A Conformational Study of Linkage Positions in Oligosaccharides Investigated by 2-D NMR Spectroscopy and Molecular Modeling

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The conformation of synthetic oligosaccharides can be elucidated by employing molecular modeling and high-field proton NMR (nuclear magnetic resonance) spectroscopy. Information with respect to the composition and configuration of saccharide residues and the sequence and linkage positions of the oligosaccharide can be obtained by employing a variety of one- and two-dimensional NMR techniques and molecular modeling. These techniques are also useful in establishing the solution conformation of the oligosaccharide moiety. This study is focused on the elucidation of linkage positions of synthetic trisaccharides, Gal(β1-4)Glc(β1-3)Glc, Gal(β1-4)Glc(β1-4)Glc and Gal(β1-4)Glc(β1-6)Glc.

Key Words: Linkage, Oligosaccharide, Conformation, 2-D NMR, Molecular modeling

Introduction

The growing interest in the biological function of oligosaccharides has stimulated the constant search for new methods for analyzing their primary and secondary structures.1,2 Linkage position among other structural parameters of carbohydrate may be the most sensitive to variance in vibrational freedom, particularly rotation, due to the different steric hindrance and degrees of freedom of motion between sugars.

NMR study combined with molecular modeling has become an increasingly important tool for the chemical and physical characterization of carbohydrates and their derivatives.3,6 The usefulness of the NMR method follows from the assignment of individual proton resonances to particular sugars. The ability to make these assignments has been enormously facilitated by the introduction of 2-D NMR techniques. A variety of 2-D 1H experiments and especially homonuclear chemical shift correlation spectroscopy (COSY), has aided signal assignments in 1H-spectra. The 2-D NOE (nuclear overhauser effect) experiment and the COSY experiment were also used for determining oligosaccharide interglycosidic linkages. The structure of different linkages can be defined in terms of NMR parameters of their structural reporter groups. The relative intensities of the signals in the NMR spectrum can be used as markers for the purity of the compound. The analysis of reducing oligosaccharides showed that the anomeric configuration of the reducing end sugar also exerts its influence on the spectral parameters of residues in its spatial neighborhood, being sometimes even the non-reducing end sugars.

The computer program used herein, modified MM2, is one of the molecular mechanics programs that optimize the atomic coordinates of a molecule to produce a structure at a local minimum on a multidimensional hypersurface of potential energy. It includes potentials for bond stretching, bending, and stretch-bending, 3-fold torsional potentials, Van der Waals interactions, and dipole-dipole interactions. A set of oligosaccharides differing only in linkage position, Gal(β1-4)Glc(β1-3)Glc, Gal(β1-4)Glc(β1-4)Glc and Gal(β1-4)Glc(β1-6)Glc, was prepared by enzymatic synthesis.7 These sets of compounds would be useful for development of structural analysis techniques geared to resolve linkage type of saccharides. Compounds like these are parts of core structures for glycolipids.1 In this study, a simple and systematic method is described to obtain the structural informations from the protons of linkage position in the synthetic oligosaccharides.

Experimental Section

Materials. Oligosaccharides were synthesized as described previously.7 Laminariobiose, gentiobiose, UDP-galactose, α-lactoalbumin and lactose synthase were purchased from Sigma Chemical Co. All other chemicals were reagent grade quality.

NMR. 1H NMR spectra of synthetic trisaccharides were recorded on a Bruker AM-400 equipped with an Aspect 3000 computer. Approximately 700 µg of the sample was used for the NMR studies. Prior to the NMR experiment, the sample was dissolved in D2O (99.9 atom% from Sigma Chemical Co.), and lyophilized to remove exchangeable hydroxyl protons. This procedure was repeated three times, at the last time, the sample was placed in the NMR tube. The sample was dissolved in 99.96 atom% D2O from MSD Isotopes to give a final concentration of about 31 mM. Both one- and two-dimensional NMR experiments were performed at 298 K. Higher temperature experiments (323 K) were performed as needed, to shift the solvent (HDO) peak away from one of the anomeric resonances. Acetone at 2.225 ppm was used as an internal reference, indirectly referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid. The homonuclear chemical shift correlated spectrometry (COSY) is obtained.
using 2048 data points in the $t_2$-domain and a spectral width of 2200 Hz. In the $t_1$ domain, 1024 data points were acquired and zero-filled to 2048 points before Fourier transformation to give a digital resolution of about 2.2 Hz/point.

