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Structure Determination of Derivatized Disaccharides by Tandem Mass Spectrometry and Molecular Modeling.

Sanford Louis Mendonca
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STRUCTURE DETERMINATION OF DERIVATIZED DISACCHARIDES BY TANDEM MASS SPECTROMETRY AND MOLECULAR MODELING

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and Agricultural and Mechanical College
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Sanford Mendonca
B.S., University of Bombay, 1988
M.S., University of Houston, 1995
December 2000
Dedicated to Monisha, Ninoshka,

Nadia, Natalia and Vinton
Acknowledgements

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wife. We have been through numerous ups and downs in our six wonderful years of marriage. I am glad I got the opportunity of sharing my life with her.

My three children Ninoshka, Nadia, and Natalia who put up with all the difficult times we had. They are a great blessing in my life and would like to thank God for three little angels. I would also like to thank God for helping me throughout my graduate career and enabling me to reach the greatest moment in my life.

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Abstract

This research entails the synthesis of derivatized disaccharides, mass spectrometry studies for structural elucidation of the synthetic disaccharides and molecular modeling of the native and derivatized disaccharides. Peralkylated (methyl to pentyl), peresterified and tert-butyl-dimethyl silylated linkage isomeric glucose-glucose disaccharides were synthesized to eventually be used in mass spectrometry studies. In this dissertation, mass spectrometry was used to examine anomeric pair discrimination of two sets of peralkylated (methyl to pentyl) disaccharides (maltose/cellobiose and isomaltose/gentiobiose). This was carried out by electrospray ionization and collision experiments on a triple quadrupole mass spectrometer. In addition, effects of alkyl substitution on cleavage of the glycosidic bond were observed. Collision energy offset voltages were plotted versus Product/Parent (D/P) ion ratios generating a trend observed for both the 1→4 and 1→6 alpha and beta isomers. The methyl derivative had the lowest D/P ion ratio followed by ethyl, propyl, butyl, and pentyl. Collision energy is converted to rotational-vibrational modes in competition with bond cleavage represented by the slope of offset energy versus D/P ion ratio. Rotational freedom at the glycosidic linkage is hypothesized to play a major role in this phenomenon. Molecular modeling was carried out using MM3 to validate differences observed in the rates of cleavage in the mass spectrometer. A flexible residue calculation was used involving rotation of the monomeric residues around the glycosidic bond having torsion angles \( \phi \) and \( \psi \). Three dimensional energy plots of \( \phi \), \( \psi \) and energy were plotted with the volumes of the wells taken as an indication of the conformational freedom of motion around the glycosidic bond. Two dimensional \( \phi-\psi \) energy surfaces of both the 1→1, 1→2, 1→3, and 1→4.
native and permethylated disaccharides were also plotted to study their characteristics. The calculated freedom of motion volumes for the β-(1→4) were smaller than for the α-(1→4) bonded glucoses, which may account for the higher rate of bond cleavage for the β-(1→4) linkage. Additionally an interesting outcome was noted from the difference maps between the permethylated and native disaccharides, which were plotted to study the effect of the methyl groups on the energy surfaces.
Chapter 1 Introduction

Carbohydrates

Carbohydrates constitute the most abundant group of natural products which is exemplified by the process of photosynthesis that produces $4 \times 10^{14}$ kg of carbohydrates each year. As their name implies, they were originally believed to consist solely of the elements carbon and water and were thus commonly designated with the generalized formula $C_x(H_2O)_y$. Later it was found that carbohydrates in fact contain hydroxyl and carbonyl groups and are polyhydroxy aldehydes or ketones. Still later it was recognized that compounds need not be aldehydes, or ketones, or have the empirical formula of a hydrate of carbon to be a carbohydrate; rather, compounds could be derived from polyhydroxy aldehydes or ketones and have the properties of a carbohydrate.

The modern definition of a carbohydrate is that it is a polyhydroxy aldehyde or ketone or compound that can be derived from them by any of several means including 1. Reduction to give sugar alcohols (alditols) 2. Oxidation to give sugar acids 3. Substitution of one or more of the hydroxyl groups by various chemical groups (for example hydrogen) to give deoxysugars, amino groups to give amino sugars, thiols or similar heteroatomic group. 4. Derivatization of the hydroxyl groups by various moieties, for example, phosphoric acid to give phospho sugars, or sulfuric acid to give sulfo sugars, or reaction of the hydroxyl groups with alcohols to give saccharides, oligosaccharides and polysaccharides. Thus the present day convention [1] is that carbohydrates are a much larger family of compounds comprising monosaccharides, oligosaccharides and polysaccharides with monosaccharides being the simplest ones which cannot be hydrolysed further to smaller constituent units.
All of these types of carbohydrates play important roles, for example, the phospho
esters of carbohydrates are intermediates in the metabolism of carbohydrates that
produces CO₂ + H₂O + energy in the process of respiration. The major compound in the
conversion and interchange of energy is the phospho sugar derivative adenosine
triphosphate (ATP). The sugar alcohols and sugar acids are involved in diverse functions,
for example, the sugar alcohol sorbitol is found in some fruits, where it imparts a
distinctive sweet taste. Sugar acids are found in plant, animal and bacterial
polysaccharides, such as pectin, hyaluronate, alginate, and bacterial capsules where they
impot important functional properties of acids and anions. Carbohydrates may also be
cova lently linked to other biopolymers such as lipids called glycolipids and to proteins
called glycoproteins. There is a growing appreciation for their role in protein solubility,
protein folding, protein turnover, cell surface receptors, cell-cell recognition, cellular
differentiation and immunological recognition.

Monosaccharides are carbohydrates that cannot be hydrolysed to simpler
compounds. Examples (shown in figure 1.1) are glucose which is a polyhyroxyaldehyde
(aldose) and polyhydroxyketone (ketose).

Disaccharides are carbohydrates that can be hydrolysed to two monosaccharides. An
eample is sucrose that is easily hydrolysed to glucose and fructose.

Sucrose → glucose + fructose

Polysaccharides are carbohydrates that can be hydrolysed to many monosaccharide
units. They are naturally occurring polymers of carbohydrates. Examples are starch and
cellulose, both biopolymers of glucose.
Figure 1.1 Structures and Fischer projections of (a) glucose; (b) fructose.
Since this dissertation is devoted to a mass spectrometric and modeling study of disaccharides, an overview of only this class of carbohydrates will be given.

Disaccharides are formed by the condensation of the anomeric hydroxyl group of one monosaccharide with the hydroxyl group of the second monosaccharide through formation of an acetal with the concomitant release of a water molecule. The bonds linking the two monosaccharide units are called the glycosidic bond and the oxygen atom connecting the two is called the "bridge" or "glycosidic" oxygen atom as shown in Figure 1.2. The monosaccharide whose anomeric carbon is involved in the glycosidic linkage is called the "non reducing" monomer and the other the "reducing" monomer.

The anomeric carbon atom of the reducing unit can either have the alpha (α) or beta (β) configuration and hence, disaccharides just like monosaccharides, can occur in the two anomeric forms and exhibit mutarotation. However, the configuration at the anomeric carbon of the nonreducing unit is fixed due to the formation of the disaccharide. If it is alpha, the linkage is α1→X depending upon the carbon atom CX of the reducing unit. If it is, beta the disaccharide linkages will be β→1X.

Two monosaccharides can also be joined in a 1→1 linkage. Here, both units are non-reducing and their anomeric configurations can be either the same or different. Depending on this, three types of 1→1 linked disaccharides can be envisaged (four types if the two units are not identical). For example the 1→1 linked disaccharides of D-glucose are α,α-trehalose, β,β-trehalose, and α,β-trehalose. Sucrose, the well known cane sugar or table sugar, is also a non-reducing disaccharide. It is made of α-D-
The configuration at this anomeric carbon atom is fixed.

"glycosidic bonds"

The configuration at this anomeric carbon atom can be either alpha or beta.

Nonreducing end (oxygen on the anomeric carbon is substituted)

"bridge" or "glycosidic" oxygen atom

Reducing end (oxygen on the anomeric carbon is unsubstituted)

**Figure 1.2** A $\beta 1\rightarrow 2$ linked disaccharide. The constituent monosaccharide units are in the $^4C_1$ conformation.
glucopyranose and β-D-fructofuranose. Thus disaccharides can be formed irrespective of whether the constituent monosaccharides are in the pyranose or furanose form. The linkage in this case is 1→2 and not 1→1 since one of the constituent monosaccharide is an aldose and the other, a ketose.

Several disaccharides can be derived from a single monosaccharide due to the possibility of a number of linkage types in contrast to amino acids and mononucleotides which can form only one type of dimer. For example, two glucopyranoses can form eleven different disaccharides, as shown in Figure 1.3. Table 1.1 gives the structures and trivial names of some disaccharides. When the non-reducing monosaccharide unit is an aldose, the linkage in the disaccharide is 1→X (X=1,2...,6). If it is a ketose the linkage in the disaccharide is 2→X (X=1,2...,6). If the conformation of the pyranose ring is known, the orientations i.e. axial or equatorial of the glycosidic bonds can also be incorporated in the description of the linkage types in the disaccharides. Accordingly they are termed Glc-α1→4-Glc for maltose and Glc-β1→4-Glc for cellobiose. Thus the series of eleven isomers is composed of eight isomers having glycosidic linkages between C-1 of one residue, in either anomic configuration, and C-2, C-3, C-4 or C-6 of the other pyranose residue. The other three isomers are obtained by acetal formation between both C-1 atoms via the glycosidic oxygen atom in α,α; α,β; β,β configuration. When the two carbohydrate residues are not identical the situation becomes even more complex since either residue can occupy the first or second position i.e. reducing or non-reducing residue. When the formation of a trisaccharide is considered the number of possible isomers increases dramatically.
Figure 1.3 The eleven possible disaccharides of D-glucopyranose with trivial names
<table>
<thead>
<tr>
<th>Common name</th>
<th>Nonreducing end sugar</th>
<th>Linkage type</th>
<th>Reducing end sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitobiose</td>
<td>D-GlcNAc</td>
<td>β1→4</td>
<td>D-GlcNAc</td>
</tr>
<tr>
<td>Chondrosine</td>
<td>D-Glucuronic acid</td>
<td>β1→4</td>
<td>D-GalNAc</td>
</tr>
<tr>
<td>Galabiose</td>
<td>D-Fructofuranose</td>
<td>α1→4</td>
<td>D-Galactose</td>
</tr>
<tr>
<td>Inulobiose</td>
<td>D-Galactose</td>
<td>β2→1</td>
<td>D-Fructofuranose</td>
</tr>
<tr>
<td>Isolactose</td>
<td>D-Galactose</td>
<td>β1→6</td>
<td>D-Glucose</td>
</tr>
<tr>
<td>Isomaltulose</td>
<td>D-Galactose</td>
<td>α1→6</td>
<td>D-Fructofuranose</td>
</tr>
<tr>
<td>Lactose</td>
<td>D-Galactose</td>
<td>β1→4</td>
<td>D-Glucose</td>
</tr>
<tr>
<td>Lactulose</td>
<td>D-Galactose</td>
<td>β1→4</td>
<td>D-Fructofuranose</td>
</tr>
<tr>
<td>Leucrose</td>
<td>D-Glucose</td>
<td>α1→5</td>
<td>D-Fructose</td>
</tr>
<tr>
<td>Levanobiose</td>
<td>D-Fructofuranose</td>
<td>β2→6</td>
<td>D-Fructofuranose</td>
</tr>
<tr>
<td>Maltulose</td>
<td>D-Glucose</td>
<td>α1→4</td>
<td>D-Fructose</td>
</tr>
<tr>
<td>Mannobiose</td>
<td>D-Mannose</td>
<td>β1→4</td>
<td>D-Mannose</td>
</tr>
<tr>
<td>Melibiose</td>
<td>D-Galactose</td>
<td>α1→6</td>
<td>D-Glucose</td>
</tr>
<tr>
<td>Sucrose</td>
<td>D-Glucose</td>
<td>α1→2</td>
<td>β— D-Fructofuranose</td>
</tr>
<tr>
<td>Trehalulose</td>
<td>D-Glucose</td>
<td>α1→1</td>
<td>D-Fructose</td>
</tr>
<tr>
<td>Turanose</td>
<td>D-Glucose</td>
<td>α1→3</td>
<td>D-Fructose</td>
</tr>
<tr>
<td>Xylobiose</td>
<td>D-Xylose</td>
<td>β1→4</td>
<td>D-Xylose</td>
</tr>
<tr>
<td>Xylosucrose</td>
<td>D-Xylose</td>
<td>α1→2</td>
<td>β— D-Fructofuranose</td>
</tr>
</tbody>
</table>
The relative orientations of the two monosaccharide units in a disaccharide can be described by the torsion angles $\phi$ and $\psi$ around the glycosidic bonds. $\phi$ represents the torsion angle about the C(anomeric)-O bond whereas $\psi$ about the O-CX' bond. (CX' is either C2', C3' or C4' depending upon the type of linkage). In the case of a non reducing disaccharide, $\phi'$ is used instead of $\psi'$ since both describe the torsion angles around the C(anomeric)-O bonds. In the case of a 1→6 linked disaccharide, the two monosaccharides are separated by an additional glycosidic bond and rotation around this is designated as $\omega$. $\phi$ is defined as earlier, $\psi$ and $\omega$ refer to C1-O-C6'-C5' and O-C6'-C5'-H5' respectively.

**Mass Spectrometry**

A mass spectrometer can be divided into three fundamental parts, namely the ionization source, the analyser, and the detector. Mass spectrometers are used primarily to provide information concerning the molecular weight of the compound, and to achieve this, the sample under investigation has to be introduced into the ionization source of the instrument. In the source, the sample molecules are ionized and these ions are extracted according to their mass (m) to charge (z) ratios (m/z). The separated ions are detected and the signal fed to a data system where the results can be studied, processed and printed out. The whole mass spectrometer (except for atmospheric pressure ionization sources) is maintained under vacuum to give the ions a good chance of travelling from one end of the instrument to the other without interference or hindrance.

A considerable impetus to the development of organic mass spectrometry has come from advances in sample handling and in methods of ion formation which have
both enormously increased the range of samples amenable to mass spectrometric analysis. The ionization methods in use are very diverse and constitute the following:

Electron Impact Ionization (EI): This source was devised by Dempster and improved by Bleakney [2] and Nier [3]. Organic molecules in the gas phase are bombarded by energetic electrons. An energy of 70eV is usually chosen to create reproducible spectra that contain odd electrons formed by ejection of an electron from the molecule. Subsequent fragmentation of the excited molecular ion produces the spectrum of fragment ions from which the structure of the original molecule can be deduced. It can be used for analyzing a wide range of volatile organic compounds.

Chemical Ionization (CI): It was developed by Munson and Field [4] in which methane was used as the reagent gas in the positive ion mode, with the major ions reacting as Bronsted acids. Molecules in the gas phase are ionized in an ion molecule reaction with a positively or negatively charged reactant ion that itself is formed as a result of electron ionization or chemical ionization reactions. The protonated or the deprotonated molecule is usually a prominent ion in the chemical ionization spectrum.

Fast Atom Bombardment: was developed by Barber et al. [5]. It consists in focusing on the sample a beam of neutral atoms or molecules that causes desorption of protonated or deprotonated molecular ions from the sample. The fast atom beams generally used are generated from xenon or argon atoms.

Field Desorption: This method was introduced by Beckey et al. [6]. The sample is deposited through evaporation of a solution containing also a salt on a tungsten or rhenium filament covered with carbon needles. A potential difference is set between the filament and an electrode. The filament is heated until the sample melts. The ions migrate
and accumulate at the tip of the needles where they are desorbed and carry along molecules of the sample.

Plasma desorption: The sample is deposited on a small aluminized nylon foil and exposed in the source to the fission fragments of $^{252}$Cf having an energy of several MeV. The shock waves resulting from the bombardment of a few thousand fragments per second induce the desorption of neutrals and ions. This technique has allowed the observation of ions above 10000 Da [7].

Inductively Coupled Plasma: It allows a rapid and simultaneous determination of several metal elements with high accuracy and sensitivity [8]. The source is made up of a flame in which a solution of a sample is introduced as a spray. Ar gas is used to support a plasma. A coil that surrounds the plasma is energized with kilowatt rf power that induces an electromagnetic field and inductively heats the plasma to temperatures exceeding 10000 K.

Thermospray: This technique developed by Vestal et al. [9] is used to desorb ions from liquids. A high percentage of the liquid flow is volatilized as it emerges from a heated metal capillary. Under these conditions the vapor that comes out from the end of the capillary has sufficient energy to transform the remainder of the liquid flow into a mist of small droplets each with a statistical distribution of excess charge. No external electric field is applied since at the lower pressure at which thermospray operates a glow discharge results.

Matrix Assisted Laser Desorption: It involves sample bombardment in a matrix containing a low concentration of sample molecules with short, intense pulses from a laser light to effect both desorption and ionization of the molecules [10]. The matrix
Figure 1.4 Schematic diagram of an electrospray source

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transforms the laser energy into excitation energy for the sample which leads to sputtering of the analyte and matrix from the surface of the mixture. In this way the energy transfer is efficient, while the sample molecules are spared from excessive energy which would lead to their decomposition.

Electrospray: Since this source is used in the experiments described in this thesis a more detailed coverage will be given. This source was devised by Yamashita and Fenn [11,12]. A schematic diagram is shown in Figure 1.4. Sample solution at flow rates between 1 and 20μL/min enters the ES chamber through a stainless steel hypodermic needle. The needle is maintained at a few kilovolts relative to the walls of the chamber and the surrounding cylindrical electrode that helps shape the distribution of potential and direct the flow of bath gas. The resulting field at the needle tip charges the surface of the emerging liquid, dispersing it by coulombic forces into a fine spray of charged droplets. Driven by the electric field, the droplets migrate to the inlet end of the glass capillary at the end wall of the chamber. A countercurrent flow of bath gas typically at 800 torr, and a flow rate of 100mL/s hasten evaporation of solvent from each droplet, decreasing its diameter as it drifts towards the end wall of the chamber. Consequently the charge density on its surface increases until the so called Rayleigh limit is reached at which the Coulomb repulsion becomes of the same order as the surface tension. The resulting instability, sometimes called a "Coulomb explosion" tears the droplet apart producing charged droplets that also evaporate. This sequence of events repeats until the radius of curvature of a daughter droplet becomes small enough that the field due to the surface charge density is strong enough to desorb ions from the droplet into the ambient gas. The desorbing ions include cations (or anions), to which are attached solvent or solute species
that are not themselves ions, thus producing so called quasi-molecular ions for mass analysis. The sequence is schematically represented in Figure 1.5.

The ions formed using any of the above sources then pass into the next region of the mass spectrometer, the analyzer, which is under vacuum. There are five different types of analysers:

Quadrupole: It was originally developed by Paul and coworkers [13]. It is a device which uses the stability of the trajectories to separate ions according to their m/z ratio [14]. A quadrupole electric field is created by electric potentials applied to four parallel rods of hyperbolic cross section. At sufficient high translational energies, all ions will pass through the quadrupole mass filter, but at energies below about 100eV, mass selectivity can be obtained using rods of 20-30cm length. Under such conditions the mass filter acts as a path stability device if opposite pairs of rods are connected electrically and a DC voltage \( U \) and a rf voltage, \( V_0 \cos \omega t \), are applied to the rods. For particular fields, ions having a small range of m/z values have stable paths through the filter and all other ions are not transmitted.

