the tyrosine a better
nucleophile as it attacks the anomic carbon. Following formation of the glycosyl-enzyme covalent intermediate, the aspartate residue deprotonates a water molecule as it attacks the anomic centre, a process that occurs simultaneously with or after the catalytic glutamic acid protonates the tyrosine. Thus, the resulting product is α-Neu5Ac which has a retained configuration at the anomic centre (Figure 1-8).

1.4. Sialidase Inhibitors

Since ~430 BC, humanity has faced the potentially fatal nature of influenza virus infections. The development of effective agents for inhibition of influenza virus has had limited success due to the ability of the influenza virus to evolve rapidly its two known antigens, neuraminidase (NA sialidase) and hemagglutinin (HA) that are present on the viral surface. Normally, the immune system copes with the gradual antigenic variation (i.e. antigenic shift) by production of new antibodies. However, the immune system cannot easily cope with antigenic shifts in the viral neuraminidases and hemagglutinins because production of new antibodies often cannot keep up with the spread of infection. Thus, a need exists for new inhibitors (and/or vaccines) for emerging strains of the virus.

Amantadine and its derivative, rimantadine shown in Figure 1.9, were the first antiviral candidates that were effective in treating influenza A viral infection by blocking the viral M2 ion-channel protein. However, these two drugs rapidly gave rise to resistant viral strains.

To design a clinically-useful inhibitor for the influenza viral sialidase, one must meet the following criteria: a) high potency, b) weak binder to human neuraminidases, c) appropriate pharmacokinetic properties, d) high therapeutic index, e) low allergenicity, f) economical to
produce and g) chemical stability. In addition to these criteria, a good inhibitor would ideally mimic the transition state structure of the enzymatic reaction.\(^{77}\) (Figure 1.8)

The original design of a transition state (TS) analogue inhibitor in which the TS was assumed to have oxacarbenium ion-character included two specific features; charge and conformation. However, it is not possible to mimic both charge and anomeric flattening simultaneously because of the presence of the anomeric carboxylate group. As a result, most inhibitors use a pseudo anomeric sp\(^2\) carbon, as seen in the inhibitor Neu2en5Ac (7) (Figure 1.10).\(^{78,79}\)

1.4.1. **Sialidase Inhibitors**

The early generation of sialidase inhibitors were based on the glycal, Neu2en5Ac (7) (5-acetamido-2,6-anhydro-3,5-dideoxy D-glycero-D-galacto-non-2-enonic acid) (Figure 1.10). Many researchers presumed that Neu2en5Ac mimicked an oxacarbenium ion-like transition state due to the presence of the double bond between C2 and C3, which flattens the ring. Inhibition studies on a viral sialidase with 7 showed that it is a good inhibitor with binding constants \(K_i \sim 1 \times 10^{-6} \text{M}\).\(^{80}\) A comparison of inhibition constants for \(\alpha\)-Neu5Ac (1) and Neu2en5Ac (7) shows that binding is increased by almost 1000-fold by incorporation of the double bond in Neu2en5Ac.

Therefore, researchers made Neu2en5Ac analogues to try and increase potency. The most potent compound among a series was the N-trifluoroacetamide analogue \((K_i = 8 \times 10^{-7} \text{M})\).\(^{81}\) However, in animal model studies, this inhibitor was shown to be not an effective antiviral due to non-selective binding and rapid excretion.\(^{76,80,82,83}\)

\(\alpha\)-Neu5Ac, 1  
Neu2en5Ac, 7

*Figure 1.10. Structure of the first class of sialidase inhibitors.*
1.4.2. **Tamiflu and Relenza**

Zanamivir, 4-deoxy-4-guanidino-Neu2en5Ac, which is also known as Relenza, is a potent and selective inhibitor for Influenza A and B virus sialidases. (Figure 1.11) Zanamivir is a derivative of Neu2en5Ac in which the C4-hydroxyl group is replaced by a guanidino moiety. As zanamivir has a very low bioavailability it needs to be administrated by inhalation.\(^7\)\(^8\)\(^4\) This compound has been proposed to be a mimic of the transition state for sialidase-catalyzed hydrolysis. The presence of the guanidino group gives a stronger interaction with the negatively charged glutamate 119 (Glu119) residue of the influenza viral sialidase and this results in a 100,000-fold higher potency than Neu2en5Ac (7) (Figure 1.7).\(^7\)

![Chemical Structure of successful sialidase Inhibitors.](image)

**Figure 1.11.** *Chemical Structure of successful sialidase Inhibitors.*

Oseltamivir (9), shown in Figure 1.11, which was marketed under the trade name Tamiflu, is another anti-influenza therapeutic that has a non-chair-based template (e.g. cyclohexene).\(^8\)\(^5\) This inhibitor (9), originally known as G4071, is a potent and selective antiviral drug. During the development of G4071 and oseltamivir (9) four concepts were used: 1) mimic an oxacarbenium ion-like transition state; 2) replace the glycerol side chain by a lipophilic group; 3) include a basic amino group at C4; and 4) esterify the carboxylic acid group to increase bioavailability.\(^8\)\(^4\)\(^,\)\(^8\)\(^6\)\(^,\)\(^8\)\(^7\) The ethyl ester of G4071 (oseltamivir 9) is a prodrug that increases the bioavailability of G4071. When oseltamivir (9) is orally administrated, it is efficiently converted to the active compound G4071 by esterases (Figure 1.11).\(^6\)\(^6\)\(^,\)\(^7\)\(^6\) This drug is widely used although the emergence of resistant influenza viral strains is decreasing its effectiveness.\(^7\)\(^6\) Thus, investigations have continued to identify potent, selective, and strong inhibitors against a variety of mutant virus strains. Peramivir (BCX-1812) (10) is another very potent inhibitor (Figure 1.12). It was shown that this drug candidate inhibited both Influenza A and B viral sialidases with few side effects and toxicity (Figure 1.12).\(^7\)\(^6\) However, the development of this compound was
stopped during phase III of the clinical trials as this material showed no activity towards strains of influenza virus that are resistant to oseltamivir.\textsuperscript{76,88} Peramivir\textsuperscript{®} (10) contains similar features to zanamivir (guanidino group) and the oseltamivir (O-isopentyl side chain).\textsuperscript{89,90}

![Chemical Structure of Peramivir](image)

Peramivir, BCX-1812, 10

\textit{Figure 1.12. Chemical structure of peramivir\textsuperscript{®}, a potent viral sialidase inhibitor but clinically ineffective.}

1.4.3. **Fluorinated Sialic Acid–Mechanism Based Inactivator**

Another class of sialidase inhibitors are fluorinated derivatives of sialic acid. It has also been shown that fluorinated carbohydrates are good tools to probe mechanisms of action.\textsuperscript{91} Ideally, the design of better inhibitors requires knowledge of the structure of the viral sialidase transition state. Figure 1.13 shows the mechanism for labelling sialidase by a fluorinated sialic acid derivative.
To stabilize the sialosyl-enzyme intermediate, it is necessary to slow down its hydrolysis.\textsuperscript{72,73,92} In this regard, Watts et al incorporated an electronegative fluorine atom adjacent to the anomeric centre (C3) to slow down both the rate of glycosylation ($k_1$) and deglycosylation ($k_2$) by destabilizing oxacarbenium ion-like transition states (Figure 1.13).\textsuperscript{72,93} To mitigate the rate decelerating effect for glycosylation a second electronegative fluorine atom, which is a good leaving group when departure is acid catalyzed, was attached to the anomeric centre (C2). Thus, the sialosyl-enzyme intermediate accumulates sufficiently to be observed and analyzed by X-ray crystallography and mass spectroscopy (MS). That is, it has been shown that fluorinated derivatives of sialic acid are capable of covalently labelling the catalytic nucleophile.\textsuperscript{94}

### 1.4.4. Reversible Inhibition

An inhibitor is a substance that is able to reduce an enzyme’s catalytic activity. Two major types of inhibitor exist and these are classified as either reversible or irreversible inhibitors. Reversible inhibition occurs when an inhibitor binds reversibly to an enzyme, which may or may
not be at the active site, through several electrostatic interactions such as hydrogen and/or ionic bonds and lipophilic interactions.\textsuperscript{95}

Reversible inhibitors are subdivided into three categories: competitive, uncompetitive and non-competitive. Non-competitive inhibition happens when an inhibitor binds to the free enzyme, and to the enzyme-substrate complex (ES). The result of this inhibitory action is the $V_{\text{max}}$ (maximum reaction velocity) is lowered and the $K_m$ (apparent dissociation constant) appears unaltered. When an inhibitor only binds to the enzyme-substrate (ES) complex, the inhibition is called uncompetitive. The overall result of this inhibitory activity is lowering of both $V_{\text{max}}$ and $K_m$.\textsuperscript{95-97} Competitive inhibition occurs when an inhibitor and substrate compete for binding to the same active site of the free enzyme.\textsuperscript{97} A more detailed description of competitive inhibition is presented below.

1.4.5. \textbf{Competitive Inhibitors}

\begin{equation}
\begin{aligned}
\text{E} + \text{S} & \xrightleftharpoons[k_{-1}]{k_1} \text{ES} \\
\text{ES} & \xrightarrow[k_{\text{cat}}]{+} \text{E} + \text{P} \\
\text{K}_i & \parallel \text{EI}
\end{aligned}
\end{equation}

\textit{Scheme 1.1. Kinetic scheme for competitive inhibition.}

The basis for competitive inhibition is shown in Scheme 1.1, where the free enzyme can bind either substrate or inhibitor but not both simultaneously. Given that substrate and inhibitor compete for the same active site, in many cases of competitive inhibition, the inhibitor resembles structural components of the substrate and/or the transition state of the enzymatic reaction. Thus, the inhibitor can interact with active site functional groups.\textsuperscript{96} A diagnostic feature of competitive inhibition is an unchanged $V_{\text{max}}$ value and an increase in that for $K_m$ (concentration of substrate at 50\% of maximum velocity).\textsuperscript{95,97} The Michaelis-Menten equation for competitive inhibition (eqn. 1.1) shows that the binding affinity term contains a factor of $(1 + [I]/K_i)$. 
1.4.6. **Irreversible Inhibition**

Inhibition that occurs due to irreversible covalent binding, which is often in the enzyme’s active site, is called irreversible inhibition. To recognize irreversible inhibition, the following criteria should be followed: i) covalent attachment of the inhibitor to the enzyme; ii) time-dependent loss of enzyme activity; and iii) no reversion to active enzyme. That is, the measured enzyme activity depends on the concentration of inhibitor and time.\(^{95,98}\) Scheme 1.2 is a generalized kinetic scheme for irreversible inhibition.

\[
\nu = \frac{V_{\text{max}}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)}
\]

Equation 1.1

**Scheme 1.2.** Kinetic scheme for irreversible inhibition.

In the overall scheme, the enzyme E and inactivator I bind with a dissociation constant \(K_i\) (concentration of inhibitor at 50% of maximal inactivation rate) to afford a reversibly formed enzyme-inactivator complex EI (Michaelis-Menten type complex). This complex EI is permanently transformed to the irreversible enzyme-inactivator complex EI* (Dead-end complex) with a first order rate constant of \(k_{\text{inact}}\) (maximal rate of enzyme inactivation).\(^{95}\)

A proposed classification for irreversible inhibitors depends on the mechanism of inhibition. The two classifications are: affinity labeling agents and mechanism-based enzyme inactivators.\(^{98}\)
1.4.7. **Affinity Labeling Agents**

\[
E + I \xrightleftharpoons[K_i]{\text{K_i}} EI \xrightarrow[k_{\text{inact}}]{\text{E-I}} E - I
\]

**Scheme 1.3. Affinity labeling scheme for irreversible inhibition**

Affinity labels are inactivators that modify the enzyme structure through the formation of an irreversibly-bound covalent adduct. Initially, an enzyme and inhibitor forms a reversible complex with a dissociation constant of \(K_i\). Then, a reactive functional group on the enzyme, such as -OH, -NH\(_2\) and -SH reacts with the inhibitor to form the covalent adduct (E-I) with an inactivation rate constant of \(k_{\text{inact}}\). The dissociation constant of \(K_i\) is the concentration of inhibitor that gives one-half of the maximal inactivation velocity, and \(k_{\text{inact}}\) is the maximum velocity for inactivation. Affinity labelling agents are used as valuable tools in biological chemistry. An example of affinity labeling agent is iodoacetamide to modify cysteine residues.