Nuclear Overhauser experiments were performed as described by Bax A. et al. with a three second presaturation pulse and a one second delay between successive pulses. The 2-D NOESY experiment was performed as described by Kumar A. et al. The spectral width was 1200 Hz. 512 data points were used in each value of $t_1$. The mixing time was 300 msec. The total accumulation time was 6 hours.

Molecular Modeling. The constitution of disaccharide, Glc($\beta$1-4)Glc, giving atom numbering and the torsional angles phi and psi, are shown in Figure 1. Phi and psi are defined by atoms H1-C1-O4'-C4' and H4'-C4'-O4'-C1, respectively.

Figure 1. The constitution of disaccharide, Glc($\beta$1-4)Glc, giving atom numbering and the torsional angles phi and psi. Phi and psi are defined by atoms H1-C1-O4'-C4' and H4'-C4'-O4'-C1, respectively.

Molecular calculations were performed on a DEC MicroVax 3500 or IBM-3090 using SYBYL and Alchemy2000 (Tripos Associates Inc., 1998) and modified MM2 software. Energy contour maps were made with TOPO and SURF programs from the SURFER package (Golden Software, Inc., Golden Co.). The first step in the modified MM2 calculations was determination of the interatomic distances, bond angles and torsional angles in the starting geometry made by SYBYL and Alchemy 2000 programs. The values obtained were used in the different potential function expressions to calculate an initial steric energy, which was simply the sum of various potential energies calculated for all bonds, bond angles, torsional angles, nonbonded pairs of atoms and so forth in the molecule. The modified MM2 program uses a block diagonal Newton-Raphson optimization. Once the optimization had converged, the program printed the final steric energy and optimized geometry. Calculation of minimized energies and optimization of geometries were repeated at each 20° increment of phi and psi torsional angles from -180° to 160°. At each 20° increment of torsion angles, the energy is minimized, providing a value for a point on the energy map.

Results and Discussion

Using lactose synthase, the product trisaccharides were Gal($\beta$1-4)Glc($\beta$1-3)Glc, Gal($\beta$1-4)Glc($\beta$1-4)Glc and Gal ($\beta$1-4)Glc($\beta$1-6)Glc. These trisaccharides have the same molecular weight, the only difference being linkage position between the second glucose and the third glucose.

The GalNAc($\beta$1-3)Gal, Gal($\alpha$1-4)Gal and Gal($\beta$1-4)Glc disaccharide fragments have very similar conformational behavior in globotetraosyl ceramide and Forssman antigen. It was suggested that the differences observed at the oligosaccharide level are probably due to the cumulative effect of minor differences at the individual disaccharide linkages. The structural details of the synthetic trisaccharides were established by NMR. Significantly, both reducing end anomers of the compounds gave good signal/noise ratios in both the one- (1D) and two-dimensional (2D) spectra. One of the anomers (the $\alpha$) was only 30-40% of the total concentration and each anomer has its own independent set of resonances. The anomeric configuration and the number

Figure 2. (A) Expansion of the 1-D $^1$H NMR spectrum and (B) 2-D COSY $^1$H NMR spectrum of Gal($\beta$1-4)Glc($\beta$1-3)Glc at 323 K.
of sugar residues were determined by measuring coupling constants and integral intensities of H-1 doublets. The 2-D NOE experiment and the COSY experiment were used for determining oligosaccharide interglycosidic linkages. Both of these approaches to establishing the interglycosidic linkage rely on proton NMR, and are often complicated because of several spectral overlap. Additionally, the effects on which those methods are based, NOE effect across the glycosidic linkage and the $4^J_HCOCH_3$ scalar coupling, are strongly conformation dependent and therefore not always unambiguous.