Sector: This comprises two kinds (i) Magnetic Sector (ii) Electric Sector

In a magnetic sector when accelerated \( m_1^+ \) ions enter a magnetic field of strength \( B_1 \), the ions follow a circular path of radius \( R \), perpendicular to the direction of the field with a velocity \( v_1 \) where \( R \) is given by

\[
R = \frac{m_1 v_1}{B_1} \quad \text{or} \quad m_1 v_1 = R B_1
\]

If \( B \) is scanned at a fixed value of \( R \), then ions of different momenta, and hence of different mass, can be made to pass through a collector slit to give a mass spectrum [15].
Figure 1.5 The electrospray ionization process
The electric sector acts both as a focusing device for diverging ion beams and as a device for dispersing ions according to their kinetic energies; it is directional focusing in that all ions of the same energy emanating from the source slit with a small angular divergence are brought to focus; it is energy dispersing in that ions having greater or lower energies follow paths of greater or lower radii and are brought to focus at different positions. Since most ion sources produce beams of ions with a significant energy spread an electric sector can be used to reduce the energy spread in the transmitted ion beam.

Quadrupole ion trap (QIT): An ion trap described by Paul and Steinwedel [16,17] is a 3-D quadrupole in which the ions of all masses are trapped on a three dimensional eight shaped trajectory. It consists of a circular electrode, and 2 end caps. A potential $\phi_0$, the sum of a direct and alternative potential is applied to the caps, and $-\phi_0$ is applied to the circular electrode. As the name suggests this instrument operates on the basis of first storing ions of different masses inside the trap, which are then expelled according to their masses to obtain a spectrum.

Fourier transform ion cyclotron resonance (FTICR): It was first developed in the mid 1970’s by Comisarow and Marshall [18]. Ions are first generated by a brief electron beam pulse and stored in the trapped ion cell. After a brief delay the trapped ions are subject to a short radio frequency pulse that increases linearly in frequency during its lifetime. After the frequency sweep is discontinued the image current induced by the various ion packets is amplified, digitized and stored in the memory. The time domain decay signal is then transformed to yield a frequency domain signal that can be converted to a mass domain signal.
Time of Flight (TOF): analysis was first described by Wiley and Mclaren [19] in 1955 and review papers were published by Cotter [20] and Wollnik [21] in 1983. The packet of ions produced by a laser pulse is extracted and accelerated by an electric field pulse of 10³-10⁴ V. The accelerated particles then pass into a field free drift tube of 1-3m in length. Since all ions entering the tube have the same kinetic energy, their velocities in the tube must vary inversely with their masses with the lighter particles arriving earlier than the heavy ones.

Mass spectrometry has for many years provided a particularly sensitive and specific method for the identification of organic compounds [22-24]. Despite the high information content of MS data, its specificity can be inadequate for structure elucidation of large molecules; the number of possible isomers goes up exponentially with increasing size. For organic mixture analysis information requirements also go up rapidly with increasing mixture complexity, raising the probability that more than one component will contribute to an individual mass peak. A method to effect an exponential increase in the amount of MS information that can be generated and retain the sensitivity, speed and accuracy of MS is the coupling of two (or more) analysers. They have the capability at their interface to fragment the unique mass ions from MS-1 to yield characteristic product ions of many masses to be separated in MS-II. Tandem mass spectrometry [24-31], for which the acronym “MS/MS” has been coined [32,33] emulates GC-MS in replacing the chromatograph by a mass separator. An advantage of this is speed; GC-MS is limited by the time required for chromatographic separation (usually many minutes), while the second MS only requires an additional ion transit time of 10⁻³-10⁻² sec. In addition to the specificity improvements in MS/MS versus MS, the sensitivity attainable in terms of
signal to noise can also be improved substantially. Although the signal is reduced by the inefficiency of post-MS-I fragmentation and MS-II transmission, the noise can be reduced much more. This can be especially significant for chemical noise arising from other parts of a large molecule in structure elucidation or from other components in complex mixture analysis. The MS/MS information for a given sample is the ion intensity for each combination of parent and daughter mass. In tandem mass spectrometry three operational modes are possible considering an instrument that combines in sequence two mass spectrometers separated by a collision cell represented in Figure 1.6.

1. **Product ion scan (daughter scan)** consists in selecting a precursor ion (or parent ion) and in determining all of the product ions (daughter ions) resulting from collision induced dissociation (CID)

2. **Precursor ion scan (parent scan)** consists in choosing a product ion (or daughter ion) and determining the precursor ions.

3. **Neutral loss scan** consists in selecting a neutral fragment and detecting all the fragmentations leading to loss of that neutral.

Collision induced dissociation is a method to activate an ion, that is add internal energy by collision with a neutral target (a gas or a solid). Often the optimum methods of ion formation do not impart sufficient energy to cause fragmentation of the molecular ion. The internal energy of the ion in these circumstances may be increased by collision activation (CA). Tandem mass spectrometry (MS/MS), in all its configurations, has emerged as the most important technique for acquiring collision activated dissociation (CAD) or collision induced dissociation mass spectra. The overall CID process can be separated into two consecutive steps occurring on
Figure 1.6 Different scan modes for a tandem mass spectrometer
well separated time scales. The first is a fast ($10^{-15}$-$10^{-14}$ sec) CA step in which some portion of the initial translational energy of the accelerated ion is converted into energy of both the ion and target (the target also acquires translational energy). The second step in this process is the dissociation of the now energized and typically isolated ion. There are different methods of ion activation: Collisions of accelerated ions with a stationary gas phase target in the (a) High energy [32-36] (keV) and (b) low energy [37-39] ranges of laboratory kinetic energy, surface induced dissociation [40,41], photodissociation [42,43] and electron impact activation.

Various instruments are used in tandem mass spectrometry. A common instrument used is the triple quadrupole mass spectrometer which will be described in detail since it is used in the experiments. Other instruments combine electric and magnetic sectors (E and B) or E, B, and Q, thus electric, magnetic sectors and quadrupoles. Time of flight instruments, or a combination of a magnetic instrument with a time of flight instrument, are also used.

The triple quadrupole mass spectrometer was developed by Yost and Enke [28,44]. It consists of three quadrupoles in tandem. The first and third quadrupoles are regular quadrupole mass filters on which a DC and RF potential is applied. The second quadrupole is not a mass filter but is a containment device for the ions undergoing collision activation with a target gas which operates in the RF-only (total ion) mode with only an RF voltage applied to it. The quadrupoles provide high transmission efficiency and unit mass resolution in the CID spectra is easily obtained. The three scans described above are carried out in the triple quadrupole as follows:
Daughter ion scan: The first quadrupole is set to transmit a selected mass while the last quadrupole is scanned to produce a spectrum of all the fragment ions from the selected precursor ion.

Parent ion scan: The last quadrupole is set to transmit the selected fragment ion while the first quadrupole is scanned to produce a spectrum of all the precursor ions that fragmented to produce the selected fragment ion.

Neutral loss scan: Both the mass filters are scanned simultaneously with a fixed difference in mass.

**Mass Spectrometry of Carbohydrates**

Mass spectrometry was first applied to carbohydrate derivatives in 1958 when Reed and co-workers [45] reported the mass spectra of D-glucose, D-galactose, methyl alpha and beta glucopyranosides and a number of disaccharides. Typically glycans [46] and glycoconjugates [46-48] were derivatized to increase their volatility and thermal stability inorder to permit determination of their molecular weights and structures by electron impact and chemical ionization [46,49]. This is accomplished by either alkylation of all hydroxy and acetamido functions with methyl groups [50] or esterification with acetyl groups. Structure determination of permethylated or peracetylated small glycans has been accomplished by electron impact ionization [46,47]. Kotchetkov and coworkers [51-53] studied permethylated disaccharides having 1→2, 1→4,1→6 linked hexose residues which led to the nomenclature of fragment ions from oligosaccharides [54,55].

Between 1965-1970 Bengt Lindberg at the Karolina Institute in Stockholm developed the procedure of linkage analysis using Corey/Hakomori methylation and
GC/MS. The saccharide is methylated at the free hydroxyl group followed by hydrolysis of the glycosidic bonds to monosaccharides, the carbonyl groups are reduced and finally the hydroxyl groups liberated are derivatized by acetylation [56]. Separation of the resulting partially methylated alditol acetates were carried out by gas chromatography and identification of the linkage position by EI-MS [57]. The feasibility of the technology was demonstrated by Hellerqvist in the structural analysis of the salmonella typhimurium liposaccharides [58], which along with a paper by Bjomdal [56] became a citation classic. The disadvantage of the methylation analysis procedure is that it requires a long and tedious protocol including several chemical degradation and derivatization steps, labor extensive, and required large sample amounts. Hence it would be necessary to develop simple and more sensitive techniques for structural analysis of oligosaccharides. Much of the relevant literature has been reviewed up through 1974 by Longren and Svensson [59].

An improvement in the sensitivity of the Lindberg method was achieved by Laine et al. using CI-MS which allowed an analysis down to 5-10ug [60]. Chemical ionization has shown to enhance the abundance of high mass ions in the spectra of derivatized carbohydrates and the overall sensitivity is comparable to EI. The disadvantage of chemical ionization is that the procedure becomes very difficult with very small amounts of sample or if sensitive functional groups are present. Californium-252 fission fragment plasma desorption TOF mass spectrometry was developed by Jardine in collaboration with Brennan [61]. However with the advent of MALDI the popularity of PDMS has declined. Field desorption (FD) mass spectrometry has been used in the structural analysis of oligosaccharides [62]. However in some cases complete structural
characterization by FD mass spectrometry is not possible since fragment ions which are structurally specific are either absent or of low intensity. Field desorption allows the detection of pseudo-molecular [M+H]^+ ions from underivatized saccharides and derivatized saccharides such as permethylated and peracetylated saccharides [63,64]. This technique has been mainly used for molecular weight determination together with prominent fragment ions resulting from cleavage of the glycosidic bond. Even though field desorption played an initial important role [65], it has been overshadowed by the ease of maintaining a stable molecular ion beam with FAB for use with sector instruments. FAB has been exploited to a great extent for the analysis of carbohydrates because it is sensitive. It does away with any force of derivatization, since it is not dependent on volatility. Underivatized glycoconjugates, which cannot be analyzed by conventional EI mass spectrometry, can be analyzed by this technique, and it gives molecular weight information.

FAB has been used extensively for determination of the interglycosidic linkages in both derivatized and underivatized carbohydrates [66-72]. FAB MS/MS is the mass spectrometric technique that is most often used in the analysis of oligosaccharides. This technique is useful in the case of pseudomolecular ions (M-H)^- [68], (M+H)^+[71-74], (M+alkali metals)^+[75,76] of native or derivatized oligosaccharides. Oligosaccharides show a tendency to yield low intensity pseudomolecular ions: peracetylation or permethylation is used to increase their hydrophobicity and allow an increase in the intensity of these ions [77]. The derivatization and/or presence of alkali metals influences the fragmentation mechanism and the fragmentation pattern.
A nomenclature suggested by Domon and Costello [78] was developed to characterize the various fragments obtained by mass spectrometry whatever the method used to produce them. The fragments retaining the charge on the non-reducing end are called A, B, C, and those retaining the charge on the reducing end are called X, Y, Z depending on whether they cut the ring or the glycosidic bond, as shown in Figure 1.7. The subscript for the B ions corresponds to the number of the ruptured glycosidic bond while the superscript at the left of the A and X fragments corresponds to bonds that were broken in order to observe these fragments, the bonds being numbered as is indicated in the figure. The letter α, β etc. that may be attached to the subscript number indicates the branch involved in the cleavage, provided the oligosaccharide is branched. The type B, C, Y, and Z derived from the cleavage of the glycosidic bond allows one to determine the sequence and branching pattern of oligosaccharides. The fragments that are most often observed in positive mode spectra correspond to cleavage of the glycosidic bond, with oxygen atom retention on the reductive part. This yields B and Y type ions. The mass difference between identical type ions allows one to deduce the sequence of oligosaccharides. The same principle may be used to determine the branching pattern.

Negative ion FAB has been used to analyze underivatized sugars with linked scanning and collision induced dissociation [66,67] or metastable ion dissociation tandem mass spectrometry [68,69] to give fragmentation patterns of branched oligosaccharides, sugar sequence, and differentiate between both linkage positions and anomeric configurations of the glycosidic bond and nature of the monosaccharide units [66-69, 74, 79]. Negative ion FAB together with metastable ion and collision activation techniques were used in differentiating isomeric aldohexoses, deoxyaldohexoses [80].

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Figure 1.7 Nomenclature suggested by Domon and Costello
and substituted glucose and glucosamine [81]. Leary and co-workers [82,83] used FAB/MS/MS to determine the linkage position of lithium cationized disaccharides in the positive ion mode. Laine et al. [84] also used fast atom bombardment and collision induced dissociation in a study on three linkage isomeric trisaccharides to ascertain whether the ion patterns could distinguish among the three possible linkage positions of the terminal fucose in otherwise identical structures. Molecular modeling was also carried out to support the suggestion that steric factors and the position of the fucose linkage relative to GlcNac contributed to bond stability during collision activation. The study [85,86] was then extended to oligosaccharides terminating in a β-D-galactose where linkage discrimination was shown among a permethylated set of six synthetic oligosaccharides. Even though FAB has been used extensively in the structural elucidation of carbohydrates it has a poor detection limit and sensitivity. These problems were overcome by matrix assisted laser desorption (MALDI) [87] and electrospray (ESI) [88] that have dramatically impacted carbohydrate analysis by allowing ion production for large thermally labile oligosaccharides. Native or derivatized oligosaccharides can be analyzed by MALDI with great sensitivity [89-91]. In MALDI-TOF the oligosaccharide ions are normally activated by post source decay [92,93] which is a spontaneous decomposition of the molecular ion in the first field free region and a subsequent analysis of the fragment ions by a reflectron instrument. MALDI in conjunction with magnetic sector instruments provided limited sequence and branching information even though the ions decomposed spontaneously in the ion source of the mass spectrometer.

Spengler et al. [93] studied native oligosaccharides under post source decay conditions where glycosidic cleavages predominated under the low energy conditions,
while Lemoine et al. [94] did a study on various permethylated, peracetylated and reductively aminated oligosaccharides. MALDI in conjunction with magnetic sector instruments provided limited sequence and branching information even though the ions decomposed spontaneously in the ion source of the mass spectrometer. Hence high energy CID spectra of underivatized oligosaccharides ionized by MALDI was carried out by Green et al. [95] using a sector instrument combined with an orthogonal tandem time of flight analyzer. This method provided good sequence, branching, and linkage information. Further work on sequence and linkage information of underivatized oligosaccharides was carried out by Librella and coworkers [96] using alkaline degradation and MALDI ionization Fourier transform mass spectrometry. They also used MALDI-FTMS to obtain a correlation between the fragment ion yield and degree of branching for oligosaccharides as well as the relationship between the size of the alkali metal ion and yield of fragment ions [97].

A very viable alternative to structural analysis of oligosaccharides is electrospray ionization which is regarded as an effective means for their characterization [98,99]. As a structural analysis technique for carbohydrates and lipids, ESI-MS is useful because processes such as desalting a sample, adding organic solvents, and applying heat to evaporate solvents, all of which are considered harsh to protein conformations, do not seriously affect the mass analysis of carbohydrates except for binding studies. In the latter case conformation plays an important role. Further, it has high sensitivity and low background noise. Electrospray has been used by several groups to characterize native [100,101] and derivatized [102,103] carbohydrates, where it has the high sensitivity that is suitable for glycan analysis. It is also useful in the analysis of carbohydrate mixtures.
This can be applied to studies of glycan heterogeneity associated with specific molecular weight intervals. Various derivatization techniques have been used such as peracetylation, permethylation [104,105], reductive amination [106].

Derivatization techniques improve the signal by using more volatile organic solvents which permit a significant increase in detection sensitivity. For the linkage analysis of glucose disaccharide isomers fragmentation rules approximately the same as those in FAB were reported in 1991. Garuzzo et al. [107] characterized fragmentational differences among glucopyranose-β-(1-C→2,3,4,6) of glucose by applying skimmer collisions (in source fragmentation) in negative ions ESI-MS. Characterization of underivatized large neutral oligosaccharide by ESI-MS was reported in 1993 on a synthetic poly-N-acetyllactosamine type pentasaccharide, which corresponds to the sugar chain of the glycoconjugate having blood group 6, activity [108]. Fura and Leary [109] analyzed three isomeric branched trisaccharides coordinated to calcium and magnesium by electrospray ionization followed by tandem mass spectrometry. Linkage positions of the glycosidic bond between the reducing and non-reducing rings were determined in all the three isomers and the linkage of the fucose ring was determined in two of the three isomers when calcium is used as the coordinating metal. A stereochemical differentiation was made by Gaucher et al. [110] using ESI-ion trap mass spectrometry between mannose, glucose, galactose and talose with zinc diethylene triamine. The same technique was used for structural analysis of complex linked glycoprotein oligosaccharides by Harvey et al. [111].

High performance liquid chromatography is a fundamental yet important technique for the separation of oligosaccharides. However, few studies using LC/MS
have been done for analysis of carbohydrates [112]. Kohler and Leary [113] used LC/MS/MS with post column addition of metal chlorides using a triaxial electrospray probe to accomplish sensitivity enhancement and structural analysis of carbohydrates. Li and Her [114] analyzed disaccharides and linear oligosaccharides labeled with a chromophore by online coupling of HPLC with ESI-CID. This approach was used to ascertain linkage position and sequence of linkages of linear oligosaccharides. Structural analysis involving sequence and branching of oligosaccharide alditols was carried out by a combination of HPLC and ESI MS/MS [115] after metal periodate oxidation and derivatization with 2 aminopyridine.