**Mechanism based enzyme inactivation**

The second type of irreversible inhibition is known as “mechanism-based inactivation”. In this case, the inhibitor (I) behaves as a substrate analogue that binds to the enzyme (E) active site with \(K_i\) dissociation constant to form an enzyme-inhibitor complex (EI). In the enzyme active site, the Michaelis complex EI undergoes aberrant catalysis and this results in formation of an irreversible modified E-I complex, which often occurs on a catalytic nucleophile in the active site. To assign an inhibitor as a mechanism-based inactivator, some criteria need to be met; i) a time-dependent enzyme inactivation, ii) a saturation kinetics, iii) a 1:1 stoichiometry ratio of inhibitor and enzyme, iv) faster rate of inactivation in absence of substrate, v) formation of highly reactive intermediate from the inhibitor that involves enzyme catalysis and vi) an active site residue that traps the reactive species. The last two are significant factors for recognition of mechanism-based inactivation. In some cases, enzyme activity returns by hydrolysis of the covalent adduct. The reversibility of some mechanism-based inactivators make their use as drug candidates desirable.

1.5. **Thesis Overview**

**Substrates used during this research:** the two substrates that were used in this research, 4-nitrophenol \(\alpha\)-D-sialoside and 4-methylumbelliferyl \(\alpha\)-D-sialoside, were synthesized by Drs.
Deepani Indurugalla and Fahimeh Shidmoossavee, respectively. I used these two substrates to monitor the remaining enzyme activity of the bacterial sialidase from *Micromonospora viridifaciens*, which was cloned, expressed and purified by Dr. Jaqueline Watson.  

Chapter two (materials and methods) contains the synthetic route used to prepare the two fluorinated inhibitors, 2,3-difluoroKdn and 2,3-difluoro sialic acid, and the kinetic experiments that I performed to measure the inactivation rate constants for these two compounds. The third chapter details the results that I obtained and a discussion of their importance by interpretation and comparison the inhibitory efficiencies and proficiencies of the these two inhibitors in order to probe the relative transition state stabilizations for the C-5 N-acetyl versus the C-5 hydroxyl group in these two inhibitors. To conclude this thesis, a discussion ensues in which these two kinetic parameters are compared with the catalytic efficiencies and proficiencies corresponding substrates.
2. Experimental Section

2.1. General Procedures, Materials, and Instrumentation

All chemical reagents were analytical grade or better; purchased from Sigma-Aldrich unless stated otherwise; and used without further purification. Solvents used for anhydrous reactions were either HPLC grade or dried and distilled prior to use. Dichloromethane and methanol were dried over calcium hydride and magnesium methoxide, respectively. For anhydrous reactions all glassware was dried overnight at 100–150 °C in an oven prior to use. Thin layer chromatography (TLC) was performed on aluminum sheets TLC plates (0.25 mm thickness) pre-coated with Merck silica gel 60 F254. Compounds were visualised with para-anisaldehyde. Flash column chromatography was performed using Fisher Scientific silica gel 60 (35-70 micron or 230-400 mesh). Melting points were recorded on OptiMelt melting point apparatus and are uncorrected. Optical rotations were determined using a Perkin-Elmer 341 polarimeter and units are reported in deg cm² g⁻¹ (concentration reported in units of g per 100 cm³). ¹H, ¹⁹F and ¹³C NMR spectra were recorded on a Bruker NMR spectrometer (either an AVAVG III 400, 500 or a TCI 600 MHz instrument). Deuterated chloroform (CDCl₃) and water (D₂O) were used as solvent and internal reference, chemical shifts (δ) were reported in parts-per-million (ppm) downfield from tetramethylsilane (TMS), and coupling constants (J) are listed in Hz. Milli-Q Water (18.2 MΩ cm⁻¹) was used all kinetic experiments. All pH values were measured using a VWR pH meter attached to a standard pH electrode. Graph Pad Prism (v4.0) was used to fit all kinetic data to the appropriate non-linear least squares equation.
2.2. Synthesis of 2,3-Difluorosialic Acid

Figure 2.1. Synthetic route to 2,3-difluorosialic acid (19). a) E. coli Neu5Ac-aldolase; pH = 8.0-8.5, 2-3 days; b) Dry MeOH; Amberlite IR 120 (H+ Form); c) (CH₃CO)₂O, pyrididine, 0 °C; separation of isomers; d) Hydrazinium acetate (CH₃CO₂⁻NH₂NH₃⁺), MeOH, CH₂Cl₂, 0 °C, 6h; e) Et₃N.3HF, CH₂Cl₂, XtalFluor-E, rt; f) NaOMe. MeOH, 0 °C, 6h; g) LiOH.H₂O, THF:H₂O, 0 °C, 1h.
2.2.1. **5-Acetamido-3,5-dideoxy-3-fluoro-D-erythro-L-manno-non-2-ulopyranosonic acid 13.**

Neu5Ac aldolase (60 mg, 30 u/mg) was added to solution of N-acetylmannosamine monohydrate (ManNAc) 11 (6.0 g, 27.5 mmol) and 3-fluoropyruvic acid sodium salt 12 (900 mg, 7.04 mmol) in water (34 mL). The pH was adjusted to 8.0-8.5 using HCl (1 M) or NaOH (1 M). The resultant mixture was left at room temperature for 3 days. The progress of the reaction was monitored by $^{19}$F NMR spectroscopy following the disappearance of the peak that corresponds to 3-fluoropyruvate (δ –233 ppm) and the appearance of the peak showing the formation of 3-fluorosialic acid 13 (δ –208.2 ppm). The protein was removed by denaturing at 50 °C for 10 min followed by centrifugation. The resulting mixture was filtered over an Amicon filter (0.2 µm). Purification of the mixture involved ion exchange column chromatography (Dowex 1x2 200 formate form) and eluting with a formic acid gradient (0 to 2 M). The fractions containing product were combined and lyophilised to afford 3-fluorosialic acid 13 as a white solid (1.86 g, 81%). mp = 157–159 °C (Lit 160 °C)\(^{101}\) 1H NMR (400 MHz, D$_2$O) 4.92 (m, 1H, $J_{3,4} = 2.6$, H-3), 4.25 (t, 1H, $J_{5,4} = 10.8$, $J_{5,6} = 10.8$, H-5), 4.16 (dd, 1H, $J_{4,5} = 10.8$, $J_{4,F3} = 30.0$, H-4), 3.85 (d, 1H, $J_{6,5} = 10.8$, $J_{6,5} = 1.1$, H-6), 3.70–3.66 (m, 2H), 3.58 (dd, 1H, $J_{7,6} = 10.8$, $J_{7,8} = 10.8$, H-7), 3.33 (d, 1H, $J = 9.8$), 2.05 (s, 3H, CH$_3$, NCOMe). $^{19}$F (376 MHz, D$_2$O) –208.13 (dd, $J_{F3,4} = 29.9$, $J_{F3,3} = 49.3$, F-3). The above NMR spectral data match those reported in the literature.\(^{101}\)

![Figure 2.2](attachment:structure.png)

**Figure 2.2. Structure of 5-acetamido-3,5-dideoxy-3-fluoro-D-erythro-L-manno-non-2-ulopyranosonic acid.**

2.2.2. **Methyl 5-acetamido 3,5-dideoxy-3-fluoro-D-erythro-L-manno-non-2-ulopyranosonate 14.**

To a solution of 3-fluorosialic acid 13 (1.54 g, 4.71 mmol) in dry methanol (29 ml) was added Amberlite IR-120 resin (H$^+$ form) (1.54 g). This mixture was left to stir at room temperature overnight. Progress of the reaction was monitored by thin layer chromatography (TLC) using EtOAc/MeOH/H$_2$O/HOAc (4/2/1/0.1, v/v) as solvent. When the reaction was
complete, it was filtered to remove the resin, which was rinsed with dry methanol (10 ml). The resultant filtrates were dried in vacuo to obtain a white solid of 3-fluorosialic acid methyl ester 14 (1.50 g, 94%). mp = 129–132 °C (Lit 131-133 °C).\(^{72}\) \(^{1}\)H NMR (400 MHz, D\(_2\)O) 4.98 (dd, 1H, J\(_{3,4}\) = 2.4, J\(_{3,F}\) = 49.2, H-3), 4.27 (t, 1H, J = 10.0, H-5), 4.17 (dd, 1H, J\(_{4,5}\) = 10.6, J\(_{4,3}\) = 2.4, J\(_{4,F3}\) = 29.5, H-4), 4.13 (dd, 1H, J\(_{6,5}\) = 10.4, J\(_{6,7}\) = 1.0, H-6), 3.81–3.87 (m, 2H, H-8, H-9'), 3.89 (s, 3H, OMe), 3.66 (dd, 1H, J\(_{9,9'}\) = 11.6, J\(_{9,8}\) = 5.9, H-9), 3.59 (dd, 1H, J\(_{7,8}\) = 8.2, J\(_{7,6}\) = 1.0, H-7), 2.06 (s, 3H, NHCOMe); \(^{19}\)F (376 MHz, D\(_2\)O) –208.13 (dd, J\(_{F3,4}\) = 29.5, J\(_{F3,3}\) = 49.1, F-3). The above NMR spectral data match those reported in the literature.\(^{101}\)

![Structure of methyl 5-acetamido 3,5-dideoxy-3-fluoro-D-erythro-L-manno-non-2-ulopyranosonate.](image)

**Figure 2.3.** Structure of methyl 5-acetamido 3,5-dideoxy-3-fluoro-D-erythro-L-manno-non-2-ulopyranosonate.

2.2.3. **Methyl 5-acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-α-L-manno-non-2-ulopyranosonate 15**

To a cooled solution of methyl ester 14 (1.51 g, 4.4 mmol) in dry pyridine (19.2 mL) at 0 °C was added acetic anhydride (22.3 mL, 240 mmol) and 4-(N,N-dimethylamino)pyridine (0.16 g, 1.3 mmol). The reaction mixture was stirred at 0 °C for 4 h and then left at room temperature for 48 h. The reaction was monitored by thin layer chromatography (TLC) using EtOAc/Hex (9/1, v/v) as solvent. The resultant mixture was extracted with dichloromethane (4 x 45 mL) and then the combined organic layers were washed with saturated sodium bicarbonate solution (100 mL), dilute sulfuric acid (10% v/v, 100 mL), water (100 mL) and brine (100 mL). The organic layer was then dried over the sodium sulfate and azeotropic distillation was used to remove the remaining pyridine. Flash chromatography (EtOAc/Hex, 9:1 v/v) was used to purify the residue to afford fully protected 3-fluorosialic acid 15 as a colorless oil (1.97 g, 81%)\(^{72,101}\). \(^{1}\)H NMR (400 MHz, CDCl\(_3\)) 5.58 (ddd, 1H, J\(_{4,F3}\) = 27.8, J\(_{4,5}\) = 10.8, J\(_{4,3}\) = 2.5, H-4), 5.36 (dd, 1H, J\(_{7,8}\) = 5.2, J\(_{7,6}\) = 2, H-7), 5.15 (ddd, 1H, J\(_{8,9}\) = 6.4, J\(_{8,F9}\) = 2.5, H-8), 4.95 (br dd, 1H, J\(_{3,F3}\) = 49.1, J\(_{3,4}\) = 2.5, H-3), 4.57 (dd, 1H, J\(_{g,9}\) = 12.5, J\(_{g,8}\) = 2.5, H-9'), 4.26 (d, 1H, J\(_{6,5}\) = 10.6, 5H-9), 4.22 (dd, 1H, J\(_{g,F9}\) = 12.5, J\(_{g,8}\) = 6.4, H-9), 4.20–4.14 (m, 1H, H-5), 3.86 (s, 3H, OCH\(_3\)), 2.20, 2.18, 2.13, 2.06, 2.05 (5
s, 15H, OAc). $^{19}$F (376 MHz, CDCl$_3$) –208.87 (dd, $J_{F3,H4} = 27.9$, $J_{F3,H4} = 49.1$, F-3) The above NMR spectral data match those reported in the literature.$^{101}$

\[ \text{15} \]

**Figure 2.4. Structure of methyl 5-acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-\alpha-L-manno-non-2-ulopyranosonate.**