Figure 2 shows the 2-D COSY spectrum of Gal($\beta_1$-$4$)Glc($\beta_1$-$3$)Glc at 323 K. The 2-D COSY spectrum provides diagnostic evidence on H couplings and with expanded spectra, confirms $J$ values. In the expansion of the 1-D spectrum on the top of its 2-D COSY spectrum, the H-1 peak of internal glucose of the trisaccharide apparently resonates as triplet instead of doublet. However, it is because the two forms of the reducing end glucose in this trisaccharide influences the H-1 of the adjacent internal glucose. Hence, the H-1 of internal glucose that apparently resonates as a triplet, is actually an overlapping doublet. Interestingly, this happens only in the 1-3 and 1-6 linked compounds. From the 1-D NMR spectrum, the synthetic oligosaccharide was confirmed as a trisaccharide. Table 1 was the summary of the chemical shifts of structural reporter group protons of constituent monosaccharides of synthetic Gal($\beta_1$-$4$)Glc($\beta_1$-$3$)Glc and Gal($\beta_1$-$4$)Glc($\beta_1$-$6$)Glc. The anomeric protons were at $\delta$ 4.45, 4.75, 4.66 and 5.23 ppm with coupling constants of 7.78, 8.19, 8.00 and 3.72 Hz, respectively (Table 1).

From coupling constant information, $4^J_HCOCH_3$ peak at $\delta$ 4.75 and peak at $\delta$ 4.66 ppm are assigned to glucose and peak at $\delta$ 4.45 ppm to galactose. Usually, integration rate of protons from monosaccharides is about 0.7-1.0. According to the integration rate, the peak at $\delta$ 4.45 ppm was galactose H-1, peak at $\delta$ 4.75 ppm was assigned to the penultimate glucose and peak at $\delta$ 4.66 ppm and the small $\alpha$-doublet ($\delta$ 5.23 ppm) together were assigned to the reducing end glucose. From the 2-D COSY spectrum, each proton of the internal glucose and of the non-reducing terminal galactose was assigned by cross peaks starting from assignments of the anomeric protons. For Gal($\beta_1$-$4$)Glc($\beta_1$-$3$)Glc, beginning at $\delta$ 4.45 ppm with a galactose H-1 resonance, and lowering the contour level somewhat it is possible to find a weak cross peak showing a connectivity to the galactose H-2 resonance at 3.55 ppm. That this connectivity is weak, is a result of a small $J_{1,2}$ coupling constant. From galactose H-2 resonance a connectivity to the galactose H-3 resonance at 3.75 ppm can be found and, likewise, from galactose H-3 to the galactose H-4 resonance at 3.90 ppm. Thus, even in this rather difficult case, it is possible to assign resonances at 4.45, 3.55, 3.75 and 3.90 ppm, to the galactose 1, 2, 3, and 4 protons of non-reducing terminal galactose residue.

By comparing several reference spectra, the chemical shifts of all protons of Gal($\beta_1$-$4$)Glc($\beta_1$-$3$)Glc (Figure 2)
were slightly changed except the internal glucose H-4 which shows a much larger shift (3.67 ppm → 3.51 ppm). The chemical shift of the internal glucose H-4 (δ 3.51 ppm) of Gal(β1-4)Glc(β1-3)Glc shifted towards lower field than an earlier reference spectrum of glucose H-4 (δ 3.67 ppm). It shows a glycosylation shift which was the diagnostic signal for the Gal(β1-4)Glc linkage position. Figure 3 is the 2-D COSY spectrum of Gal(β1-4)Glc(β1-3)Glc. The chemical shift of the middle glucose H-4 (δ 3.51 ppm) of Gal(β1-4)Glc(β1-3)Glc also shifted towards lower field than the reference spectrum. Each internal glucose H-4 of Gal(β1-4)Glc(β1-3)Glc and Gal(β1-4)Glc(β1-6)Glc shows a glycosylation shift which was the diagnostic signal for the Gal(β1-4)Glc linkage position.

Figure 4 is the 1-D NOE 1H NMR spectrum of Gal(β1-4)Glc(β1-6)Glc at 298 K. (a) pre-irradiation of AH-1, (b) pre-irradiation of BH-1 and (c) pre-irradiation of CH-1.

Table 2. Interresidue and Intraresidue NOE contacts of synthetic trisaccharides, Gal(β1-4)Glc(β1-3)Glc and Gal(β1-4)Glc(β1-6)Glc

<table>
<thead>
<tr>
<th>Contact Type</th>
<th>Gal(β1-4)Glc(β1-3)Glc</th>
<th>Gal(β1-4)Glc(β1-6)Glc</th>
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</thead>
<tbody>
<tr>
<td>Interresidue</td>
<td>AH-1/BH-4</td>
<td>AH-1/BH-4</td>
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<tr>
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<td>AH-1/BH-3</td>
<td>BH-1/CβH-6</td>
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<td></td>
<td>BH-1/CβH-6</td>
<td>BH-1/CβH-6'</td>
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<tr>
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<td>BH-1/CβH-4</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>BH-1/CβH-4</td>
</tr>
</tbody>
</table>

Figure 5. 2-D NOESY 1H NMR spectrum of Gal(β1-4)Glc(β1-3)Glc at 323 K.