**Molecular Modeling of Carbohydrates**

In both the industrial and biological functions of carbohydrates, the three-dimensional characteristics of carbohydrates are important. For three-dimensional structure the natural questions that arise are whether the relatively minor changes in the chemical structure produce meaningful changes in conformation and if so whether the environment (crystalline, solution or protein) plays a role in bringing about and stabilizing these changes. To address these questions, it is important to find the amount of energy required to alter the various geometrical parameters within a molecule. Many of these stereochemical features are described for carbohydrates in the classic text by Stoddart [116]. The importance of stereochemistry is underscored by the unique physical and chemical properties of the individual sugars, many of which are configurational isomers. Stereochemistry also plays a role in determining the properties of polysaccharides. Molecular shape is as significant for the properties of an industrially modified starch as it is for the recognition of one particular blood type and the rejection
of others. Molecular shape is defined by three different types of molecular parameters: bond lengths, bond angles and torsion angles. Variations in the molecular geometry of a molecule are defined as changes in these parameters: bond stretching or compression, bond bending or deformation and bond twisting or torsion. Coincident with the increased interest in carbohydrates, techniques for studying molecular shape have increased. Single crystal diffraction studies can give a fast and precise description of molecules in the solid state. Recent advances in nuclear magnetic resonance provide increased detailed conformational information about the solid state as well as on solutions. However, the structural characteristics of many carbohydrate molecules remain unknown. It is often difficult to obtain single crystals needed for crystallography, even if the required amount of pure material is available to attempt crystal growth. Some carbohydrates persist as syrups, and oligomers and polymers often form microcrystalline particles or fibers that yield inadequate data for a complete structural determination by diffraction methods alone. In 1969, D.W. Jones supplemented fiber diffraction data from cellulose with a computer model, a list of proposed atomic coordinates that were stored in a digital computer [117]. He then calculated the diffraction intensities that would arise from the models and compared them with the observed intensities. The model was repeatedly readjusted, with intensities calculated and compared at each adjustment, in a trial and error study. While inconclusive, this was one of the first reports of computer modeling of a carbohydrate. More recently the interpretation of coupling constants and nuclear overhauser effects from Nmr spectra has been expedited by computer models that provide a ready source of distances and angles. Since these early efforts, computer modeling has become an integral part of some procedures for structural determination. Theory is better
combined with experiment, so that each can support the other. However, some problems are not amenable to experiment. Acyclic glucose, for example occurs in such small concentrations that experimental data are overwhelmed by data from the pyranose forms. In contrast a model is easily built and studied. A good modeling study provides a framework for integrating the experimental results from various techniques to provide a good overall understanding.

Molecular modeling calculations attempt to predict physical properties for molecular systems based on the numerical solution of the equations that embody the physical laws that govern their behavior [118,119]. At the most fundamental level, this approach involves the direct solution of Schrodinger’s equation for the nuclear and electronic degrees of freedom. Since these studies determine energies directly from first principles, they are referred to as ab initio or nonempirical calculations. Such calculations rapidly become impossible in the practical sense for systems containing more than a few atoms heavier than hydrogen, and it becomes necessary to invoke various additional approximations to extend these calculations to systems containing more than two dozen atoms.

Molecular mechanics is one such approach to treat the motions of atomic nuclei as classical particles, since most of the quantal character of molecules resides in their electronic motions. It is then possible to use the Born-Oppenheimer approximation to solve for the electronic energies at fixed nuclear positions, and to treat these electronic energies as the potential energy field for the nuclear motions. After this separation, analytic, empirical energy functions may be used to approximate the way in which the molecular energy changes with the coordinates of the constituent atoms. Any technique
will be called a “molecular mechanics” (mm) calculation if it uses such analytic energy functions to predict changes in a system’s energy arising from variations in its atomic coordinates [118,119]. The empirical energy functions used in mm calculations usually consist of sums of terms representing various easily conceptualized contributions to the total energy of a molecule. For example, such energy functions generally contain terms to represent the energy of stretching or compressing chemical bonds, bending bond angles and changing torsion angles which are intramolecular motions of a molecule. These functions also generally contain terms to represent Van der Waals (non bonded) interactions, hydrogen bonding and electrostatic interactions between the various partially charged atoms and/or dipoles in a molecule. These energy contributions contribute to the total energy of the molecule and are represented by equation 1.

\[ E_{\text{tot}} = E_b + E_a + E_{\text{tor}} + E_{\text{nb}} + E_{\text{hb}} + E_{\text{elst}} \]  

\[ E_b = \text{bond stretching} \]

\[ E_a = \text{angle bending} \]

\[ E_{\text{tor}} = \text{internal rotation} \]

\[ E_{\text{nb}} = \text{nonbonded interaction} \]

\[ E_{\text{hb}} = \text{hydrogen bonded interaction} \]

\[ E_{\text{elst}} = \text{electrostatic interaction} \]

MM3 is one such molecular mechanics program developed by Allinger et al. [120] that is used for the molecular modeling described in this thesis. It has several advantages in that it takes into account lone pair effects. It has separate parameters for the ring oxygen, glycosidic oxygen and anomeric carbon, which differ for the \( \alpha \) and \( \beta \) anomers in the calculation of the energy terms. Further, it compensates for the anomeric
effect, and an attempt is made to account for the bond length distance changes arising from torsional changes in anomeric (O-C-O-C) sequences. It also has a dihedral driver facility that accepts the initial, final and increment size values of the two torsion angles $\phi$ and $\psi$.

**Scope of Work**

The focus of this dissertation is the investigation and development of a method to differentiate stereoisomeric synthetic peralkylated disaccharides using electrospray ionization in conjunction with tandem mass spectrometry. Molecular modeling of the permethylated derivatives is carried out to rationalize the fragmentation ratios observed in the mass spectrometer. Our research is targeted at not only differentiating glucose-glucose anomers but in understanding the mechanism by which the differentiation is made and to examine the effects of the alkyl groups on glycosidic bond cleavage. Studies to understand carbohydrate structure is important because of the growing interest in the biological function attributed to these moieties. Carbohydrates and their glycoconjugates are found to be crucial participants in the biochemical basis of cell-cell interaction. The evidence for these interactions has been growing and is strongly supported by the use of carbohydrate inhibitors, deglycosylating enzymes and site directed mutagenicity of participating glycans. From these studies carbohydrates appear to serve as the “Velcro” of adhesion, decoys against bacterial invasion, the modulators of protein structure and epitopes for molecular targeting and trafficking. Numerous human parasites through molecular mimicry have capitalized on these intricate functional roles to subvert immune surveillance, invade and thrive within human cells. Wherever confining membrane surfaces occur, and signal transduction is carried out, the diversity and specificity of the
carbohydrate molecule are likely to be utilized as a conduit for this communication. Thus in order to better understand the biological functional roles of carbohydrates a rapid method for the anomeric configuration differentiation and an understanding of how the differentiation is made is important. The anomeric configuration determination is crucial because prior knowledge of it gives information of which anomer plays a more important role in its biological functions and thus enable design of more effective drugs based on the interactions of the carbohydrates with receptors like proteins. Further the geometry and torsion angles about the glycosidic linkage are the most important geometrical parameters in defining the three dimensional structure of oligosaccharides. Since the latter determine their biological functions, the stereochemistry of this linkage is important.

The disaccharides investigated were the anomers of the 1→4 and 1→6 linked disaccharides that were derivatized with alkyl groups ranging from methyl to pentyl. In Chapter 2 the synthesis of these disaccharides and other linkage isomeric glucose-glucose disaccharides viz. 1→1, 1→2, 1→3 derivatized with the same alkyl groups were synthesized. Further the 1→4 and 1→6 linkage disaccharides were esterified with acetyl, pivaloyl and mesitoyl functional groups and last of all silylated with tert-butyl-dimethylsilyl groups (TBDMS). A large number of derivatives were synthesized with the goal of carrying out electrospray collision induced studies. However, in this thesis, the results of the mass spectrometric studies of only the peralkylated 1→4 and 1→6 linkage isomeric disaccharides will be presented.

In chapter 3, the electrospray collision induced dissociation studies on the disaccharides mentioned above will be discussed to examine their anomeric configuration differentiation and the effect of alkyl groups on glycosidic bond cleavage. The
conformational analysis of the permethylated disaccharides of the 1→4 anomeric pairs will also be discussed to rationalize the mass spectrometric results.

Finally Chapter 4 presents a conformational analysis of both the native and permethylated 1→1, 1→2, 1→3 and 1→4 glucose-glucose disaccharides. The 1→4 anomeric pair is explained here in a different light with a discussion of the 2-dimensional energy surfaces, the minimum energy structures and the effect of methylation through difference plots of the energy surfaces between the methylated and native surfaces.

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Chapter 2

Synthesis of Sterically Crowded Derivatives of Anomeric Pairs of Glucose-Glucose Disaccharides

Introduction

This chapter discusses the synthesis of the various sterically crowded derivatives of glucose-glucose disaccharides to be used for the mass spectrometry studies to differentiate the anomeric configuration of the glycosidic bond. The preparation of derivatized carbohydrates is of interest because they serve as synthetic intermediates in which they temporarily protect those functional groups not planned to be involved in a desired manipulation [1]. Further they are very useful in structural studies in mass spectrometry [2]. The synthesis is mainly focussed on etherification, esterification and silylation of the hydroxyl groups of the disaccharides. The disaccharide ethers synthesized are the methyl, ethyl, propyl, butyl and pentyl ethers. Methylation can be used for confirming any evidence of the chemical structure [3-6]. With increasing recognition of the significance of complex carbohydrates, roles played in the determination of specificities of hormones, immunity, biological transport, recognition and in various pathological phenomena, it has become more urgent to find rapid and sensitive methods for discerning the chemical structure. For this purpose permethylation on a microscale is important. The higher ethers of carbohydrates are also important to study since they could possibly influence the conformational behaviour, making the glycosidic linkages more sterically crowded hence comparative steric information can be obtained.

The disaccharide esters synthesized are the peracetylated, perpivaloylated and the permesitoylated derivatives. Peracetylated oligosaccharides have been used extensively
in the structural determination of oligosaccharides [7-13] because they are of interest not only in relation to the conformational data of fully acetylated polymeric derivatives such as cellulose acetate [14], but also because many naturally occurring polysaccharides particularly those of microbial origin are partially acetylated [15]. Although the biological functions of these acetyl groups are not well understood, it is known in some instances, e.g. gellan, their presence greatly affects the rheological properties of aqueous solutions. Similarly, it might be possible that pivaloylated and mesitoylated derivatives have some biological activities.

Several approaches have been developed for the preparation of methylated derivatives. The early methods were those of Purdie [16] and Haworth [17]. The disadvantage of the Purdie and Haworth techniques is that they require several remethylations to obtain complete etherification. Strong bases such as sodium hydride or potassium tert-butoxide in dipolar aprotic organic solvents are now used. Using methyl iodide in N,N-dimethyl formamide with silver oxide [18,19], barium hydroxide [20], or sodium hydride [21] as the basic agent, the reaction rates increased but full methylation was not achieved. The use of sodium hydride has a disadvantage in that it requires careful handling. Corey and co-workers developed a new synthesis using the methyl sulfinyl carbanion, the conjugate base of dimethyl sulfoxide [22]. The Wittig type synthesis was achieved with great ease [23,24]. Hakomori adapted Cory’s methylsulfinyl carbanion [25] reagent to the methylation of even complex carbohydrates. In spite of the low yields (0.3 mol of permethylated derivative per mol of sugar), the Hakomori method has been used extensively in structural investigations of carbohydrates. The use of potassium tert-butoxide [26] instead of sodium hydride improved the stability of the reagent but did not
substantially increase the yield of the permethylated product [27,28]. In 1984 Ciucanu and Kerek [29] reported a simple and rapid method for the permethylation of carbohydrates by using dimethyl sulfoxide, sodium hydroxide and methyl iodide. This approach has the advantage of readily available starting materials, potential compatibility with a wide range of protecting groups, and a very straightforward experimental procedure. Thus, we envisioned this as an ideal method for making the different peralkylated derivatives efficiently, as it avoids the experimental difficulties of the Hakomori method (e.g. low yields). Herein we show that this method works very well for the preparation of protected disaccharides with alkyl groups. Additionally, perethylated, perpropylated, perbutylated and perpentylated glucose-glucose disaccharides have not been reported and can be prepared in good yields with this straightforward chemistry. The advantage of this method is the greater generality of protecting groups that can be used. However for the higher derivatives complete etherification of the disaccharides was not obtained if the alkylating agent was added in its entirety all at once. The procedure worked if it was added in incremented amounts on a daily basis for about a week.

The per-esterified derivatives prepared were the acetylated, pivaloylated and the mestitoylated derivatives. Peracetylated derivatives have been synthesized previously [30]. The hydroxyl groups of a carbohydrate material react readily with acetic anhydride in the presence of a basic catalyst like pyridine. However, the pivaloyl and mesitoyl derivatives of carbohydrates have not been reported and were prepared in good yields. No difficulties were encountered in the synthesis of the acetylated and pivaloylated
derivatives. For the mesitoylated derivatives a large excess of mesitoyl chloride had to be added to ensure complete derivatization of the disaccharides.

One of the most widely used protective groups for alcohols since its introduction by Corey and Venkateswarlu is the tert-butyl-dimethyl silyl (TBDMS) group [31]. The chemical properties of these hindered silyl ethers make them desirable intermediates for a large number of synthetic transformations involving multifunctional compounds. The established procedure for the preparation of TBDMS ethers involves reaction of an alcohol with t-butyl dimethylchlorosilane (1.1eq) in the presence of imidazole (2.2eq) in N,N-dimethylformamide (DMF) at room temperature. However, this procedure was not efficient in completely silylating all the eight hydroxyls of the disaccharides. The tert-butyl-dimethyl silyl (TBDMS) derivatives of disaccharides have been reported for the first time in this dissertation using the procedure of E.J. Corey [32] in which TBDMS triflate is used as the silylating agent.

**Experimental**

The protected disaccharide derivatives were prepared according to methods below. The yields are shown in Table 2.1 and Rf values in Table 2.2. Table 2.3 shows exact mass measurements of the various synthetic derivatized disaccharides. Figure 2.1 is the exact mass measurements of perbutylated maltose which serves as a representative spectrum. Figure 2.2-2.5 are the GC-MS spectra of the 1→4 and 1→6 methylated anomers. Figure 2.6 is the thin layer chromatography of the permethylated derivatives. The thin layer chromatograms of the higher derivatives like the penty1 one however, revealed a number of spots because of the formation of partially derivatized products.
General Conditions for Alkylation

The underivatized disaccharide (5mg, 0.015mmol) was introduced into a dried tube. DMSO (2mL) was added and the solution stirred till it dissolved. Powdered sodium hydroxide (200mg, 5mmol) was added and the suspension was stirred for 10 minutes. Finally methyl iodide (0.21g, 1.4mmol) was added and the solution was stirred at room temperature for 24 hours. The product was extracted with chloroform (3mL), washed several times with distilled water (4x10ml) and dried with sodium sulfate and then at high vacuum to yield a yellowish oil. The same reagents were used for preparation of the higher derivatives from ethyl to pentyl with differences in the proportions of alkyl iodide added. For ethylation 500uL of ethyl iodide was added in 100uL (0.19g, 1.25mmol) aliquots over a period of 5 days after which the products were extracted in chloroform as for methylation. For propylation 600uL of propyl iodide was added in 100uL (0.17g, 1.03mmol) increments over a period of 6 days after which the product was extracted. Butylation was carried out over a period of 7 days in 100μL (0.16g, 0.87mmol) increments. Preparation of the ethyl, propyl and butyl derivatives were all carried out at room temperature. The pentyl derivatives were synthesized by first heating the reaction mixture at 55-60°C for 24 hours and then for 8 days at room temperature. 2ml of pentyl iodide was added to the reaction mixture in aliquots of 200μL (0.3g,1.5mmol) for 9 days, after which the product was extracted with chloroform.

General Conditions for Acetylation

The underivatized disaccharide (10mg,0.02mmol) was introduced in a dried flask. It was dissolved in dry pyridine (1mL). To this acetic anhydride (1.5mL) was added and the reaction mixture was then stirred, heated to 60°C, and stirred for 24 hours. The product
Table 2.1 Product Yields for synthetic derivatized disaccharides

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Table 2.2  Rf Values for 1→4 & 1→6 synthetic derivatized disaccharides

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Table 2.3 Exact mass measurements of $1\rightarrow4$ and $1\rightarrow6$ derivatized disaccharides

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<td>701.485</td>
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<tr>
<td><strong>Perpivaloylated</strong></td>
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Figure 2.1 Exact mass measurement of perbutylated maltose
Figure 2.2 GC-CI spectra of permethylated maltose
Figure 2.3  GC-CI spectra of permethylated cellobiose
Figure 2.4  GC-CI spectra of permethylated isomaltose
Figure 2.5  GC-CI spectra of permethylated gentiobiose
Figure 2.6 Thin layer chromatography of permethylated (a) maltose (b) cellobiose (c) isomaltose (d) gentiobiose (Ethyl Acetate: Hexane:: 3:1)
was then extracted with ethyl acetate (5mL) and washed with water (10mL). It was then washed with 10% HCl (2x10mL) to remove the pyridine. It was further washed with NaHCO₃ (2x10mL) and finally with water (10mL). It is then dried with Na₂SO₄ and under high vacuum.

**General Conditions for Pivaloylation**

The underivatized disaccharide (5mg, 0.015mmol) was introduced into a dry flask. It was dissolved in dry pyridine (230μL). Dimethyl amino pyridine (DMAP) (17.6mg, 0.0049mmol) was added to catalyze the reaction. Finally, trimethylacetyl chloride (0.18g, 1.46mmol) was added to the reaction mixture. The reaction mixture was then stirred at room temperature for two days. The product was then extracted with ethyl acetate (10mL) and washed with water (2x10mL), followed by 10%HCL (2x10mL), then NaHCO₃ (2x10mL) and finally again with water (10mL). The product was an oil which was vacuum rotor evaporated and then dried under high vacuum.

**General Conditions for Mesitoylation**

The underivatized disaccharide (100mg, 0.29mmol) was introduced into a dry, 2-necked flask under Ar. Dry pyridine (10mL) was added and the solution stirred until it dissolved. Dimethyl amino pyridine (350mg, 2.9mmol) was then added followed by trimethylbenzoyl chloride (5.34g, 29.2mmol). The solution was heated to 80°C and stirred for 3 days. The product was then extracted with ethyl acetate (20mL) and washed with water (10mL) followed by 10%HCL (2x10mL), then saturated NaHCO₃ (2x10mL) and finally by water (10mL). The product was then rotor evaporated and finally dried with vacuum to yield a yellowish solid.

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**General Conditions for Silylation**

The underivatized sugar (100mg, 0.29mmol) was introduced in a dry flask under Ar. Dry methylene chloride (10mL) was added and the solution stirred until most of the sugar dissolved. Dry 2,6-lutidine (0.75g, 6.9mmol) was then added followed by tert-butyl-dimethyl triflate (0.93g,3.5mmol). On addition of the triflate the undissolved sugar dissolved completely and the reaction mixture became clear. The reaction mixture was stirred at room temperature for a day. The product was extracted with ether (20mL) and washed with water (20mL). The aqueous layer was then extracted with ether (3x10mL). The product in the combined ether extracts was rotor evaporated and dried under high vacuum to yield a clear oil.

