A Systematic NMR Determination of α-D-Glucooligosaccharides, Effect of Linkage Type, Anomeric Configuration and Combination of Different Linkages Type on ^{13}C Chemical Shifts for the Determination of Unknown Isomaltooligosaccharides

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Prebiotic isomaltooligosaccharide preparations contain α-D-glucooligosaccharides comprising isomaltooligosaccharides (IMOs) and non-prebiotic maltooligosaccharides (MOs). They are both glucose oligosaccharides characterized by their degree of polymerization (DP) value (from 2 to ~10), linkages types and positions (IMOs: α-(1→2, 3, 6 and in a lower proportion internal 1→4) linkages, MOs: α-(1→4) linkages). Their structure is the key factor for their prebiotic potential. In order to determine and elucidate the exact structure of unknown IMOs and MOs, unambiguous assignments of ^{13}C and ^{1}H chemical shifts of commercial standards, representative of IMOs and MOs diversity, have been determined using optimized standard one and two-dimensional experiments such as ^{1}H NMR, ^{13}C NMR, APT and ^{1}H-^{1}H COSY, TOCSY, NOESY and ^{1}H-^{13}C heteronuclear HSQC, HSQC-TOCSY, and HMBC. Here we point out the differential effect of substitution by a glucose residue at different positions on chemical shifts of anomeric as well as ring carbons together with the effect of the reducing end configuration for low DP oligosaccharides and diasteroisotopic effect for H-6 protons. From this study, structural ^{13}C specific spectral features can be identified as tools for structural analysis of isomaltooligosaccharides.

Key Words: Isomaltooligosaccharides, ^{13}C NMR, Glycosylation effect, Effect of anomeric configuration, 2D NMR

Introduction

Isomaltooligosaccharides are low digestibility oligosaccharides, considered as prebiotics which can be used to selectively feed probiotics indigenous to the human colon.[1-5] Recent data obtained on human subjects, support the involvement of dietary oligosaccharides in physiological processes in the different intestinal cell types and also outside the gastrointestinal tract (e.g. hormone production, lipid and carbohydrate metabolism).[6] The composition (DP and linkage types) of prebiotic mixtures is the key factor for their efficiency throughout the structure/function relationship and the possible partial digestion by indigenous enzymes that can occur before reaching the colon. IMOs are enzymatically produced glucooligosaccharides linked by at least one α-(1→6) linkage, or in a lower proportion α-(1→3) (nigerooligosaccharides) or α-(1→2) (kojioligosaccharides) glucosidic linkages depending on the substrate and the origin of the transglucosidase which specifies its tendency to form specific linkages.[7-13] The transglucosidase removes a Glc*p* residue linked through a α-(1-4) linkage from the non-reducing end of a glucooligosaccharide (preferably maltose) and transfers it to the non-reducing end of any glucooligosaccharide present in the medium This flexibility of the transglucosylation mechanism gives rise to a large variety of structurally close molecules in IMO preparations characterized at the same time by their DP value (from 2 to ~10), linkage types (α-1→2, 3, 4 or 6) and the proportion and position of each type of linkage.

In the case of homooligosaccharides, such as IMOs and MOs, with different types of linkages, the NMR structural analysis is more complicated than for heterooligosaccharides with a repeating unit which generally have well-separated anomeric ^{1}H and ^{13}C signals for each residue of the repeating unit.[14] The full ^{1}H and ^{13}C assignments must thus be obtained by employing a variety of 1D and 2D NMR techniques[14,15] including ^{1}H-^{1}H COSY, TOCSY, NOESY, ROESY and ^{1}H-^{13}C heteronuclear HSQC, HSQC-TOCSY, and HMBC.

Moreover, taking into account that oligosaccharide chemical shifts are more influenced by slight structural changes than polysaccharides, the structural origin of chemical shifts differences and the study of substitution patterns, effect of anomeric configuration and diastereotopic effect can give us potential tools for unknown IMO structural determination. The anomeric effect is the tendency of an electronegative substituent at the anomeric carbon to assume the axial rather than the equatorial conformation,[16,17] thus, in our case, α-form rather

than β-form for reducing end residue. As a consequence, independent sets of resonances arise for the two anomeric forms. Effects of anomeric configuration can also be observed, at a certain extent, on the directly linked residues.[15]

Recently, Van Leeuwen *et al.*[14] reported a ^{1}H NMR structural reporter-group concept for α-glucan analysis after assignments of similar commercial standards using various 2D experiments. In our study, as a preliminary work, their ^{1}H assignments are confirmed at a slightly different temperature and by a wider range of techniques making them unambiguous. These assignments can then be used in 2D heteronuclear spectra to confirmed ^{13}C assignments but moreover as a support for ^{13}C chemical shift deviation interpretations as proton configuration and interactions highly influences these deviations. ^{13}C chemical shifts can then be used as IMO structural determination tools, as they are more spread out, therefore less overlapped, and chemical shifts changes due to anomeric configuration and glycosylation are more marked than in the case of ^{1}H. These last considerations are of a great importance taking into account the wide variety of linkage type combinations found in IMO preparations.