### 2.2.4. Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-\alpha-L-manno-non-2-ulopyranosonate 16

Hydrazinium acetate (615 mg, 6.7 mmol) was dissolved in dry methanol (12 mL) and this solution was added to a solution of 15 (1.23 g, 2.2 mmol) in dry dichloromethane (12 mL). This reaction mixture was stirred at 0 °C for 6 h. Progress of the reaction was monitored by thin layer chromatography (TLC) using CH$_2$Cl$_2$/MeOH (9.5/0.5, v/v) as solvent and when complete the reaction mixture was concentrated *in vacuo* and the residue was extracted with ethyl acetate (3 x 50 mL). The organic solution was washed with water (100 mL) and then it was dried over anhydrous sodium sulphate (Na$_2$SO$_4$). The solvent was removed under vacuum and the resulting residue was purified by flash chromatography CH$_2$Cl$_2$/MeOH (9.5/0.5 v/v) to afford the hemiketal as a colorless syrup (1.03 g, 91 %). $^1$H NMR (400 MHz, CDCl$_3$) 5.58 (d, 1H, $J_{\text{NHAc,5}} = 9.9$, NH), 5.36 (br dd, 1H, $J_{4,F3} = 27.9$, $J_{4,5} = 10.8$, H-4), 5.23–5.35 (m, 2H), 4.85 (br dd, 1H, $J_{3,F3} = 49.8$, $J_{3,4} = 1.9$, H-3), 4.32–4.48 (m, 1H), 4.24 (d, 1H), 4.02–4.14 (m, 2H), 3.85 (s, 3H, OCH$_3$), 2.14, 2.08, 2.02, (3 s, 12H, OAc), 1.91 (s, 3H, NHCOCH$_3$). $^{19}$F (376 MHz, CDCl$_3$) –204.83 (dd, $J_{F3,H4} = 27.8$, $J_{F3,H4} = 49.7$, F-3). The above NMR spectral data match those reported in the literature.$^{93}$
2.2.5. **Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-β-L-manno non-2-ulopyranosyl fluoride 17**

Diethylaminodifluorosuminium tetrafluoroborate (XtalFluor-E) (1.3 g, 6.1 mmol) and hemiacetal 16 (1.03 g, 2.03 mmol) were added to a mixture of triethylamine trihydrofluoride (Et₃N.3HF) (1.4 mL, 8.1 mmol) and anhydrous dichloromethane (10 mL). The resultant mixture was left to stir at room temperature for 4 h. Reaction progress was followed by using thin layer chromatography with EtOAc/Hex (8.5/1.5, v/v) as solvent. The reaction mixture was then neutralized by the addition of aqueous solution of sodium bicarbonate (~35 mL, 5%) and extracted with dichloromethane (3 x 50 mL). The combined organic layer was washed with brine (100 mL) and dried over anhydrous sodium sulphate (Na₂SO₄). The resultant residue was passed through a pad of silica using EtOAc/Hex (8.5/1.5, v/v) and then evaporation of the solvent gave difluorosialic acid 17 and an isomer (919 mg, 89%). The ratio of the two major isomers of per-O-acetylated-2,3-difluorosialic acid was measured using ¹⁹F NMR spectroscopy (β/α, 2/1). Crystallization from ethyl acetate and methyl tert-butyl ether solution gave pure per-O-acetylated-2,3-difluorosialic acid 17 as long white needles. mp = 66–69 °C (Lit 68-70 °C). ¹H NMR (500 MHz, CDCl₃): 5.49 (br dd, 1H, J₄,F₃ = 25.5, J₄,5 = 10.8, H-4), 5.42–5.35 (m, 2H), 5.29 (m, 1H, H-7), 5.13 (dt, 1H, J₃,F₃ = 50.7, J₃,4 = 2.6, H-3), 4.31–4.40 (m, 2H, H-6, H-9'), 4.31 (dd, 1H, J₉,9' = 12.6, J₉,8 = 5.1, H-9), 4.01–4.09 (m, 1H, H-5), 3.91 (s, 3H, OCH₃), 2.17, 2.12, 2.02, (4 s, 12H, OAc), 1.94 (s, 3H, NHCOCH₃). ¹³C NMR (151 MHz, CDCl₃): 20.77, 20.86, 20.90, 21.06 (OAc), 23.48 (NHaC), 45.96 (C-5), 53.94 (OME), 62.04 (C-9), 67.26 (C-7), 68.61 (dd, J₄,F₃ = 17.4, J₄,F₂ = 6.0, C-4), 69.09 (C-8), 71.95 (d, J₆,F₃ = 3.9 Hz, C-6), 85.71 (dd, J₃,F₂ = 19.4, J₃,F₃ = 194.6 Hz, C-3), 104.77 (dd, J₂,F₂ = 17.4, J₂,F₂ = 225.6 Hz, C-2), 164.43 (br d, J₁,F₂ = 29.8 Hz, C-1), 169.91, 170.43, 170.49, 170.67, 170.71 (s, C=O). ¹⁹F (471 MHz, CDCl₃) –216.93 (ddd, J₉₃,H₃ = 50.5, J₉₃,H₄ = 25.5, J₉₃,F₂ = 11.2, F-3), –123.36 (d, J₉₂,F₃ = 11.3, F-2). HRMS-FAB (m/z): [M+H⁺]
calcd for C_{20}H_{27}F_{2}N_{1}O_{12}, 512.1574; Found, 512.1582. The above NMR spectral data match those reported in the literature.\textsuperscript{72}

![Figure 2.6](image)

**Figure 2.6.** Structure of methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride.

### 2.2.6. Methyl 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride 18

To a solution of 17 (189.4 mg, 0.37 mmol) in dry methanol (10 mL) at 0 °C was added sodium methoxide (20 mg, 0.37 mmol) and this mixture was stirred for 6 h. The reaction mixture was neutralized by addition of Amberlite IR-120+ resin (H\(^+\) form). This solution was then filtered and the resin was washed with dry methanol. The combined filtrate was concentrated to afford the desired product as a colorless oil 18 (109.1 mg, 86%). The resultant product was used in the next step without further purification.\(^1\)H NMR (400 MHz, D\(_2\)O) 5.22 (dt, 1H, \(J_{3,F3} = 51.4, J_{3,F2} = 2.6, J_{3,A} = 2.6, H\)-3), 4.29 (t, 1H, \(J_{5,A} = 10.3, H\)-5), 4.17 (ddd, 1H, \(J_{4,F3} = 26.4, J_{4,F5} = 10.7, J_{4,F5} = 2.04, H\)-4), 3.84–3.90 (m, 2H, H-6, H-8), 3.67–3.60 (m, 1H, H-9), 3.56 (d, 1H, \(J = 8.9, H\)-7), OCH\(_3\)), 2.05 (s, 3H, NHCOCH\(_3\)).\(^{19}\)F NMR (471 MHz, CDCl\(_3\)) \(-216.93\) (ddd, \(J_{F3,H1} = 50.5, J_{F3,H4} = 25.5, J_{F3,F2} = 11.2, F\)-3), \(-123.36\) (d, \(J_{F2,F3} = 11.3, F\)-2). The above NMR spectral data match those reported in the literature.\textsuperscript{94}

![Figure 2.7](image)

**Figure 2.7.** Structure of methyl 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride.
2.2.7. 5-Acetamido-2,3,5-trIDEOXY-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride 19

Compound 18 (109.10 mg, 0.32 mmol) was dissolved in THF/H2O (3/1 v/v) followed by the addition of LiOH.H2O (13.5 mg, 0.32 mmol). The resultant mixture was stirred at 0 °C for 1 h, when the reaction was complete as shown by thin layer chromatography (TLC) analysis using EtOAc/MeOH/H2O/HOAc (4/2/1/0.1, v/v) as solvent. The reaction mixture was neutralized by adding Amberlite IR-120+ resin (H+ form), which was then filtered to remove the resin. The resin was washed with dry methanol and then the combined filtrate was concentrated in vacuo. The remaining aqueous residue was then lyophilized to afford the final product 2,3-difluorosialic acid 19 (92.7 mg, 88%). mp = 21–25 °C (Lit 21-25 °C)72. [α]D20 = −29 (c = 0.1, H2O) 1H NMR (600 MHz, D2O) 5.22 (dt, 1H, J3,F3 = 51.4, J3,F2 = 2.6, J3,A = 2.6, H-3), 4.29 (t, 1H, J3,A = 10.6, H-5), 4.17 (br dd, 1H, J4,F3 = 28.4, J4,A = 10.3, H-4), 3.84–3.91 (m, 2H, H-8, H-9), 3.82 (br d, 1H, J5,A = 10.6, H-6), 3.62 (br dd, 1H, J6,F = 11.8, J6,F2 = 6.2, H-9), 3.57 (br d, 1H, J7,F = 9.2, H-7), 2.05 (s, 3H, NHCOCH3). 13C NMR (151 MHz, D2O) 21.48 (s, 1C, NHCOCH3), 46.36 (d, J5,F = 3.3, C-5), 62.46 (C-9), 67.38 (C-7), 68.63 (br dd, J4,F2 = 5.9, J4,F3 = 18.0, C-4), 69.98 (C-8), 72.10 (d, 1C, J6,F2 = 4.1, C-6), 88.35 (br dd, 1C, J3,F3 = 183.9, J3,F2 = 18.5, C-3), 106.07 (dd, 1C, J2,F = 219.5, J2,F3 = 14.9, C-2), 168.80 (dd, J1,F2 = 26.9, C-1), 174.56 (s, C=O). 19F (376 MHz, D2O) –217.95 (ddd, JF3,H3 = 51.5, JF3,H4 = 28.5, JF3,F2 = 11.2, F-3); −121.27 (d, JF2,F3 = 11.6, F-2). HRMS-FAB (m/z): [M+H+] calcd for C11H17F2N1O8, 330.0995; Found, 330.0997. The above NMR spectral data match those reported in the literature.72

![Structure of 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride.](image)

Figure 2.8. Structure of 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonyl fluoride.
2.3. Synthesis of 2,3-Difluoro-Kdn

Figure 2.9. Synthetic route to 2,3-DifluoroKdn. a) E. coli Neu5Ac-aldolase; pH = 8.0-8.5, 3-4 days; b) Dry MeOH; Amberlite IR 120 (H⁺ Form); c) (CH₃CO)₂O, pyridine, 0 °C, separation of isomers; d) Hydrazinium acetate (CH₃CO₂⁺NH₂NH₂⁺); MeOH, CH₂Cl₂, 0 °C, 6h; e) Et₃N.3HF, CH₂Cl₂, XtalFluor-E, rt; f) NaOMe, MeOH, 0 °C, 3h; g) LiOH.H₂O, THF:H₂O, 0 °C, 1h.

e) (ii) XtalFluor-E = Et⁺N=SF₂BF₄⁻
2.3.1. 3-Deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonic acid 23

Neu5Ac aldolase (30 mg, 30 u/mg) was added to solution of D-mannose (Man) (21) (3.0 g, 16.7 mmol) and 3-fluoropyruvic acid sodium salt (12) (450 mg, 3.49 mmol) in water (17 mL). The pH was adjusted to 8.0-8.5 using HCl (1 M) or NaOH (1 M). The resultant mixture was left at room temperature for 4 days. The progress of the reaction was monitored by $^{19}$F NMR spectroscopy following the disappearance of the peak that corresponds to 3-fluoropyruvate ($\delta$ – 233 ppm) and the appearance of the peak showing the formation of 3-fluoro-Kdn (23) ($\delta$ –208 ppm). The protein was then removed by denaturation at 50 °C for 10 min followed by centrifugation. The resulting mixture was filtered over an Amicon filter (0.2 µm). Purification of the mixture involved ion exchange column chromatography (Dowex 1x2 200 formate form) and eluting with a formic acid gradient (0 to 2 M). The fractions containing product were combined and lyophilised to afford 3-fluoro-Kdn (23) as a white powder (828 mg, 83%). $\text{mp} = 150–152 ^\circ \text{C}$, $^1$H NMR (400 MHz, D$_2$O) 4.90 (dd, 1H, $J_{3,4} = 2.1$, $J_{3,F3} = 49.2$, H-3), 4.04 (dd, 1H, $J_{6,7} = 2.0$, $J_{6,5} = 10.3$, H-6), 3.82–3.78 (m, 2H), 3.74–3.67 (m, 2H), 3.55 –3.49 (m, 2H). $^{19}$F (376 MHz, D$_2$O) –208.13 (dd, $J_{F3,4} = 32.2$, $J_{F3,3} = 49.2$, F-3). The above NMR spectral data match those reported in the literature.$^{72}$

![Structure of 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonic acid](image)

**Figure 2.10. Structure of 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosonic acid**

2.3.2. Methyl 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ululosonate 24

To a solution of 3-fluoro-Kdn (23) (541 mg, 1.9 mmol) in dry methanol (30 mL) was added Amberlite IR-120 resin (H$^+$ form) (600 mg). This mixture was stirred at room temperature overnight. Progress of the reaction was monitored by thin layer chromatography (TLC) using EtOAc/MeOH/H$_2$O/HOAc (4/2/1/0.1, v/v) as solvent. When the reaction was completed, it was filtered to remove the resin, which was rinsed with dry methanol (10 ml). The resultant filtrates
were dried \textit{in vacuo} to obtain a white solid of 3-fluoro-Kdn methyl ester (24) (482 mg, 85%). $^1$H NMR (400 MHz, D$_2$O) 4.93 (dd, 1H, $J_{3,4} = 2.5$, $J_{3,F} = 49.2$, H-3), 4.05 (d, 1H, $J_{6,5} = 10.4$, H-6), 4.01 (ddd, 1H, $J_{4,5} = 9.6$, $J_{4,3} = 2.4$, $J_{4,F3} = 30.2$, H-4), 3.75–3.95 (m, 4H, H-5, H-7, H-8, H-9), 3.85 (s, 3H, OMe), 3.68 (dd, 1H, $J_{9,9'} = 11.9$, $J_{9,8} = 5.8$, H-9); $^{19}$F (376 MHz, D$_2$O) –207.25 (dd, $J_{F3,4} = 30.2$, $J_{F3,3} = 49.2$, F-3).