**References**


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Chapter 3

Collision Induced Discrimination of the Glucose 1→4 Disaccharide Anomeric Pair with Incremented Alkyl Substitution

Introduction

Oligosaccharides attached to proteins or lipids participate in a wide spectrum of biological roles determined by their primary structure [1,2]. An increasing number of reports show that carbohydrates exhibit a variety of important biological functions, such as anti-clotting agents, adhesion ligands, immunomodulators, antigenic microbial recognition factors and inflammatory response addressins, among others [1,2]. Rapid methods for detailed characterization of their structures have not been fully developed. The wide variety of positional and anomeric structures makes it possible for saccharides to form as many as $10^{12}$ distinct structures from as few as six different monosaccharide units [3,4]. Therefore a complete description of carbohydrate structures requires singling out a structure from a potentially large heterogeneous set of individual discrete molecular isomers. The problems encountered in their structural elucidation far exceed those encountered with proteins. A complete structural analysis of carbohydrates requires a determination of composition, molecular mass, sugar sequence, branching, molecular mass, ring size, anomeric configuration and interglycosidic linkages. Currently there is no universal method to obtain complete structural information of glycans. Methods that are currently being pursued for oligosaccharide analysis include combinations of enzymatic degradation [5-8], chemical degradation such as acid hydrolysis [9], periodate
oxidation [10-12], reduction and methylation [13-18], chromium trioxide oxidation [19] and nuclear magnetic resonance (nmr) [20-23]. Nmr requires micromole quantities of pure material for complex 2-D experiments. For high sensitivity, mass spectrometry has become an important method for structural characterization of oligosaccharides [24-27]. Tandem mass spectrometry allows the analyte molecule to be selectively analyzed ignoring all other impurities except isobars (at low resolution) of the same mass in the sample. Hence, it can be used in mixture analysis, which is always encountered with real-world biological samples [28]. Mass spectrometry has been used in the determination of monosaccharide type [29-32], sequence [33-37], and linkage position [38-42]. Differentiation of the anomeric configuration of the glycosidic bond has long been the stronghold of nmr spectroscopy, since traditionally mass spectrometry was not considered a technique to differentiate stereoisomers. However, mass spectrometry is emerging as a technique capable of differentiating stereoisomeric compounds with rapid advancements in instrumentation and software [43]. Among other attributes, the anomeric configuration of the glycosidic bond has resisted discernment with rapid and sensitive methods.

**Previous MS work on Differentiation of the Anomeric Configuration of Carbohydrates**

Mass spectrometry was tested as a tool for differentiation of stereoisomeric saccharides as early as the late nineteen fifties when Reed and coworkers [44] reported the results of electron impact appearance potentials on the $\text{C}_6\text{H}_{11}\text{O}_5^+$ ion derived from alpha and beta methyl glucopyranosides and four disaccharides. The alpha glycosides had a slightly lower appearance potential as
compared to the beta glycosides (a sign of a difference in the energy level of the most easily removed electron). Heyns [45] distinguished between a few stereoisomeric permethylated monosaccharides by specific ion ratios, which stimulated Ridder [46] to investigate stereoisomeric trimethylsilyl derivatives of the disaccharides α-lactose, β-lactose, β-cellobiose and the equilibrium mixtures α,β-lactose and α,β-cellobiose which were distinguished from each other by measuring one particular peak intensity ratio for each anomer. The configuration of the anomeric C-atom of the reducing unit was found to be the most important factor. However, no differentiation of the inter-glycosidic bond configuration was made. Chemical ionization has been used by several researchers to attempt differentiation between stereoisomeric saccharides, since EI-MS employs fragmentation conditions that are frequently too drastic to unambiguously discern stereoisomers of many saccharides. Murata [47] discriminated between anomeric TMS derivatives of the per-trimethyl silyl ethers of the monosaccharides D-glucose, D-galactose, and D-mannose using ammonia as the reagent gas. He found that the β anomers gave ions at m/z 558 showing intensities 3-12 times those of the α-D anomers. Discrimination of a few anomeric permethylated disaccharides was attempted by De Jong et al. [48]. Some glycosidic linkage positions could be estimated and in some cases differences were found between spectra of anomers. However, several disaccharides could still not be uniquely identified even by their collision activation spectra. A continuation of these studies [49] of permethylated 1→1, 1→2, 1→3, 1→4, 1→5, 1→6 disaccharides was preferred using chemical ionization (CI) with ammonia and isobutane as
reagent gases in a reversed geometry double focusing mass spectrometer, using helium as the collision gas. Even though there was an improvement in the number of anomeric disaccharides discriminated, a disadvantage of this method is that in some cases experiments with two different reagent gases were needed for discrimination of some disaccharides that could not be discriminated using one reagent gas. The advent of low energy ionization techniques like FAB, ESI and MALDI which are capable of generating ions from the condensed phase, have enhanced the analysis of carbohydrates in general. FAB and ESI in particular have been used for studies on anomeric configuration differentiation.

Positive ion fast atom bombardment (FAB) in conjunction with low energy collision induced dissociation (CID) was first used by Laine et al. [40,41,42] for linkage analysis position in both the reducing and non reducing terminals of linkage isomeric sets of trisaccharides. Domon et al. [50,51] assigned the anomeric configuration of non reducing end glucopyranosyl ions derived from larger oligoglycosides. The study was later extended to anomeric peracetylated glucose-glucose disaccharides [52,53]. In these studies, low energy CID was carried out on precursor ions of peracetylated anomers of 1→2, 1→3, 1→4, 1→5 linkage type disaccharides that were generated by FAB or DCI. The C-1 carbenium precursor ions were used. Both linkage and anomeric configuration differentiation were attempted. A weak distinction was made based on the abundance of the parent and daughter ions for anomeric configuration differentiation. Differences in ion abundance were rather minor and only certain fingerprint ions were chosen to enable the discrimination while ignoring major
daughter ions. In the case of linkage position a small discrimination was made for the 1→2, 1→3, 1→4 positions but not the 1→6. Differentiation of the same anomers was also carried out in the high energy regime [53]. Again a differentiation was possible based on the difference in ratios of two ions without consideration given to the ratios of other ions. Khoo and Dell [54] used fast atom bombardment for assigning the anomeric configurations of pyranose sugars in oligosaccharides. The method is based upon a FAB-MS analysis of deuteroacetylated derivatives before and after oxidation with chromium trioxide which oxidizes the beta pyranosides to keto esters leaving the alpha pyranosides largely intact. FAB was also used to examine the anomeric configuration in a series of 1→3, 1→4 and 1→6 glucosyl-glucose disaccharides through the mass-analyzed, ion kinetic energy spectra of the metastable complexes [55]. Leary et al. [55] reported that the alpha linked disaccharide complexes consistently displayed a 20-30 percent greater kinetic energy release value than the corresponding beta disaccharide complexes. This is reminiscent of the very early work on appearance potentials, however Leary et al. state that it was difficult to correlate the kinetic energy release data (KER) with structural characteristics of the individual Co(acac)_2/disaccharide complexes without detailed information on the geometry of the disaccharide complex with bis acetyl acetonate. Positive ion FAB-MS/MS data of derivatized xylobiosides showed some distinction between alpha and beta configurations of the interglycosidic linkage in the unimolecular decomposition spectra of the [M + H]^+ ions of the peracetylated methyl glycosides [56]. The ratio of m/z 259/475 was used as a gauge to differentiate alpha and beta anomers. The
ion at m/z 259 corresponded to cleavage of the glycosidic bond while m/z 475 corresponded to loss of methanol from the parent ion. The comparison of beta/alpha values of the 259/475 ratios for the 1→2, 1→3 and 1→6 anomers gave 1.43, 2.84, and 3.06 respectively, with the beta anomer having a consistently higher ratio than the corresponding alpha anomer. Fast atom bombardment was also used together with mass-analyzed ion kinetic energy (MIKE) spectra and collision induced dissociation in both the positive and negative ion mode to attempt differentiation of unsaturated and saturated anemic C glycosides [57,58].

Negative ion FAB together with metastable ion and collision activation techniques were used to examine anemic linkages for all glucose-glucose disaccharides [59]. This analysis was made based on the abundance of the monosaccharide ion at m/z 221. Differences between the anomers were small and did not always allow unambiguous identification. Negative ion electrospray was used to determine directly the anemic configuration in a complete series of underivatized glucopyranosyl disaccharides based on “in-source” CID in the nozzle-skimmer region rather than tandem mass spectrometric analysis [60]. The discrimination was based on the ratio of m/z 161/341 monosaccharide/disaccharide at different settings of the nozzle to skimmer voltage difference. In-source CID was also used to differentiate between the anemic configuration of the glycosidic bond in disaccharides and linear oligosaccharides labeled with p-aminobenzoic ethylester chromophore [61]. While little to no discrimination was found for the 1→3 and 1→6 linkage,
anomeric configuration discrimination was made for the 1→4 linkage glucose-glucose disaccharides. More recently, Nilsson et al. [62] reported that both the linkage position and anomeric configuration in glucose containing disaccharide alditols could be estimated by multivariate analysis mass spectrometry. Their results indicated that the axial glycosidic bond (α-D) is more easily cleaved than a corresponding equatorial bond (β-D). Their models were restricted only to glucose containing disaccharides or disaccharides with a non-reducing terminal galactose residue. A theoretical study of deprotonated glucopyranosyl disaccharide fragmentation was carried out in which it was shown that the stereochemistry at the 2-position of the non-reducing ring could have a significant effect on disaccharide fragmentation especially with regard to determination of the anomeric configuration [63].

Differentiation of the stereochemistry of the glycosidic bond by FAB-MS has a number of disadvantages including a relatively poor detection limit and sensitivity, due to poor ionization efficiency and considerable in-source fragmentation. In contrast, electrospray has more efficient ionization, which produces intense molecular ion beams and high sensitivity, as well as lower background chemical noise. Derivatization of carbohydrates by permethylation, peracetylation and reductive amination with an aromatic or an aliphatic amine have been reported to increase mass spectral response. Burlingame [64] and Reinhold [65] have both stated that chemical derivatization was the usual approach adopted in structural analysis of carbohydrates and that it enhanced sensitivity in ESI. We have used electrospray in our work because of its above-
stated advantages to examine the effect of alkyl derivatization on decomposition spectra of a series of peralkylated disaccharides.

In previous FAB-CID-MS/MS studies of linkage position discrimination in a set of linkage isomeric neutral or permethylated trisaccharides it was previously hypothesized that steric factors played an important role in the determination of fragmentation patterns. For sterically crowded linkages there were higher glycosidic bond cleavage rates for some linkages when compared with others under collision activation conditions which was rationalized by molecular modeling [40,41,42]. Later Leary et al. [55] also proposed the importance of steric factors as related to energy differences in an attempt to explain differences in kinetic energy release values between the alpha and beta anomers in a set of metal ion coordinated glucose-glucose disaccharides. Ohashi et al. [66] stated that one of the future challenges would be to obtain steric information by ES-MS, especially in its CID-MS/MS mode which is a focus of our project.

In the present investigation we examine the importance of steric factors by incrementing alkyl substitution. We hypothesize that higher alkyl substitution may increase differentiation not only among linkage positions but in the anomeric configuration of the glycosidic bond. An alkyl series (ranging from methyl to pentyl) of two pairs of glucose-glucose disaccharides maltose\cellobiose and isomaltose\gentiobiose were synthesized as described elsewhere [67]. The goal of the work to be presented in this paper is to differentiate the stereochemistry of the glycosidic bond in these anomeric pairs of disaccharides and to study the effect of
the alkyl groups on collision-induced glycosidic bond cleavage. To the best of our knowledge electrospray in conjunction with tandem mass spectrometry of this extensive set of protonated peralkylated disaccharides used here for anomeric configuration differentiation has not yet been investigated under the low energy regime. This work will demonstrate that differentiation of the alpha (α) and beta (β) anomers of glucose-glucose disaccharides by positive ion electrospray tandem mass spectrometry is possible, depending on the ability of the molecule to rotationally diffuse the energy attained in the collision event. The spectra obtained are strongly dependent on the freedom of rotation of the monosaccharide rings about the glycosidic bond.

Experimental

Materials

The native disaccharides maltose, cellobiose, isomaltose and gentiobiose were purchased from Sigma Chemicals (St. Louis MO). All materials were used as received without further purification. Solutions for ESI-MS were prepared by mixing 100uL of the peralkylated disaccharide of 2mM in methanol with 1-2% trifluoroacetic acid.

High-Resolution Measurements

High resolution nanoelectrospray-MS was performed on all precursor ions prior to low energy CID studies to unambiguously ascertain the molecular ion. Accurate mass measurements were made with an Ion Spec FTICR.
**Instrumentation**

Electrospray ionization mass spectrometry was performed using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass Inc., Manchester, England). The collision cell is an rf only quadrupole and is the reaction region. Collision induced dissociation is carried out in this reaction region between the first and third quadrupole. The sample is introduced via direct infusion at a flow rate of 2-3μL/min. Ions formed in the source are transmitted into the first quadrupole where the precursor ion is mass selected. The precursor ion is then transmitted into the second quadrupole where it undergoes collision induced decomposition by collision with argon target gas which is introduced into this quadrupole. The pressure of the collision gas is at 1x10^{-5} torr which is kept constant throughout the experiment. The threshold laboratory collision energy is 5eV for each derivative, which is then incremented by 4, 5, 6, 7, 8 eV for the methyl, ethyl, propyl, butyl, and pentyl derivatives respectively. Incrementation of the collision energy has been calculated in the center of mass frame of reference based on the following equation

\[ E_{\text{com}}=E_{\text{lab}}[m_t/m_i+m_t] \]

\( E_{\text{com}} \) = collision energy in the center of mass frame

\( E_{\text{lab}} \) = collision energy in the lab frame

\( m_i \) = mass of ion

\( m_t \) = mass of target

The third quadrupole is then scanned for the product ions. The product ion spectra resulted from the signal averaging of 25 scans.
Results and Discussion

Four alpha and beta glucose-glucosyl disaccharides viz. 1→4 linked maltose (α) and cellobiose (β) and 1→6 linked isomaltose (α) and gentiobiose (β) derivatized with alkyl groups (methyl to pentyl) (as shown in figure 3.1) were analyzed by low energy ESI-MS/MS since this regime allows one to probe a subtle variation of ion internal energies. The peralkylated disaccharides preferentially yield intense sodium cationized adducts, which give ions due to both cleavage of the glycosidic bond and sugar ring cleavages and generally require a higher energy to fragment [65,68]. The protonated molecular ion of permethylated oligosaccharides had previously been shown to provide facile fragmentation of the glycosidic bond in studies of sugar sequence and branching [68]. The protonated molecules (MH)+ were generated in high abundance by addition of 2% trifluoroacetic acid after desalting of the compound with a cation exchange column. The on-line reverse phase desalting step adopted by Viseux et al. [68] prior to MS analysis appears to be more time consuming.

Rationale for derivatization of disaccharides with a homologous series of alkyl groups

Derivatization of the saccharide in our study with an increasing size of alkyl substituent provided not only an increase in the steric crowding around the glycosidic bond but a very clear reflection of the importance of steric factors on fragmentation. The steric encumberance on the disaccharides was incremented by simple increase of the carbon chain length, to examine the effects of crowding on glycosidic bond rotation, while keeping constant the number of potential
Figure 3.1 1→4 linked disaccharides (Maltose and Cellobiose) and 1→6 linked disaccharides (Isomaltose and Gentiobiose)

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nucleophylic charge sites. Moreover crowding will restrict rotation of the
disaccharide rings causing differences in the manner in which the molecules in the
homologous series take up collision energy. The molar fraction of cleavage of the
glycosidic bond will show an effect of increasing size of the alkyl groups.

Alkyl substitutions on the disaccharides do not possess nucleophilic sites
for ionization, which could compete with ionization of the glycosidic bond
oxygen. This is unlike the case with acyl groups [33], which possess a carbonyl
group having nucleophilic oxygen that can easily protonate and compete with the
glycosidic bond or ring oxygen for acceptance of a proton.

By increasing the length of the alkyl chain the basicity and the
polarizability of the alkyl groups is increased and stabilization of the charge on
the ether oxygen is enhanced.

Higher sensitivity and an added advantage of directing fragmentation
along a limited number of well-defined pathways is obtained since derivatization
greatly influences the fragmentation process. EI, CI, FAB have been extensively
used for methylated saccharides. [69,70,71]

Identity and Mechanistic Pathway for Ion Formation in the Collision Process

Electrospray tandem mass spectra of methylated maltose and isomaltose at
13eV (Figure, 3.2 –3.5) and 29eV (Figure, 3.6-3.9) are illustrated. In separate
experiments high resolution exact mass measurements were performed for each
derivative to unambiguously determine the molecular formula of the precursor ion
Figure 3.2  Electrospray tandem mass spectra of permethylated maltose at 13eV
Figure 3.3  Electrospray tandem mass spectra of permethylated cellobiose at 13eV
Figure 3.4 Electrospray tandem mass spectra of permethylated isomaltose at 13eV
Figure 3.5  Electrospray tandem mass spectra of permethylated gentiobiose at 13eV
Figure 3.6  Electrospray tandem mass spectra of permethylated maltose at 29eV
Figure 3.7  Electrospray tandem mass spectra of permethylated cellobiose at 29eV
Figure 3.8  Electrospray tandem mass spectra of permethylated isomaltose at 29eV
Figure 3.9  Electrospray tandem mass spectra of permethylated gentiobiose at 29eV
and eliminate the possibility of isobaric ions being present. Figure 3.10 is a representative spectrum of permethylated isomaltose with the exact mass experimentally determined on an Ion Spec FTMS instrument as 455.2640. This value deviates from the theoretical value of 454.2603 by 3.7 millimass units. The ES-CID spectrum of the permethylated derivatives has the [M+H]+ ion at m/z 455. The MS/MS spectrum is characterized by significant fragmentation corresponding to cleavage of the interglycosidic bond. The mechanistic pathway is depicted in Scheme 3.1. According to the Domon and Costello nomenclature [72] this results in the formation of a B type oxonium ion with retention of the glycosidic oxygen atom by the species formed from the reducing end as shown by Pathway a. The B ion loses a second methanol (or higher alcohol) via 1,2-elimination to form a diene, an intense E type ion. The E ion can undergo a further loss of methanol to form the triene at m/z 155, an E' type ion. Loss of the alcohol and charge retention on the sugar ring moiety indicates that its acidity is greater than the corresponding ether. Cole et al. [73] used similar reasoning to explain the ejection of the trienic system C_{16}H_{16} from the sugar moiety. To determine the fragmentation pathway, a daughter ion (Figure 3.11) and parent ion scan (Figure 3.12) was carried out on each product ion of the parent ion at m/z 455 (using in this case methylated maltose as an example). It was established that the ions at m/z 187 and m/z 155 may be formed via a consecutive methanol loss from m/z 219. The other monosaccharide ions of importance are ions at m/z 141 and m/z 111. The ion at m/z 111 is formed by a decomposition of the ion at m/z 187 by elimination of two methoxy groups through an intermediate at m/z141 (Pathway
Figure 3.10  Exact mass measurement spectrum of permethylated maltose
Figure 3.11 Daughter ion scan of permethylated maltose at m/z 187
Figure 3.12 Parent ion scan of permethylated maltose at m/z 187
b). A similar fragmentation pathway was earlier observed by Chizov et al. [74].