Experimental

Preparation of standard samples. Prior to the NMR experiments, the standards were dissolved in D_2O (99.9 atom% from Sigma Chemical Co.) under a N_2 atmosphere and lyophilized to remove exchangeable hydroxyl protons. This procedure was repeated three times and the sample was finally dissolved in 99.97 atom% D_2O and placed in the NMR tube. According to their amounts, standards were either analyzed in normal NMR tubes (10 mg of the standard in 600 μL of D_2O) or in Shigemi NMR tubes (2 - 5 mg in 200 μL of D_2O). D-Glucose (Glc), maltose/α-D-Glc*p*-(1→4)-D-Glc*p* (**B→A**; **MT2**) and panose/ α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p* (**C→B→ A**; **PAN**) were purchased from Sigma-Aldrich (Bornem, Belgium), isomaltotriose/α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)- D-Glc*p* (**C→ B→A**; **IMT3**) from Medac (Wedel, Germany), kojibiose/α-D-Glc*p*-(1→2)-D-Glc*p* (**B→A**; **KOJ**), nigerose/ α-D-Glc*p*-(1→ 3)-D-Glc*p* (**B→A**; **NIG**) and maltotriose/α- D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p* (**C→B→A**; **MT3**) from Wako chemicals GMBH (Neuss, Germany) and nigerotriose/α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-D-Glc*p* (**C→B→A**; **NGT3**) from Dextra Laboratories (Reading, England). The purity of these standards was checked by HPAEC-PAD and was > 95% for all of them.

NMR spectroscopy. All spectra were recorded at 30 °C on a JEOL ECX-400 MHz NMR spectrometer equipped with broadband autotune universal probe with field gradients. Spectrometer operates at ^{1}H frequency of 399.782 MHz. The instrument is controlled by a PC running Delta 4.3 JEOL software used to acquire and process the NMR data. The spectra were calibrated with internal acetone (δH 2.225 ppm and δC 31.45 ppm). Dante's presaturation was applied when the HOD peak was too high and deleterious for the sensitivity of the ^{1}H experiments.

Eight scans and 2 prescans were used for 1D proton experiments as well as a 1 s relaxation delay and 1024 data points for abundant samples. Decoupled ^{13}C spectra were recorded with a relaxation delay of 0.1 s and at least 1000 scans and 16384 data points were acquired. For APT experiments, 2000 scans, 16384 data points and a 0.1 s pulse were applied. A mixing time of 1 s, 16384 data points, 16 scans were used in 1D NOE difference experiments.

The parameters used for 2D homonuclear experiments were as follows: COSY and TOCSY (512 × 256 data matrix; 4 and 8 scans, respectively, per t_1 value; spectral width 1799.1 Hz; relaxation delay 0.7 s; mixing time 50 ms); NOESY and ROESY (1024 × 256 data matrix; 16 scans per t_1 value; spectral width 1799.59 Hz; mixing time 500 ms). In all homonuclear experiments, the data matrix was zero-filled in the F1 dimension to give a matrix of 1024 × 512 points and was resolution enhanced in both dimensions by a shifted sine-bell processing function before Fourier Transformation.

HSQC, HSQC- TOCSY and HMBC heteronuclear experiments were measured in the ^{1}H-detected mode with proton decoupling in the ^{13}C domain and were carried out in the phase-sensitive mode. The parameters were as follows: HSQC and HSQC-TOCSY (512 × 256 data matrix; zero-filled to 512 data points in t_1; 32 scans per t_1 value; spectral width in t_1 1801. 7 Hz and in t_2 6032.8 Hz; recycle delay 1.0 s; mixing time 50 and 100 s, respectively; shifted sine-bell filtering processing in t_1 and t_2); HMBC (512 × 256 data matrix; zero-filled to 512 data points in t_1; 32 scans per t_1 value; spectral width in t_1 1845.4 Hz and in t_2 17006.8 Hz; recycle delay 1.2 s; shifted sine-squared filtering in t_1 and t_2).