\begin{center}
\begin{tikzpicture}
\node[anchor=west] (O) at (0,0) {Figure 2.11.}
\node[anchor=west] (H) at (0,0) {Structure of methyl 3-deoxy-3-fluoro-\(\text{D-erythro-\(\beta\)-L-manno}\)-non-2-ulosonate.}
\end{tikzpicture}
\end{center}

2.3.3. \textbf{Methyl 2,4,5,7,8,9-hexa-O-acetyl-3-deoxy-3-fluoro-\(\text{D-erythro-\(\alpha\)-L-manno}\)-non-2-ulosonate 25}

To a cooled solution of methyl ester (24) (482 mg, 1.61 mmol) in dry pyridine (3.4 mL) at 0 °C was added acetic anhydride (3.9 mL, 42 mmol) and 4-(N,N-dimethylamino)pyridine (28.1 mg, 0.23 mmol). The reaction mixture was stirred at 0 °C for 4 h and then left at room temperature for 48 h. The reaction was monitored by thin layer chromatography (TLC) using EtOAc/Hex (1/1, v/v) as solvent. The resultant mixture was extracted with dichloromethane (5 x 30 mL) and then the combined organic layers were washed with saturated sodium bicarbonate solution (75 mL), dilute sulfuric acid (10% v/v, 75 mL), water (75 mL) and brine (75 mL). The organic layer was then dried over the sodium sulfate and azeotropic distillation was used to remove the remaining pyridine. Flash chromatography (EtOAc/Hex, 1/1 v/v) was used to purify the residue to afford per-acetylated 3-fluoro-Kdn (25) as a colorless syrup (776.1 mg, 87.5%). $^1$H NMR (400 MHz, CDCl$_3$) 5.38 (dd, 1H, $J_{7,8} = 5.5$, $J_{7,6} = 2.3$, H-7), 5.25–5.35 (m, 2H, H-4, H-5), 5.17 (td, 1H, $J_{8,7} = 5.8$, $J_{8,9'} = 5.95$, $J_{8,9} = 2.4$, H-8), 4.97 (br dd, 1H, $J_{3,F3} = 48.7$, $J_{3,4} = 2.2$, H-3), 4.55 (dd, 1H, $J_{9,9'} = 12.4$, $J_{9',8} = 2.4$, H-9), 4.18 (dd, 1H, $J_{9,9'} = 12.5$, $J_{9',8} = 6.2$, H-9’), 4.10 (d, 1H, $J_{6,5} = 9.2$, H-6), 3.84 (s, 3H, OCH$_3$), 2.19, 2.13, 2.09, 2.05, 2.04, 2.03 (6 s, 18H, OAc). $^{13}$C NMR (151 MHz, CDCl$_3$): 20.08, 20.12, 20.15, 20.26, 20.39, (6 s OAc), 53.09 (OMe), 61.40 (C-9), 63.13 (C-5), 66.49 (C-7), 69.27 (d, $J_{4,F3} = 16.8$, C-4), 70.31 (C-8), 71.13 (C-6), 86.98 (br d, $J_{3,F3} = 185.6$ Hz, C-3), 94.67 (d, $J_{2,F3} = 29.1$, C-2), 164.28, 166.46, 168.74, 169.45, 169.59, 170.08 (7C,
C-1, C=O). $^1$H NMR (400 MHz, CDCl$_3$) 5.38–5.18 (m, 4H, H-4, H-5, H-7, H-8), 4.93 (br dd, 1H, $J_{3,F3} = 49.0$, $J_{3,4} = 2.3$, H-3), 4.55 (dd, 1H, $J_{9,9'} = 12.4$, $J_{9,8} = 2.4$, H-9) 4.18 (dd, 1H, $J_{9,9'} = 12.5$, $J_{9',8} = 6.2$, H-9'), 4.10 (d, 1H, $J_{6,5} = 9.2$, H-6), 3.85 (s, 3H, OCH$_3$), 2.11, 2.08, 2.06, 2.03, 2.01 (5 s, 15H, OAc). $^{19}$F (376 MHz, CDCl$_3$) –204.57 (dd, $J_{F3,3} = 49.3$, $J_{F3,4} = 28.1$, F-3). The above NMR spectral data match those reported in the literature.$^{72}$

**Figure 2.12.** Structure of methyl 2,4,5,7,8,9-hexa-O-acetyl-3-deoxy-3-fluoro-D-erythro-α-L-manno-non-2-ulosonate.

### 2.3.4. Methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-α-L-manno-non-2-ulosonate 26

Hydrazinium acetate (125 mg, 1.36 mmol) was dissolved in dry methanol (15 mL) and this solution was added to a solution of (25) (705 mg, 1.28 mmol) in dry dichloromethane (15 mL). This reaction mixture was stirred at 0 °C for 6 h. Progress of the reaction was monitored by thin layer chromatography (TLC) using EtOAc/MeOH (1/1, v/v) solvent. Following completion of the reaction, volatiles were removed in vacuo and the residue was extracted with ethyl acetate (3 x 50 mL). The organic solution was washed with water (100 mL) and then it was dried over anhydrous sodium sulphate (Na$_2$SO$_4$). The solvent was removed under vacuum and the resulting residue was purified by flash chromatography EtOAc/Hex (1/1, v/v) to afford the hemiketal as a colorless syrup (26) (482 mg, 74%). $^1$H NMR (400 MHz, CDCl$_3$) 5.38–5.18 (m, 4H, H-4, H-5, H-7, H-8), 4.93 (br dd, 1H, $J_{3,F3} = 49.0$, $J_{3,4} = 2.3$, H-3), 4.55 (dd, 1H, $J_{9,9'} = 12.4$, $J_{9,8} = 2.4$, H-9) 4.18 (dd, 1H, $J_{9,9'} = 12.5$, $J_{9',8} = 6.2$, H-9'), 4.10 (d, 1H, $J_{6,5} = 9.2$, H-6), 3.85 (s, 3H, OCH$_3$), 2.11, 2.08, 2.06, 2.03, 2.01 (5 s, 15H, OAc). $^{19}$F (376 MHz, CDCl$_3$) –204.57 (dd, $J_{F3,3} = 49.3$, $J_{F3,4} = 28.1$, F-3). The above NMR spectral data match those reported in the literature.$^{72}$
Figure 2.13. Structure of methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-α-L-manno-non-2-ulosonate.

2.3.5. Methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride 27

Diethylaminodifluorosufinium tetrafluoroborate (XtalFluor-E) (390 mg, 1.7 mmol) and hemiacetal (26) (290 mg, 0.6 mmol) were added to a mixture of triethylamine trihydrofluoride (Et₃N.3HF) (0.38 mL, 2.3 mmol) and anhydrous dichloromethane (3 mL). The resultant mixture was left to stir at room temperature for 4 h. Reaction progress was followed by using thin layer chromatography with EtOAc/Hex (1/1, v/v) as solvent. After completion the reaction mixture was neutralized by the addition of an aqueous solution of sodium bicarbonate (5%) and extracted with dichloromethane (3 x 40 mL). The combined organic layer was washed with brine (100 mL) and dried over anhydrous sodium sulphate (Na₂SO₄). The solvent was removed under vacuum and the resulting residue was purified by flash chromatography EtOAc/Hex (1/1, v/v) to afford the per-O-acetylated-2,3-difluoro-Kdn (27) as a colorless oil (159.6 mg, 55%). ¹H NMR (400 MHz, CDCl₃) 5.40–5.33 (m, 2H, H-8, H-7), 5.29 (dd, 1H, J₅,₆ = 9.6, J₅,₄ = 9.6, H-5), 5.19 (dd, 1H, J₄,₅ = 9.6, J₄,F₃ = 24.9, H-4), 5.12 (br dd, 1H, J₃,F₃ = 49.9, J₃,₄ = 2.35, H-3), 4.33 (dd, 1H, J₉,₉' = 12.6, J₉,F₄ = 2.1, H-9), 4.22–4.14 (m, 2H, H-9, H-6), 3.89 (s, 3H, OCH₃), 2.12, 2.10, 2.07, 2.05, 2.04 (5s, 18H, OAc). ¹³C NMR (151 MHz, CDCl₃): 53.39 (OMe), 61.21 (C-9), 63.03, 65.55, 68.07, (C-5, 7, 8), 69.44 (dd, J₄,F₃ = 17.1, J₄,F₂ = 5.5, C-4), 71.52 (d, J₆,F₃ = 4.0 Hz, C-6), 85.18 (dd, J₃,F₂ = 19.6, J₃,F₃ = 194.2 Hz, C-3), 104.03 (dd, J₂,F₃ = 17.7, J₂,F₂ = 228.1 Hz, C-2), 163.64 (br d, J₇,F₂ = 29.9 Hz, C-1), 168.65, 169.12, 169.30, 169.45, 170.06 (s, C=O). ¹⁹F (376 MHz, CDCl₃), -215.6 (ddd, J₉,F₃,₈' = 49.8, J₉,F₄,₈ = 24.6, J₉,F₃,F₂ = 10.9, F-3), -124.5 (d, J₉,F₂,F₃ = 10.3, F-2). HRMS-FAB (m/z): [M+H⁺] caleed for C₂₀H₂₂F₂O₁₃, 513.1414; Found, 513.1398. The above NMR spectral data match those reported in the literature.⁷²
2.3.6. **Methyl 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride 28**

To a solution of 27 (110 mg, 0.22 mmol) in dry methanol (5 mL) at 0 °C was added sodium methoxide (12 mg, 0.22 mmol) and this mixture was stirred for 3 h. The reaction mixture was neutralized by addition of Amberlite IR-120+ resin (H⁺ form). This solution was then filtered and the resin was washed with dry methanol. The combined filtrate was concentrated to afford the desired product as a colorless oil (28) (61.7 mg, 95 %). The resultant product was used in the next step without further purification.

2.3.7. **3-Deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride 29**

Compound 28 (61.7 mg, 0.21 mmol) was dissolved in THF/H₂O (3/1 v/v) (4 mL) followed by the addition of LiOH.H₂O (8.2 mg, 0.21 mmol). The resultant mixture was stirred at 0 °C for 1 h, when the reaction was complete as shown by thin layer chromatography (TLC) analysis using EtOAc/MeOH/H₂O/HOAc (4/2/1/0.1, v/v) as solvent. The reaction mixture was
then neutralized by adding Amberlite IR-120+ resin (H\(^+\) form), which was subsequently filtered to remove the resin. The resin was washed with dry methanol and then the combined filtrate was concentrated. The remaining aqueous residue was then lyophilized to afford the final product 2,3-difluoro-Kdn (29) (54 mg, 91%). \(^1\)H NMR (600 MHz, D\(_2\)O) 5.21 (br dt, 1H, \(J_{3,F3} = 51.3, J_{3,F2} = 3.1, J_{3,4} = 2.9, \text{H-3}\)), 4.10–4.02 (m, 1H, \text{H-4}), 3.98–3.85 (m, 4H, \text{H-5, H-7, H-8, H-9'}), 3.74 (d, 1H, \(J_{6,5} = 10.1, \text{H-6}\)), 3.70 (dd, 1H, \(J_{9,9'} = 12.4, J_{9,8} = 6.5, \text{H-9}\)). \(^{13}\)C NMR (151 MHz, D\(_2\)O) 62.51 (s, C-9); 64.86 (s, C-5), 67.11 (s, C-7), 70.29 (s, C-8), 70.79 (dd, \(J_{4,F2} = 5.4, J_{4,F3} = 17.4, \text{C-4}\), 73.28 (d, 1C, \(J_{6,F2} = 3.4, \text{C-6}\)), 89.24 (dd, \(J_{5,F3} = 182.6, J_{3,F2} = 18.5, \text{C-3}\)), 106.30 (br dd, \(J_{2,F} = 219.4, J_{2,F3} = 14.8, \text{C-2}\)), 169.24 (d, \(J_{1,F2} = 26.6, \text{C-1}\)). \(^{19}\)F (376 MHz, D\(_2\)O) –216.8 (ddd, \(J_{F3,H3} = 51.4, J_{F3,F2} = 11.2, F-3\)), –121.3 (d, \(J_{F2,F3} = 11.2, F-2\)). HRMS-FAB (m/z): [M+H\(^+\)] calcd for C\(_9\)H\(_{14}\)F\(_2\)O\(_8\), 289.0729; Found, 289.0723. \(\alpha_D^{20} = -31.5 \) (c = 0.008, CH\(_2\)Cl\(_2\)) The above NMR spectral data match those reported in the literature.\(^\text{72}\)

\[
\text{Figure 2.16. Structure of 3-deoxy-3-fluoro-D-erythro-\(\beta\)-manno-non-2-ulopyranosyl fluoride.}
\]
2.4. NMR data of DiFluoro Compounds

