Characterization of methyl-, 3-deoxy-, and methyl-deoxysugars in marine high molecular weight dissolved organic matter

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ABSTRACT

Nuclear magnetic resonance spectroscopy of marine high molecular weight dissolved organic matter (HMWDOM) in surface waters show that >50% of the carbon is a compositionally well-defined family of acylated-polysaccharides that are conserved across ocean basins. However, acid hydrolysis of HMWDOM followed by chromatographic analyses recover only 10-20% of the carbon as neutral, amino, and acidic sugars. Most carbohydrate in HMWDOM therefore remains uncharacterized. Here we use acid hydrolysis followed by Ag^+ and Pb^{2+} cation exchange chromatography to separate HMWDOM hydrolysis products for characterization by 1-D and 2-D NMR spectroscopy. In addition to neutral sugars identified in past studies, we find 3-*O*-methylglucose, 3-*O*-methylrhamnose, 2-*O*-methylrhamnose and 2-*O*-methylfucose. We also find 3-deoxysugars to be present, although their complete structures could not be determined. Methyl sugars are widely distributed in plant and bacterial structural carbohydrates, such as cell wall polysaccharides, and their presence in HMWDOM suggests that structural carbohydrates may contribute to DOM in surface seawater. We find most HMWDOM carbohydrate is not depolymerized by acid hydrolysis, and that the nonhydrolyzable component includes 6-deoxysugars.

1. INTRODUCTION

Dissolved organic matter (DOM) is a large reservoir of carbon that affects light

penetration (Nelson et al., 1998; Nelson and Siegel, 2002), microbial activity (Williams,

1981; Azam et al., 1983; Ducklow and Carlson, 1992), carbon-nitrogen-phosphorus

storage (Benner, 2002; Bronk, 2002; Karl and Björkman, 2002; Mopper and Kieber,

2002), trace metal bioavailability (Rue and Bruland, 1997, Wells, 2002), and the

retention of hydrophobic organic contaminants (Guo and Santschi, 1997; and references

therein) in seawater. Although the influence of the dissolved organic matter reservoir on

ocean biogeochemical cycles has been widely recognized, we have only a broad

understanding of its chemical composition, sources or fates. Molecular level analyses of

DOM hydrolysis products (simple sugars, amino acids and lipids) in the surface and deep

ocean recover only 4-11% and 1-2% of the total carbon respectively, and most DOM

remains unidentified at this level of characterization (Lee and Bada, 1977; Wicks et al.,

1991; Benner, 2002; Eglinton and Repeta, 2004).

The introduction of large volume ultrafiltration techniques was a major advance

to DOM studies and has made gram quantities of the high molecular weight fraction of

DOM (HMWDOM) available for wet chemical and spectral analyses. Ultrafiltration

through a 1 nm pore size membrane nominally retains organic matter of >1 kDa MW,

and routinely isolates 20-35 % of total marine DOM. Marine HMWDOM is chemically

distinct from humic substances and is enriched in carbohydrates (Benner et al., 1992).

Chromatographic and ^{13}C nuclear magnetic resonance (^{13}CNMR) spectral analysis of

HMWDOM in surface waters show that >50% of the carbon consists of compositionally

well-defined acyl-polysaccharides (APS) that are conserved across ocean basins

(Aluwihare et al., 1997; Eglinton and Repeta, 2004). However, after acid hydrolysis, only 10-20% of HMWDOM carbon (HMWDOC) can be recovered as simple neutral sugars (arabinose (**I**), fucose (**II**), galactose (**III**), glucose (**IV**), mannose (**V**), rhamnose (**VI**) and xylose (**VII**); see Appendix 1) using gas or high pressure liquid chromatography (GC, HPLC; Panagiotopoulos and Sempéré, 2005 and references therein). Another 5-8% of HMWDOC is recovered as acetic acid (Aluwihare et al., 1997), and a further 3-5% of HMWDOC is recovered as amino acids (McCarthy et al., 1996). Lipids represent < 2% HMWDOC (Mannino and Harvey, 1999; Wang et al., 2004). It is notable that the distribution of major sugars released by acid hydrolysis of HMWDOM is nearly the same irrespective of where the sample was collected or the specific hydrolysis conditions used for depolymerization (Sakugawa and Handa, 1985; McCarthy et al., 1996; Aluwihare et al., 1997; Borch and Kirchman, 1997; Panagiotopoulos and Sempéré, 2005). Most of the carbohydrate in HMWDOM is therefore not characterized by conventional HPLC or GC techniques. Reconciling the discrepancy between the amount of carbohydrate measured by ^{13}CNMR spectroscopy, and the amount of carbohydrate hydrolysis products measured by chromatographic techniques represents a major challenge to HMWDOM characterization.

Other approaches such as direct temperature-resolved mass spectroscopy (DT-MS) and ^{15}N-NMR indicate the presence of a much broader range of sugars than have been observed by GC and HPLC, including methylsugars, methyl-deoxysugars, aminosugars, and furfurals (Boon et al., 1998; Minor et al., 2001; 2002; Aluwihare et al., 2005). DT-MS studies characterize classes of sugars by diagnostic fragment ions but do not allow the full identification of specific sugars or their abundance. ^{15}N-NMR spectroscopy can be used to quantify nitrogen-containing sugars indirectly, but likewise

does not give information on the presence or amounts of specific aminosugars. For example, Aluwihare et al. (2005) inferred from ^{15}N- and ^{1}HNMR analyses that 25-30% of HMWDOM occurs as unspecified N-acetyl-amino sugars. Chromatographic analyses have identified glucosamine and galactosamine in HMWDOM, but at concentrations of $\leq$ 2% HMWDOC (Aluwihare et al., 1997; 2002). Similar inconsistencies have also been reported for plankton and sinking particulate organic matter (Hedges et al., 2001; 2002). Although a clear explanation for these discrepancies has not yet been found, two likely scenarios exist.

First, sugars other than neutral sugars, such as amino sugars, sugar alcohols, methylated sugars, uronic acids, sulfonated sugars, furfurals etc, may constitute a considerable fraction of HMWDOM carbohydrate but lie outside the analytical window of the HPLC or GC techniques used for HMWDOM characterization. A second likely explanation is that HMWDOM carbohydrate is simply not depolymerized by acid hydrolysis. In this study, we use Ag^+ and Pb^{2+} ion chromatography to show that acid hydrolysis does not depolymerize most carbohydrate in HMWDOM. We also report the spectroscopic analyses of new methyl-, deoxy-, and methyl-deoxysugars isolated from HMWDOM hydrolysis products. These sugars represent 2-5% of HMWDOM carbohydrate, but may also be present in the unhydrolyzed carbohydrate fraction.

2. METHODS

2.1　Sampling

Seawater was drawn from the 15 m seawater intake at the Natural Energy Laboratory of Hawaii Authority in Kona, Hawaii in February 2002. The samples were

filtered to remove bacteria and small particles using a cleaned (10% HCl) Suporflow dual stage (0.8μm and 0.2 μm) Gelman polyether sulfone cartridge filter (Chisolm Corp., Lincoln, RI) fitted to an Advanta stainless steel housing. High molecular weight DOM samples were collected using a large volume cross flow ultrafiltration system consisting of a stainless steel centripetal pump and membrane housings and a fluorinated high density polyethylene reservoir. The system was plumbed with teflon tubing and fitted with PVDF valves. We used four ultrafiltration membranes (90 ft^2 (8.4 m^2) each; Separation Engineering, Escondido, CA) with a 1 nm pore size that nominally retain organic matter of a molecular weight greater than 1000 Da (> 98% rejection of vitamin B$_{12}$). Membranes were cleaned using isopropanol, detergent (0.01% micro), HCl (0.01N) and NaOH (0.01N), stored in sodium azide (0.55mM), and rinsed with water and seawater immediately before use. The four filters were plumbed in parallel. Surface seawater (28900 L) were concentrated over several days to approximately 40 L, frozen and returned to Woods Hole for further processing. The HMWDOM samples were concentrated to 2 L using a smaller system fitted with a 1 nm pore size, 10 ft^2 (0.93 m^2) membrane, desalted by repeated (10x) diafiltration with high purity, low carbon, deionized water (Milli-Q water, 2L), and reduced to 2 L. The final concentrate gave no visible precipitate (AgCl) when an aliquot of permeate was mixed with a 5 mg/50 ml solution of AgNO$_3$. After diafiltration was complete, the sample (2 L) was lyophilized to a fluffy off-white powder (9.4 g) that was 35% by weight carbon with a C/N ratio of 13.