For the derivatives higher than methyl viz. ethyl, propyl, butyl, and pentyl the representative ion at m/z 111 (for methyl) could be formed by Pathway b or through an intermediate at m/z 183 or higher depending on the alkyl group by a hydride shift (Pathway c). However the methylated derivatives can follow only mechanism b. The major monosaccharide ion at m/z 141 is formed by decomposition of the ion at m/z 391 as revealed in the product ion spectrum of m/z 391. The ion at m/z 141 is formed by a beta cleavage of the ion at m/z 391 (Pathway d). A similar fragmentation pattern was proposed by Dell et al. [75] in FAB mass spectrometry. Derivatives higher than methyl could follow a similar pathway (Pathway d) or by elimination of the alkyl chain as an alkene (Pathway e) for alkyl groups higher than methyl. The minor ions at m/z 423, 391 and 359 (Figure 3.1) are formed by successive losses of methanol (or the corresponding alcohol in the case of the higher derivatives) which is a continuous pathway as shown by the MS/MS/MS experiment (Pathway f).

**Effect of Increasing Masses of the Ions**

To maintain collision events as isoenergetic one must compensate for the increasing mass of the precursor ions that change from methyl to pentyl derivatives. This is accomplished by holding the collision energy constant in the center of mass frame of reference [76].

In the center of mass frame the maximum energy available for transfer depends on the mass of the projectile ion (m_i) and the target gas (m_t) (eqn 1)

\[ E_{com} = E_{lab} \frac{m_t}{m_i + m_t} \]  

**eqn 1**
which initiates decompositions. $E_{lab}$ is the translational energy of the precursor ion before collision. The collision energy in the center of mass frame of reference ($E_{com}$) represents the maximum amount of kinetic energy available for conversion into internal energy for an analyte ion of mass $m_i$ colliding with a stationary gas atom of mass $m_t$. $E_{lab}$ is incremented with increasing mass of $MH^+$ such that the center-of-mass collision energies remain nearly constant. This allows a direct comparison between cleavage rates for the different alkyl derivatives. Table 3.1 shows a comparison of the center-of-mass energy ($\Delta E_{com}$) which is nearly constant with the incremented collision energy ($\Delta E_{lab}$) for each alkyl derivative. Note that only integer increments of laboratory collision energy are possible on the employed instrument. Compensation for the increase in mass of the target ions is important since only a fraction ($E_{com}$) of $E_{lab}$ is available for internal excitation [77]. The energy in the center of mass frame of reference also decreases with increase in mass of the projectile ion, with a consequent decrease in collision efficiency. Collision with a target gas molecule such as Ar will transfer proportionately more energy to smaller, in comparison with larger, molecules. In this study, anomeric configuration discrimination will be based upon a comparison of the relative rates of competitive and consecutive decompositions. The methylated derivatives would be expected to fragment more completely than the perpentylated derivatives at the same center-of-mass energy on collision with stationary argon. However the glycosidic bond may cleave more easily in the perpentylated derivative as described below. No correction is made for subtle differences in collision cross-section. If the conversion of $E_{lab}$ to $E_{com}$ according to

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Scheme 3.1  Mechanistic pathway of ions formed in the electrospray ionization process (contd.)
(contd.)
(contd.)
equation 1 is not taken into account investigators would be inadvertently observing the incorrect relative effect of collision energy. Special attention was also paid to the adjustment of the of collision gas pressure, such that single or low number collision conditions were maintained in the r.f. only collision hexapole [76]. Maintaining a low constant pressure of \(1 \times 10^{-5}\) torr at zero offset so that the main ion beam was attenuated by 10% was used as the criterion for low number collision conditions [78,79].

**Methodology Used to Differentiate Stereoisomers**

The MS-MS spectra of each anomer derivatized with the same alkyl group and of the same linkage type show the same parent ion and fragment ions at a certain collision energy. Even though the fragment ions are identical, the abundances of the ions differ significantly between the two anomers to an extent that makes it possible to differentiate the stereochemistry of the glycosidic bond in each anomer. Anomeric configuration information is thus obtained through differential ion abundance of the glycosidic fragments, which provides the most promising criteria for usage of the methodology to be described for differentiation of the stereochemistry and rates of cleavage of the glycosidic bond. The MS-MS spectra of decompositions of the \([M+H]^+\) at m/z 455 for the four methylated glucose-glucose disaccharide anomers for the \(1\rightarrow4\) and \(1\rightarrow6\) linkage positions at a collision offset energy of 13eV and 29eV (Fig. 3.2-3.9) serve as representative spectra for the entire disaccharide series. The complete fragmentation pathway of all the ions formed in the spectrum of methylated isomaltose is summarized in Scheme 3.2. This depiction of one possible pathway is deduced by carrying out an
Table 3.1 Comparison of $\Delta E_{\text{com}}$ and $\Delta E_{\text{lab}}$ for disaccharide derivatives

<table>
<thead>
<tr>
<th>R Group</th>
<th>$\Delta E$ Center of Mass</th>
<th>$\Delta E$ Lab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>0.32</td>
<td>4eV</td>
</tr>
<tr>
<td>Ethyl</td>
<td>0.33</td>
<td>5eV</td>
</tr>
<tr>
<td>Propyl</td>
<td>0.33</td>
<td>6eV</td>
</tr>
<tr>
<td>Butyl</td>
<td>0.34</td>
<td>7eV</td>
</tr>
<tr>
<td>Pentyl</td>
<td>0.34</td>
<td>8eV</td>
</tr>
</tbody>
</table>
MS-MS-MS experiment involving a sequential dissociation of the daughter ions (daughter ion scan) in the spectrum as stated above to obtain their individual product ions. To ascertain if the same pathway is followed by the anomer of a different linkage position viz. permethylated maltose, and for a higher derivative viz. perbutylated maltose the product ion spectra of all the daughter ions were obtained, and a parent ion scan on each daughter ion was carried out. The fragmentation pathway for permethylated maltose (Scheme 3.3) was similar to that for permethylated isomaltose except that the formation of m/z 111 from m/z 141 is of minor abundance. In the case of perbutylated maltose (Scheme 3.4) the mechanistic pathway is similar to that of permethylated maltose except for the presence of an enhanced pathway from 239→183. Thus, each alkyl derivative forms homologous sets of ions, with their formation proceeding at different rates through alternative pathways.

The internal energy deposited in an ion and hence the degree of fragmentation is dependent on the collision energy [80]. This is evident from the MS/MS spectra for permethylated isomaltose which is used as a model compound that the collision energy has a dramatic effect upon the character of the spectra because of increased energy deposition, as shown in Fig. 3.8.

In our experiments, the collision energy is varied by incrementing the voltage on a lens before the RF only quadrupole. Plots of the change in collision offset voltage against the ion ratio of the combined intensity of relevant daughter ions divided by the intensity of the parent ion were constructed. Figure 3.13 is an example. Then plots of the ratio of the daughter to parent ion intensity for each
anomer are constructed. To differentiate between alpha and beta anomers, a comparison between the absolute intensities of the product ions related to cleavage of the glycosidic bond is not as useful as taking the daughter to the parent ratio which minimizes effects due to variations in instrumental parameters over time. According to the MS-MS-MS spectra (Scheme 3.2) fragmentation of the glycosidic bond takes place resulting in a cascade of relevant product ions. These product ions are formed through a consecutive pathway of alcohol losses directly related to cleavage of the glycosidic bond. Van der Kerk et al. [56] used the daughter ion ratios to judge the difference between the alpha and beta anomers in a series of peracetylated β-xylobiosides after ascertaining that there were no factors adding to, or subtracting from, the intensity of the glycosidic bond ion other than from the [M+H]⁺ ion. The MS-MS-MS spectra of our compounds revealed pathways that were related to glycosidic bond cleavage. These ions taken together comprise the intensity of the glycosidic ion. An explanation of the daughter ions taken into account and summed up is shown in Scheme 2 using methylated isomaltose as a representative compound. The very minor disaccharide ions at m/z 423, m/z 391, m/z 359 are not considered since they do not involve cleavage of the glycosidic bond. The ion at m/z 423 could form m/z 391 or m/z 219 through two different pathways each with a different rate constant. If the rate constant for the pathway 423 → 391 is much greater, then the formation of the ion at m/z 219 would be negligible and formation of the disaccharide ion would be very facile, giving no information of the glycosidic bond. Not knowing the rate constant for the two pathways leaves some amount of ambiguity,
Scheme 3.2  Fragmentation pathway for permethylated isomaltose
Scheme 3.3  Fragmentation pathway for permethylated maltose
Scheme 3.4  Fragmentation pathway for perbutylated maltose
Figure 3.13 Plot of the collision offset voltage versus daughter/parent ion ratio
therefore warranting non-consideration of the disaccharide ions. The daughter ions summed up are m/z 219, the glycosidic bond ion and monosaccharide ions at m/z 187, m/z 155, m/z 141 and m/z 110. Even though some of the ions are formed by two different pathways and the ion at m/z 187 in turn fragments to form other ions by two pathways (Scheme 3.2) they are considered since they are monosaccharide ions which are formed only after cleavage of the glycosidic bond. Similarly the ion at m/z 141 is a monosaccharide ion formed from the disaccharide ion at m/z 391. The ions obtained after glycosidic bond cleavage are related to it and thus considered to arise only beyond threshold energy for cleavage. In summary the daughter ion value is obtained by summing up the abundance for all the daughter ions formed once the glycosidic bond is cleaved.

Fragmentations taking place a number of steps after cleavage of the glycosidic bond are related to it and information regarding the glycosidic bond could be obtained from such distant fragmentations since they are formed with a range of internal energies because of the excess energy contained in the glycosidic bond ion and are thus influenced by the energy contained in it. It is known that the internal energy acquired by the parent and product ions is related to the structure of the parent ion [81]. Thus it is reasonable to say that a “memory effect” is retained in distant fragment ions which is related to the structure of the disaccharide ion. The ions that are formed after glycosidic bond cleavage could also be formed from precursor ion structures that lead directly to the transition state and thus posses characteristics of their precursors. The relation of the “memory effect” of the fragment ions formed due to glycosidic bond cleavage
with structure can be rationalized as follows: [i] At the temperatures of the mass spectrometer a large number of conformations are possible for each anomer. Differences in the distribution of conformers present would be expected to contribute to differences in the rate of cleavage of the glycosidic bond for the two anomers. [ii] Since we are operating under near single collision conditions it results in fragment ions that are not produced from high energy dissociation pathways, providing information specific to ion structure.

This particular methodology is used to determine the anomerity of the glycosidic bond for the following reasons: It takes into account specific decompositions initiated by a favorable stereochemistry at the glycosidic site thus giving information on stereochemical effects. Incrementing of the collision energy and evaluation of the data within a range of collision energy will enhance the stereochemical differentiation window. This is because it is difficult to predict in advance the optimum collision energy to enable maximum stereochemical differentiation. For a particular alkyl group, both the anomers of a particular linkage isomer have all identical parameters except differences in anomerity.

Relative daughter ion abundances in collision activation spectra are determined by the internal energy acquired by the molecule during activation. We hypothesize that the amount of internal energy acquired by a molecule and thus the propensity for cleavage of the glycosidic bond are linked to its anomerity, in addition to the parameters defined by equation 1. This is because stereoisomers are known to have different bond dissociation energies. Relative ion abundances are known to be a measure of internal energy [82]. It is thus conceivable to say
that the slopes of the daughter to parent ion ratio plots reflect the stability of the glycosidic bond thus giving information on its anomery since the D/P plots reflect the differences in stability between the two anomers. Further the collision offset voltage can be better controlled than the pressure of the collision gas and would be a more effective way of controlling the internal energy imparted to the molecule. Thus the information obtained would be much more reliable.

Incrementation of the collision energy will allow a stepwise increase of internal energy acquired by the molecule, and will thus reflect threshold fragmentation as well as complete decomposition of the molecular ion and the influence of collision energy on fragmentation of the molecule. It will ensure an optimal differentiation of anomers. These plots can be compared to a breakdown curve because of the relationship between the internal energy deposition and collision energy [79]. They contain a lot of information just as breakdown curves do and as such can be expected to be useful in the characterization of isomeric ions because the plots are a function of ion abundance and a parameter related to internal energy.

The dihedral angles phi (\(\phi\)) and psi (\(\psi\)) are known to be important degrees of freedom in disaccharides [83,84]. We hypothesize that the dihedral angles traversed energetically are determined by the freedom of rotation of the molecule about the glycosidic bond. Thus as energy is imparted to a molecule during collision induced dissociation, the more sterically crowded molecule would cleave more readily at the glycosidic bond rather than dissipate the energy in its internal modes, partially compensated by larger derivatives that have a higher number of
oscillators. We expect there to be an enhancement in the rate of cleavage of the glycosidic bond with increase in derivative mass due to hindrance of rotation, since rotation about carbon-carbon single bonds is impeded by torsional energy barriers with increasing size of the substituent attached. These plots would thus be able to reflect the rotational freedom of a molecule and the effect of steric factors on it.

**Anomeric Configuration**

1→4 Linkage Pair

Figures 3.14-3.18 are the plots of the ratio of the daughter to parent ion intensities of the beta anomer to the alpha anomer versus the collision offset voltage of the permethylated to perpentylated derivatives for the 1→4 linkage compounds. Figure 3.19 is a consolidated plot of the 1→4 linkage anomers. From the five plots, it is evident that discrimination between the two stereoisomers is made for all pairs of alkylated derivatives, which increase as the size of the alkyl group increases from methyl to pentyl. This can be attributed to the fact that for each derivative there is a certain degree of steric crowding for a molecule, different for the alpha and beta anomers. This causes the freedom of motion to be different for the two anomers. Low energy collision induced dissociation reflects these differences, as shown in this work, but it is small for the methylated derivative since the differences in the degree of steric crowding between the two anomers is not as pronounced as compared to the other higher derivatives. The conformations of the two anomers might thus range from being most similar in the case of the methylated derivative to being increasingly different as one goes to
Figure 3.14 Anomeric configuration differentiation of 1→4 linked permethylated derivatives

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Figure 3.15 Anomeric configuration differentiation for 1→4 linked perethylated derivatives
Figure 3.16 Anomeric configuration differentiation for 1→4 linked perpropylated derivatives.
Figure 3.17 Anomeric configuration differentiation for 1→4 linked perbutylated derivatives
Figure 3.18 Anomeric configuration differentiation for 1→4 linked perpentylated derivatives
Figure 3.19 Consolidated plot for anomeric configuration differentiation of 1→4 derivatives

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The pentylated derivative. The barriers to internal rotation and thus the conformational freedom greatly depend on the location of substituents in conformational space. Thus, as the size of the alkyl substituent is increased, especially to the largest alkyl group, pentyl, the probability of neighbouring pentyl groups occupying a close conformational space is much higher than for methyl. With each molecule having a unique degree of steric crowding, one of the anomers in the two pentylated stereoisomers will have a much lesser degree of freedom of motion than the other and thus a very different chemical free energy. Low energy collision induced dissociation is able to reflect these differences in free energy thus providing a greater discrimination for the pentylated anomers.

The differences in collisional cross-sections of the particular conformation undergoing cleavage for each of the ions of the permethylated derivative anomers might be very close. Thus the rate constant for dissociation of the glycosidic bond will be very similar, which is indicated by the similar abundances of the daughter ions and a small differentiation between the anomers. In contrast, for the perpentylated derivatives the conformation of the individual anomers undergoing cleavage might possibly have vastly different collision cross-sections allowing one anomer to undergo a more frequent collision with the target gas molecule, and thus a higher rate constant for glycosidic bond cleavage and formation of the related fragment ions.

Another aspect which is evident from the plots is that the beta anomer consistently has a higher daughter to parent ion ratio (D/P) than the corresponding alpha anomer in all the five pairs of the 1→4 linkage isomer. A higher D/P ion
ratio is reflective of the fact that the beta anomer is more readily cleaved than the alpha anomer. We had anticipated that the beta anomer would be more resistant to decomposition than the corresponding alpha anomer since the beta bond is equatorial and should be more stable. Unexpectedly, a reverse trend is observed.

It is thought that the transition state could be governed by the steric crowding of the substituents. Thus, it could be possible that the permethylated cellobiose is more sterically restricted than permethylated maltose, which will be corroborated by descriptions of modeling these derivatives below. Another possible explanation for this unusual phenomena is a raising of the ground ionic state of the beta anomers thus lowering the activation barrier, which in turn enhances the rate for glycosidic bond cleavage of the beta anomer. Thus the beta anomer is kinetically more labile than the corresponding alpha anomer. It is reasonable to consider the activation energy because heterolytic bond dissociation energies are known to correlate well with activation energies as noted by Maccoll et al. [85].

We can also attribute this trend to the anomeric effect (figure 3.20) which is a dipole-dipole repulsion between exocyclic and endocyclic C-O bonds [86]. In a beta isomer there is a dipole from the anomeric carbon to the sugar ring oxygen and to the glycosidic bond oxygen in the same direction. On the other hand in the alpha anomer the dipole from the anomeric carbon to the sugar ring oxygen and to the glycosidic bond are in opposite directions. Thus there must be a greater dipole present in the beta anomer causing it to be at a higher energy than the alpha anomer. Relieving of this excess dipole moment is an energetic consequence
causing the beta isomer to have a higher intrinsic chemical energy and thus fragment more easily in the collision chamber.

According to a second school of thought [87], it has been shown that the anomeric effect is due to stabilizing 2-electron delocalizations (hyperconjugation) from the oxygen lone pairs into adjacent polarized antiperiplanar $\sigma^*\text{CO}$ bonds $n(O)\rightarrow\sigma^*\text{CO}$ interactions. In this conformation the $\sigma^* \text{CO}$ orbital is anti to one of the lone pairs on the ring oxygen making it suitable for sigma electron delocalization. Sigma delocalization of electrons is known to stabilize a molecule and stabilize the charge on the molecule since sigma interactions are analogous to pi interactions as stated by Dewar et al. [88]. Thus the rate of cleavage of the alpha anomer is reduced by through bond interaction. In contrast, the beta anomer has the $\sigma^* \text{CO}$ orbital gauche to the lone pairs on the ring oxygen thus impeding sigma electron delocalization and hence charge stabilization, causing a faster rate of cleavage than the corresponding alpha anomer. Thus stereo-electronic factors in addition to steric crowding of the freedom of motion of the glycosidic linkage, also play a major role in the differences in the rate of cleavage. The anomeric effect is known to have a 1-2 kcal/mol effect in bringing about an energy (enthalpy) difference between the alpha and beta anomers of $-1$ to $-2$ kcal/mol (4kJ/mol) [56]. Such an enthalpy difference is sufficient to bring about a difference in the rate of cleavage of the glycosidic bond allowing the anomeric effect to play a major role in the rates of formation of the glycosidic bond ion. Vanderkerk et al. [56] also attributed rates of formation of the glycosidic bond ion for the alpha and beta anomers of peracetylated xylobiosides (alpha to beta ratio of 2.84) to an enthalpy.
Figure 3.20 The Anomeric effect
Figure 3.21 Consolidated plot for anomeric differentiation of 1→6 derivatives
difference of 4kJ/mol. Thus, the 1→4 daughter ion mass spectra of the 1→4 linkage anomers show ion abundances, which are characteristic of each anomer

1→6 Linkage Pair

The 1→6 derivatives, as shown in Fig. 3.21 which is a consolidated plot of the center of mass collision energy versus the alpha D/P ion ratio/beta D/P ion ratio, exhibit little or no discrimination between the alpha and beta anomers for all the five pairs of derivatives.