Figure 2.17. $^1$H NMR spectrum of 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-$\beta$-L-manno non-2-ulopyranosonyl fluoride (19) (in $D_2O$).
Figure 2.18. $^{19}$F NMR spectrum of 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-$\beta$-L-manno-non-2-ulopyranosonyl fluoride (in D$_2$O).
Figure 2.19. $^{13}$C NMR spectrum of 5-acetamido-2,3,5-trideoxy-3-fluoro-D-erythro-$\beta$-L-manno-non-2-ulopyranosonyl fluoride (19) (in D$_2$O).
Figure 2.20. $^{13}$C NMR spectrum of methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (17) (in CDCl$_3$).
Figure 2.21. $^{19}$F NMR spectrum of methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (27) (in CDCl$_3$).
Figure 2.22. $^1$H NMR spectrum of methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (27) (in CDCl$_3$).
Figure 2.23. $^{13}$C NMR spectrum of methyl 4,5,7,8,9-penta-O-acetyl-3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (27) (in CDCl$_3$).
Figure 2.24. $^1$H NMR spectrum of 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (29) (in $D_2$O).
Figure 2.25. $^{19}$F NMR spectrum of 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (29) (in $D_2O$).
Figure 2.26. $^{13}$C NMR spectrum of 3-deoxy-3-fluoro-D-erythro-β-L-manno-non-2-ulopyranosyl fluoride (29) (in D$_2$O).
2.5. Measurement of the Kinetic Parameters for Inactivation of *Micromonospora viridifaciens* sialidase by Difluoro-Kdn (29)

All kinetic experiments were performed in 1.0 cm path-length quartz cuvettes using a Varian Cary 3E UV-visible spectrophotometer equipped with a temperature controller. For all experiments, *Micromonospora viridifaciens* sialidase (MvS) was incubated in a buffer (NaOAc-HOAc 100 mM, pH 5.25 containing BSA 0.1% w/v) with 2,3-difluoro-Kdn at 25 °C for various times ranging from 0 to 60 min.

Then the remaining activity was measured at pH 7.00. Specifically, the stock solution of MvS (0.92 μM) with various concentrations of 2,3-difluoro-Kdn (29) (0.25, 0.5, 1, 3, 5, 10, 15, 30, 50 and 100 μM) was made up to a final volume of 500 μL in the inactivation buffer. At various time intervals an aliquot (50 μL) of this solution was added to a cuvette containing 450 μL buffer (pH 7.00) containing KCl (100 mM), BSA (0.01% w/v) and PNP-αNeu5Ac (80 μM). The pseudo first order rate constants for inactivation of MvS by 2,3-difluoro-Kdn (29) was calculated by fitting the residual activity versus incubation time data to a standard first-order exponential decay (Graph Pad Prism v4.0). Their first order rate constants (K_{obs}) graphs at various (29) are illustrated in Figures 2-38 to 2-47.

To determine the inactivation rate constant (k_{inact}) and the pseudo dissociation constant (K_i) for the enzyme-inhibitor complex, the values for the first-order rate constants (K_{obs}) and the inactivator concentrations (29) were fit to a Michaelis-Menten-type equation $K_{obs} = k_{inact}[I]/(K_i + [I])$ using the computer program Graph Pad (Figure 2-37).

2.6. Measurement of the Kinetic Parameters for Inactivation of *Micromonospora viridifaciens* sialidase by Difluorosialic Acid (19)

All kinetics experiments were performed at 25 °C in MOPS buffer (100 mM, pH 7.00) containing KCl (100 mM) and BSA (0.01 %) using an Applied Photophysics SX20 stopped-flow spectrophotometer that was interfaced to an external RMS 6 Lauda temperature controller. Changes in fluorescence intensity of the solution were measured by using an excitation wavelength of 365 nM, while emission was measured with a photomultiplier tube after the light
had passed through a cut off filter (440 nM). Several repeat measurements were averaged for each concentration and incubation time.

Specifically, the activity of MvS after incubation with various concentration of 2,3-difluorosialic acid (19) was determined by monitoring the intensity of fluorescence due to liberation of 4-methylumbelliferone (4-MU) that results from sialidase-catalyzed hydrolysis of 4-MU-αNeu5Ac. The stopped-flow spectrometer was used in double mixing mode (Figure 2.27), in which four drive syringes are used. The first pneumatic mixing combines equal volumes of reactants (A + B) in mixer 1 (total volume 220 ± 10 µL) which results in a bolus of enzyme and inhibitor solution filling delay loop D (Figure 2.27). Following aging of this solution for various time intervals (10–3000 ms) a second pneumatic drives pushes equal volumes from syringes C and D (total volume 180 ± 10 µL) in which the solution from syringe D pushes the incubation mix to Mixer 2 where it is combined with substrate (syringe C). The contents of the four syringes are as follows: i) syringe A, MvS (0.23-1.80 µM) and BSA (0.02 %) in MOPS buffer (200 mM, pH 7.00) with KCl (200 mM); ii) syringe B, various concentrations of (19) (2.0, 3.0, 6.0, 10.0, 20.0, 40.0, 80.0, 160.0 µM); iii) syringe C, 4-MUαNeu5Ac 140 µM; and iv) syringe D, water.

The residual activity of MvS (aged solution) was recorded for each concentration of (19) at various inactivation times from 10 ms to 3.0 s (Figures 2.29) The pseudo first order rate constants for inactivation of MvS by 2,3-difluorosialic acid (19) were calculated from the residual activity versus incubation time data by fitting to a standard first order exponential (Graph Pad Prism v4.0) and plots of this data are illustrated in Figures 2.30 to 2.38. Of note, the residual activity at each incubation time was calculated as the mean (and standard deviation) from several initial rate measurements at each inactivator concentration.

Control experiments were performed in which it was shown that: 1) when water was placed in syringe B, no decrease in activity of MvS was observed; and 2) the observed inactivation rate constant ($k_{obs}$) was independent of the concentration of MvS.
To determine the inactivation rate constant ($k_{\text{inact}}$) and the dissociation constant ($K_i$) for the enzyme-inhibitor complex, the values for the first-order rate constants ($k_{\text{obs}}$) and the inactivator concentration ($I$) were fit to a Michaelis-Menten-type equation $k_{\text{obs}} = k_{\text{inact}} [I]/(K_i + [I])$ using the computer program Graph Pad (Figure 2.28).
2.7. Results for the Measurement of Rate Constants for the Inhibition of $MvS$ by DiFluorosialic Acid

DiFluoro-Sialic Acid Inhibition of MvNA

\[ K_i = (1.7 \pm 0.4) \, \mu M \]

\[ k_{\text{inact}} = (6.70 \pm 0.32) \, s^{-1} \]

\[ k_{\text{inact}}/K_i = (3.9 \pm 0.8) \times 10^6 \, M^{-1} \, s^{-1} \]

Figure 2.28. Inactivation of $MvS$ by 2,3-difluorosialic acid in 100 mM MOPS buffer at a pH of 7.00 and $T = 25 \, ^\circ C$. The dashed line is the best fit to the Michaelis-Menten equation by Graph Pad Prism v4.0.
Figure 2.29. Representative traces for the time-dependent inhibition of MvS with 2,3-Di fluorosialic acid (5 μM) at three different Incubation times the reactions were performed at pH = 7.00 (100 mM MOPS buffer, 100 mM KCl) and T = 25 °C.
Figure 2.30. Apparent first-order rate constant at each inhibitor concentrations for MvS with 2,3-difluorosialic acid. The reaction were done at pH = 7.00 (100 mM MOPS buffer, 100 mM KCl) and T = 25 °C.

Figure 2.31. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (1 µM) in 100 mM MOPS buffer at a pH of 7.00 and T = 25 °C.
Figure 2.32. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (1.5 µM) in 100 mM MOPS buffer at a pH of 7.00 and T = 25 °C.

Figure 2.33. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (2.5 µM) in 100 mM MOPS buffer at a pH of 7.00 and T = 25 °C.
Figure 2.34. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (5 µM) in 100 mM MOPS buffer at a pH of 7.00 and $T = 25 \, ^\circ C$.

Figure 2.35. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (10 µM) in 100 mM MOPS buffer at a pH of 7.00 and $T = 25 \, ^\circ C$. 
$k_{obs} = (5.71 \pm 0.53) \, s^{-1}$
$k_{obs} = (5.23 \pm 0.56) \, s^{-1}$
Figure 2.36. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (20 µM) in 100 mM MOPS buffer at a pH of 7.00 and $T = 25 ^\circ C$.

Figure 2.37. Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (40 µM) in 100 mM MOPS buffer at a pH of 7.00 and $T = 25 ^\circ C$. 
Figure 2.38.  Time dependent inactivation of MvS activity during incubation with 2,3-difluorosialic acid (80 µM) in 100 mM MOPS buffer at a pH of 7.00 and T = 25 °C.
2.8. Results for the Measurement of Rate Constants for the Inhibition of \( MvS \) by DiFluoro-Kdn

\[
\begin{align*}
K_i & = (40.8 \pm 12.0) \, \mu\text{M} \\
K_{\text{inact}} & = (0.118 \pm 0.011) \, \text{s}^{-1} \\
\frac{k_{\text{inact}}}{K_i} & = (2.92 \pm 0.89) \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1}
\end{align*}
\]

**Figure 2.39.** Apparent dissociation constant (\( K_i \)) for \( MvS \) with 2,3-difluoroKdn), inactivation at \( pH = 5.25 \) and measured at \( pH = 7.00 \) in 100 mM MOPS buffer, 100 mM KCl, and \( T = 25 \, ^\circ\text{C} \).

\[
\begin{align*}
5 \, \mu\text{M Rel Reactivity}\% \\
0.25 \, \mu\text{M Rel Reactivity}\% \\
0.5 \, \mu\text{M Rel Reactivity}\% \\
1.5 \, \mu\text{M Rel Reactivity}\% \\
35 \, \mu\text{M Rel Reactivity}\% \\
100 \, \mu\text{M Rel Reactivity}\%
\end{align*}
\]

**Figure 2.40.** Time-dependent-inactivation of \( MvS \) with increasing concentrations of 2,3-difluoroKdn, inactivation at \( pH = 5.25 \) and measured at \( pH = 7.00 \) in 100 mM MOPS buffer, 100 mM KCl, and \( T = 25 \, ^\circ\text{C} \).
$k_{obs} = (4.91 \pm 1.30) \times 10^{-4} \text{ s}^{-1}$

Figure 2.41. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (0.25 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and $T = 25^\circ C$.

$k_{obs} = (8.09 \pm 3.5) \times 10^{-4} \text{ s}^{-1}$

Figure 2.42. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (0.5 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and $T = 25^\circ C$. 

60
Figure 2.43. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (1.5 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{\text{obs}} = (4.04 \pm 0.475) \times 10^{-3} \text{ s}^{-1} \]

Figure 2.44. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (3 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{\text{obs}} = (5.09 \pm 0.30) \times 10^{-3} \text{ s}^{-1} \]
Figure 2.45. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (5 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{obs} = (7.56 \pm 1.36) \times 10^{-3} \text{ s}^{-1} \]

Figure 2.46. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (10 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{obs} = (3.06 \pm 0.20) \times 10^{-2} \text{ s}^{-1} \]
Figure 2.47. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (35 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{\text{obs}} = (4.84 \pm 0.16) \times 10^{-2} \text{ s}^{-1} \]

Figure 2.48. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (50 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{\text{obs}} = (7.24 \pm 0.35) \times 10^{-2} \text{ s}^{-1} \]
Figure 2.49. Time dependent inactivation of MvS activity during incubation with 2,3-difluoroKdn (100 µM), inactivation at pH = 5.25 and measured at pH = 7.00 in 100 mM MOPS buffer, 100 mM KCl, and T = 25 °C.

\[ k_{obs} = (8.32 \pm 0.31) \times 10^{-2} \text{ s}^{-1} \]
3. Results and Discussion

3.1. Synthesis

The synthesis of 2,3-difluorosialic acid (19) followed the reported literature procedure of Watts et al., except that a different reagent was used for fluorination of the anomeric centre. The synthetic scheme shown in Figure 2.1, starts by using N-acetylneuraminic acid aldolase to catalyze the formation of 3-fluorosialic acid (13) from N-acetylmannosamine (11) and 3-fluoropyruvate (12). The optimal reaction conditions for the Neu5Ac aldolase enzyme occurs at a pH between 6 and 9 and a temperature of 37 °C. As the enzymatic production of 3-fluorosialic acid (13) is an equilibrium reaction, one of the reagents N-acetylmannosamine was used in excess in order to improve the yield of the desired product, 3-fluorosialic acid (13). The progress of this enzymatic reaction was monitored by 19F NMR spectroscopy by following the disappearance of the fluorine signal that corresponds to 3-fluoropyruvate (12) (δ –233 ppm) and the appearance of the fluorine peak for the product, 3-fluorosialic acid (13) (δ –208 ppm).