2.2   Sample preparation

HMWDOM samples (200-300 mg) were hydrolyzed in 2M trifluoroacetic acid (TFA). Approximately 14.5 g of TFA was added to 43 g of water in a 250 mL round

bottom flask. The solution was heated and HMWDOM added as a solution in approximately 2-3 mL of water. The flask was flushed with He, sealed, and placed in a sand bath at 120°C. After 4 hours, the dark brown solution was cooled, frozen at −20°C, and freeze-dried. The dried sample was dissolved in 2-3 mL of water and applied to a 6-8 mm i.d. packed with 4 g anion exchange resin (OH⁻ form , Bio-Rex® 5 resin, Bio-Rad Laboratories, Hercules, CA). The column was preconditioned by rinsing with 15 mL of NaOH (0.5 M) followed by Milli-Q water until the pH returned to approximately 7. The column was drained of water and the sample was applied. Carbohydrates were eluted with 20-40 mL of water, collected, concentrated to a small volume (few ml) by rotary evaporation and freeze dried. Recovery of the sample (by weight) after hydrolysis and ion exchange was 87%.

2.3   Chromatography

Chromatography was performed on a Waters pump (Model 510) and a Shodex RI-71 refractive index detector. Sample injection was performed with a manual Rheodyne injector fitted with a 100 μL sample loop. Carbohydrates were separated by degree of polymerization on sulfonated styrene-divinylbenzene gel analytical columns (30 cm x 7.8 mm I.D.; particle size 8 μm; cross linking 8%) in the silver or lead form (Supelcogel™ Ag or Pd, Supelco, Inc., Bellefonte PA). Column temperature was controlled using an oven (Tiberline TL-50) set at 80 ºC. Sugars were eluted with water at a flow rate of 0.5 ml min⁻¹. Prior to use, the mobile phase was degassed with He for 30 min. The total weight of the sample injected onto the column did not exceed 10 mg/injection. Columns were regenerated at the end of each day by washing overnight with water at 0.2 ml min⁻¹. Retention times were measured using arabinose (**I**), fucose

(**II**), galactose (**III**), glucose (**IV**), mannose (**V**), rhamnose (**VI**) and xylose (**VII**), 3-*O*-methylglucose (**VIII**), and DP (degree of polymerization, or number of monosaccahrides) 2-7 maltooligosaccharides (**IX** through **XII**; Sigma, St. Louis, MO, USA). Data acquisition and processing were performed with Agilent ChemStation software (Agilent Technologies).

2.4   Mass spectrometry

Matrix assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF-MS) analyses were performed at the Center for Complex Carbohydrate Research in Athens, GA (USA). MALDI-TOF-MS analyses were performed with a Voyager DE mass spectrometer (Applied Biosystems) operated in the positive ion mode. Aqueous solutions of samples were diluted 1:1 with 2, 5 dihydroxybenzoic acid, applied to a MALDI plate, and desorbed with a nitrogen laser having a guide wire reading of 0.005% and a laser intensity of approximately 2630. Ions formed by the pulsed laser beam were accelerated through 20 kV with an acquisition range of 149-2000 Da. The mass spectrometer was calibrated with angiotensin I.

2.5   Nuclear magnetic resonance (NMR) spectroscopy

All spectra were recorded on a Bruker Avance DPX 400 MHz spectrometer using XWIN-NMR 3.0 software and an inverse broadband (BBI) 5 mm probe tuned for ^{13}C with a Z-gradient coil. Approximately 1-2 mg of the isolated compounds were dissolved in 1-2 ml of D_2O (99.9 atom% D, Aldrich) and dried under an N_2 stream. Dissolution/drying was repeated to ensure complete replacement of all exchangeable hydrogen with deuterium. Samples were finally dissolved in 0.75 ml of D_2O (freshly

opened ampoules, > 99.96 atom% D) and transferred to 5 mm glass NMR tubes (Model 535-PP, Wilmad) using combusted (450 ºC) NMR pipettes. ^{1}H chemical shift was set against the residual HOD signal (δ 4.80 ppm). ^{13}C chemical shifts were derived from the transmitter frequency.

The proton spectral width and file size were set to achieve ca. 0.3Hz resolution, with subsequent equivalent zero filling bringing the final resolution to ca. 0.14 Hz. Experiments employed some suppression of the residual water signal, either a simple 30° pulse and presaturation during a 1 s relaxation or WATERGATE (WATER suppression by GrAdient Tailored Excitation; Bruker sequence "p3919gp") sequence with gradient strengths reduced for minimal collateral suppression (particularly of the anomeric proton signals). Acquisition times ranged from 10 min to several days depending on the amount of material available. ^{1}H-^{1}H gs-COSY (gradient-selected COrrelation SpectroscopY; Bruker sequence "cosygp") spectra were acquired with a 45° mixing pulse to produce 512 or 1024 FIDs (Free Induction Decay) of 32 or 64 summed scans resulting in 2 x 8 Hz raw resolution and 2 x 2 Hz final resolution after zero filling and symmetrization. Interscan relaxation time was 3 s. Acquisition times were 15 - 30 h. ^{1}H-^{13}C gs-HSQC (phase sensitive echo/antiecho-TPPI gradient-selected Heteronuclear Single Quantum Coherence; Bruker sequence "invietgpsi") spectra were acquired with delays optimized for $^1J_{13C1H}$ = 145 Hz. 1024 FIDs of 144 summed scans and no zero filling, resulting in 2 x 24 Hz final resolution. Interscan relaxation time was 2 s, and acquisition times were 4 days. All gradient experiments used 1500 µS 100 point sine shaped gradient pulses.

3. RESULTS AND DISCUSSION

3.1   Isolation of mono- and oligosaccharides from HMWDOM

To further investigate the composition of HMWDOM carbohydrates, we used Ag

and Pb cation exchange chromatography to separate oligosaccharides from

monosaccharides released by acid hydrolysis. The mechanism of separation is based on

the strength of the complex formed between the hydroxyl groups of the sugars and the

metal (Ag^+ or Pb^{2+}), and the technique easily separates glycans by their degree of

polymerization (DP) as shown in Fig. 1A (R_t monosaccharides: (glucose (**IV**)) **>**

disaccharides (maltose (**IX**)) > trisaccharides (maltotriose (**X**))> oligosaccharides

(maltopentaose (**XI**), maltoheptaose (**XII**))> polysaccharides (laminarin (**XIII**)).

Monosaccharides are resolved by the sequence of axial and equatorial (ax, eq) vicinal

hydroxyl groups that form the complexes with the metal cation (Angyal, 1989). The

column selectivity can be manipulated by changing the complexing cation (K^+, Ca^{2+},

Ag^+, Pb^{2+}) and resolution is generally improved when large cations are used (e.g. Pb^{2+} or

Ca^{2+}), because stronger complexes are formed.

Figure 1B depicts a typical chromatogram of hydrolyzed HMWDOM using two

Ag columns in series. Unhydrolyzed or partially hydrolyzed HMWDOM fractions F_1

(59%, 11-18.5 min) and F_2 (15%, 18.5-23.6 min) were eluted before disaccharides and

methyl sugars (F_3; 5%, 23.6-26.8 min), neutral sugars (F_4; 20%, 26.8-35 min), and other

hydrolysis products (F_5; 2%, 35-40 min). The seven major neutral sugars characteristic

of HMWDOM are eluted in fraction F_4, and collected in three subfractions (F_{4a}-F_{4c}).

Fraction F_{4a} includes glucose (27.8 min) and rhamnose (28.5 min), fraction F_{4b} includes

xylose (29.5 min), galactose (30.5 min) and mannose (30.5), while fraction F_{4c} contains fucose and arabinose which co-eluted at 33.1 min.

The ^{1}HNMR spectrum of fraction F_3 shows it to be a complex mixture of methylated, 6-deoxy- and 3-deoxysugars, and the fraction was further separated into 10 additional fractions using four Pb-cation exchange columns in series. The three largest peaks were collected as fractions F_3A-C (Fig. 2A) and were purified one more time using four Ag columns in series. Figure 2B depicts a typical separation of the F_3B fraction purification. Only the largest peaks were collected for detailed compositional analyses (see below).

3.2   Characterization of methyl, methyl-deoxy, and deoxysugars in HMWDOM

3.2.1 Fraction F_3A: Separation of fraction F_3A on four Ag columns yields three peaks. The major peak was collected as fraction F_3A2, and had an ^{1}HNMR spectrum with signals for the anomeric protons at δ 5.27 ppm (H-1, α anomer: $J_{1,2}$=3.6 Hz) and 4.69 ppm (H-1, β anomer, $J_{1,2}$=7.9 Hz), **H**-C-OH protons at 3.34-3.93 ppm and one *O*-methyl (-OCH$_3$) group at 3.65 ppm (Table 1). Protons H-2 through H-6 were assigned from COSY experiments, while HSQC experiments indicated the presence of seven carbon atoms consistent with an O-methylated sugar. On the basis of its NMR spectrum, fraction F_3A2 was identified as a mixture of α– and ß-3-*O*-methylglucose (**VIII**). Additional confirmation was made by examination of the first order coupling constants, and co-elution with authentic 3-*O*-methylglucose on HPLC using four Ag columns connected in series (retention time of 46.2 min).