The alpha anomer, if anything, has a marginally higher D/P ion ratio than the beta anomer. The 1→6 derivatives have much more rotational freedom of motion around the glycosidic bond than the corresponding 1→4 derivatives. This is validated by Cummings and Carver [89], in which the glycosidic linkages of pyranose rings involving primary hydroxyls as in 1→6 linkages are known to be much more flexible than linkages involving bonds to secondary hydroxyls groups as in 1→4 linkages. In 1→6 linkages there is a three-bond rotation that diminishes inter-residue interactions due to increased distance between the monosaccharide sugar rings. French et al. [90] have demonstrated that both isomaltose and gentiobiose prefer extended orientations in which the two rings are placed trans relative to the O1-C6' bond. Therefore, both the anomers for all the different alkyl derivatives are able to rotationally diffuse the collision energy obtained during the collision event. Even though the steric crowding around the glycosidic bond increases, especially as one goes to the pentyalted derivatives, the conformational freedom of the molecule predominates over the hindrance provided by the steric bulk of the molecule precluding any sort of discrimination in all the 1→6
anomeric pairs. In comparison the 1→4 linkage derivatives are more sterically hindered due to two-bond rotation between the monomeric residues allowing greater inter-residue interactions for the 1→4 derivatives. Further, the orientations of the two anomers towards the colliding molecule might be more similar so that they both receive an equal amount of kinetic energy that is converted to a corresponding equal amount of internal energy. Since the amount of internal energy acquired during a collision is related to structure, as stated before, with the only differences in structure being the anomeric configuration of the glycosidic bond, no discernment of the glycosidic bond configuration is possible for all the 1→6 derivatives. We have stated above that the alpha anomer might possibly have a higher D/P ion ratio than the corresponding beta if there is any discrimination in the 1→6 linkage anomers at all. This could be rationalized by invoking the exo-anomeric phenomenon [91,92] in which the electron density of the lone pair of the exocyclic oxygen atom is transferred to the anti bonding orbital of the endocyclic C-O bond. Essentially this effect is maximized when the p orbital for the unshared pair of electrons is anti-periplanar to the C-1 ring oxygen. There are two conformations for the equatorially substituted anomer in comparison to only one for the axially substituted one that are stabilized by the exo-anomeric effect. This causes it to have a marginally lower D/P ion ratio than the corresponding alpha anomer. Dowd and French [90] have located minima having $\phi$ values between $-18^\circ$ and $-46^\circ$ for isomaltose in conformity with the exo-anomeric effect.
Figure 3.22 Plot of effect of alkyl groups on glycosidic bond cleavage for α-(1→4) linked derivatives
Figure 3.23 Plot of effect of alkyl groups on glycosidic bond cleavage for β-(1→4) linked derivatives
Figure 3.24 Plot of effect of alkyl groups on glycosidic bond cleavage for α-(1→6) linked derivatives.
Figure 3.25 Plot of effect of alkyl groups on glycosidic bond cleavage for β-(1→6) linked derivatives
Effect of the Size of the Alkyl Group on Glycosidic Bond Cleavage

Figures 3.22-3.25 are plots showing the daughter to parent ion ratios versus the collision offset voltage for the methylated to pentylated $1\rightarrow 4$ and $1\rightarrow 6$ linkage anomers. These plots indicate that the stability of the derivatives decreases in the order of methyl $>$ ethyl $>$ propyl $>$ butyl $>$ pentyl. This trend is attributed to a gradation in crowding by alkyl groups with the larger alkyl disaccharide derivatives dissociating faster to relieve additional steric strain and to relieve the extra torsional strain. Substitution by smaller alkyl group can more efficiently dissipate acquired energy by intramolecular vibrational-rotational energy redistribution because the rotational barrier is not as high as the higher derivatives thus the allowable number of rotamers is larger.

Dissociation pathways can be described in terms of coupling of molecular vibrations. When the number of rotamers is large there is a greater number of vibrational states that can couple and dissipate the energy. Even though the number of vibrational modes is increased by an increase in the number of methylenes and they are able to explore more states, the pentylated derivatives are unable to dissipate the internal energy as freely as the methylated derivatives among these excess vibrational modes. This is due to the large hindrance in rotation. Cleavage of the glycosidic bond will therefore be favored because of the predominance of a small number of the total possible conformations of the molecular ion that enables focusing of the available energy on the glycosidic bond. Thus, attaining the transition state will reduce the number of free rotamers because of an enhanced rotational barrier. The normal and bend vibrational modes
and rotational modes connected to these "frozen" rotamers as a result become inaccessible.

Since some of the rotamers are "frozen" for the higher derivatives the transition state leading to the cleavage products must be more ordered with a reduced entropy of activation. In contrast, a smaller derivative like the permethylated one will have a less ordered transition state with a higher entropy of activation due to the higher number of available conformational states. The formation of a conformationally flexible transition state will not lock up the free rotations of the ion which will allow dissipation of the acquired collision energy and thus slow down its rate of cleavage in comparison to the perpentylated derivative. If the difference in the rotational barrier between the permethylated and perpentylated derivatives (presuming they have the same ground state energy) were small enough it could preclude any predominance of kinetic effects, resulting in an enhanced rate of cleavage of the permethylated over the perpentylated derivative. Thus cleavage of the perpentylated derivative is statistically favored.

The permethylated derivatives might also be undergoing an enhanced secondary rotation of the methyl side chain in comparison to the pentyl which further reduces the steric interactions between the methyl side chains during rotation of the glycosidic bond, and enhancing its freedom of motion. An alternative explanation is that the different derivatives might be lying in different ground state conformations as R varies. Also, as one goes to the higher alkyl group derivatives there is an increase in the surface area of the molecule
enhancing the probability of collision with the target. This increased collision cross-section increases the tendency for cleavage of the glycosidic bond.

It is also known that the general trend among alkyl ethers upon protonation is a lengthening of the O-CR bond with R=ethyl to pentyl by 0.14 Å and R=methyl 0.08 Å [93]. This would cause a further increase of intramolecular interaction of alkyl groups steric crowd for the larger derivatives augmenting their rates of glycosidic cleavage. The gas phase basicities and proton affinities of alkyl ethers are known to decrease with a decrease in size of an alkyl group [93]. Hence a longer chain alkyl group which is more polarizable will stabilize the positive charge better by an inductive effect than a methyl group. Thus the protonated methyl ether group would be more easily eliminated than a corresponding pentyl ether group since the electron donation from the sigma bond attached to the ring would less effectively stabilize the protonated methyl ether. Hence the perpentylated derivative would undergo a more facile cleavage at the glycosidic bond and at a faster rate than the corresponding permethylated derivative which would preferentially cleave to form a neutral methanol since the methyl ether is a more likely location of the charge as compared to the glycosidic bond oxygen. This is indicative from the higher abundances of the disaccharide ions formed for the permethylated derivative by 3 consecutive losses of methanol in comparison to the perpentylated derivative.

**Modeling of 1→4 Linkage Anomers Permethylated Maltose and Cellobiose**

Computer modeling studies were undertaken for assessing the conformational freedom of motion around the glycosidic bond for the anomic
pair of permethylated maltose and cellobiose. Chem-X was used to generate phi-psi energy surfaces for 360° rotations in 20° increments about the φ and ψ bonds. 162 starting structures were generated via a rules based search which enabled taking into account a limited number of starting structures. The collision induced dissociation experiments were carried out in vacuum, hence the modeling was carried out without solvation effects.

The modeling studies supported the fragmentation ratios and hence the degree of freedom of motion observed under collision activation conditions in the triple quadrupole mass spectrometer for both the anomers. Figure 3.26 and Figure 3.27 show the three dimensional energy wells of permethylated maltose and cellobiose, which are plots of φ, ψ and energy that are derived from MM3 calculations with a 10kcal mol⁻¹ cut off above the minima. Permethylated cellobiose exhibited a smaller volume of 1.059e⁶ than its corresponding anomer permethylated maltose which had a volume of 1.132e⁶. The exact values of the volume have arbitrary units and are computed by the Surfer program. The volumes of the energy wells correspond to the conformational freedom of motion of the two monosaccharide sugar rings about the glycosidic bond. The freedom of motion is correlated with the ability of the molecule to dissipate the energy obtained in the collision event. Thus permethylated maltose, which has a greater volume, will dissipate the added collision energy amongst its internal modes more easily than Permethylated Cellobiose and thus exhibits a greater stability in the mass spectrometer. However, the values of the volumes are very close to each other, which corroborates with the fragmentation ratios obtained in the mass
spectrometer where a small discrimination is made between the two anomers due to a very similar degree of motional freedom.

Conclusions

Electrospray tandem mass spectrometry is a useful analytical approach for obtaining structural information of derivatized disaccharides. This methodology has the potential of differentiating 1→4 linkage anomeric pairs of disaccharides but not 1→6. Although further studies of model systems will be required before this approach can be routinely used we believe that this methodology will be very useful in understanding the complexities of the structural problems in carbohydrates.

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Figure 3.26 $\phi, \psi$, Energy plot of permethylated maltose
Figure 3.27 $\phi, \psi$, Energy plot of permethylated cellobiose
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Chapter 4

Conformational Analysis of Permethylated Disaccharides Using MM3

Introduction

Mass spectrometry has been used to determine the various aspects of the primary structure of carbohydrates namely sugar sequence, branching pattern, identification of the constituent monosaccharide units, molecular mass, ring size, anomeric configuration and interglycosidic linkages. However, even with the advent of new techniques like fast atom bombardment (FAB) and electrospray (ESI) ionization, obtaining a good sensitivity for underivatized sugars has been a daunting task. This is because of the weak acidity of the hydroxyl protons and lack of surface activity. Derivatization overcomes these problems and allows mass spectrometric identification. Permethylated carbohydrates have been especially useful in the field of mass spectrometry. The first synthesis of a permethylated sugar was reported by Hakomori et al. [1] who adapted Cory's methyl sulfinyl carbanion reagent. Since then several approaches to their synthesis have been reported [2].

Electrospray–CID studies [3] were carried out on a series of peralkylated disaccharides with the intent to discriminate among the anomeric configuration. The present modeling study is a conformational analyses of both the permethylated and native two-bond linked glucose-glucose disaccharides to try to see if we could explain the behavior of the permethylated disaccharides and understand their breakdown in the mass spectrometer by determining their conformational freedom. This was discussed in Chapter 3 with reference to permethylated maltose and cellobiose. A second objective of interest is to see the effect of the methyl groups on the energy surfaces. Of prime interest is to see the effect of suppression of the hydrogen bonds which are often present in the
experimental crystal structures and computer models of native disaccharides. A third objective is to study the characteristics of the energy surfaces for both the permethylated and native disaccharides at one dielectric constant $\varepsilon = 1.5$. A comparison is made between the minimum energy structures for the permethylated derivatives and between the $\phi$-$\psi$ maps for the native disaccharides at $\varepsilon = 1.5$. For each particular native dimer a comparison between the $\phi$-$\psi$ energy surfaces at three different dielectric constants is made.

Results from this study are valuable because the native and permethylated disaccharides are the smallest building blocks of analogous oligo- and polysaccharides. The conformations are described by the degrees of rotation of the monomeric residue about their bonds to the glycosidic oxygen atom as indicated by the value of angles $\phi$ and $\psi$ as shown in Figure 3.1. The torsion angle referring to rotation about the glycosidic C-O bond is denoted by $\phi$, and that for the O-C bond to the aglycon is denoted by $\psi$. The energy for each $\phi,\psi$ combination can be plotted on a grid of $\phi$ and $\psi$ and the resulting $\phi$-$\psi$ plots are called Ramachandran maps [4]. These energies can be calculated in various ways. In this case we have used the molecular mechanics program MM3 developed by Allinger et al. [5]. This is a highly detailed force field which in addition to the classical terms, includes anisotropy of hydrogens, corrections for stereoelectronic effects, cross-term effects like torsion stretch, torsion bend and bend-bend interactions that contribute to the energy of a particular conformer, a Buckingham type potential for non-bonded interactions and explicit terms for hydrogen bonding. MM3 has been used previously with apparent success for many disaccharides [6,7].
Brief Historical Perspective of Disaccharide Modeling

Computerized molecular modeling of disaccharides has been carried out for nearly 35 years [8]. In the mid 1960's Ramachandran and coworkers [9] developed a classic approach for disaccharides. They computed the conformational energy of the disaccharides as a function of $\phi$ and $\psi$ of the interglycosidic bond. In their calculations they treated the atoms as incompressible spheres of a set diameter. In these initial studies, such as one on amylose, the only variables were $\phi$ and $\psi$; the sugar rings were not allowed to change their internal coordinates [10]. The energy value reported included only nonbonding and electrostatic terms. Since then Ramachandran maps have been used extensively for conformational analysis of disaccharides. Rees and Skerrett [11] performed conformational analyses of cellobiose, cellulose and xylan by assuming the ring conformations and C1-O-C4' angle for each pair of residues to be fixed and derivable from known crystal structures. They used three different potential energy functions, as proposed by Liquori and coworkers [12], by Brant and Flory [13] and by Kitaygorodsky [14] to calculate the conformational energies. Subsequently Rao and coworkers [15,16] examined the $\alpha$-linked disaccharides kojibiose, nigerose and maltose and the $\beta$-linked disaccharides sophorose, laminaribiose, and cellobiose. First a contact approach was used in which the energy was either 0 or $\infty$. Then potential energies for generation of the $\phi$-$\psi$ maps were computed by contributions from Van der Waal interactions, hydrogen bonds, and torsional potentials. The maps showed that the sterically allowed region is only 5% of the $\phi$-$\psi$ space indicating limited conformational freedom. Rees and Scott [17] reported similar maps. Later Giacomini [18] carried out semiempirical quantum mechanical calculations using the PCiLO method.
Several disaccharide-specific rigid residue programs like HSEA [19,20] and PFOS [21] have been used for conformational analysis. These programs considered the exo-anomeric effect. Brady and coworkers [22] performed a rigid rotation conformational analysis on the disaccharide maltose that is the repeating unit of amylose using a CHARMM like energy function. That paper also had a relaxed-residue map. Several other rigid rotation maps were published using other energy functions [23-26]. Rigid residue methods have disadvantages in that inappropriately high energies are encountered when traversing from one low-energy region to another. The high energies result because pyranoid rings are not completely rigid. The internal degrees of freedom of motion should be allowed to relax for disaccharide molecules during rotation about the glycosidic bond, lowering the conformational energy [27]. Rigid maps depend on the exact choice of the starting model [21]. Also, important minima on the energy surface are likely to be ignored since energies are high on most of the surface except near the starting shape [28]. This limitation of rigid residues does not exist in computational software developed in the 1980’s for doing conformational analysis. Relaxed residue modeling methods for generation of the φ–ψ energy surfaces include force fields like CHARMM [29], Amber [30], MM2CARB [21], CVFF [31], TRIPOS [32], MM3 [33].

Rasmussen and Melberg [34,35] presented the first relaxed residue conformational analysis of maltose [34] and cellobiose [35]. They found four minima within 5 kcal/mol for maltose and five conformers within 3 kcal/mol for cellobiose using the PEF300 force field program through energy minimization in which all internal degrees of freedom are allowed to relax. The calculations correlated with those carried out by Tvaroska et al. [36,37]. However, not all of φ–ψ space was systematically...
searched and no attempt was made to find the global minimum. In a subsequent paper Rasmussen [38] investigated the conformational flexibility of the β-(1→6) linkage in β-gentiobiose using for the first time a newly optimized set of potential energy functions PEF9IL. The minimized coordinates from one grid point were used as the initial set for the following grid point and no rigid rotations were performed when going from one grid point to another letting the harmonic drag change the glycosidic conformation. Tvaroska and Perez [39] carried out a conformational analysis on maltose and cellobiose using MM2CARB, which demonstrated the variation of internal disaccharide geometry with change of conformation around the glycosidic linkage. Subsequently, Tvaroska and Vaclavik [40] reported the conformational analysis of α,α-trehalose, β,β-trehalose and α,β-trehalose to aid in understanding the anomeric and exo-anomeric effects.

Several papers compare the φ–ψ maps obtained by rigid residue and flexible residue analysis. Brady [22] reports a comparison for sucrose using the CHARMM parameters. French [41] et al. reports a similar comparison for cellobiose using MM2. Tran [42] et al. reports a comparison for maltose using MM2 and MM2CARB energy functions. Dowd et al. [43,44] later carried out more complete flexible residue conformational analysis on the α-linked disaccharides maltose, kojibiose, and nigerose and the β-linked disaccharides sophorose, laminaribiose, and cellobiose using the MM3 force field. The α and β anomers were done separately, and as many as 24 combinations of side group orientations were used in making the starting models. The maps illustrated the importance of the exoanomeric effect and the linkage type in determining the conformational flexibility of the respective disaccharides. In another paper [45] they generated isoenergetic surfaces for the 1→6 dimers isomaltose and gentiobiose. These
anomers showed a greater number of low energy minima than the corresponding two-bond linked structures. Different orientations of the hydroxyl hydrogens were considered for each anomer. In some other papers they generated energy surfaces for the pyranosyl rings of α,α-, α,β and β,β trehalose [46] and analogues by MM3, and for the disaccharides leucrose and turanose [47].

Relaxed potential energy surfaces have been computed for other disaccharides. Tran [48] and Perez [49] have computed them for sucrose, Edge [50] and Perez [51] for α-(1→2) linked mannobiose, Imberty [52] and Carver [53] for α(1→3) linked mannobiose, Jimenez-Barbero [54] for β(1→4) linked mannobiose, Calub [55] for inulobiose using MMP2(85) in which the fructofuranose rings initially had the low-energy $4\beta T$ conformation that was retained except when the linkage conformations created severe inter-residue conflicts, and Waterhouse [56] for levanbiose.

Methods

We used Chem-X and Sybyl to sketch and display structures. Molecular mechanics energy surfaces of the native and derivatized molecules were prepared using MM3-96 [57]. This force field compares favorably with other molecular mechanics force fields [58,59]. The single exception appears to be sucrose, where difficulties with adjacent anomeric centers appear to cause errors in the calculated energy [60,61]. Three different dielectric constants ($\varepsilon$) were used. The default dielectric constant of 1.5 was used to mimick molecules in vacuum (e.g. the mass spectrometer) and 3.5 and 7.5 were used to see the effect of decreased hydrogen bonding strength on the maps. Block Diagonal minimization was applied with energy optimization to the default convergence value (3.6 cal/mol for the native disaccharides). The conformational analysis was carried
out in 20° intervals, with the torsion angles defined as $\phi = O5-C1-O1-C4'$ and $\psi = C5'-C4'-O1-C1$. In all optimizations, only these angles were constrained and all other geometric properties were relaxed. The maps show the lowest energy calculated irrespective of the starting model at each $\phi$, $\psi$ point. Unix shell scripts were written to read in the structures from Chem-X for MM3 minimizations. The $\phi$, $\psi$ maps were plotted with Surfer (Golden software). Contours are plotted out to 10 kcal/mol above the respective global minima. The native and permethylated disaccharides studied are: $\alpha,\alpha$-trehalose, $\beta,\beta$-trehalose, kojibiose, sophorose, nigerose, laminaribiose, maltose, cellobiose.