Results and Discussion

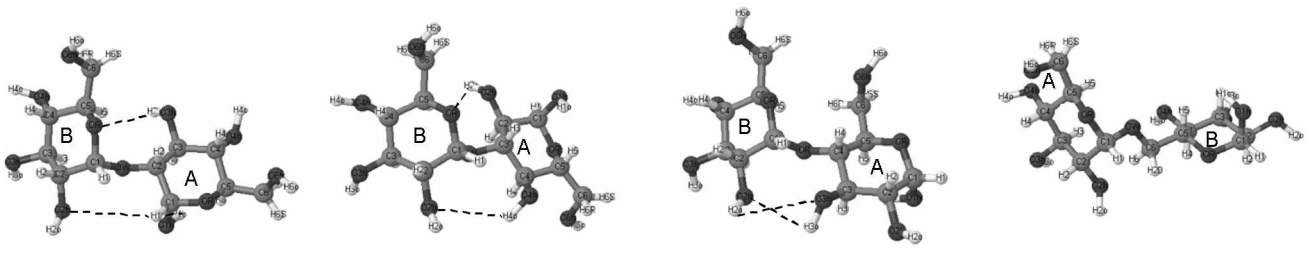
The following residue naming conventions were used: Aα and Aβ for reducing end residues in the α- and β-anomeric form, respectively, B(α) and B(β) for the adjacent residue linked to Aα and Aβ, respectively and C in case of a trisaccharide for the non-reducing end residue. Figure 1 presents the atoms and residue names for the following disaccharides: kojibiose, nigerose, maltose, isomaltose.

COSY, TOCSY, HSQC and HSQC-TOCSY were used to identify most of the ^{1}H and ^{13}C signals that belong to the same sugar unit.[15]

Inter-residue cross-peaks were identified in 2D NOE and HMBC spectra to determine the glycosidic linkage, the sequence of residues, and to ascertain the anomeric configuration of linkages[19-22] Anomeric configuration of the linkage is determined by the presence or absence of specific NOESY and HMBC correlation peaks between atoms involved in the linkage.

Assignments of ^{1}H-^{13}C chemical shifts of commercial standard IMOs and MOs. ^{1}H and ^{13}C complete assignments referenced to acetone (acetone δC 31.45 ppm and δH 2.225 ppm) for the eight standard glucooligosaccharides are shown in Table 1. ^{1}H assignments are in accordance with previous studies[7-15,18,23-30] including the most recent one.[14]

Two approaches are available for determination of anomeric configuration. In the first, using proton integrals, the ratio of α- to β-form is approximately 2:3 for free reducing glucose in D_2O. For substituted glucose, the predominance of the β-form is also present with the exception of a position 2 substitution



(1) (2) (3) (4)

Figure 1. Structure of studied disaccharide with residues and atoms naming: (1) kojibiose (α-D-Glc*p*-(1→2)-D-Glc*p*), (2) nigerose (α-D-Glc*p*-(1→3)-D-Glc*p*), (3) maltose (α-D-Glc*p*-(1→4)-D-Glc*p*), (4) isomaltose (α-D-Glc*p*-(1→6)-D-Glc*p*). In each case, reducing residue is referred to as residue A and B as non-reducing end residue. Atom numbering follows the standard convention with HX_O for hydroxyl hydrogen and OX_h for hydroxyl oxygen and OR for cycle oxygen. Intra-molecular hydrogen bonds are depicted in dashed lines.

Table 1. ^{13}C and ^{1}H NMR chemical shift data for 8 standard α-glucooligosaccharides referenced to internal acetone (δC = 31.45 ppm and δH = 2.225 ppm) for residue A, B and C.