Next, the 3-fluorosialic acid (13) was esterified by treating with methanol and Amberlite IR-120 resin (H⁺) to give the 3-fluorosialic acid methyl ester (14), which was then converted to the per-O-acetylated 3-fluorosialic acid methyl ester 15 in a yield of 81%. Selective deacetylation of 15 at the anomeric centre occurred on treatment with hydrazinium acetate to afford hemiacetal 16 in a yield of 90%. Compound 16 was then treated with diethylaminodifluorosulfonium tetrafluoroborate (XtalFluor-E) at room temperature to afford the β-fluoro compound 17 in high yield. The stereochemical assignment for 17 was based on 19F NMR coupling constants, that is, a small coupling constant of 11 Hz is expected for an equatorial F2 and an axial F3. Whereas, according to a literature report, a larger coupling constant of 20 Hz should be observed for an axial F2 and axial F3. Deprotection of 17 was achieved in two steps; 1) deacetylation using sodium methoxide; and 2) hydrolysis of the methyl ester using lithium hydroxide. The resultant final compound is 2,3-difluorosialic acid 19 (shown in Section.2.2).
The synthesis of 2,3-difluoroKdn (29), which is shown in Figure 2.4, was accomplished by using the same procedures as those for the synthesis of 2,3-difluorosialic (19) acid except that the starting material N-acetylmannosamine (11) was replaced by D-mannose (21) (shown in Section 2.3).

The literature procedure reported by Watts et al involved fluorination at the anomeric carbon using DAST (diethylaminosulfur trifluoride) (Figure 3.1) at –30 °C. In contrast, in our work, DAST was replaced with diethylaminodifluorosulfinium tetrafluoroborate (XtalFluor-E), a fluorinating reagent developed by Heureux et al which can be used at room temperature. Xtalfluor-E is superior to DAST for several reasons: (i) it is more convenient for handling as it is a crystalline reagent, whereas DAST is a liquid; (ii) Xtalfluor-E has a higher thermal stability; and (iii) unlike DAST, Xtalfluor-E does not generate free HF so that standard glassware can be used as reaction vessels.

![Figure 3.1. Structure of fluorinating reagents using in this study (XtalFluor-E) and by Watts et al (DAST).](image)

3.2. Tabulation of Kinetic Data

Kinetic analysis of the inactivation of MvS by 2,3-difluorosialic acid (19) was accomplished by measuring the time-dependent rate of inactivation at various inhibitor concentrations. As the inactivation reaction is rapid, I used a stopped-flow spectrophotometer in order to measure the inactivation rate constants. These kinetic experiments were done at 25 °C and pH of 7.0, a non-optimal pH for enzyme activity (section 2.5) was used in order to improve the sensitivity of the assays. The apparent first order rate constant \(k_{\text{obs}}\) at each concentration of inactivator was calculated by plotting the residual MvS activity, measured using the substrate 4-MUαNeu5Ac (31) at different incubation times. The table below shows all measured \(k_{\text{obs}}\) values and the corresponding standard errors for inactivation of MvS by 2,3-difluorosialic acid (19) (Table 3.1). A comparison of the \(k_{\text{obs}}\) values shows that increasing the inactivator concentration causes an initial rise in the inactivation rate constant followed by a leveling off. All graphs that
correspond to these first-order rate constants are shown in Figures 2.17 to 2.25. Of note, the life time of the enzyme-inactivator complex even at low concentrations of 19, is less than a second. It was not possible to measure the reactivation rate constants, which is the first-order reaction for hydrolysis of the covalent E–I product, because these experiments require the inactivated enzyme to be freed of excess inhibitor and this process is slower than reactivation and this process has been shown to be rapid.105

<table>
<thead>
<tr>
<th>[DFSIA] (µM)</th>
<th>(k_{\text{obs}}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2.16 ± 0.25</td>
</tr>
<tr>
<td>1.5</td>
<td>2.87 ± 0.15</td>
</tr>
<tr>
<td>2.5</td>
<td>4.07 ± 0.20</td>
</tr>
<tr>
<td>5.0</td>
<td>5.81 ± 0.58</td>
</tr>
<tr>
<td>10</td>
<td>5.23 ± 0.56</td>
</tr>
<tr>
<td>20</td>
<td>6.47 ± 0.30</td>
</tr>
<tr>
<td>40</td>
<td>6.79 ± 0.30</td>
</tr>
<tr>
<td>80</td>
<td>5.96 ± 0.86</td>
</tr>
</tbody>
</table>

Table 3.1. The measured pseudo first-order rate constants (\(k_{\text{obs}}\)) for inactivation of MvS versus concentration of difluorosialic acid (19).

Reaction condition: in 100 mM MOPS buffer at a pH of 7.00 and \(T = 25 ^\circ\)C.

The kinetic data for MvS inactivation by 2,3-difluoro Kdn (29) were measured at the pH optimum for catalysis (pH 5.25 and at \(T = 25 ^\circ\)C). The crucial role of the functional group at C-5 of the sialic acid in substrate binding and catalysis was revealed from the kinetic and structural studies of previous researchers.106,107 The two fluorinated Kdn-based inhibitors, (19) and (29), are different at C-5 with the \(N\)-acetyl group of 19 being replaced by a hydroxyl group in 29. This change caused the loss of significant binding interactions and this results in destabilization of the enzymatic transition state for hydrolysis. Inactivation of MvS by 2,3-difluoroKdn (29) was measured at the optimal pH (5.25) and 25 °C. However, to increase the sensitivity of the assay for remaining activity, the hydrolysis of 4-nitrophenyl \(\alpha\)-sialoside was measured at a pH of 7.0. The apparent first order rate constant \(k_{\text{obs}}\) at each inactivator concentration (29) was obtained from plotting the residual MvS activity versus incubation time. The fit of the resulting data to a first order decay yields \(k_{\text{obs}}\). These graphs are shown in Figures 2-26–2-36 and a summary of the results are given in Table 3.2.
Table 3.2. The measured pseudo first-order rate constants ($k_{obs}$) for inactivation of MvS versus concentration of difluoroKdn (29).

<table>
<thead>
<tr>
<th>[DFKdn] (µM)</th>
<th>$10^3 \times k_{obs}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>0.5</td>
<td>0.81 ± 0.35</td>
</tr>
<tr>
<td>1.5</td>
<td>4.00 ± 0.48</td>
</tr>
<tr>
<td>3.0</td>
<td>5.09 ± 0.30</td>
</tr>
<tr>
<td>5.0</td>
<td>7.6 ± 1.4</td>
</tr>
<tr>
<td>10</td>
<td>30.6 ± 2.0</td>
</tr>
<tr>
<td>35</td>
<td>48.4 ± 1.6</td>
</tr>
<tr>
<td>50</td>
<td>72.4 ± 3.5</td>
</tr>
<tr>
<td>100</td>
<td>83.2 ± 3.1</td>
</tr>
</tbody>
</table>

Reaction condition: 100 mM acetate buffer at a pH of 5.25 and T = 25 °C.

3.3. Definition Parameters

To discuss the results contained this thesis, it is necessary to define the various kinetic parameters. One of the significant kinetic parameters for Michaelis–Menten kinetics is $k_{cat}$, the turnover number of the enzyme, which represent the maximum number of substrates converted to product per enzyme active site per unit of time. For example, the simple reaction mechanism of Michaelis–Menten shown in the following scheme 

$$E + S \stackrel{k_m}{\longrightarrow} ES \stackrel{k_{cat}}{\longrightarrow} EP,$$

$k_{cat}$ is the first order rate constant for conversion of Michaelis ES complex to EP. However, when the reaction is more complicated, the first order rate constant $k_{cat}$ can be a complex rate constant.

The next parameter, $K_m$, is defined as the concentration of substrate at which the enzymatic activity is one half of the maximum. As for most enzyme mechanisms, $K_m$ is not equal to $K_s$ (the dissociation constant of the initial Michaelis complex), and there is no correspondence between the binding affinity of substrate and the Michaelis constant $K_m$. Therefore, the ratio $k_{cat}/K_m$ is a better parameter to use as it expresses the catalytic efficiency of the enzyme.95

The ratio of $k_{cat}/K_m$ represent the second order rate constant for reaction between free enzyme and free substrate to form the first irreversible transition state ES‡ complex. A comparison of catalytic efficiencies ($k_{cat}/K_m$) of specific enzymes toward different substrates
reflects the differences in transition state free energy for ES‡, which result from differences in substrate specificities of the enzyme.\textsuperscript{95}

\[
E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_{\text{inact}}}{\rightarrow} EP \rightarrow \text{Dead}
\]

\textit{Scheme 3.1. Basic kinetic scheme for time-dependent inactivation for a mechanism based irreversible inhibitor.}

The scheme above corresponds to enzyme inactivation that results from covalent bonding between an enzyme active site residue and a reactive centre on the inhibitor. The product of this enzymatic reaction is the covalent adduct of enzyme-inhibitor (EP). A plot of \(k_{\text{obs}}\), the first-order rate constant for inactivation as a function of inhibitor concentration will fit to a hyperbolic curve defined by the expression of \(k_{\text{obs}} = k_{\text{inact}} [I] / (K_i + [I])\). The term \(K_i\) represents the required inhibitor concentration to reach one-half of the maximal inactivation rate, which is given by the term \(k_{\text{inact}}\). As it was mentioned previously, the ratio of \(k_{\text{cat}}/K_m\) is the catalytic efficiency of an enzyme and for irreversible inhibition, the ratio of \(k_{\text{inact}}/K_i\) reports on the inhibitory efficiency (\textit{Scheme 3.1}).\textsuperscript{95,96}

Most inhibitors that are irreversibly bound to the enzyme active site do so in two steps. First, a reversible binding of inhibitor to the enzyme active site (\(K_i\)) occurs and then a slower step of inactivation happens (\(k_{\text{inact}}\)) (\textit{Scheme 3.1}).\textsuperscript{95}

The following section shows how to derive an equation for the Scheme 3.1 corresponding to a reaction of mechanism-based irreversible inhibitor:

Where free enzyme (E) and free inhibitor (I) are in rapid equilibrium with enzyme-inhibitor complex (EI), i.e., \(k_i \& k_{-1} \gg k_{\text{inact}}\), and EI is an intermediate that accumulates.