Fraction F_3A3 was an α/β mixture of at least three different sugars. The major anomers appeared as a broad singlet at 5.34 ppm (α anomer) and 4.82 ppm (β anomer). We were unable to measure the coupling constant for the β anomer due to interference with the solvent peak (water, 4.80 ppm). We observed a sharp O-methyl singlet at 3.68 ppm, and the ^{1}H COSY NMR spectrum allowed us to partially assign two coupled spin systems: αH-1 (5.34 ppm) $\rightarrow$ αH-2 (4.29 ppm) $\rightarrow$ αH-3 (2.54, 1.88 ppm) $\rightarrow$$\alpha$H-4 (4.17 ppm) and βH-1 (4.82 ppm) $\rightarrow$ βH-2 (3.60 ppm) $\rightarrow$ βH3 (2.32, 1.67 ppm) $\rightarrow$ βH-4 (3.58 ppm) (Table 1). Unlike other sugar fractions we isolated from HMWDOM, for this sugar we observe strong cross peaks in the COSY spectrum between the equatorial proton (H_{eq}) αH-3$_{eq}$ at 2.59 ppm (14.9, 5.5 Hz), and the axial proton (H_{ax}) at αH-3$_{ax}$ at 1.90 ppm (13.0, 5.5 Hz) with αH-2 at 4.29 ppm, and between βH-3$_{eq}$ at 2.34 ppm (1.7, 14.4 Hz) and βH-3$_{ax}$ at 1.70 ppm (1.2, 12.5 Hz) with βH-2 at 3.35 ppm that are characteristic of a 3-deoxysugar. Figure 3 compares the ^{1}H COSY NMR spectrum for 3-deoxyglucose (top; **XIV**) and fraction F_3A3 (bottom), and highlights the presence of the **CH**$_2$ protons as cross peaks in the 3.5-4.5 ppm x1.5-2.6 ppm region (compare to Fig. 4). The large coupling constants of 12.5-14.4 Hz are characteristic of H_{eq}/H_{ax} coupling, while the smaller coupling constants are typical of H_{eq}/H_{ax} coupling to neighboring (H-2, H-4) protons. We were unable to identify the major compound in F_3A3 beyond the level of an O-methyl-3-deoxysugar. Comparison with the NMR spectra for authentic 3-deoxyglucose shows that F_3A3 is not O-methyl-3-deoxyglucose based on differences in cross peaks at 3.5-4.5 ppm x 1.5-2.6 ppm (Fig. 3). We also identified a third deoxysugar, which from the ^{1}H COSY NMR spectrum appears to have the system: βH-1 (4.69 ppm) $\rightarrow$ βH-2 (3.34 ppm) $\rightarrow$ βH-

3 (3.54 ppm) → βH-4 (2.62, 1.52 ppm). From the COSY spectrum, we assigned this as a

4-deoxysugar, but our assignment is not definitive.

     3.2.2 Fraction F₃B: Separation of F_3B on four Ag columns resulted in two major

peaks that were collected as fractions F_3B1 and F_3B2 (Fig. 2B). The ^{1}HNMR spectra of

the two fractions showed they were closely related. Fraction F_3B1 was obtained as a

mixture of α and β anomers, for which we were able to assign protons H 2-6 by the

COSY spectrum, and all carbons through HSQC experiments. The ^{1}HNMR spectrum of

fraction F_3B1 displayed signals for two anomeric protons at δ 5.21 ppm (H-1, α-anomer;

d, $J_{1,2}$=1.6 Hz), and 4.91 ppm (H-1, β-anomer; d, $J_{1,2}$=1.0 Hz), **H**-C-OH protons at 3.37-

4.25 ppm, one *O*-methyl group at 3.50 ppm, and two methyl groups at 1.32 ppm (αH-6,

$J_{5,6}$= 5.4 Hz) and 1.34 ppm (βH-6, $J_{5,6}$= 6.4 Hz) (Table 1). We noted the very small

coupling constant for the β anomer, the α/β anomeric ratio of > 1, and the smaller

coupling constant of $J_{5,6}$ for the β relative to the α anomer, which are all characteristics

of rhamnose (**VI**). First order coupling constants for α F_3B1 (H-1, 1.6 Hz; H-2, 3.6 Hz;

H-3, 9.7 Hz; H-4, 9.7 Hz; H-5, 6.4 Hz) were within 1-2 Hz of authentic rhamnose (α-6-

deoxymannose) reported by Bock and Thøgersen (1985) (H-1, 1.6 Hz; H-2, 3.5 Hz; H-3,

9.5 Hz; H-4, 9.5 Hz; H-5, 6.2 Hz). We also noted the approximately 10 ppm downfield

shift in the HSQC spectrum for C-3 (αC-3, 79.9; βC-3, 82.3) relative to rhamnose (αC-3,

71.1; βC-3, 73.8 ppm), indicating methylation at this site (Deepak et al., 2003). In the

HSQC spectrum we observed a correlation between βH-3 (3.37 ppm) and a carbon at

82.4 ppm, which we assigned as βC-3, further establishing the site of methylation. There

is a cross peak between H-4 (3.45 ppm) and a carbon at 71.4 ppm, which we assigned as

βC-4, in good agreement with the values assigned by Popper and Fry (2003) and Popper

et al. (2004) for 3-*O*-methylrhamnose released by hydrolysis of lower land plant primary cell walls. We therefore identified F_3B1 as a mixture of α– and β-3-*O*-methylrhamnose (**XV**; Table 1). Mass spectrometry of F_3B1 showed a molecular ion at 176 Da in agreement with our identification. However, for αF_3B1, protons αH-3 and αH-4 have the same chemical shift, and for this anomer, we are unable to establish the site of *O*-methylation (3-*O*-methyl *vs* 4-*O*-methyl). Our assignment of 3-*O*-methylation therefore rests on our assignments for the β anomer.

We used the same reasoning to identify F_3B2 as a α/ß mixture of 2-*O*-methylrhamnose (**XVI**) (Zdorovenko et al. 2001; Schäffer et al. 2002). Fractions F_3B1 and F_3B2 have very similar NMR spectra, but fraction F_3B2 was obtained as a mixture of at least four different sugars, leading to some ambiguities in our interpretation of the COSY and HSQC spectra. We were only able to make full assignments on the most abundant α anomer (α F_3B2). However, due to the complexity of the spectrum, our assignments of H-3 to H-5 should be viewed as tentative. We found two anomeric protons at 5.32 ppm (H-1, α-anomer; d, $J_{1,2}$=1.6 Hz) and 4.92 ppm (H-1, β-anomer; broad singlet), **H**-C-OH protons at 3.39-3.87 ppm, one O-methyl group at 3.51 ppm, and two methyl groups at 1.31 ppm ($J_{5,6}$ = 5.3 Hz) and 1.33 ppm ($J_{5,6}$= 6.7 Hz) for the α and β anomers respectively. We found good agreement between our spectral data and 2-*O*-methylrhamnose as the terminal sugar in a glycan polymer (Schäffer et al., 2002). Because the 2-*O*-methylrhamnose reported by Schäffer et al. (2002) is glycosylated at C-1, we expect a difference in the chemical shift for H-1, and for the C-1 chemical shift to appear downfield by 6-7 ppm of our value for F_3B1 (Deepak et al., 2003). Other ^{1}H and ^{13}C values should remain near those for α-2-*O*-methylrhamnose (Table 1). 2-*O*-

methlyrhamnose was previously identified in HMWDOM from the NE Atlantic Ocean by gas chromatography mass spectrometry of the alditol peracetate (Aluwihare et al., 2002). The mass spectrum of fraction F$_3$B2 has a molecular ion at 176 Da, as expected for a methylated deoxysugar.