Residue[a]		C-1	H-1	C-2	H-2	C-3	H-3	C-4	H-4	C-5	H-5	C-6	H-6S	H-6R
α-C	NGT3	100.38	5.37	72.71	3.59	74.06	3.77	70.68	3.46	73.00	4.03	61.70	3.87	3.79
	IMT3	98.95	4.95	72.60	3.53	74.55	3.70	70.72	3.42	73.03	3.70	61.70	3.84	3.77
	PAN	99.47	4.93	72.87	3.54	74.48	3.73	70.95	3.41	73.21	3.70	61.89	3.83	3.75
	MT3	101.20	5.39	73.10	3.57	74.30	3.66	70.70	3.42	74.10	3.69	61.90	3.85	3.73
α-B (α)	KOJ	97.62	5.08	72.71	3.54	74.09	3.78	70.72	3.44	73.14	3.93	61.65	3.83	3.76
	NIG	100.14	5.35	72.77	3.55	73.97	3.74	70.55	3.45	72.82	4.00	61.47	3.83	3.77
	NGT3	100.04	5.39	71.46	3.69	81.15	3.91	71.01	3.67	72.90	4.04	61.37	3.84	3.74
	IMT3	99.20	4.96	72.65	3.54	74.30	3.70	70.72	3.47	71.44	3.91	66.80	3.96	3.77
	PAN	101.19	5.38	73.1	3.58	74.48	3.67	70.82	3.49	72.6	3.93	67.33	3.98	3.71
	MT2	100.84	5.38	72.96	3.57	74.05	3.68	70.54	3.41	73.87	3.71	61.90	3.85	3.76
	MT3	100.95	5.38	73.00	3.61	74.70	3.93	78.20	3.63	72.60	3.83	61.90	3.84	3.81
α-B (β)	KOJ	99.01	5.37	72.75	3.53	74.09	3.74	70.65	3.45	72.97	4.01	61.65	3.82	3.77
	NIG	100.09	5.33	72.71	3.57	73.97	3.74	70.40	3.43	72.82	4.00	61.47	3.83	3.77
	NGT3	100.38	5.38	71.46	3.68	81.15	3.91	70.91	3.66	72.90	4.07	61.22	3.80	3.72
	IMT3	99.10	4.96	72.69	3.54	74.30	3.69	70.72	3.47	71.40	3.90	66.73	3.96	3.76
	PAN	101.1	5.38	73.02	3.58	74.48	3.67	70.82	3.49	72.72	3.93	67.33	3.98	3.71
	MT2	100.76	5.38	72.86	3.57	74.05	3.68	70.54	3.41	73.87	3.71	61.90	3.85	3.76
	MT3	100.80	5.38	72.90	3.61	74.70	3.93	78.20	3.63	72.60	3.83	61.90	3.84	3.81
α-A	Glc	93.28	5.22	72.63	3.53	73.96	3.68	70.85	3.40	72.65	3.83	61.81	3.83	3.74
	KOJ	90.74	5.42	77.23	3.62	72.51	3.81	70.89	3.44	72.60	3.86	61.90	3.83	3.76
	NIG	93.30	5.21	71.05	3.64	80.77	3.84	71.18	3.65	72.60	3.82	61.31	3.85	3.75
	NGT3	93.41	5.25	71.53	3.66	80.89	3.88	71.30	3.65	72.38	3.86	61.58	3.86	3.76
	IMT3	93.40	5.24	72.60	3.54	74.20	3.71	70.82	3.50	71.16	3.99	67.05	4.00	3.71
	PAN	93.3	5.22	72.87	3.57	74.58	3.97	78.81	3.64	71.37	3.93	62.09	3.86	3.79
	MT2	93.07	5.22	72.49	3.56	74.40	3.96	78.21	3.63	71.16	3.94	61.90	3.85	3.79
	MT3	93.30	5.22	72.70	3.56	74.60	3.96	78.60	3.61	71.40	3.93	62.00	3.85	3.80
β-A	Glc	97.09	4.62	75.33	3.23	76.95	3.48	70.80	3.39	77.13	3.44	61.96	3.89	3.73
	KOJ	97.53	4.78	79.89	3.37	75.75	3.56	71.06	3.40	77.04	3.45	62.10	3.88	3.67
	NIG	97.02	4.65	73.92	3.32	83.28	3.64	71.10	3.62	76.70	3.46	61.64	3.88	3.71
	NGT3	97.14	4.68	74.06	3.36	83.42	3.68	71.16	3.66	76.81	3.49	61.70	3.90	3.72
	IMT3	97.29	4.67	75.27	3.26	77.20	3.46	70.67	3.49	75.42	3.65	66.99	3.96	3.76
	PAN	97.17	4.64	75.38	3.26	77.57	3.76	78.59	3.64	75.97	3.62	62.22	3.89	3.77
	MT2	96.96	4.62	75.19	3.27	77.39	3.77	78.00	3.65	75.77	3.61	62.10	3.90	3.75
	MT3	97.20	4.64	75.40	3.26	77.60	3.75	78.40	3.65	76.00	3.61	62.10	3.90	3.74

[a]α-A and β-A stand for the anomeric configuration of residue A; α-B (α) and α-B (β) stand for residue B connected to α- and β-anomer of residue A, respectively. Glc: glucose. KOJ: koibiose, NIG: nigerose. NGT3: nigerotriose. IMT3: isomaltotriose. PAN: panose, MT2: maltose, MT3: maltotriose. A: Reducing end residue. B: Non-reducing end residue or in case of a trisaccharide central residue. C: Non-reducing end residue in case of a trisaccharide.

Table 2. Inter-residue and intra-residue nOe cross peaks for maltotriose, panose, nigerose and kojibiose.[a]

	α-(1-4)/α-(1-4) maltotriose[a,b]	α-(1-6)/α-(1-4) panose[a,b]	α-(1-3) nigerose[a,b]	α-(1-2) kojibiose[c]
Inter-residue nOe interactions		C H-1/$B_{(c)}$ H-6S		
	C H-1/$B_{(c)}$ H-4	C H-1/$B_{(\alpha)}$ H-6R		
	$B_{(\alpha)}$ H-1/A_{α} H-4	$B_{(\alpha)}$ H-1/A_{α} H-4	$B_{(\alpha)}$ H-1/A_{α} H-3	$B_{(c)}$ H-1[d]
	C H-1/$B_{(\beta)}$ H-4	C H-1/$B_{(\beta)}$ H-6S	$B_{(\beta)}$ H-1/A_{β} H-3	A_{β} H-1[e]
	$B_{(\beta)}$ H-1/A_{β} H-4	C H-1/$B_{(\beta)}$ H-6R		A_{β} H-2[e]
		$B_{(\beta)}$ H-1/A_{β} H-4		
Intra-residue nOe interactions	A_{α} H-1/A_{α} H-2	A_{α} H-1/A_{α} H-2		
	$B_{(c)}$ H-1/$B_{(c)}$ H-2	A_{α} H-1/A_{α} H-3	A_{α} H-1/A_{α} H-2	
	C H-1/C H-2	$B_{(c)}$ H-1/$B_{(\alpha)}$ H-2	$B_{(c)}$ H-1/$B_{(\alpha)}$ H-2	$B_{(\beta)}$ H-2[e]
	A_{β} H-1/A_{β} H-2	C H-1/C H-2	A_{β} H-1/A_{β} H-2	A_{α} H-2[d]
	$B_{(\beta)}$ H-1/$B_{(\beta)}$ H-2	A_{β} H-1/A_{β} H-2	$B_{(\beta)}$ H-1/$B_{(\beta)}$ H-2	
		$B_{(\beta)}$ H-1/$B_{(\beta)}$ H-2		