**Disaccharide Starting Models**

The initial structures were based on glucose fragments provided by Chem-X that had $4C_1$ rings. To decrease the number of structures to be considered, Chem-X supports a rule-based method based on conformational energies. From approximately 500 million potential structure combinations of methyl group orientations, only a small certain number of starting structures were selected based on the Chem-X rules-based search. Conformations are rejected by the rules if the torsion angles are known to cause a high energy. When applying the rules to a systematic search, the calculation time is significantly decreased and a much smaller results file containing only low energy conformations is generated. The time taken for the search is roughly proportional to the number of search variables.

For permethylated (maltose, cellobiose, kojibiose, sophorose, nigerose, laminaribiose) 162 starting structures were found. For permethylated $\alpha,\alpha$-trehalose and
permethylated ββ–trehalose 81 and 243 starting structures were used respectively. All the starting structures were used at each Φ, Ψ point for each disaccharide.

The starting structures for the native disaccharides were obtained in a different manner, because hydroxyl groups are not covered by the Chem-X rules. (i) Orienting the hydroxyl groups on each monosaccharide residue in a clockwise orientation by setting the torsion angles H-O-Cn-Cn+1=180° (c conformer) or (ii) Orienting the hydroxyl groups on each monosaccharide residue in a counterclockwise fashion (r conformer) with H-O-Cn-Cn+1=180° or (iii) The same counterclockwise set except H-O-C2-H=180° (x conformer). Thus 9 different combinations of (i), (ii) and (iii) viz. cc, cr, cx, rc, rr, rx, xc, xr, xx were obtained. The clockwise (c) and reverse clockwise(r) exocyclic orientations of the secondary hydroxyl groups for each glucose ring are chosen because they form a partial network of weak hydrogen bonds around the pyranosyl ring [28] and provides a way to give all the Φ, Ψ conformations the lowest possible calculated energy. For each of the 9 different starting structures and for the intial starting structure with orientations from the database starting structures were then generated using the rules base search that included Φ,Ψ and the primary alcohol groups.

Results and Discussion

Description of Starting Structures

In this section permethylated maltose will represent molecules with 162 starting structures. The default structure that results from using the glucose residues provided by Chem-X has all the methyl groups either (+) or (-) gauche to the methine hydrogens. The methyl groups at the C6 position (Cm-O6-C6-') on both the nonreducing and reducing monomers are in a trans orientation to C5. The O6 is trans to the ring oxygen and gauche
to C₄. The other starting structures generated by the rules based search exhibit a certain degree of similarity to the starting structure. In most of the structures the C₆ chain is either in 180° or 120° orientations to C₅ on both residues of the disaccharide. The methyl groups on the C₁, C₂ or C₃ are either in the eclipsed (0°) or gauche orientation to the respective methine hydrogen on the reducing sugar of the disaccharide. On the nonreducing sugar the methyl groups at the C₂ and C₃ positions are in similar nearly eclipsed orientations while the one on C₄ is at various varying positions for the different starting structures. In an ab initio study of the conformational behavior of α and β glycosidic linkages on axial and equatorial 2-methoxy tetrahydropyranosans as models Tvaroska and Carver et al. [62] state that conformers having the methyl group on C1 in a gauche position to the ring oxygen are preferred over those having it in the trans position due to the exo-anomeric effect. In a previous study [63] of the conformational properties and the gauche effect of the methoxymethyl in hexopyranosides, methylation of the hydroxymethyl group oxygen resulted in the tg conformer being favored. The starting structures generated by Chem-X have the methyl groups in a nearly eclipsed orientation to the methine hydrogen and some of them have the O6 methyl in a tg conformation. Tvaroska and Carver [64] also carried out an ab initio study of the conformational behaviour of aglycon O-C bonds and rotameric distribution in O-methylated axial and equatorial 1→2, 1→3, 1→4- dimethoxy tetrahydropyranosys as models. The 1→4 and 1→3 models had their lowest energy minima when the methyl groups were either + or – gauche to the methine hydrogen atom. The kojibiose model is characterized by two equivalent minima, plus a slightly higher gg minimum, two of which are in the gt and tg orientation at the same energy. Only the (+) gauche model of sophorose had a low
energy. These quantum mechanics results suggest that our starting structures are reasonable.

**Description of Minimum Energy Structures**

The minimum energy structures of the two bond linked permethylated disaccharides as shown in Figure 4.1, exhibit both similarities and differences. Similarities of all the disaccharides are that the torsion angles for the exocyclic methyl groups are gauche to eclipsing the methine hydrogen atom. The primary alcohol (C6) chains on both the residues are trans to the C5 atom. The O6 atoms are both either in gt ot gg orientations. The only exception is permethylated kojibiose where the O6 atom is in a gg orientation on the non-reducing sugar and in a gt orientation on the reducing sugar. The glycosidic angles are approximately the same in all the axial-equatorial reducing sugars kojibiose, nigerose and maltose at 116-117° and in the diequatorial reducing sugars sophorose, laminaribiose and cellobiose at 114°. The difference between the axial-equatorial versus diequatorial is that the glycosidic angle has opened up for the axial-equatorial by approximately 2-3° to prevent the C6 group from colliding with the second residue. Thus the steric strain of the axial linkages causes the bond angle to open up. The non-reducing disaccharides α,α–trehalose and β,β–trehalose have similar glycosidic angles of 113.8° and 114° respectively. The global minima for each of the permethylated disaccharide molecules are located at the φ,ψ values shown in Table 4.1.

**Characteristics of Maps with Different Linkage Configurations**

MM3 energy surfaces at ε=1.5 for the 8 disaccharides are presented in figures. 4.2-4.9 and Table 4.2 gives the positions of the global minima on each surface along with their flexibility values. We define the flexibility over the Ramachandran surface as
Figure 4.1 Minimum energy structures of permethylated disaccharides (a) α, α-trehalose (b) β,β-trehalose (c) kojibiose (d) sophorose

(contd.)
(e) nigerose (f) laminaribiose (g) maltose (h) cellobiose
the probability volume in degrees squared, with the probability calculated by a Boltzman relationship, \( p_i = e^{-\Delta E/RT} \). Therein, \( \Delta E \) is the electronic energy relative to the global minimum determined by quantum mechanics, \( R \) is the universal gas constant, and \( T \) is 298 K. This probability volume is related to the partition function, a summation of the probability values over the surface. A completely flexible molecule would have a relative energy of 0 kcal (and \( p_i = 1 \)) everywhere on the surface. On a grid with a 1° spacing there are \( 360 \times 360 = 129,600 \) points. A completely rigid molecule would have a \( p_i \) of 1 at only one point and a value of zero at all others. Thus the maximum possible value of the partition function would be 129,600, and the minimum on that basis would be 1. Similarly, the maximum and minimum probability volumes would be 129,600 deg x deg and 1 deg x deg respectively. Numerical values of the dimensionless partition function were essentially the same as the probability volume for the analog surfaces when using a 1° grid spacing and the Trapezoidal rule, Simpson’s rule or Simpson’s 3/8 rule. At larger grid increments, the probability volume represents a reasonable fine quadrature approach to the partition function calculated for a 1° grid, but values of the partition function for other grid increments were, of course, quite dependent on the number of grid points in the summation.

The main energy wells for the symmetric non-reducing disaccharides \( \alpha,\alpha \)-trehalose and \( \beta,\beta \)-trehalose differ in character from the reducing disaccharides: kojibiose, nigerose, maltose, sophorose, laminaribiose and cellobiose. The non-reducing disaccharides have a single low-energy region with different positions. The different positions result from the different order of atoms seen during counterclockwise rotation. While the reducing disaccharides have elongated regions in the \( \psi \) direction within the 1 kcal contour, the
alpha and beta disaccharides are similar in the region of the global minimum. The beta disaccharides viz. sophorose, laminaribiose and cellobiose have an alternate secondary minimum at $\phi=60^\circ$ while the alpha anomers do not. The flexibility values Table 4.2 show in comparison to the other molecules that $\alpha,\alpha$-trehalose has the smallest flexibility value and is the most sterically hindered molecule while the least sterically hindered anomers are the diequatorial disaccharides of sophorose, laminaribiose and cellobiose. The restricted flexibility arises from the fact that there are two anomic centers in $\alpha,\alpha$-trehalose and from the extra stabilization given to the minimum energy form due to the exo-anomeric effect. An unusual result is that $\beta,\beta$-trehalose is more flexible than maltose and nigerose that have one anomic center and are axial equatorial linkages. The flexibility values indicate that the alpha–equatorial reducing disaccharides are less flexible than the corresponding diequatorial reducing disaccharides. This is confirmed by the 1 kcal contours that are smaller for the axial-equatorial disaccharides than the corresponding diequatorial disaccharides.

Comparison of Maps with Different Dielectric Constants

The $\phi-\psi$ maps at $\varepsilon=1.5, 3.5, and 7.5$ are shown in figures 4.2-4.25. In the case of $\alpha,\alpha$-trehalose the maps look very similar at all the three dielectric constants. The maps are symmetric since $\alpha,\alpha$-trehalose has rotational symmetry. There is no shift in the position of the global minimum as the dielectric constant is increased for either $\alpha,\alpha$- or $\beta,\beta$-trehalose. The energy surface becomes flatter as one goes to higher $\varepsilon$ for $\alpha,\alpha$-trehalose while for $\beta,\beta$-trehalose the energy surface becomes steeper. For the $1 \rightarrow 2$ and $1 \rightarrow 3$ linked disaccharides both the axial-equatorial disaccharides kojibiose and nigerose have their low energy regions lying in the same trough.
Table 4.1 Energy minima for MM3-generated relaxed residue analysis of permethylated disaccharides

<table>
<thead>
<tr>
<th>Disaccharide (Permethylated)</th>
<th>Conformer</th>
<th>Location of Minimum $\phi$</th>
<th>Location of Minimum $\psi$</th>
<th>Flexibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha,\alpha$-trehalose</td>
<td>A</td>
<td>79.71</td>
<td>79.52</td>
<td>468.9</td>
</tr>
<tr>
<td>$\beta,\beta$-trehalose</td>
<td>A</td>
<td>-80.37</td>
<td>-80.21</td>
<td>712.43</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>67.69</td>
<td>-77.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-82.77</td>
<td>62.64</td>
<td></td>
</tr>
<tr>
<td>Kojibiose</td>
<td>A</td>
<td>71.29</td>
<td>-164.98</td>
<td>818.96</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>108.64</td>
<td>-73.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>72.72</td>
<td>83.66</td>
<td></td>
</tr>
<tr>
<td>Sophorose</td>
<td>A</td>
<td>-70.57</td>
<td>-95.17</td>
<td>1398.09</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-66.11</td>
<td>86.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>57.93</td>
<td>-118.34</td>
<td></td>
</tr>
<tr>
<td>Nigerose</td>
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<td>76.17</td>
<td>104.86</td>
<td>1242.97</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100.28</td>
<td>-66.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>115.2</td>
<td>162.30</td>
<td></td>
</tr>
<tr>
<td>Laminaribose</td>
<td>A</td>
<td>-80.07</td>
<td>142.05</td>
<td>815.55</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<td>92.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-86.84</td>
<td>-65.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>80.24</td>
<td>-161.05</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>A</td>
<td>66.81</td>
<td>-166.13</td>
<td>870.04</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>91.45</td>
<td>70.81</td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>A</td>
<td>-77.07</td>
<td>-106.34</td>
<td>1328.455</td>
</tr>
<tr>
<td></td>
<td>B</td>
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</tr>
<tr>
<td></td>
<td>C</td>
<td>62.04</td>
<td>-115.35</td>
<td></td>
</tr>
</tbody>
</table>
at $\phi = 80^\circ$ and $93^\circ$ respectively. They otherwise exhibit different characteristics. In kojiibiose the regions around the global minima (< 5 kcal) look very similar for the $\phi, \psi$ maps at $\varepsilon = 1.5$ and 7.5, while the three nigerose maps have little similarity at any of the 3 dielectric constants. In kojiibiose at all three dielectric constants the energy increases gradually to 3 kcal and then steeply to 10 kcal. However in nigerose the contours become steeper as one goes from $\varepsilon = 1.5$ to 3.5 but remain the same at 7.5. In the case of the diequatorial linkages sophorose and laminaribiose the 1 kcal contour is longer on the $\varepsilon = 7.5$ surface than on the $\varepsilon = 1.5$ surface. There is no shift in the position of the global minima as the dielectric constant is increased but the position of the secondary minimum at $\phi = 80^\circ$ and $\psi = -75^\circ$ is shifted to the left in laminaribiose. In the case of nigerose, however the global minimum is shifted.

In the case of the 1→4 linked disaccharides both native maltose and cellobiose the area surrounded by the 1 kcal contour increases as the dielectric constant is increased from 1.5 to 3.5 to 7.5. However for cellobiose it remains the same from $\varepsilon = 3.5$ to 7.5 and the $\phi, \psi$ maps at $\varepsilon = 3.5$ and 7.5 look very similar. The energy increases more gradually as one goes from the lowest to highest dielectric constant for both the molecules. At dielectric constant 1.5 the 10 kcal contour around the global minimum is separate from the 10 kcal contour around the secondary minimum. However as one increases the dielectric constant the contours join forming a trough that encloses the saddle point. There is no shift in the position of the secondary minimum as the dielectric constant is increased for both maltose and cellobiose. There is a slight shift in the position of the global minimum for maltose and no shift in the case of cellobiose as $\varepsilon$ is increased.
Characteristics of the Individual Native Disaccharides

The discussion of the individual native disaccharides pertains to those at ε=1.5. The disaccharides at ε=3.5 and 7.5 will not be discussed but the energy surfaces will be given as supplementary material.

α,α trehalose

The map of α,α trehalose is shown in Fig. 4.2. Their conformational features along with their flexibility values are listed in Table 4.2. This molecule has chemical symmetry and thus structures drawn on one side of the diagonal line on which ϕ=ψ could be plotted on the other side. The global minimum (A) is located at ϕ,ψ= 79.9°,79.8°. Each residue is stabilized by a crown of intra-residue hydrogen bonds. The global minimum is a single round well in ϕ,ψ space. The map indicates that this molecule is the most sterically hindered among all the 2-acetal-bond linked disaccharides. The MM3-generated map of α,α-trehalose was reported previously [45]. Similarities between both the maps can be found. The only difference between the maps is that the present maps are created with a greater variety of starting structures.

β,β-trehalose

The relaxed residue map of ββ-trehalose generated by MM3 is shown in Figure 4.3 and the location of the low energy conformers are in Table 4.2. The appearance of this map differs considerably from that of α,α—trehalose. In addition the ϕ,ψ map indicates that it is more flexible than α,α—trehalose. There are three low energy regions. They are contained in one single region extending vertically across the map in the ψ direction. The two secondary minima at ϕ,ψ = -81.9 ° , 53.2 ° and −120.6 ° , 62.2° are symmetry related. The global energy minimum is at ϕ,ψ = -80.3 °, -79.5 ° and is at the
center of symmetry. It is stabilized by intramolecular hydrogen bonds on both residues and by an inter residue hydrogen bond from O2-O6'. The two other minima which are at a substantially higher energy are at $\phi, \psi = -81.9^\circ, 53.2^\circ$ that has the hydroxyl groups in a near-eclipsed orientation and $\phi, \psi = -120.6^\circ, 62.2^\circ$ that is stabilized by an inter-residue bond at O2-O2'.

**Kojibiose**

The MM3-generated relaxed residue map of kojibiose is shown in Figure 4.4 and the optimized minima are listed in Table 4.2. A deep trough of low energy conformations running along the $\psi$ direction is present. The global minimum is located at $\phi, \psi = 101.3^\circ, -162.18^\circ$ which is a near eclipsed orientation. It is stabilized by an inter-residue hydrogen bond between O2 and O1'. The second minimum is at $\phi, \psi = 94.09^\circ, -76.94^\circ$. There is a small energy difference of <1 kcal that exists between these two conformers. The third minimum is at $\phi, \psi = 81.72^\circ, 58.04^\circ$. It is < 2 kcal higher in energy than the global minimum. It is stabilized by an inter residue hydrogen bond between O2-O3'. For all the three minima the $\phi$ angle is centered around 80°.

**Sophorose**

The MM3 $\phi, \psi$ map of sophorose is shown in Figure 4.5 with isoenergy contour lines at 1 kcal/mol increments where four minima are denoted and represented in Table 4.2. There are two intersecting troughs containing the low energy regions with some of the energy barriers between the low energy regions larger than 8 kcal/mol. The lowest energy minimum lies within the 1 kcal contour that extends in the $\psi$ direction from $-180^\circ$ to $-120^\circ$ at $\phi, \psi = -84^\circ, -172.4^\circ$. It has an inter residue hydrogen bond between O2-O3'. The other two secondary minima lie within the same trough defined by the 5 kcal contour that
extends in the $\psi$ direction from $20^\circ$ to $80^\circ$. The two minima are found at $\phi, \psi = -118.861.5^\circ$ that is stabilized by an interresidue bond between O2-O1' and $\phi, \psi = -82^\circ, 42^\circ$, that is in a staggered orientation one on each side of the gauche orientation at $\psi = 60^\circ$. The energy difference between the two minima is very small. The fourth minimum is in the intersecting trough at $\phi, \psi = 66.9^\circ, -110^\circ$ stabilized by an interresidue hydrogen bond between O2-O1'.

**Nigerose**

The map of nigerose is shown in Figure 4.6 and the optimized minima are listed and represented in Table 4.2. There are two low-energy regions, both of which are contained in a single low-energy trough extending vertically in the $\psi$ direction. The global minimum is at $\phi, \psi = 83.7^\circ, 157.8^\circ$ and is stabilized by an inter residue bond between O2 and O4' and the secondary minimum is at $\phi, \psi = 117.7^\circ, -59.2^\circ$ with an intramolecular hydrogen bond that could be formed between O1' and O2'. A plateau is found at $\phi, \psi = 60^\circ, 60^\circ$.

**Laminaribiose**

The relaxed residue map of laminaribiose as generated by MM3 is shown in Figure 4.7 along with the representation and location of the minima in Table 4.2. Two low energy minima A and B are located parallel to the $\psi$ axis along the same line and centered around $\phi = -80^\circ$. The global minimum is elongated and is located at $\phi, \psi = -83.7^\circ, 82.4^\circ$. It is stabilized by a crown of both intra residue and inter residue hydrogen bonds. The secondary minimum is <3 kcal higher in energy than the global minimum and is located at $\phi, \psi = -83^\circ, -78.3^\circ$ that is stabilized by an inter residue bond between O2 and O2'. A second lowest minimum (<1 kcal/mol) is separated from the other two minima in
another trough at $\phi, \psi = 66.3^\circ, 132.9^\circ$. It is stabilized by an interresidue bond between O2 and O4'.