   3.2.3 Fraction F$_3$C: Separation of F$_3$C on four Ag columns yielded one large peak that was collected as fraction F$_3$C1. The ^{1}HNMR spectrum has major signals for the anomeric protons at δ 5.49 ppm (H-1, α anomer: $J_{1,2}$=1.4 Hz) and 4.64 ppm (H-1, β anomer, $J_{1,2}$=8.0 Hz), **H**-C-OH protons at 3.23-4.24 ppm, two *O*-methyl groups at 3.54 and 3.66 ppm, and two methyl groups at 1.26 and 1.29 ppm ($J_{5,6}$= 6.5 Hz; Table 1). The strong coupling between the methyl group at 1.26 ppm and the upfield quartet ($J_{5,6}$ = 6.5 Hz,) at 4.24 ppm is typical of an α fucose-like configuration. Protons H-2 through H-6 were assigned from COSY experiments, while HSQC experiments indicated the presence of fourteen carbon atoms consistent with a mixture of α and β anomers of methylfucose (Fig. 4). In Table 1 we compare chemical shift data for F$_3$C1 with ^{1}HNMR data we acquired for authentic fucose. We find excellent agreement between fucose and F$_3$C1, with the greatest difference at H-2, suggesting 2-*O*-methylation in F$_3$C1. We measured first order coupling constants for βF$_3$C1 of H-1 (8.0 Hz), H-2 (9.6 Hz), H-3 (3.4 Hz), H-4 (1 Hz), H-5 (6.6 Hz) compared to H-1 (8.2 Hz), H-2 (10.0 Hz), H-3 (3.6 Hz), H-4 (0.8 Hz), H-5 (6.6 Hz) for authentic β-fucose (Bock and Thøgersen, 1983). Integration of the *O*-methyl and 6-methyl groups gave a 1:1 quantitative relationship, and we note that C-2 signals (α C-2 = 77.7; β C-2 = 81.9) are shifted approximately 10 ppm downfield from C-2 in fucose (α C-2 = 68.6 ppm, β C-2 = 72.2 ppm), confirming O-methylation at C-2. On the basis of its NMR spectrum, we identified F$_3$C1 as a mixture of α and β 2-*O*-

methylfucose (**XVII**). To the best of our knowledge, there are no reports of 2-*O*-methylfucose ^{1}H- and ^{13}CNMR spectra with which to compare our data. However, NMR data for 2-*O*-methylfucosyl-containing heptasaccharide isolated from red wine rhamnogalacturonan II are in good agreement with our values (Glushka et al. 2003; Table 1).

3. 3   O-methyl and deoxysugars in structural polysaccharides

2-*O*-methylrhamnose and 3-*O*-methylrhamnose have been reported in structural polysaccharides from a large number of cyanobacteria (Painter, 1983; Shekharam et al. 1987; Ogawa et al. 1997), and vascular plants (Bacon and Cheshire, 1971; Casagrande et al. 1985; Popper and Fry, 2003; Popper et al. 2004; Sassaki et al., 2005). These two sugars, together with 3-*O*-methylglucose and 2-*O*-methylfucose are common in lipopolysaccharides, part of the outer cell membrane of gram-negative bacteria (Kennedy and White, 1983; Brade et al. 1988). 2-*O*-methylfucose is also a constituent of rhamnogalacturonan II pectic polysaccharides, part of the primary walls of dicotyledenous and monocotyledenous plants and gymnosperms (Glushka et al., 2003). 2- and 3-*O*-methylrhamnose along with 2- and 3-*O*-methylfucose have been putatively reported in cyanobacterial mats and in recent marine sediments, where they comprise up to 5-15 wt% of the total neutral monosaccharide yield (Klok et al. 1983; 1984), and in humic and fulvic acids extracted from soils (Ogner, 1980). Given the wide distribution of these methylsugars in biological and geological materials, they provide little information about the specific source of HMWDOM carbohydrate. However, to the best of our knowledge these methylsugars have only been reported to occur in structural

polysaccharides, and not in storage polysaccharides. Their occurrence in HMWDOM suggests that specific degradation resistant structural polysaccharides contribute to DOM.

Only one methyl sugar, 2-O-methylrhamnose, has been previously reported as a trace constituent in HMWDOM (Aluwihare et al., 2002), and we are not aware of reports of 3-deoxysugars in HMWDOM. Methyl and 6-deoxysugars may nevertheless be important in the uncharacterized fraction of HMWDOM, and might serve to give the ^{1}HNMR spectrum of HMWDOM its characteristic appearance (Aluwihare et al. 1997). Fractions F_1 and F_2 from our Ag column separation of acid hydrolyzed HMWDOM were eluted with $\geq$ DP 3 glucans, which have a nominal molecular weight of >0.5 kD (Fig. 1B). ^{1}HNMR spectra of F_1 (Fig. 5) and F_2 display none of the spectral fine structure characteristic of DP 1-4 mono- and oligosaccharides that would be expected if these fractions contained significant amounts of degraded simple sugars. The two fractions represent 74% of the total HMWDOM, demonstrating that commonly used hydrolytic protocols (2N TFA; 1-6N HCl (Repeta unpublished results)) do not depolymerize most HMWDOM. The recalcitrance of HMWDOM carbohydrate towards acid hydrolysis explains most of the discrepancy between NMR measurements of HMWDOM carbohydrate and GC or HPLC measurements of simple sugars. Previous GC and HPLC analyses of HMWDOM hydrolysis products have also missed methyl sugars identified in our current study, further lowering the amount of carbohydrate reported by these methods.

3.4   Hydrolysis resistant carbohydrate in HMWDOM

Qualitative information on the composition of HMWDOM carbohydrate that is resistant to acid hydrolysis can be obtained from NMR spectra of F_1 fraction. The COSY

spectrum of hydrolyzed HMWDOM fraction F_1 (Fig. 1B) shows strong cross peaks for 6-deoxysugars (1.2 to1.4 ppm x 3.3 to 4.4 ppm; Fig. 5). The most intense cross peaks appear at 1.34 x 4.32 ppm and 1.23 x 3.79 ppm which are similar to α and β fucose (1.26 x 4.24 ppm and 1.29 x 3.83 ppm respectively). The COSY spectrum further shows at least eight distinct α anomeric, and four distinct β anomeric protons. These protons could represent a mixture of different α- and β-linked sugars or positional isomers, but nevertheless suggest a structurally complex polysaccharide.

We did not observe cross peaks in the COSY spectrum that are characteristic of lipids. No cross peaks were observed between 1.3 ppm (-CH_2-) and signals < 1 ppm (CH_3) or 1.5 ppm to 3 ppm (-CH_2-OH; -CH_2-COOH) that we expect to see if ether or ester bound lipids were present in the sample. The COSY spectrum of HMWDOM does not suggest a major contribution by lipids to the peak at 1.3 ppm in the ^{1}HNMR spectrum. In support of this, Quan (2005) noted the production of acetic acid as major oxidation product of previously hydrolyzed HMWDOM (F_1) treated with sodium periodate, and ascribed this to the oxidation of 6-deoxysugars at C-5. Quan (2005) did not recover lipids from HMWDOM periodate oxidation products, even though lipids should have survived the reaction conditions. Quan's data support the COSY NMR assessment that lipids are only a minor component of HMWDOM. If we assume that all the carbon represented by the 1.3 ppm peak is from 6-deoxysugars, then 6-deoxysugars represent 25-30% of the total uncharacterized HMWDOM carbohydrates.

4. CONCLUSIONS

Previous characterization of HMWDOM by DT-MS has shown that HMWDOM carbohydrate includes methyl- and methyl-deoxysugars. We confirm these results with the identification of 2- and 3-*O*-methylrhamnose, 2-*O*-methylfucose, and 3-*O*-methylgucose in HMWDOM. We also isolated fractions that have NMR spectral characteristics of 3- and 4-deoxysugars, although the full structure of these components could not be determined. We are not aware of reports of *O*-methylrhamnose and *O*-methylfucose in algal or bacterial storage polysaccharides, but these sugars are commonly found in structural polysaccharides from higher plant cell walls, and our analyses point to degradation resistant cell wall polysaccharides as an important component of HMWDOM. Separation of HMWDOM after acid hydrolysis using Ag^+ and Pb^{2+} cation exchange chromatography show that most carbohydrate is not depolymerized by commonly used hydrolysis protocols. New strategies need to be developed if HMWDOM is to be characterized at the level of constituent simple sugars. 2-D NMR techniques provide some insights into the composition of the nonhydrolyzed component, and show it to be a heterogeneous mixture containing up to 25-30% 6-deoxysugars, most likely rhamnose and fucose.

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REFERENCES

Aluwihare, L. I., Repeta, D. J., Chen, R. F., 1997. A major biopolymeric component to dissolved organic carbon in surface water. Nature 387, 166-169.

Aluwihare, L. I., Repeta, D. J., Chen, R. F., 2002. Chemical composition and cycling of dissolved organic matter in the Mid-Atlantic Bight. Deep-Sea Research II 49, 4432-4437.

Aluwihare, L. I., Repeta, D. J., Pantoja, S., Johnson, C. G., 2005. Two chemically distinct pools of organic nitrogen accumulate in the Ocean. Science 308, 1007-1010.

Angyal, S. J., 1989. Complexes of metal cations with carbohydrates in solution. Advances in Carbohydrate Biochemistry 47, 1-43.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L., Thingstand, F., 1983. The ecological role of water column microbes in the sea. Marine Ecology Progress Series 10, 257-263.

Bacon J. S. D., Cheshire M. V., 1971. Apiose and mono-O-methyl sugars as minor constituents of the leaves of deciduous trees and various other species. Biochemical Journal 124, 555-562.