[a]NOESY experiment. [b]ROESY experiment. [c]Difference NOE experiment. [d]Irradiation of Aα H-1. [e]Irradiation of $B_{(\beta)}$ H-1.

where the ratio is close to 1:1. The second method employs a comparison of the NOESY and HMBC spectra. In the HMBC spectra, H_1/C_3 and H_1/C_5 over three-bonds intra-residue correlation peaks are found for the α-form but not for the β-form. This is due to the corresponding bond angle (45° and 180°) which is inducing small and large coupling constants, respectively. In NOESY, correlation peaks between H_1 and axial H_2, H_3, and H_5 which are close in space are present for the β-form while only the H-1/H-2 correlation peak is found for the α-form.

Glycosidic linkage position determination was then considered. The inter-residue and intra-residue nOe cross peaks for maltotriose, panose and nigerose (NOESY and ROESY spectrum) and kojibiose (1D Difference NOE spectrum) are compiled in Table 2.

Concerning NOESY and ROESY inter-residue signals in the maltotriose spectrum, the C H-1 and B H-1 peaks (δ 5.39 and 5.38 ppm) are dipole-dipole coupled with respectively B H-4 (δ 3.63) and A H-4 (3.61 for the α-form and 3.65 for the β-form). These intense signals indicate clearly a (1→4) linkage position between the three glucose units. The HMBC spectrum contains specific inter-residual cross peaks between H-4 and C-1 of the previous residue (A H-4/B C-1 and B H-4/C C-1 for both α and β forms) and H-1 and C-4 of the next residue (B H-1/A C-4 and C H-1/B C-4 for both α and β forms), confirming the presence of (1→4) linkages.

$B_{(\alpha)}$H-1/A_{α}H-3 and $B_{(\beta)}$H-1/A_{β}H-3 inter-residue cross peaks found in nigerose NOESY and ROESY spectra suggest (1→3) linkage. As for maltotriose, for the α-form glucose residue, H-1/H-2 are the most intense intra-residue cross peaks, corresponding to intra-residue hydrogen bondings, H1o/O2h and H2o/O1h being two of the shortest (2.22 Å).[31]

Two 1D Difference NOE spectra were collected for kojibiose corresponding to the two anomeric forms of the reducing end H-1. For the first one, reducing end A H-1α (δ 5.38 ppm) was irradiated, which generated two signals at δ 5.04 and 3.58 ppm in the spectrum. They correspond to the inter-residue nOe to the non-reducing (B) H-1 and the intra-residue nOe to the reducing (A) H-2, respectively. The δ 5.04 ppm nOe signal between the two anomeric protons indicates their spacial proximity, thus the presence of the α-(1→2) linkage. For the second difference nOe experiment, B H-1(β) was irradiated and generated three separate signals at δ 4.74, 3.33 and 3.49 ppm. The first two correspond to the inter-residue A H-1β and A H-2β respectively. The nOe to AH-2β was the most intense due to the proximity of the corresponding protons in space. The third signal corresponds to the intra-residue B H-2(β) nOe. The intense nOe between A H-2β and B H-1(β) confirms the presence of the α-(1→2) linkage.

The effects of anomeric configuration. Independent sets of resonances can arise for the two anomeric forms. The effects of anomeric configuration can also be observed, at a certain extent, on the directly linked residues (data not shown) as already reported.[15] Indeed, in our case, B C-1 shifts of α-glucooligosaccharides all exhibit a split due to the reducing residue configuration whatever the linkage, with the highest and positive value of $\Delta_{\beta\text{-}\alpha} = \delta C_{\beta} - \delta C_{\alpha}$ for the α-(1→2) linkage ($\Delta_{\beta\text{-}\alpha}$ = 1.39). α-(1→2) linked glucooligosaccharides also exhibit a split for B C-5 ($\Delta_{\beta\text{-}\alpha}$ = −0.17), while α-(1→3) for B C-4 and B C-6 ($\Delta_{\beta\text{-}\alpha}$ = −0.10 and −0.15, respectively) and α-(1→4) linked glucooligosaccharides for B C-2 ($\Delta_{\beta\text{-}\alpha}$ = −0.10). The presence or absence of slight splits can constitute a diagnostic tool for the type of linkage determination.