**Maltose**

The MM3-generated relaxed residue map of maltose is shown in Figure 4.8 along with the details and representations of the low energy conformations. There are two low energy minima that are in separate energy wells. The global minimum is located at $\phi, \psi = 107^\circ, -140^\circ$ and is stabilized by an interresidue hydrogen bond between O3'-O2. The secondary minimum is located at $\phi, \psi = 87.2^\circ, 76.3^\circ$. It is stabilized by an interresidue hydrogen bond between O3'-O5.

**Cellobiose**

The relaxed residue $\phi, \psi$ map of cellobiose is shown in Figure 4.9 and the exact location and representation of each minimum is shown in Table 4.2. The energy surface has three low energy minima with the global and one secondary minimum located along the same line parallel to the $\psi$ axis between $\phi = -60^\circ$ and $-180^\circ$. The global minimum is at $\phi, \psi = -82.3^\circ, -138.7^\circ$ and is stabilized by an interresidue bond between O3-O5' and the secondary minimum (B) is located at $\phi, \psi = -82.3^\circ, 57^\circ$. A third minimum ($< 2 \text{kcal}$) is located in a separate well at $\phi, \psi = 63.9^\circ, -122.1^\circ$ and is stabilized by the O3-O2 hydrogen bond.

**Individual Maps for Permethylated Disaccharides**

**Permethylated $\alpha, \alpha$-Trehalose**

The MM3 generated flexible residue map of permethylated $\alpha\alpha$-trehalose is shown in Figure 4.26. The $\phi, \psi$ map is very similar to the map of the native molecule at $\varepsilon = 7.5$ because in the permethylated derivative there is no hydrogen bonding and at $\varepsilon = 7.5$ there
Table 4.2  Energy minima for MM3-generated relaxed residue analysis of 2 bond linked nonreducing and reducing disaccharides.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Conformer</th>
<th>Location of minimum</th>
<th>Flexibility</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>$\phi$</td>
<td>$\psi$</td>
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</tr>
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<td>$\alpha,\alpha$-trehalose</td>
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<td>B</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
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<td>62.29</td>
<td></td>
</tr>
<tr>
<td>$\beta,\beta$-trehalose</td>
<td>A</td>
<td>-80.35</td>
<td>-79.50</td>
<td>934.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-81.92</td>
<td>-53.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
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<td>62.29</td>
<td></td>
</tr>
<tr>
<td>Kojibiose</td>
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<td>101.34</td>
<td>-162.18</td>
<td>1153.6</td>
</tr>
<tr>
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<td>B</td>
<td>94.09</td>
<td>-76.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>81.72</td>
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<td>Sophorose</td>
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<td>C</td>
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</tr>
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<td></td>
<td>D</td>
<td>66.99</td>
<td>-110.07</td>
<td></td>
</tr>
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<td>Nigerose</td>
<td>A</td>
<td>83.65</td>
<td>157.81</td>
<td>845.2</td>
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<td></td>
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<td>83.65</td>
<td>-59.29</td>
<td></td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>A</td>
<td>-83.72</td>
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</tr>
<tr>
<td></td>
<td>B</td>
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<td>C</td>
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<tr>
<td>Maltose</td>
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<tr>
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<td>B</td>
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<tr>
<td>Cellobiose</td>
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<td>63.9</td>
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<td>C</td>
<td>-82.3</td>
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</tr>
</tbody>
</table>
Figure 4.2 MM3 $\phi-\psi$ map at $\epsilon = 1.5$ for $\alpha,\alpha$-trehalose
Figure 4.3 MM3 $\phi-\psi$ map at $\epsilon = 1.5$ for $\beta,\beta$-trehalose
Figure 4.4 MM3 $\phi-\psi$ map at $\varepsilon = 1.5$ for kojibiose
Figure 4.5  MM3 $\phi$–$\psi$ map at $\varepsilon = 1.5$ for sophorose
Figure 4.6 MM3 $\phi-\psi$ map at $\epsilon = 1.5$ for nigerose
Figure 4.7 MM3 $\phi-\psi$ map at $\varepsilon = 1.5$ for laminaribiose
Figure 4.8 MM3 $\phi$–$\psi$ map at $e = 1.5$ for maltose
Figure 4.9 MM3 $\phi-\psi$ map at $\varepsilon = 1.5$ for cellobiose
Figure 4.10 MM3 $\phi-\psi$ map at $\varepsilon = 3.5$ for $\alpha,\alpha$-trehalose
Figure 4.11 MM3 $\phi$–$\psi$ map at $\varepsilon = 3.5$ for $\beta,\beta$-trehalose
Figure 4.12 MM3 $\phi$-$\psi$ map at $\varepsilon = 3.5$ for kojibiose
Figure 4.13 MM3 $\phi-\psi$ map at $\varepsilon = 3.5$ for sophorose
Figure 4.14 MM3 φ−ψ map at ζ = 3.5 for nigerose
Figure 4.15 MM3 $\phi$-$\psi$ map at $\epsilon = 3.5$ for laminaribiose.
Figure 4.16 MM3 $\phi$–$\psi$ map at $\varepsilon = 3.5$ for maltose
Figure 4.17 MM3 φ-ψ map at ε = 3.5 for cellobiose
Figure 4.18 MM3 $\phi-\psi$ map at $\varepsilon = 7.5$ for $\alpha\alpha$-trehalose
Figure 4.19 MM3 $\phi$-$\psi$ map at $\varepsilon = 7.5$ for $\beta\beta$-trehalose
Figure 4.20 MM3 $\phi$-$\psi$ map at $\varepsilon = 7.5$ for kojibiose
Figure 4.21 MM3 $\phi$--$\psi$ map at $\varepsilon = 7.5$ for sophorose
Figure 4.22 MM3 $\phi-\psi$ map at $\varepsilon = 7.5$ for nigerose
Figure 4.23 MM3 $\phi-\psi$ map at $\varepsilon = 7.5$ for laminaribiose
Figure 4.24 MM3 $\phi$-$\psi$ map at $\varepsilon = 7.5$ for maltose
Figure 4.25 MM3 $\phi-\psi$ map at $\varepsilon = 7.5$ for cellobiose

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is very little strength accorded to hydrogen bonding. Like the native molecule there is only one low energy minimum at a $\phi,\psi = 79.7^\circ, 79.5^\circ$. It is different from maltose and cellobiose which have very viable secondary minima. The energy surface is symmetrical about the diagonal where $\phi=\psi$. This is the most sterically restricted of all the 2-acetal-bond linked glucose-glucose disaccharides.

**Permethylated $\beta,\beta$-trehalose**

The relaxed residue map of permethylated $\beta,\beta$-trehalose is shown in figure 4.27. Like for the native molecule there are three low energy regions, with one of the secondary minima lying in an intersecting trough. The 1 kcal contour is much smaller than the corresponding contour at $\varepsilon=1.5$ because of the inability of the permethylated disaccharide to form hydrogen bonds in contrast to the native molecule. The global minimum is at $\phi,\psi = -80.3^\circ, -80.2^\circ$. The second minimum lies at a higher energy within the same trough as the global minimum at $\phi,\psi = -82.8, 62.6$. The third minimum lies in the intersecting trough at $\phi,\psi = 67.7^\circ, -77.1^\circ$.

For the reducing disaccharides shown in figures 4.28-4.33 the general trend is that they all have three low energy regions with the exception of maltose that has two low energy regions and laminaribiose has four low energy regions. The axial-equatorial disaccharides kojibiose, nigerose and maltose have their low energy regions lying in the same trough. In the case of kojibiose all the three low energy regions lie along the same line in the $\psi$ direction while for nigerose and maltose they do not. The diequatorial disaccharides sophorose, laminaribiose and cellobiose also exhibit a similar trend. Sophorose and cellobiose have two of the three low energy minima lying in the same trough with a third minimum in an intersecting trough. Laminaribiose has three of the
four low energy minima lying in the same trough with the fourth minimum lying in an intersecting trough.

**Difference Maps**

To see the effect of the methyl groups on the energy surfaces a difference in the zeroed out map for the permethylated derivative and the zeroed out map for the native disaccharide is taken. The solid lines in the map show where the permethylated derivative had a higher relative energy. The dashed lines show where the native disaccharides had a higher relative energy than the permethylated derivatives. At zero energy the permethylated and the native disaccharides have the same relative energy, indicating that the minimum energy rotamers have excess space for the methyl groups.

**α,α-trehalose**

The Surfer generated difference map is shown in figure 4.34. In the $\phi,\psi$ difference map of $\alpha,\alpha$-trehalose the region of interest is $\phi = 20^\circ$ to $180^\circ$ and $\psi = 0^\circ$ to $180^\circ$ because the global minimum was situated in this region in both the permethylated and native $\phi,\psi$ map. The $\phi,\psi$ difference map shows that all the differences in energy are positive hence addition of the methyl groups restricts the freedom of motion of the molecule.

**β,β-trehalose**

The Surfer generated difference map is shown in figure 4.35. The global minimum is at the same place as the derivatized and native molecule. The difference in energy between the permethylated and native molecule is essentially positive except in the high energy region. This indicates that the methylated derivative is more sterically
hindered than the corresponding native molecule. Native and permethylated α,α trehalose is however more restricted than native and permethylated β,β-trehalose.

Kojibiose

The Surfer generated difference map is shown in figure 4.36. The region of interest is between $\phi = 80^\circ$ to $180^\circ$ and $\psi = -140^\circ$ to $-180^\circ$. This difference plot shows more positive values of relative energy which shows that addition of methyl groups makes the molecule more sterically restricted. Further, the position of the global minimum is not shifted upon methylating the molecule.

Sophorose

The Surfer generated difference map is shown in figure 4.37. The region of interest is between $\phi = 0^\circ$ to $-180^\circ$ and $\psi = 0^\circ$ to $-180^\circ$ since the global minimum for both permethylated and native sophorose lie in this region. As for kojibiose there are more regions of positive difference than negative indicating that methylating the molecule has a restrictive effect on the freedom of motion of the molecule.

Nigerose

The Surfer generated difference map is shown in figure 4.38. There are more regions where native nigerose has a higher relative energy than the corresponding methylated derivative. Thus methylation makes the molecule more flexible.

Laminaribiose

The Surfer generated difference map is shown in figure 4.39. The $\phi, \psi$ difference map has a larger region of positive relative energies indicating that methylation decreases the conformational freedom of the molecule.
**Maltose**

The Surfer generated difference map is shown in figure 4.40. The \( \phi,\psi \) map clearly indicated a shift in the global minimum due to methylation by 40° in \( \phi \). The global minimum for native maltose is at \( \phi,\psi = 107°, -140° \). At that point the permethylated molecule has a relative energy of 2 kcal/mol. The global minimum for permethylated maltose is at \( \phi,\psi = 66.8°, -166.1° \). At this point the native molecule has a relative energy of 6 kcal/mol as indicated in the difference map. Hence the region of interest is \( \phi = 0° \) to 180° and \( \psi = -100° \) to -180°. The difference map has a majority of negative values of energy which indicates that native maltose has a higher energy than the corresponding methylated derivative. Thus methylation increases the flexibility of the molecule.

**Cellobiose**

The Surfer generated difference map is shown in figure 4.41. The region of interest is between \( \phi = -40° \) to -180° and \( \psi = -60° \) to -180° since the global minima of permethylated and native cellobiose are present in this region. The \( \phi,\psi \) space in this difference map like that for maltose has a greater number of negative values for the energy represented by the dashed lines which indicates that native cellobiose has a higher flexibility than permethylated cellobiose. Thus addition of methyl groups has little to no effect on the energy surface. There are few regions where permethylated cellobiose has a higher relative energy.

**Conclusions**

MM3 generated relaxed residue \( \phi,\psi \) maps of the nonreducing and reducing two bond linked glucose-glucose disaccharides were computed except for \( \alpha,\beta \)-trehalose.
Figure 4.26 MM3 φ-ψ map for permethylated αα-trehalose
Figure 4.27 MM3 φ–ψ map for permethylated ββ-trehalose
Figure 4.28 MM3 $\phi$–$\psi$ map for permethylated kojibiose
Figure 4.29 MM3 $\phi$–$\psi$ map for permethylated sophorose

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Figure 4.30  MM3 $\phi-\psi$ map for permethylated nigerose
Figure 4.31 MM3 $\phi$–$\psi$ map for permethylated laminaribiose
Figure 4.32 MM3 $\phi-\psi$ map for permethylated maltose
Figure 4.33 MM3 $\phi-\psi$ map for permethylated cellobiose
Figure 4.34 Difference maps for $\alpha,\alpha$-trehalose
Figure 4.35 Difference maps for β,β-trehalose
Figure 4.36 Difference maps for kojibiose
Figure 4.37 Difference maps for sophorose
Figure 4.38 Difference maps for nigerose
Figure 4.39 Difference maps for laminaribiose
Figure 4.40 Difference maps for maltose
Figure 4.41 Difference maps for cellobiose
Addition of methyl groups for \( \alpha,\alpha \) and \( \beta,\beta \)-trehalose, kojibiose and sophorose makes the molecule more conformationally restricted. In contrast permethylation does not have much of an effect on the energy surfaces of nigerose, laminaribiose, maltose and cellobiose. However methylation causes a shift in the position of the global minimum for both the \( 1 \rightarrow 3 \) and \( 1 \rightarrow 4 \) linked disaccharides. The flexibility values for the various disaccharides calculated indicate that diequatorial linkages have a greater flexibility than the corresponding axial-equatorial linkages.

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Chapter 5
Conclusions and Future Research

Conclusions

The research presented in this dissertation can be divided into three major headings. The first goal was to synthesize the derivatized anomeric pairs of linkage isomeric disaccharides to be used in the mass spectrometry studies. The second goal was to use electrospray ionization in conjunction with a triple quadrupole mass spectrometer to develop a method for anomeric discrimination of the glycosidic bond and to study the effects of the added functional groups on glycosidic bond cleavage. The third goal was to use molecular modeling to rationalize the fragmentation ratios obtained in the mass spectrometer.

As described in Chapter 2 peralkylated (methyl to pentyl), peresterified and tert-butyl-dimethyl silyl derivatives of the different linkage isomers of glucose-glucose disaccharides were synthesized to increase the steric crowding in the glycosidic bond. Further derivatization enhances the surface activity of the molecule and counteracts the hydrophylicity of the molecule enhancing its ability to protonate. The aim was to use these derivatives to discriminate the anomeric configuration and to study the effects of the different functional groups on cleavage of the glycosidic bond by mass spectrometry. The compounds had synthetic yields ranging from 45%-92%. The mass spectrometry studies were limited in this study to only the peralkylated derivatives of the 1→4 and 1→6 linkage isomers.

In chapter 3 electrospray-collision induced dissociation studies showed that a discrimination of the anomeric configuration could be attained for the 1→4 anomers but
In chapter 3 electrospray-collision induced dissociation studies showed that a
discrimination of the anomeric configuration could be attained for the 1→4 anomers but
predictably not the 1→6 linkage pair. The research shows conclusively the dependence of
the spectra on the rotational freedom of motion of the disaccharide around the glycosidic
bond which directly influenced its cleavage. This was true not only for anomeric
configuration discrimination but also for the effect of the alkyl groups on glycosidic bond
cleavage. Thus a more sterically crowded derivative cleaved more readily in comparison
to a flexible molecule because of its inability to dissipate the added collision energy
within its rotational and vibrational modes and concentrated the energy on the weakest
bond in the molecule the glycosidic bond. The perpentylated molecule being the most
sterically restricted cleaved at the highest rate among all the peralkylated derivatives. In
addition to rotational freedom of the molecule other factors such as the basicity of the
alkyl groups, the conformation of the molecule and collision crossection also played a
major role in frangibility and fragmentation. Parent ion and daughter ion scans were
carried out to elucidate the fragmentation pathway. Chapter 3 also describes molecular
modeling of the permethylated 1→4 linkage disaccharides to corroborate their
fragmentation behavior in the mass spectrometer.

Chapter 4 described the conformational analysis of permethylated derivatives of
1→1, 1→2, 1→3, 1→4, 1→6 linked disaccharides. The research shows that methylation
makes only some of the disaccharides like α,α-trehalose, β,β-trehalose, kojibiose, and
sophorose more conformationally restricted. It, however, makes nigerose, laminaribiose,
maltose and cellobiose more flexible. It also shows that some of the energy surfaces of
the permethylated molecules are very alike the energy surface of the native molecule at

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the highest dielectric constant \( \varepsilon = 7.5 \) because of the decreased hydrogen bonding. This research is important since it can enhance our understanding of the properties of oligosaccharides.

**Future Research**

The results of the studies in this dissertation show that mass spectrometry can be used for stereoisomeric differentiation of certain compounds, which has long been the domain of Nmr spectroscopy. The idea was to begin development of a sensitive method that can identify the anomeric configuration of small oligosaccharides. Therefore, the next set of experiments to be performed is to study the other sterically hindered linkage isomers of glucose-glucose disaccharides (\( 1\rightarrow1, 1\rightarrow2, 1\rightarrow3 \)) to test the applicability of the methodology described in this dissertation to anomeric configuration differentiation. Then we propose to study heterogeneous residue oligosaccharides like glucose-galactose or glucose-fucose and see whether this methodology can differentiate not only anomeric configuration but also the type of linkage involved. This method would be used to complement methods like the methylation GC-MS analysis developed by Bjorndal et al. when amounts are too small to conduct anomeric studies. Experiments should also be later directed at larger sugars such as tri- and tetra saccharides. Further studies can be performed on the disaccharides already derivatized with other functional groups such as esters or silyl, the synthesis of which have been described in this thesis. Thus, the effect of other functional groups on cleavage of the glycosidic bond can be studied. After carrying out these various experiments, a database could be built. For the anomeric pairs that are not distinguishable by this method we suggest, instead of derivatization, the use of metals coordinated with the native compound since metals are known to promote
alternative fragmentation pathways and could provide additional discrimination. Molecular modeling is currently being carried out by our group on the higher alkyl derivatives to see whether their more facile fragmentation in the mass spectrometer can be rationalized.
Appendix

List of Abbreviations

EI  Electron Impact Ionization
CI  Chemical Ionization
FAB Fast atom Bombardment
MALDI Matrix Assisted Laser Desorption Ionization
ESI Electrospray Ionization
CID Collision Induced Dissociation
CA Collision Activation
CAD Collision Activation Dissociation
Sanford Mendonca was born in Bombay, India. He is the third of four children. He graduated from Campion high school, a very elite school, with honors. He completed his bachelor’s degree from St. Xavier’s College affiliated with the University of Bombay. He completed his master’s degree from the University of Houston. He joined Louisiana State University in fall 1996 to pursue a doctorate degree under the direction of Professor Roger Laine. He will receive the degree of Doctor of Philosophy in December of 2000. He has three adorable children Ninoshka, Nadia, and Natalia.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Sanford Mendonca

Major Field: Chemistry

Title of Dissertation: Structure Determination of Derivatized Disaccharides by Tandem Mass Spectrometry and Molecular Modeling

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August 30, 2000

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