So at any time in reaction:

\[
K_{eq} = \frac{[EI]}{[E][I]} = \frac{k_i}{k_{-1}} = \frac{1}{K_i}
\]
\[
\frac{d[EP]}{dt} = k_{\text{inact}} [EI], \quad [EI] = K_{eq} [E][I],
\]

From the stoichiometric relationship: where \([E]_0\) was the amount of enzyme added at time = 0 (note the precise definition of \([E]_0\);

\([E]_0 = [E] + [EI] + [EP] \Rightarrow \text{i.e. } [E]_0 - [E] - [EI] = [EP];

so \[E] = \frac{[EI]}{K_{eq}[I]} \Rightarrow [EP] = [E]_0 - \frac{[EI][1 + K_{eq}[I]]}{K_{eq}[I]}

and \([EI] = \frac{K_{eq}[I]}{1 + K_{eq}[I]}( [E]_0 - [EP])\)

given that \([E]_0 = [EP]_\infty\) (i.e., the reaction goes to completion).

\[
\frac{d[EP]}{dt} = \frac{k_{\text{inact}} K_{eq} [I]}{1 + K_{eq}[I]} ([EP]_\infty - [EP])
\]

Consequently, \(k_q = k_{\text{obs}} = \frac{k_{\text{inact}} K_{eq}[I]}{1 + K_{eq}[I]}\)

when we divide the top and bottom by \(K_{eq}\), we get, \(k_{\text{obs}} = k_{\text{inact}}\frac{[I]}{\left(\frac{1}{K_{eq}} + [I]\right)}\)

as \(K_i = \frac{1}{K_{eq}}, \quad k_{\text{obs}} = k_{\text{inact}}\frac{[I]}{(K_i + [I])}\) (Equation 2.1)

3.4. Analysis of the Michaelis-Menten Kinetic Parameters for Inactivation of \(Mv\). Sialidase by Difluorosialic Acid and Difluoro-Kdn

Table 3.3 shows the second order rate constants for inhibition of \(MvS\) by the two fluorinated inhibitors and the \(MvS\) catalyzed hydrolyses for the substrates, \(MU\alphaKdn\) (30) and \(MU\alphaNeuAc\) (31).
Table 3.3. Kinetic parameters for Mv-sialidase catalyzed hydrolysis of MUαKdn and MUαNeuAc and the inhibition of this enzyme by 2,3-difluoroKdn and 2,3-difluorosialic acid.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{inact}/K_i$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUαKdn</td>
<td>$(7.2 ± 2.8) \times 10^{-1}$</td>
<td>$(3.9 ± 0.8) \times 10^{-1}$</td>
</tr>
<tr>
<td>MUαNeuAc</td>
<td>$(1.03 ± 0.36) \times 10^{-1}$</td>
<td>$(2.9 ± 0.9) \times 10^{-1}$</td>
</tr>
</tbody>
</table>

Note: a) Data taken from reference 47; conditions pH 5.2 T = 37 °C. b) Data taken from references 106,107, conditions pH 5.25 and T = 37 °C. c) pH 5.25 and T = 25 °C. d) pH 7.0 and T = 25 °C.
Table 3.3 lists the kinetic data for two pairs of substrates and the corresponding inhibitors. A comparison of the catalytic efficiencies \( (k_{\text{cat}}/K_m) \) shows that the \( \text{MvS} \) hydrolyzes \( \text{MU} \alpha \text{Neu5Ac} \) (31) about 7000-fold more efficiently than does \( \text{MU} \alpha \text{Kdn} \) (30).\(^{47,108}\) A comparison of the two inhibitory potencies \( (k_{\text{inact}}/K_i) \) shows that 2,3-difluorosialic acid (19) is about a 1000-fold more potent inactivator of \( \text{MvS} \) than is 2,3-difluoroKdn (29). These differences in activity are remarkably similar, although the reaction conditions are slightly different.

To interpret the kinetic data, need to pay attention to the structural differences between the pairs of substrates and inhibitors. The difference in the structures of \( \text{MU} \alpha \text{Kdn} \) (30) and \( \text{MU} \alpha \text{Neu5Ac} \) (31) occurs at C-5 which is a hydroxyl group in the case of \( \text{MU} \alpha \text{Kdn} \) (30) and an acetamido function in the \( \text{MU} \alpha \text{Neu5Ac} \) (31). The same difference occurs in the two inhibitors, 2,3-difluoroKdn (29) and 2,3-difluorosialic acid (19). Based on the kinetic results, it is clear that the presence of an acetamido function at C-5 increases catalysis as well as the inhibition for 31 and 19 versus the alcohol at C-5 for 30 and 29, respectively. Based on the crystal structure of soil bacterium \( \text{MvS} \), Gaskell et al., showed that \( \text{MvS} \) has a polar active site with charged residues of arginine, aspartic acid and glutamic acid as well as a hydrophobic pocket that includes five residues alanine (Ala93), valine (Val148), two phenylalanines (Phe155, Phe203) and leucine (Leu170).\(^{109}\) When \( \text{MU} \alpha \text{Neu5Ac} \) or 2,3-difluorosialic acid (19) interacts with enzyme active site, the three conserved arginine residues (Arg68, Arg276, Arg342) are responsible for the important electrostatic interactions with the carboxylate group.\(^{109}\) Arg68 is stabilized through the hydrogen bond interaction with conserved Glu386 residue. The presence of the 5-acetamido group at C5 creates several important interactions; which hydrogen-bonding interactions between an aspartic acid (Asp131) residue and amidic nitrogen, and the accommodation of the 5-acetamido in the hydrophobic pocket that engender hydrophobic interactions to the amidic methyl group.\(^{109}\) However, when \( \text{MU} \alpha \text{Kdn} \) (30) and 2,3-difluoroKdn (29) interact with the \( \text{MvS} \) active site, all interactions are expected to be to those for 31 and 19, except the interactions at C5. The loss of hydrophobic interactions of the amidic methyl at C5 results in lower activities for 30 and 29 (Figure 3.2).\(^{109}\) Binding of a 5-hydroxyl group in the hydrophobic pocket leaves spaces which are filled by water molecules.\(^{48}\) Thus, the data shown in Table 3.3 for \( \text{MvS} \) reveals that the catalytic efficiency \( (k_{\text{cat}}/K_m) \) for 31 is similar to the inhibitory potency \( (k_{\text{inact}}/K_i) \) for 19 (about \( 10^6 \text{ M}^{-1} \text{ s}^{-1} \)) and the catalytic efficiency \( (k_{\text{cat}}/K_m) \) for 30 is similar to the inhibitory potencies \( (k_{\text{inact}}/K_i) \) for 29 (about \( 10^3 \text{ M}^{-1} \text{ s}^{-1} \)).
3.5. Relative Intrinsic Reactivities of 31 and 19.

To compare the enzymatic rate constant for hydrolysis of MUαNeu5Ac \( (k_{cat}/K_m) \) and inactivation by 2,3-difluorosialic acid \( (k_{inact}/K_i) \), it is necessary to estimate the intrinsic reactivities for these two compounds. To this end, kinetic data from several research groups were tabulated in Tables 3-4, 3-5 and 3-6. Figure 3.3 shows the pathways (A, B, C) that are used to predict the intrinsic reactivity difference between compounds 19 and 31.
Figure 3.3. The hypothetical pathway used to estimate the relative hydrolytic rate constants for reaction of 2,3-difluorosialic acid (19) and MUαNeu5Ac (31).
Table 3.4. Extrapolated rate constants for the hydrolysis of aryl α-sialosides\textsuperscript{110}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pK\textsubscript{a} (ROH)</th>
<th>( k\text{obs} ) or ( k\text{calc} ) (s(^{-1}))</th>
<th>Relative Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPNeu5Ac (32)</td>
<td>7.15</td>
<td>( 1.81 \times 10^{-5} )</td>
<td>7.00</td>
</tr>
<tr>
<td>DNPNNeu5Ac (33)</td>
<td>4.10</td>
<td>( 1.67 \times 10^{-1} )</td>
<td>6.5 \times 10^4</td>
</tr>
<tr>
<td>4MUNeu5Ac (31)</td>
<td>7.80</td>
<td>( 2.59 \times 10^{-6} )</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Reaction Conditions: Neutral monobasic Buffer 0.30 M, sodium perchlorate to maintain ionic strength at 0.30 M, pH = 6.67, T = 50 °C.

To predict the relative rates for path A in Figure 3.3, the kinetics data is taken from Ashwell and Sinnott’s research.\textsuperscript{110} In Table 3.4, the rate constants for hydrolysis of a series of aryl α-sialoside are tabulated. According to Ashwell et al, the first order rate constant (\( k\text{obs} \)) for hydrolysis of PNP-Neu5Ac was measured at different pH values and at T = 50 °C.\textsuperscript{110} The measured \( k\text{obs} \) for spontaneous hydrolysis of PNP-Neu5Ac at pH 6.67 is \( 1.81 \times 10^{-5} \) s\(^{-1}\). These authors measured the pseudo-first order rate constants (\( k\text{obs} \)) for hydrolysis of five different aryl sialosides and they plotted \( \log (k\text{obs}) \) as a function of pK\textsubscript{a} of the leaving groups. The linear relationship obtained gave a \( \beta\lg \) value of –1.3. Thus, it was used these data to calculate the expected rate constants \( k\text{calc} \) for the hydrolysis of MUαNeu5Ac (31) and it was extrapolated a value for the hypothetical substrate 2,4-dinitrophenyl α-D-sialoside (DNPNeu5Ac, 33). This calculation is shown below:

\[
k_{\text{ArNeu5Ac}} = k_{\text{PNPNeu5Ac}} \times 10^{(\beta\lg \times \Delta pK\text{a})}
\]

Where \( \Delta pK\text{a} \) is \( pK\text{a}(\text{4-Nitrophenol}) - pK\text{a}(\text{ArOH}) \)

and (\( pK\text{a}(4\text{MU}) = 7.8 \), \( pK\text{a}(\text{PNP}) = 7.15 \) and \( pK\text{a}(\text{DNP}) = 4.10 \)

\[
k_{\text{calc}}(\text{4MUNeu5Ac}) = k_{\text{obs}}(\text{PNPNeu5Ac}) \times 10^{(\beta\lg (pK\text{a}(\text{4MU}) - pK\text{a}(\text{PNP}))}
\]

\[
k_{\text{calc}}(\text{4MUNeu5Ac}) = 1.81 \times 10^{-5} \times 10^{(-1.3 \times 0.65)}
\]

\[
k_{\text{calc}}(\text{4MUNeu5Ac}) = 2.30 \times 10^{-6} \text{ s}^{-1}
\]
\[
k_{\text{calc}}(\text{DNPNeu5Ac}) = k_{\text{obs}}(\text{PNPNeu5Ac}) \times 10^{(\beta \log(pK_a(\text{DNP}) - pK_a(\text{PNP}))}
\]

\[
k_{\text{calc}}(\text{DNPNeu5Ac}) = 1.81 \times 10^{-5} \times 10^{-1.3 \times (-3.05)}
\]

\[
k_{\text{calc}}(\text{DNPNeu5Ac}) = 1.53 \times 10^{-1} \text{ s}^{-1}
\]

The comparison of rate constants for the two non-enzymatic reactions shows that the first order rate constant \(k_{\text{calc}}\) for the hydrolysis of 2,4-DNPNeu5Ac (33) is expected to be \(6.5 \times 10^4\) times faster than that for MU\(\alpha\)Neu5Ac (31) (Figure 3.3).\(^{110}\) Of note, the measurement by Ashwell were made at a different temperature (50 °C) than the temperature used in our studies.\(^{110}\)

In order to predict the relative rate constants for non-enzymatic hydrolysis of the two compounds (33 and 34) in path B of Figure 3.3, kinetic data were taken from the Withers’ research group.\(^{111}\) According to these authors, the rate of spontaneous hydrolysis of 2,4-dinitrophenyl \(\beta\)-D-glycosides was measured with different substituents at C2. These authors used Hammett \(\sigma\)-correlation, that is, plotted \(\log k_{\text{obs}}\) as a function of \(\sigma\) (inductive effect substituent constant) for the three (H, OH, F) substituents.\(^{111}\) Table 3.5 shows the reported non-enzymatic rate constant for hydrolysis of 2dDNPmannoside (35) and 2FDNPmannoside (36). Thus, the relative rate constant \(k_{\text{rel}}\) for hydrolysis of 2FDNPmannoside (36) is \(4.5 \times 10^{-6}\). (Figure 3.4)\(^{111}\)

<table>
<thead>
<tr>
<th>Substrate/ Inhibitor</th>
<th>(k_{\text{obs}} \text{ (s}^{-1}\text{)})</th>
<th>(k_{\text{rel}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2dDNPmannoside (35)</td>
<td>(6.82 \times 10^{-3})</td>
<td>1.00</td>
</tr>
<tr>
<td>2FDNPmannoside (36)</td>
<td>(3.05 \times 10^{-8})</td>
<td>(4.5 \times 10^{-6})</td>
</tr>
</tbody>
</table>

Reaction Condition: \(T = 37 \degree \text{C}\)
A comparison of 2FDNPMannoside (36) with compound 34 and 2dDNPMannoside (35) with compound 33 shows that the both sets have similar substituents adjacent to the anomeric carbon and both have the same leaving group. Based on these kinetics data and the similarity in structural features at C2 adjacent to the anomeric centre, the predicted relative rate constant for non-enzymatic hydrolysis of compound 34 is expected to be approximately (about) 4.5 x 10^{-6} times slower than compound 33. Of note, these reaction were performed at the slightly higher temperature (37 °C) than our studies.\textsuperscript{111}

The research by Sinnott et al on the hydrolysis of β-D-galactopyranoside derivatives was used to predict the relative rate constants for the non-enzymatic hydrolyses of the two compounds, 19 and 34, (Figure 3.3).\textsuperscript{112} As shown in following Figure 3.5, the pH independent hydrolyses of the two β-D-galactopyranoside derivatives was measured at 25 °C. In Table 3.6, the pH-independent rate constant for hydrolysis of galactosyl fluoride (38) and 2,4-dinitophenol galactopyranoside (37) is tabulated. Based on these results, the estimated rate ratio for hydrolysis of fluoride from 19 is 27.9-fold faster than the rate of hydrolysis of 2,4-dinitrophenyl from 34.\textsuperscript{112}

![Image](image1.png)

**Figure 3.4.** The comparison of non-enzymatic reaction for release of DNP from 2dDNPMannoside and 2FDPMannoside at 37 °C.\textsuperscript{111}

![Image](image2.png)

**Figure 3.5.** The comparison of non-enzymatic reaction for hydrolysis of compound 37 and 38 at 25 °C.\textsuperscript{112}
Table 3.6. Kinetics data for the hydrolysis of anomeric substituted of β-D-galactopyranosides\textsuperscript{112}.