Inversion of an equatorial O to axial O is associated with increased shielding of the ^{13}C nucleus to which it is bonded, of adjacent ^{13}C nuclei, and of those in the nextposition. The anomeric effect does not arise through instability of the equatorial anomeric C-O bond since the destabilizing interaction appears to cause polarization of most C-H bonds in the molecules, ^{13}C becoming more shielded, and ^{1}H less shielded, suggesting a concerted means for delocalizing the instability. Moreover, free-energy difference between the α and β anomers is related to the degree of axial asymmetry of the chemical shift tensor in the plane containing the O-5-C-1-O-1 bonds, showing that

the anomeric effect has an electronic origin.

For residue A, since the effects of anomeric configuration arise principally from interaction of the electron charge clouds between the C-1-O-5 and the C-1-O-1 bonds.[16] the most significant effect is observed for A C-5 (4.4 ppm in average) and A C-1 (4.16 ppm in average). C-2 and C-3 exhibit lower but still significant effects, respectively 2.65 and 2.89 ppm due to overall compensatory mechanism tending to dissipate the destabilizing action of the axial group over several carbon atoms and the protons bonded to them.[32] Only nuclei in the 4-position are unaffected materially by the configurational change.

As already reported, ^{13}C nucleus has been found in several instances to be accompanied by a deshielding of the appended proton.[33-37] Our data supported well these findings with negative Δ(β − α) for the majority of ^{1}H except for H-4.[36,37] Additionally, anomeric configuration acts on the H-bond strength as reported by Deshmukh *et al.*[31] and Lopez de la Paz *et al.*[38] who noticed an increase in H-bond strength with an increasing number of axial −OH groups. Furthermore, the adjacent axial-equatorial H-bonds between A H1o/A O2h and A H2o/A O1h such as in the α-formare seen to be stronger than equatorial-equatorial same H-bonds such as in the β-form.[31,38] Moreover, in some cases, ^{13}C chemical shift differences due to anomeric configuration can be highly influenced by proton interactions. This last consideration is well illustrated by the exceeding Δ (β − α)(6.79 ppm) value found for A C-1 of the α-(1→2) linked kojibiose because of the spatial proximity of A H1α and B H1(α).

Concerning the effects of glycosylation at different positions on ^{13}C Δ(β − α) the overall feature is a decrease of Δ (β − α) values for the ^{13}C involved in the linkage (approximately −0.04, −0.47, −0.16 and −0.21 for α-(1 → 2, 3, 4 and 6) linkages, respectively). In addition, α-(1 → 2) linkage is increasing all Δ (β − α) values, with A C-2 being the only exception, keeping in mind the substitution impact for the ^{13}C involved in the linkage. This is essentially due to the spatial proximity of A H1α and B H1(α) reported above. For other substitution positions, the linkage seems to have a little effect on Δ (β − α) values.

Substitution effect: the key for linkage position determination. As already reported by Lipkind *et al.*,[37] the formation of a glycosidic linkage leads to changes in ^{13}C and ^{1}H chemical shifts which will depend on the type of linkage and the stereochemistry of the monosaccharide residues connected. Thus, together with the use of the HMBC, 1D NOE difference and NOESY techniques, correction values of ^{1}H and moreover ^{13}C chemical shift due to the glycosidic linkage (in comparison with glucose) are useful tools for structural determination of oligosaccharides. These correction values for our standard molecules for ^{1}H and ^{13}C chemical shifts are given in Table 3. Results are in good accordance with those of Lipkind *et al.* obtained for polysaccharides, considering the fact that in our case oligosaccharide chemical shifts are more influenced by slight structural changes than in polysaccharides.[37]

Results for the ^{1}H chemical shifts deviation due to substitution have already been discussed in a recent quality paper and permitted the development of structural-reporter-groups.[14] Our results are in very good agreement with this study. However, in the present study we focus on ^{13}C chemical shift changes as a tool for structural determination as ^{13}C signals are more spread out over a wider range of chemical shifts and chemical shift

Table 3. Effect of substitution on glucose residue ^{13}C and ^{1}H chemical shifts resonances for residues A, B and C depending on glycosidic linkage type (absolute value under 0.2 ppm for ^{13}C and 0.04 ppm for ^{1}H chemical shifts deviation were considered not significant).