Benner, R., 2002. Chemical composition and reactivity. In: Hansell, D., Carlson, C. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, pp. 59-90.

Benner, R., Pakulski, J. D., McCarthy, M., Hedges, J. I., Hatcher, P. G., 1992. Bulk chemical characteristics of dissolved organic matter in the ocean. Science 255, 1562-1564.

Bock, K., Thøgersen, H., 1985. Nuclear magnetic resonance spectroscopy in the study of

    mono- and oligosaccharides. Annual Reports on NMR Spectroscopy 13**,** 1-57.

Boon, J. J., Klap, V.A ., Eglinton, T. I., 1998. Molecular characterization of microgram

    amounts of oceanic colloid organic matter by direct temperature-resolved

    ammonia chemical ionization mass spectrometry. Organic Geochemistry 29**,**

    1051-1061.

Borch, N. H., Kirchman, D. L., 1997. Concentration and composition of dissolved

    combined neutral sugars (polysaccharides) in seawater determined by HPLC-

    PAD. Marine Chemistry 57**,** 85-95.

Brade, H., Brade, L., Rietschel, E. TH.,1988. Structure-activity relationships of bacterial

    lipopolysaccharides (endotoxins). Zentralblatt fur Bakteriologie Mikrobiologie

    und Hygiene A. 268**,** 151-179.

Bronk, D., 2002. Dynamics of DON. In: Hansell, D., Carlson, C. (Eds.),

    Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, pp. 153-

    247.

Casagrande, D. J., Ferguson, A., Boudreau, J., Predny, R., Folden, C., 1985. Organic

    geochemical investigations in the Okefenokee swamp, Georgia: The fate of fatty

    acids, glucosamine, cellulose, and lignin. In : Cross, T. (Ed.), Proceedings of the

    9eme Congres International de Stratigraphie et de Géologie du Carbonifere, 1979.

    pp. 193-204.

Deepak, D., Arun, S., Sapna, S. S., Anakshi, K., Sanjay. S., 2003. Structure elucidation

    of oligosaccharides containing 2-deoxy sugars by NMR techniques.Trends in

    Carbohydrate Chemistry 4**,** 25-32.

Ducklow, H, W., Carlson, C. A., 1992. Oceanic bacterial production. In: Marshall, K. C.
(Ed.), Advances in Microbial Ecology. Plenum Press, pp. 113-181.

Eglinton, T.I., Repeta, D.J., 2004. Marine Organic Geochemistry. In : Elderfield, H. (Ed.),
Treatise on Geochemistry, Volume 7. Elsevier, pp. 145-180.

Glushka, J. N., Terrell, M., York, W. S., Malcolm, A. O'Neill., Gucwa, A., Darvill, A. G.,
Albersheim, P., Prestegard, J. H., 2003. Primary structure of 2-O-methyl-α-L-
fucose-containing side chain of the pectic polysaccharide, rhamnogalacturonan II.
Carbohydrate Research 338, 341-352.

Guo, L., and Santschi, P. H., 1997. Composition and cycling of colloids in marine
environments. Reviews of Geophysics 35, 17-40.

Hedges, J. I., Baldock, J. A., Gélinas, Y., Lee, C., Peterson, M., Wakeham. S. G., 2001.
Evidence for non-selective preservation of organic matter in sinking marine
particles. Nature 409, 801-804.

Hedges, J. I., Baldock, J. A., Gélinas, Y., Lee, C., Peterson, M., Wakeham. S. G., 2002.
The biochemical and elemental compositions of marine plankton: A NMR
perspective. Marine Chemistry 78, 47-63.

Karl, D. M., Björkman, K. M., 2002. Dynamics of DOP. In: Hansell, D., Carlson, C.
(Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press,
pp. 249-366.

Kennedy, J. F., White. C. A., 1983. Bioreactive carbohydrates. In: Horwood, E. (Ed.),
Chemistry, Biochemistry and Biology of Carbohydrates. Halsted Press, pp. 331.

Klok, J., van der Knaap, J. M. M., de Leeuw, J. W., Cox, H. C. Schenck, P. A., 1983.
Qualitative and Quantitative characterization of the total organic matter in a recent

marine sediment. In: M. Bjory (Ed.), Advances in Organic geochemistry. J. Wiley
& sons Ltd., pp. 813-818.

Klok, J., Cox, H. C., Baas, M., de Leeuw, J. W., Schenck. P. A., 1984. Carbohydrates in
recent marine sediments-II. Occurrence and fate of carbohydrates in a recent
stromatolitic deposit: Solar lake, Sinai. Organic Geochemistry 7**,** 101-109.

Lee, C., Bada, J.L., 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso
Sea, and Biscayne Bay. Limnology Oceanography 22, 502-510.

Mannino, A., Harvey, H.R., 1999. Lipid composition in particulate and dissolved organic
matter in the Delaware Estuary: Sources and diagenetic patterns. Geochimica et
Cosmochimica Acta 63**,** 2219-2235.

McCarthy, M., Hedges, J. I., Benner, R., 1996. Major biochemical composition of
dissolved high molecular weight organic matter in seawater. Marine Chemistry
55**,** 281-297.

Minor, E. C., Boon, J. J., Harvey, H. R., Mannino, A., 2001. Estuarine organic matter
composition as probed by direct temperature-resolved mass spectrometry and
traditional geochemical techniques. Geochimica et Cosmochimica Acta 65**,** 2819-
2834.

Minor, E. C., Simpouw, J-P., Boon, J. J., Kerkhoff, A. E., van der Horst, J., 2002.
Estuarine/marine UDOM as characterized by size-exclusion chromatography and
organic mass spectrometry. Marine Chemistry 78**,** 75-102.

Mopper, K., Kieber, D. J., 2002. Photochemistry and the cycling of carbon, sulfur,
nitrogen and phosphorus. In: Hansell, D., Carlson, C. (Eds.), Biogeochemistry of
Marine Dissolved Organic Matter. Academic Press, pp. 455-489.

Nelson, N.B., and Siegel, D.A., 2002. Chromophoric DOM in the Open Ocean. In: Hansell, D., Carlson, C. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, pp. 547-578.

Nelson, N. B., Siegel, D. A., Michaels, A. F., 1998. Seasonal dynamics of colored dissolved material in the Sargasso Sea. Deep-Sea Research I. 45, 931-957.

Ogawa, K., Yamaura, M., Maruyama, I., 1997. Isolation and identification of 2-O-methyl-L-rhamnose and 3-O-methyl-L-rhamnose as constituents of an acidic polysaccharide of Chlorella vulgaris. Bioscience, Biotechnology and Biochemistry 61, 539-540.

Ogner, G., 1980. Analysis of the carbohydrates of fulvic and humic acids as their partially methylated alditol acetates. Geoderma 23, 1-10.

Painter, T. J., 1983. Algal Polysaccharides. In : Aspinall, G.O. (Ed.), The Polysaccharides Vol. 2. Academic Press, pp. 195–285.

Panagiotopoulos, C., Sempéré, R., 2005. Analytical methods for the determination of sugars in marine samples: A historical perspective and future directions. Limnology Oceanography Methods 3, 419-454.

Popper, Z. A., Fry, S. C., 2003. Primary Cell Wall Composition of Bryophytes and Charophytes. Annals of Botany 91, 1-12.

Popper, Z. A., Sadler, I. H, and Fry, S. C., 2004. 3-O-Methylrhamnose in lower land plant primary cell walls. Biochemical Systematics and Ecology 32, 279-289.

Quan, T. M., 2005. Chemical Characterization of Dissolved Organic Matter (DOM) in Seawater: Structure, Cycling, and the Role of Biology. Ph.D. thesis. MIT/WHOI.

Rue, E. L., and Bruland, K. W., 1997. The role of organic complexation on ambient iron chemistry in the Equatorial Pacific Ocean and the response of a mesoscale iron addition experiment. Limnology Oceanography 42, 901-910.

Sakugawa, H., and Handa. N., 1985. Chemical studies on dissolved carbohydrates in water samples collected from North Pacific and Bering Sea. Oceanologica Acta 8, 185-196.

Sassaki, G. L., Gorin, P. A. J., Reis, R. A., Serrato, R. V., Elífio, S. L., and Iacomini, M., 2005. Carbohydrate, glycolipid, and lipid components from the photobiont (Scytonema sp.) of the lichen, *Dictyomema glabratum*. Carbohydrate Reserach 340, 1808-1817.

Schäffer, C., Wugeditsch, T., Kählig, H., Scheberl, A., Zayni, S., Messner, P., 2002. The surface layer (S-layer) glycoprotein of *geobacillus stearothermophilus* NRS 2004/3a. Journal Biological Chemistry 277**,** 6230-6239.