<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>$k_{\text{obs}}$ (s\textsuperscript{-1})</th>
<th>$k_{\text{rel}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-DNP β-D-galactopyranoside (37)</td>
<td>$2.15 \times 10^{-6}$</td>
<td>1.00</td>
</tr>
<tr>
<td>Galactosylfluoride (38)</td>
<td>$6.0 \times 10^{-5}$</td>
<td>$2.79 \times 10^{+1}$</td>
</tr>
</tbody>
</table>

Reaction condition: $T = 25^\circ C$, $[\text{NaClO}_4.] = 1 \text{ M}$

Again, compounds 37 and 38 have similar structural changes to those of 34 and 19. Overall, based on the analysis given above the expected relative rate constants for the hydrolysis of 2,3-difluorosialic acid (19) is about 8 fold more facile than is that for MUαNeu5Ac (31).

Based on Figure 3-3, the corresponding calculations are:

$$\frac{k_{33}}{k_{31}} \times \frac{k_{34}}{k_{33}} \times \frac{k_{19}}{k_{34}} = \frac{k_{19}}{k_{31}}$$

Where, the $k_{31}$, $k_{33}$, $k_{34}$ and $k_{19}$ accounts as the intrinsic reactivity rate constant for MUαNeu5Ac (31), 2,4-DNPNeu5Ac (33), 2DNP3FNeu5Ac (34) and 2,3-difluorosialic acid (19). Therefore, the result shows that the expected non-catalyzed reactivity of compound 19 is about 8-fold higher than for compound 31.

$$(6.46 \times 10^{+4}) \times (4.5 \times 10^{-6}) \times (2.79 \times 10^{+1}) = \frac{k_{19}}{k_{31}} = 8.06$$

3.6. Transition-State Stabilization during Catalysis

To understand the parameters that were measured in this chapter an analysis based on enzymatic transition-state (TS) theory is required. For an enzymatic reaction pathway, the TS structure for the first irreversible step (ES\textsuperscript{i}) is compared to that (S\textsuperscript{i}) for a non-enzymatic reaction (Figure 3.6). The hypothetical dissociation of (ES\textsuperscript{i}) to (S\textsuperscript{i}) and free enzyme is given by $k_{\text{cat}}/K_m \times 1/k_{\text{uncat}}$ (Figure 3.6). This term is called the catalytic proficiency of an enzyme, which is comparing $k_{\text{cat}}/K_m$ (second order rate constant) with the rate of intrinsic reactivity (non-
enzymatic reaction). Transition state theory correlates the rate of reaction to the Gibbs free energy differences between ground and transition state.

\[
E + S \xrightarrow{k_{\text{cat}}/K_m} ES^+
\]

\[
\Delta G^+ = -RT \ln k \quad \text{Equation 3.1}
\]

In above equation, \( \Delta G \) stands for the change in Gibbs free energy (kJ mol\(^{-1}\)); R stands for gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)); and T is absolute temperature (Kelvin).

Based on data taken from our research and two other literature sources, the catalytic efficiencies of \( MvS \) for the various substrates and inhibitors were tabulated in Table 3.7. The catalytic efficiencies of \( MvS \) for those corresponding compounds also has been calculated by dividing the catalytic efficiency by the rate constant (\( k_{\text{uncat}} \)) for the uncatalyzed reaction. The catalytic efficiency (\( k_{\text{cat}}/K_m \)) of \( MvS \) while cleaving 4-MU leaving group from \( MU\alpha Neu5Ac \) (31) is about 7000 fold greater than the corresponding value for \( MU\alpha Kdn \) (30). In addition, the inhibitory efficiency (\( k_{i\text{inact}}/K_i \)) of \( MvS \) shows the ability of the enzyme to cleave the fluoride leaving group from 2,3-difluorosialic acid (19) is about 1000 times greater than it is for 2,3-difluoroKdn (29). The similarity between the catalytic efficiency of \( MvS \) for \( MU\alpha Neu5Ac \) (31) and its inhibitory efficiencies for 2,3-difluorosialic acid (19) and the similarity between the efficiencies of \( MvS \) for \( MU\alpha Kdn \) (30) and 2,3-difluoroKdn (29) illustrate that the presence of N-acetamido group at C5 has a significant effect on the affinity of substrate 31 and inhibitor 19 compared to the 5-hydroxyl group in 31 and 29.
Table 3.7. Kinetic parameters for uncatalyzed and catalyzed hydrolysis of MUαKdn and MUαNeu5Ac and two inhibitor of this enzyme 2,3-difluoroKdn and 2,3-difluorosialic acid.

<table>
<thead>
<tr>
<th>Substrate/ Inhibitor</th>
<th>Catalytic efficiency (M⁻¹ s⁻¹)</th>
<th>Catalytic proficiency (M⁻¹)</th>
<th>k_(uncat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Difluorosialic acid (19)</td>
<td>(3.9 ± 0.8) x 10⁶</td>
<td>3.45 x 10⁺¹¹</td>
<td>2.09 x 10⁻⁵</td>
</tr>
<tr>
<td>2,3-DifluoroKdn (29)</td>
<td>(2.92 ± 0.89) x 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUαKdn (30)</td>
<td>(1.03 ± 0.36) x 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUαNeuAc (31)</td>
<td>(7.2 ± 2.8) x 10⁶</td>
<td>2.78 x 10⁺¹²</td>
<td>2.59 x 10⁻⁶</td>
</tr>
</tbody>
</table>

Note: a) Data taken from reference 48; conditions pH 5.2; T = 37 °C. b) Data taken from references 106,107,74,92,108 conditions pH 5.2 and T = 37 °C. c) pH 5.25 and T = 25 °C. d) pH 7.0 and T = 25 °C.

The diagram below shows the reaction coordinate for the hydrolysis of substrate in an enzymatic and a non-enzymatic reaction. The significant parameters are catalytic efficiency (kcat/Km), uncatalyzed hydrolysis rate constant (kuncat) and catalytic proficiency (kcat/Km x 1/kuncat) (Figure 3.6). For example, MUαNeu5Ac (31) has a kcat/Km of 7.2 x 10⁶ M⁻¹ s⁻¹ which can be related to the differences in Gibbs free energy between the transition state ES² and the ground state. The catalytic proficiency of MVs for MUαNeu5Ac (31) is about 10¹² M⁻¹ which correlates to the differences in Gibbs free energy between ES² and S². This value of catalytic proficiency shows the enzymatic hydrolysis reaction of 31 occurs with the same rate as that of the uncatalyzed hydrolysis of 31 in the presence of approximately 1 pmol of enzyme (Table 3.7 and Figure 3.6).
The next diagram shows the reaction coordinates for the catalyzed and uncatalyzed inhibition reactions. The important terms and parameters are shown in Figure 3.7 such as inhibitory efficiency \( \frac{k_{inact}}{k_i} \), uncatalyzed rate constant \( k_{uncat} \) and inhibitory proficiency \( \frac{k_{inact}}{k_i \times 1/k_{uncat}} \). Based on our results (Table 3.7), the inhibitory efficiency \( \frac{k_{inact}}{k_i} \) of MvS for 2,3-difluorosialic acid (19) is about \( 3.6 \times 10^6 \) M\(^{-1}\) s\(^{-1}\) which correlates the Gibbs free energy difference between the transition-state EI\(^\ddagger\) and ground state. In addition, the inhibitory proficiency of MvS for 19 is about \( 6.5 \times 10^{10} \) M\(^{-1}\) which represents the difference in Gibbs free energy between catalyzed EI\(^\ddagger\) and uncatalyzed I\(^\ddagger\) transition states (Figure 3.7).
The catalytic proficiency of an enzyme is given by the expression \( \frac{k_{\text{cat}}}{k_m} \times \frac{1}{k_{\text{uncat}}} \) which relates the catalytic efficiency (enzymatic second-order rate constant) to the intrinsic reactivity of the compound in absence of enzyme. For example, Table 3.7 shows that \( \text{MvS} \) has a catalytic proficiency of \( 2.78 \times 10^{12} \text{ M}^{-1} \) for hydrolysis of \( \text{MU\alphaNeu5Ac} \) (31) and \( 3.45 \times 10^{11} \text{ M}^{-1} \) for 2,3-difluorosialic acid (19). Clearly, the two transition states are stabilized inhibition by \( \text{MvS} \) to similar degrees and 2,3-difluorosialic acid (19) is a bona fide Mechanism-based inhibitor.

In addition, the two Kdn derived compounds \( \text{MU\alphaKdn} \) (30) and 2,3-difluoroKdn (29) behaves similarly and we can conclude that interactions with the 5-aceamido group provide about 22 kJ mol\(^{-1}\) in free energy stabilization at the \( \text{MvS-catalyzed transition states} \) (TSs).
\[ \Delta \Delta G = -RT \ln \left( \frac{k_{KDN}}{k_{Neu5Ac}} \right) \]

Where \( \Delta \Delta G \) = change in Gibbs free energy, \( R \) (gas constant) = 8.314 J mol\(^{-1}\) K\(^{-1}\) and \( T \) = absolute temperature (Kelvin), \( k_{KDN} \) is \( (k_{cat}/K_m)_{KDN} \) and \( k_{Neu5Ac} \) is \( (k_{cat}/K_m)_{Neu5Ac} \). All data are shown in Figure 3.7.
4. Conclusion and Future Work

In conclusion, the synthesis of two fluorinated inhibitors, 2,3-difluorosialic acid (19) and 2,3-difluoroKdn (29) allowed us to measure the kinetic parameters for Micromonospora viridifaciens sialidase inhibition.

Comparison of the two inhibitory efficiencies ($k_{\text{inact}}/K_i$) of $MvS$ for 2,3-difluorosialic acid (19) ($3.6 \times 10^6$ M$^{-1}$ s$^{-1}$) and 2,3-difluoroKdn (29) ($2.92 \times 10^3$ M$^{-1}$ s$^{-1}$) with the two catalytic efficiencies ($k_{\text{cat}}/K_m$) of same enzyme for MU$\alpha$Neu5Ac (31) ($7.2 \times 10^6$ M$^{-1}$ s$^{-1}$) and MU$\alpha$Kdn (30) ($1.3 \times 10^3$ M$^{-1}$ s$^{-1}$) respectively, allowed us to conclude that inhibition of $MvS$ is mechanism-based.\(^7^2\)

In addition, the $N$-acetyl group at C5, increases catalysis which by virtue of several important electrostatic interactions such as hydrogen-bonding and hydrophobic interactions with the residues in the enzyme active site, gives rise to transition state stabilizations of the complex of $MvS$ with 2,3-difluorosialic acid (19) and MU$\alpha$Neu5Ac (31). That is, the replacement of the $N$-acetyl group by a hydroxyl functionality at C5 results in the loss of a several hydrophobic interactions in the active site (Figure 3.2). For example, the loss of these interactions; interaction between Asp131 residue and amidic NH, which is replaced by an OH and the hydrophobic interactions of Leu170, S Val147 and Phe203 with the acetamido methyl group results in transition state destabilization.\(^4^8,1^0^5\)

The kinetic studies from Watts et al were performed with the inactivation and reactivation studies on the sialidase from Trypanosoma rangeli (TrSA) with 2,3-difluoroKdn (29); however, they were unsuccessful in measuring the inhibitory efficiency for 19 because of the rapidity of inhibition. The result for the inhibitory efficiency of TrSA for 2,3-difluoroKdn (29) is $4.53 \times 10^{-1}$ M$^{-1}$ s$^{-1}$ while $MvS$’s inhibitory efficiency for 29 is about $2.92 \times 10^3$ M$^{-1}$ s$^{-1}$.\(^4^8,1^0^5\) The comparison of the two inhibitory efficiencies corresponds to the transition state for inhibition of $MvS$ by 2,3-difluoroKdn (29) being stabilized by $\sim21.8$ kJ mol$^{-1}$ in comparison to that for TrSA.
A possible extension of this work is to study the enzyme from *Aspergillus fumigatus* (AfS), which has been shown to be a Kdnase, which preferentially recognizes Kdn substrates over the homologous Neu5Ac substrates. This will be an important comparison because AfS and MvS are structurally analogous despite their substrate specificities. The proposed kinetic studies on AfS will need to measure the time-dependent inactivation and inhibitory efficiency of AfS with 2,3-difluoroKdn (29) and 2,3-difluorosialic acid (19). These results can then be used to compare with those from the current inactivation studies on MvS.
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