Residue		C-1	H-1	C-2	H-2	C-3	H-3	C-4	H-4	C-5	H-5	C-6	H-6S	H-6R
α-C	α-(1-3)	**7.1**	0.15		0.06		0.09	0.31	0.06	0.35	0.2		0.04	0.05
	α-(1-6)	**5.67**	−0.27			0.59				0.38	−0.13			
	α-(1-4)	**7.92**	0.17	0.47	0.04	0.34				1.45	−0.14			
α-B (α)	α-(1-2)	**4.34**	−0.14		0.1		0.1		0.04	0.49	0.1			
	α-(1-3)	**6.76**	0.17	−1.17	0.23	**7.19**	0.23		0.27	0.25	0.21	−4.4		
	α-(1-6)	**5.92**	−0.26			0.34			0.07	−1.21	0.08	**4.99**	0.13	0.07
	α-(1-4)	**7.67**	0.16	0.37	0.25	0.74	0.25	**7.35**	0.23					
α-B (β)	α-(1-2)	**5.73**	0.15		0.06		0.06	−0.2	0.05	0.32	0.18			0.03
	α-(1-3)	**7.10**	0.16	−1.17	0.23	**7.19**	0.23		0.25	0.25	0.24	−0.59		−0.02
	α-(1-6)	**5.82**	−0.26			0.34			0.07	−1.25	0.07	**4.92**	0.13	0.02
	α-(1-4)	**7.52**	0.16	0.27	0.25	0.74	0.25	**7.35**	0.23					0.07
α-A	α-(1-2)	-2.54	0.2	**4.6**	0.13	−1.45	0.13		0.04					
	α-(1-3)			−1.1	0.2	**6.93**	0.2	0.45	0.25	−2.7		−0.23		
	α-(1-6)					0.24			0.1	−1.49	0.16	**5.24**	0.17	
	α-(1-4)				0.28	0.64	0.28	**7.75**	0.21	−1.25	0.1			0.06
β-A	α-(1-2)	0.44	0.16	**4.56**	0.08	−1.2	0.08	0.26						−0.06
	α-(1-3)			−1.27	0.16	**6.47**	0.2	0.36	0.27	−3.2	0.05	−0.26		
	α-(1-6)		0.05			0.25			0.1	−1.71	0.21	5.03	0.07	
	α-(1-4)		0.06		0.27	0.65	0.27	**7.60**	0.26	−1.13	0.17			

changes are more marked compared to ^{1}H chemical shifts deviations. Glycosidic effects are considered significant for a minimum difference value of 0.2 ppm.

Substition effect of α-(1 → 2) linkage present in kojibiose. For both residues B(α) and B(β), substitution effect on C-1 is small in comparison with other type of linkage, 4.34 and 5.73 ppm, respectively. These values reflect the spatial proximity of B H-1 and A H-2 for both residue illustrated by the presence of a hydrogen bonding (A H3o/B O5h) and A H-1α and B H-1(α) for α- conformation of the reducing residue. C-5 also exhibit a small changes (0.49 and 0.32 for B(α) and B(β), respectively).[39]

For both Aα and Aβ configurations, C-1, C-2 an C-3 chemical shifts are affected by substitution. A C-1 carbons don't participate in linkages but present a change in chemical shifts especially for the α-form which exhibits a negative value. This is due to the above mentioned spatial proximity between A H-1α and B H-1(α). A C-2 chemical shift changes reflect its participation in the linkage. A C-3 negative change arises from the presence of an inter-residue hydrogen bonding between B O5h and A H3o, see Figure 1.[39,40]

Substition effect of α-(1→3) linkage present in nigerose and nigerotriose. Nigerose and nigerotriose B C-1 carbons exhibit high substitution correction values between 6.76 and 7.1 ppm where A C-1 presents no significant change. A negative change is found for C-2 due to the presence of hydrogen bonding between C O5h and B H2o) for nigerotriose and B O5h and A H2o) for nigerotriose and nigerose.[39,40] Substitution at C-3 leads to an important chemical shift deviation of the corresponding carbon between 6.47 and 7.19 ppm. Slight positive deviations are observed for A C-4 because of the presence of a hydrogen bonding between A H4o and B O2h[39] as well as for B C-5 and C C-5 together with a negative deviation for A C-5.

Substitution effect of α-(1→4) linkage present in maltose, maltotriose and panose. Effect of substitution on maltotriose is presented. For both residue B(α) and B(β), with position 4 substituted, the effect on A C-1 chemical shift change is the highest with 7.67 and 7.52 ppm, respectively. Substitution at A C-4 leads to the most important chemical shift deviation observed for a substituted carbon, 7.35 for both B(α) C-4 and B(β) C-4, 7.75 and 7.6 for Aα C-4 and Aβ C-4. For Aα and A β, the position 4 substitution causes shielding of A C-5 -1.25 and -1.13 ppm, respectively. However, for both B(α) and B(β) residues, the involvement of their anomeric carbons in a linkage is causing a deshielding effect on C-5 which counterbalances the above mentioned shielding. This last deshielding effect is observed on C residue displaying a 1.45 ppm shift. On the other hand, C-3 from A and B residues, undergo a slight deshielding between 0.64 - 0.74 ppm due to the presence of an inter-residual hydrogen bond between B O2h and A H3o and between B H2o and A O3h.[39-42] As a consequence, C C-2 and B C-2 also exhibit a slight deshielding between 0.27 - 0.47 ppm.