Shekharam, K. M., Venkataraman, L .V., Salimath, P. V., 1987. Carbohydrate composition and characterization of two unusual sugars from the blue green alga, *spirulina platensis.* Phytochemistry 26**,** 2267-2269.

Wang, X-C., Altabet, M.A., Callahan, J., Chen, R. F., 2004. Stable carbon isotopic compositions of high molecular weight dissolved organic matter from four U.S. estuaries. Geochimica et Cosmochimica Acta 68**,** 2681-2691.

Wells, M. L., 2002. Marine colloids and trace metals. In: Hansell, D., Carlson, C. (Eds), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, pp. 367-404.

Wicks, R. J., Moran, M. A., Pittman, L. J., and Hodson, R. E., 1991. Carbohydrate signatures of aquatic macrophytes and their dissolved degradation products as

determined by a sensitive high-performance ion Chromatography method. Applied Environmental Microbiology 57, 3135-3143.

Williams, P. J. le B., 1981. Microbial contribution to overall marine plankton metabolism: Direct measurements of respiration. Oceanologica Acta 4, 359-364.

Zdorovenko, E. L., Ovod, V. V., Zatonsky, G. V., Shashkov, A. S., Kocharova, N. A., Knirel, Y. A., 2001. Location of the O-methyl groups in the O polysaccharide of *Pseudomonas syringae* pv. phaseolicola. Carbohydrate Research 330**,** 505-510.

TABLE AND FIGURE CAPTIONS

Table 1. ^{1}H and ^{13}CNMR chemical shifts for HMWDOM sugars. Numbering of C 1-6 and H1-6 are given in Appendix 1. Chemical shifts for fractions isolated from HMWDOM are given in bold face type.

Figure 1. (A) Separation of glucose (**IV**; 28.2 min), maltose (**IX**; 22.6 min), maltotriose (**X**; 19.3 min), maltopentaose (**XI**; 15.8 min), maltohepatose (**XII**; 14.2 min), and laminarin (**XIII**; 12.8 min) by Ag$^+$ cation exchange chromatography at 80 ºC using two columns connected in series. Sugars were eluted with high purity water at 0.5 ml min^{-1} and detected with a refractive index (RI) detector. (B) Typical separation of hydrolyzed HMWDOM using the same columns and conditions.

Figure 2. (A) Separation of fraction 3 (F3; Fig. 1B) by cation exchange HPLC-RI. Analytical conditions were four Pb columns in series; column temperature, elution mode, flow rate, and detection were the same as described for Fig. 1. (B) Separation of the F$_3$B fraction on four Ag columns in series. The figure shows an expansion of the 35-55 min section of the analysis. No peaks were detected before 35 min. Analytical conditions were the same as described in Figure 1.

Figure 3. ^{1}HNMR COSY spectra of 3-deoxyglucose (top panel) and fraction F$_3$A2 (bottom panel). The 1-D ^{1}HNMR spectrum of 3-deoxyglucose (**XIV**) is projected on the

X and Y axes of the top panel for reference. Cross peaks due to the coupling of axial and equatorial protons H-3 between 1.5-2.6 ppm are characteristic of 2-, 3-, and 4-deoxysugars. Cross peaks in this region of the spectrum are not observed in the spectra of hexoses (glucose, galactose) and 6-deoxysugars (see Fig. 4 for comparison).

Figure 4. ^{1}HNMR COSY spectra (top) and HSQC spectrum (bottom) of fraction F_3C1 (2-*O*-methylfucose; **XVII**). The 1-D ^{1}HNMR spectrum of F_3C1 is projected on the X and Y axes of the top panel for reference. Cross peaks in the COSY spectrum are due to coupling between adjacent protons and are used to assign signals in the 1-D spectrum. ^{1}HNMR spectra assignments are then used to determine the carbon chemical shifts in the HSQC spectrum (lower panel).

Figure 5. ^{1}HNMR COSY spectrum of fraction F1 (11.8-18.5 min). The spectrum displays numerous cross peaks for α and β anomeric protons (αH-1, 5.23-5.52; βH-1, 4.54-4.64) and α/βH-2 protons (3.22-3.80 ppm) indicate heterogeneity in either the monosaccharide composition or glycosidic linkages of HMWDOM carbohydrate. Strong cross peaks are also apparent between 1.23-1.42 ppm and 3.79-4.38 ppm from 6-deoxysugars that are part of the nonhydrolyzable fraction of HMWDOM carbohydrate.

Appendix 1. Chemical structures of methyl, deoxy, and methyl-deoxysugars discussed in the text. Postions 1-6 are noted in bold numerals on structures **II** and **XVII** . H_{ax} and H_{eq} in ß--3-deoxyglucose (**XIV**) denote the configuration (axial and equatorial) of the H-3 protons.

Table 1

Fraction/Compound	Nucleus	1	2	3	4	5	6; 6'	*O*-methyl
α-F3-A2	**H**	**5.27**	**3.63**	**3.53**	**3.53**	**3.88**	**3.88; 3.81**	**3.65**
	C	**92.5**	**71.4**	**83.1**	**69.4**	**71.9**	**60.9**	**60.3**
3-O-methyl-α-glucose[1]	H	5.25	3.64	3.53	3.53	3.89	3.88; 3.81	3.68
	C	92.4	71.4	83.1	69.4	71.9	60.9	60.3
β-F3-A2	**H**	**4.69**	**3.34**	**3.34**	**3.53**	**3.53**	**3.93; 378**	**3.65**
	C	**96.3**	**73.9**	**85.8**	**69.4**	**76.3**	**61.1**	**60.3**
3-O-methy-β-glucose[1]	H	4.70	3.35	3.34	3.53	3.53	3.94; 3.77	3.68
	C	96.3	73.9	85.7	69.4	76.2	61.1	60.3
α-F3-A3	**H**	**5.34**	9	4; 1.88[2] **4.17**		**ND**	**ND**	**3.68**
	C	**92.9**						**60.5**
β-F3-A3	**H**	**4.82**	**3.60**	2; 1.67[2] **3.58**		**ND**	**ND**	**3.68**
α-F3-B1	**H**	**5.21**	**4.23**	**3.53**	**3.53**	**3.94**	**1.32**	**3.50**
	C	**94.3**	**66.8**	**79.9**	**71.6**	**68.8**	**17.2**	**56.5**
α-3-*O*-methylrhamnose[3]	H	5.16	4.18	3.48	3.46	3.89	1.27	3.45
	C	94.3	66.9	79.9	71.6	68.7	17.2	56.5
β-F3-B1	**H**	**4.91**	**4.25**	**3.37**	**3.45**	**3.47**	**1.34**	**3.50**
	C	**94.0**	**67.3**	**82.4**	**72.5**	**ND**	**17.2**	**56.5**
β-3-*O*-methylrhamnose[3]	H	4.86	4.18	3.31	3.39	3.41	1.29	3.45
	C	93.9	67.4	82.3	71.3	72.5	17.2	56.5
α-F3-B2	**H**	**5.32**	**3.60**	**3.87**	**3.39**	**3.87**	**1.30**	**3.51**
	C	**91.1**	**81.2**	**70.1**	**73.0**	**68.6**	**17.2**	**59.1**
α-2-*O*-methylrhamnose[4]	H	5.17	3.70	3.89	3.37	3.81	1.26	3.45
	C	99.8	81.0	70.6	73.1	70.0	17.4	59.4
α-F3-C1	**H**	**5.49**	**3.52**	**3.93**	**3.86**	**4.24**	**1.26**	**3.54**
	C	**89.8**	**77.7**	**68.9**	**72.4**	**66.6**	**15.8**	**57.8**
α-2-O-methylfucose[5]	H	5.65	3.45	4.00	3.81	4.37	1.17	3.47
	C	97.6	79.9	70.7	74.4	68.8	17.9	59.8
α-fucose[6]	H	5.25	3.83	3.92	3.85	4.25	1.27	
	C	92.7	68.5	69.7	71.3	66.8	16.0	

β-F3-C1	**H**	**4.64**	**3.23**	**3.73**	**3.79**	**3.83**	**1.29**	**3.66**
	C	**96.4**	**81.9**	**73.0**	**71.9**	**71.2**	**15.8**	**60.6**
β-fucose[6]	H	4.60	3.50	3.70	3.79	3.86	1.29	
	C	96.7	72.2	73.4	71.9	72.3	16.0	