The computer program used herein, modified MM2, is one of the molecular mechanics programs that optimize the...
atomic coordinates of a molecule to produce a structure at a
local minimum on a multidimensional hypersurface of
potential energy.\textsuperscript{1,15} The MM2 molecular modeling pro-
grams was modified for use on an IBM3090 and were also
used on DEC Microvax equipment to calculate minimum
energy structures and freedom of motion volumes near the
minima.\textsuperscript{3,16} It includes potentials for bond stretching, bend-
ing, and stretch-bending, 3-fold tortional potentials, Van der
Waals interactions, and dipole-dipole interactions. The
modified MM2 program was chosen for this work as
following reasons: first, carbohydrate has a number of
possible ring conformers such as \( ^4C_1, ^1C_4, ^1S_5 \), etc.\textsuperscript{14} The
MM2 program can alter the ring geometry to a low energy
form for a particular saccharide shape. Secondly, the MM2
version, automatically provides the anomeric effects that are
important for sugars. Also, the lone pairs are treated as if

Figure 6. The phi and psi plots of the total energies and energy wells derived from the modified MM2 calculation. The drawings were made
with the SURF program for SURFER from Golden software. (a) Gal(\( \beta 1-4 \))Glc(\( \beta 1-3 \))Glc, (b) Gal(\( \beta 1-4 \))Glc(\( \beta 1-4 \))Glc and (c) Gal(\( \beta 1-4 \))Glc(\( \beta 1-3 \))Glc.
they are atoms because modified MM2 requires lone pairs of electrons on all ether and hydroxyl oxygen atoms to fit the data on alcohols and ethers which are major components of carbohydrate. Finally, this version has “dihedral driver” facility which accepts the initial, final, and increment size values of two torsional angles and energy-minimization at each increment of these torsion angles. It permits conformational analysis of a disaccharide by rotating of glycosidic bond and was further modified to give a rigid dihedral driver option that starts with the same intra-residue geometry at bond and was further modified to give a rigid dihedral driver.

Molecular modeling of the oligosaccharides supported the relative stability of each glycosidic linkage by examining the degree of rotational freedom around the isomeric linkage. Figure 6 shows 2-D energy maps and 3-D energy wells derived from the modified MM2 calculations on neutral, uncharged molecules which depict degrees of phi-psi rotational freedom. The Gal(β1-4)Glc(β1-3)Glc, the most rigid isomer, generates a volume which I will depict as 1.0, while the Gal(β1-4)Glc(β1-4)Glc generates an intermediate volume of 1.2. The Gal(β1-4)Glc(β1-6)Glc, being the most flexible with its three rotational bonds generates a much larger well (1.8). Comparing the coupling constants (Table 1) of internal glucose between Gal(β1-4)Glc(β1-3)Glc and Gal(β1-4)Glc(β1-6)Glc, the most rigid isomer (Gal(β1-4)Glc(β1-3)Glc) has 7.76 Hz, whereas the most flexible one (Gal(β1-4)Glc(β1-6)Glc) has 8.19 Hz. Calculated linkages from conformations whose energies were based on the modified MM2 parameters gave results in good accord with the conformational properties of oligosaccharides in solution as reflected from NMR data. We are currently studying the dynamic kinetics of the linkage-isomeric oligosaccharides.

The trisaccharides should be linear, not stacked underneath the second or third Glc rings, because there is no intra-NOE signals from hydrogen bonding between the second or third Glc hydroxyl protons. Therefore synthetic trisaccharides, Gal(β1-4)Glc(β1-3)Glc and Gal(β1-4)Glc(β1-6)Glc, have linear structures, not folded structures. The results derived from these techniques are complementary and are integrated with each other to arrive at the final model. A fine example is the use of interatomic distance information gathered from NMR studies in force field calculation to determine the solution conformation.

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References

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