Substitution effect of α-(1→6) linkage present in isomaltotriose and panose. Effect of substitution on isomaltotriose is presented. Substitution at position 6 causes a deshielding between 4.92 and 5.24 ppm for B(α) C-6, B(β) C-6 and C C-6 which is lower compared to deshieldings observed for B C-3 and B C-4 involved in position 3 and 4 substitution and slightly higher than for B C-2 involved in position 2. The anomeric carbons B(α) C-1, B(β) C-1 and C C-1 involved in the linkage undergo a deshielding between 5.67 and 5.92 ppm. C-5 chemical shifts are influenced by the change in local electron density and are shifted upfield (−1.13 to −1.25 ppm). As C-1, C-3 and C-5 may be regarded as in one plane, C-3 is shifted downfield by 0.59 to 0.74 ppm. Best *et al.*[42] and Pereira *et al.*[39] found no inter-residue hydrogen bonding for isomaltotriose.

These results indicate that substitution on a glucose residue in position 2 influences the chemical shifts of C-1, -2 and -3, in position 3: C-2, -3, -4, -5 and -6, in position 4: C-3, -4 and -5 and finally in position 6: C-3, -5 and -6.

Moreover, as already observed by Van Leeuwen *et al.* for protons, comparison of the ^{13}C chemical shifts of residue B in trisaccharides (maltotriose, nigerotriose, isomaltotriose and panose) with those in disaccharides (maltose and nigerose) shows that the size and direction of the shifts (upfield or downfield) upon substitution are similar to the size and direction of the shifts of residue Aα of all the standards in reference to free α-D-Glc.[14]

The diastereotopic effect in α-(1→6) linked IMOs. Chemical shifts of diastereotopic protons H-6S and H-6R can diverge due to phenomena such as restriction of the conformational freedom.[21] A significant diastereotopic effect (Δdia = δH6S – δH6R) between 0.12 and 0.21 is observed for each Aβ residue but not for the α-form of the reducing residue (except for α-(1-6) linked oligosaccharides). Perlin *et al.* found that the inversion of the equatorial to the axial anomer (β-form to α-form) causes the introduction of a destabilization interaction that may lead to widespread alterations of bond angles and dimensions, states of hybridization *etc.*[17] This can explain the more important conformational freedom of A H-6Sα and A H-6Rα and as a consequence, the absence of a significant diastereotopic effect for the α-form.[30]

On the other hand, a strong diastereotopic effect is observed for H-6 protons when the corresponding C-6 is involved in the glycosidic linkage, which causes an important conformational freedom restriction. This phenomenon is observed for A and B residue of isomaltotriose and for B residue of panose.

Conclusions

For the analysis of α-glucooligosaccharides (in particular isomaltooligosaccharides) the full and unambiguous assignments of ^{1}H an ^{13}C chemical shifts of standard molecules has been completed through the use of various 1D and 2D NMR experiment such as ^{1}H NMR, ^{13}C NMR, APT and COSY, TOCSY, NOESY or ^{1}H-^{13}C HSQC, HSQC-TOCSY, and HMBC. Additionally, the effects of oligosaccharide structure on ^{13}C chemical shifts have been fully studied and specific spectral features are compile in Table 4. Indeed, structural characteristics such as anomeric configuration, substitution pattern, as well as the diastereotopic effect act on ^{13}C chemical shifts giving specific deviations usefull for unambiguous structure determination. These specific deviations will represent key tools for further structural determination of unknown α-

Table 4. Special ^{13}C spectral features (deviation from free glucose chemical shifts) for α-glucooligosaccharide structural determination.

Type of linkage	Effects on Glycosylated Glc	Effects on Glycosylating Glc	Other effects
α-(1-2)	C-1α upfield (2.54)	C-1 relatively low downfield (4.34 -5.73)	Strong effect of anomeric configuration Δ = 6.79
	C-2 downfield (4.56 - 4.6)		Significant split of B C-1(α) and B C-1(β) peaks
	C-3 upfield (1.2 - 1.45)		
α-(1-3)	C-2 relativey low downfield (1.1 - 1.27)	C-1 relatively high downfield (6.76 - 7.10)	
	C-3 downfield (6.47 - 7.19)		
α-(1-4)	C-4 downfield (7.35 -7.75)	C-1 very high downfield (7.52 - 7.92)	
	C-5 upfield (1.13 - 1.25)		
α-(1-6)	C-5 upfield (1.21 - 1.41)	C-1 relatively low downfield (5.67 - 5.92)	strong diastereotopic for H-6 protons
	C-6 downfield (4.92 - 5.24)		

D-glucooligosaccharides such as IMOs. IMO preparations can thus be fully characterized through the structural identification of each component individually. Moreover, in order to predict an overall prebiotic potential of industrial IMO mixtures, the determination of different linkage type ratio can be achieved through the use of the proton integrals of the anomeric region in the 1D ^1H spectra of IMO mixtures.

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