1. From authentic 3-*O*-methylglucose.
2. H(equatorial); H(axial)
3. Popper et al. (2004) [Data for pure α and β-3-*O*-methylrhamnose; trivial name acofriose].
4. Schäffer et al. (2003) [Data for α-2-*O*-methylrhamnose as terminal sugar in a glycan polymer].
5. Glushka et al. (2003) [Data for α-2-*O*-methylfucoheptasaccharide].
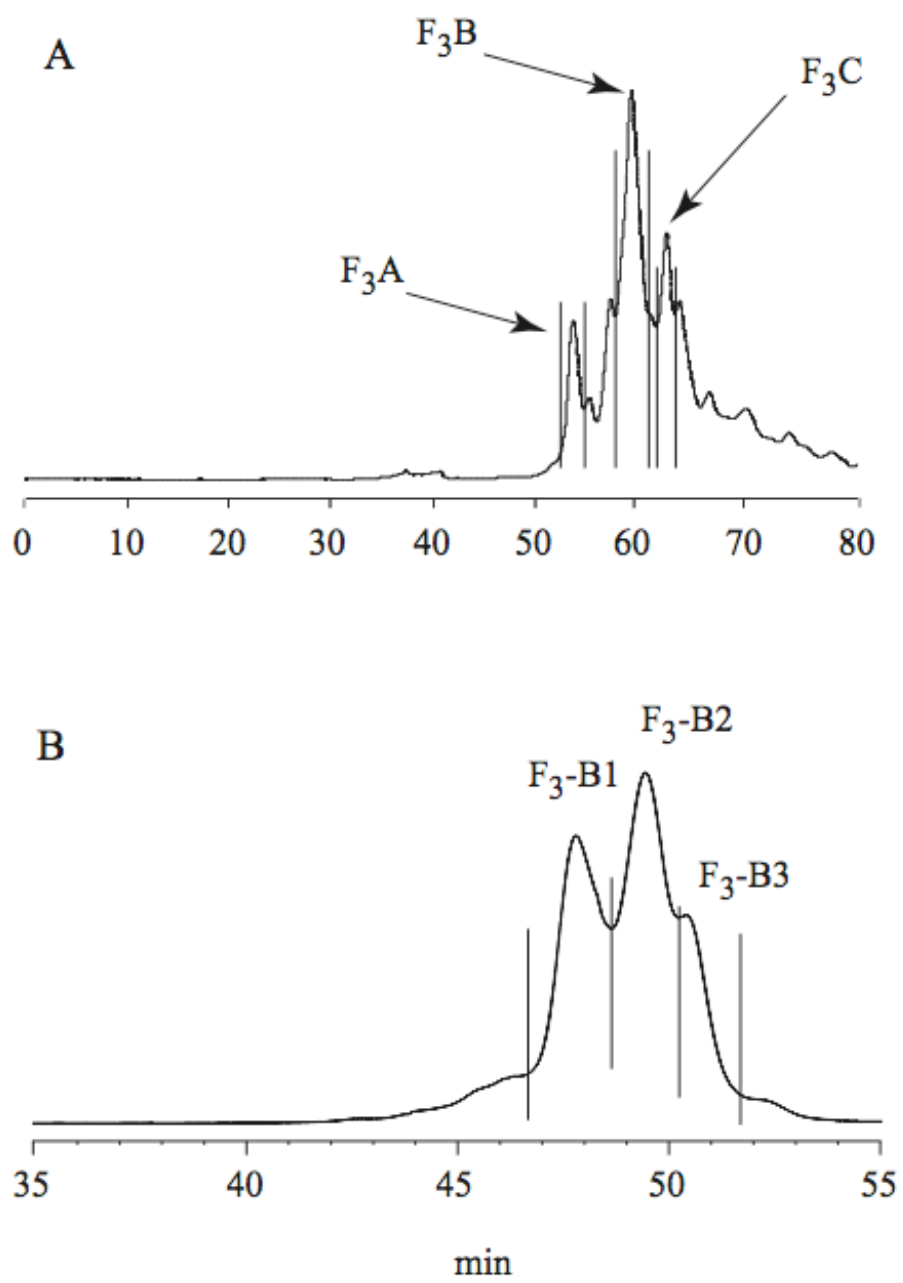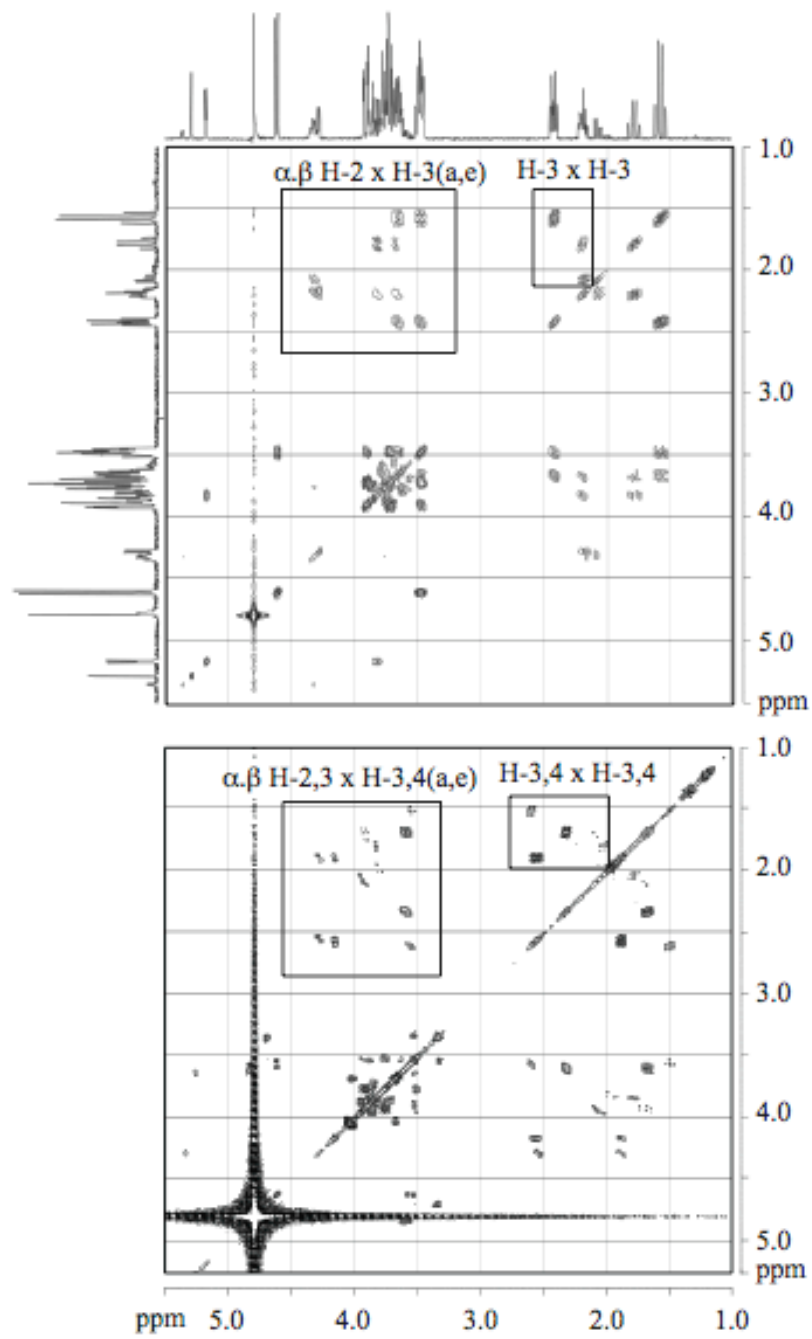6. From authentic fucose.
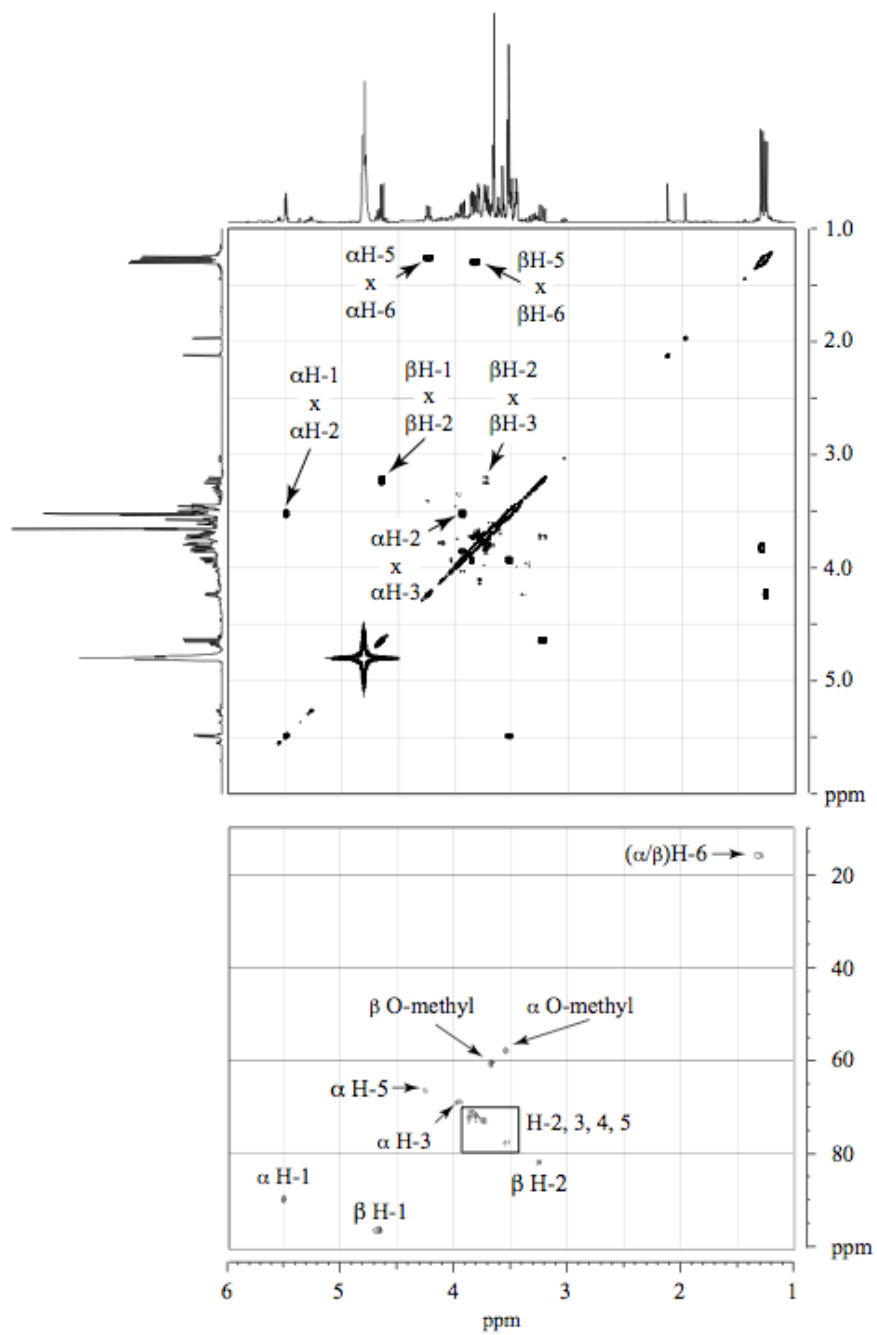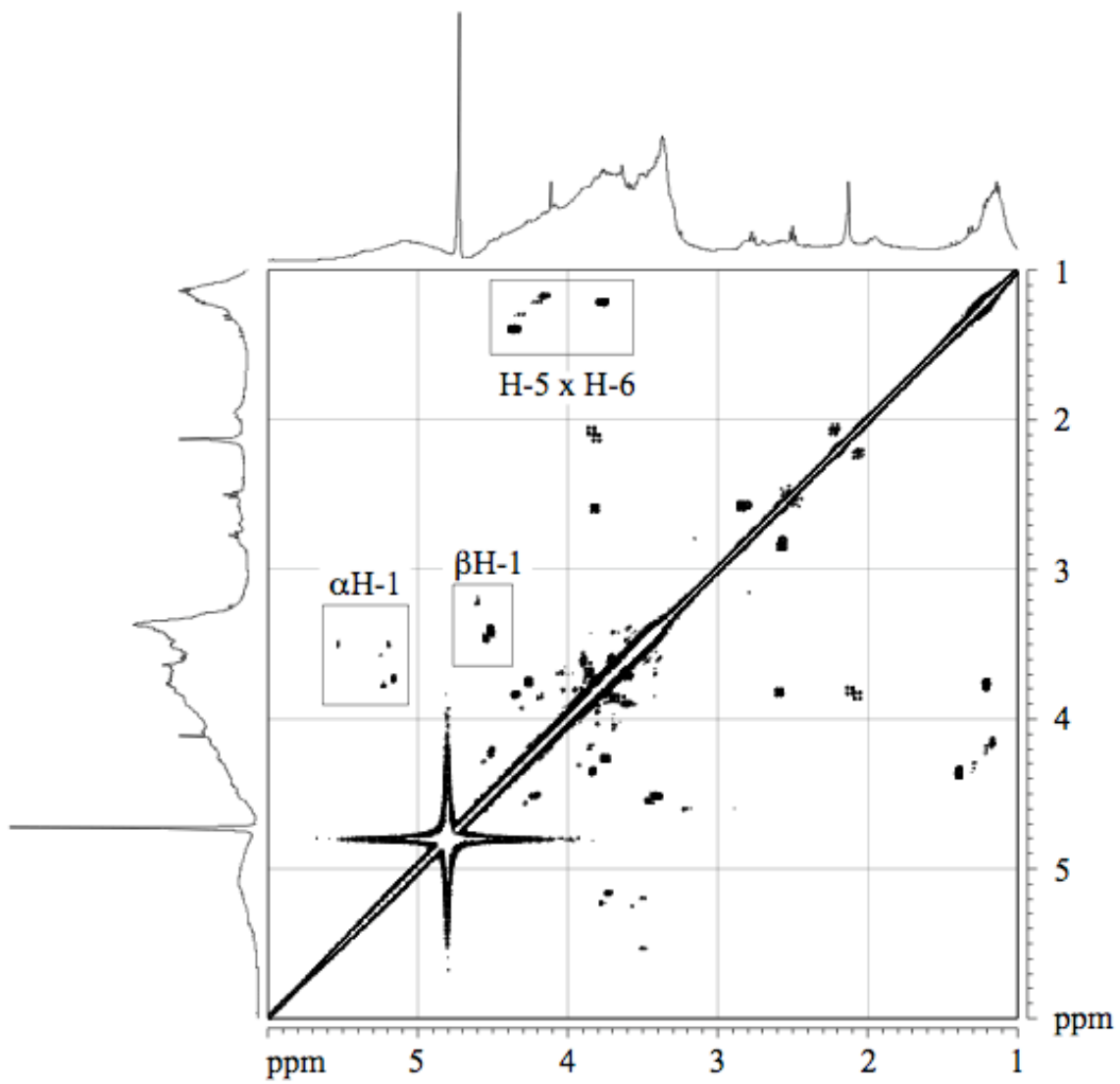7. ND not determined
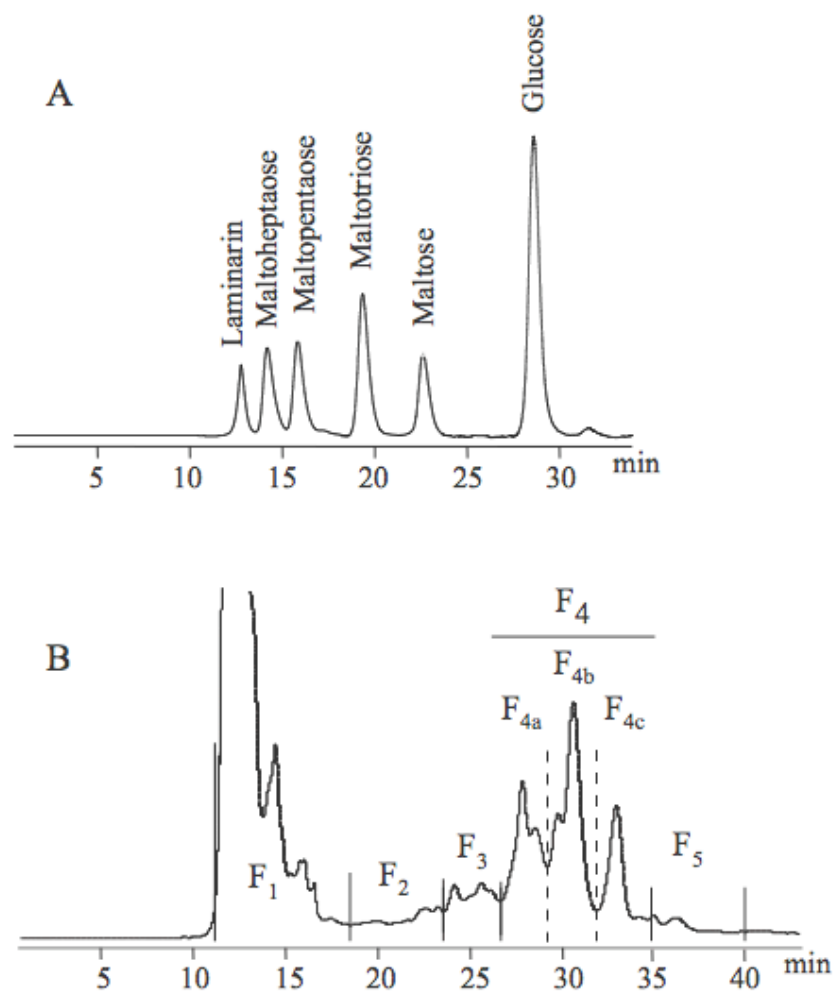
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5