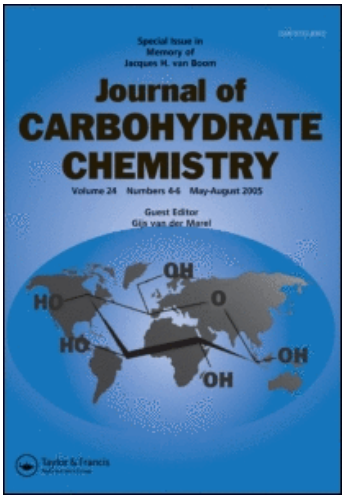

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Hellebrandt, Wilfried , Haselhorst, Thomas , Köhli, Thies , Bäuml, Englbert and Peters, Thomas(2000) 'Deuterated Disaccharides for the Investigation of Protein-Carbohydrate Interactions-Application of Bioaffinity-and STD-NMR', Journal of Carbohydrate Chemistry, 19: 6, 769 — 782
To link to this Article: DOI: 10.1080/07328300008544115
URL: http://dx.doi.org/10.1080/07328300008544115

J. CARBOHYDRATE CHEMISTRY, 19(6), 769–782 (2000)

DEUTERATED DISACCHARIDES FOR THE INVESTIGATION OF PROTEIN-CARBOHYDRATE INTERACTIONS - APPLICATION OF BIO-AFFINITY- AND STD-NMR

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Received July 23, 1999 - Final Form March 16, 2000

ABSTRACT

NMR spectroscopic analysis of carbohydrates often suffers from severe overlap of resonance signals, especially in ^{1}H NMR spectra. Therefore, we synthesized four 2,3,4-trideuterio-α-L-fucose containing disaccharides, α-L-Fuc-(1→6)-β-D-GlcNAc-OMe **1**, α-L-Fuc-(1→4)-β-D-GlcNAc-OMe **2**, α-L-Fuc-(1→3)-β-D-GlcNAc-OMe **3**, and α-L-Fuc-(1→2)-β-D-Gal-OMe **4**. Compounds **1** to **4** are well suited to be subjected to NMR conformational analysis because their ^{1}H NMR spectra show almost no overlap of signals. The deuterated disaccharides **1** to **4** will therefore be used as NMR probes for the exploration of fucose-binding proteins. With a mixture of the corresponding non-deuterated disaccharides it is demonstrated that recently developed parallel NMR screening protocols, Bio-Affinity NMR and STD-NMR, deliver fast and robust tools to assay the compounds synthesized for protein-binding affinity.

INTRODUCTION

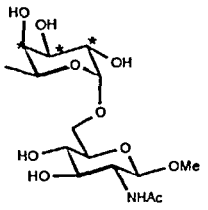
Carbohydrate ligands are involved in a number of biological processes that are based on molecular recognition reactions.[1] It is clear that an understanding of protein-

carbohydrate interactions at a molecular level is the key for controlling and manipulating the biological functions linked to the recognition event. NMR spectroscopy has proved to be a valuable tool for the investigation of the molecular details of protein-carbohydrate binding reactions, especially for the elucidation of bioactive conformations of saccharide ligands.[2] Nevertheless, NMR-spectroscopic analysis of carbohydrate ligands suffers from severe overlap of resonance signals, especially in ^{1}H NMR spectra. Although multi-dimensional techniques resolve some of the problems, difficulties remain, especially since ^{13}C-enriched saccharides are difficult to obtain,[3] and multi-dimensional NMR experiments remain limited to homo-nuclear variants. Another approach for the simplification of ^{1}H NMR spectra involves selective deuterium labeling.[4] Spectral simplifications resulting from selective deuteration give extra information for conformational analyses which is especially useful for the investigation of protein-bound carbohydrates. In general, the study of protein-carbohydrate interactions requires the identification of bioactive carbohydrate ligands in a first step. For this, we have developed a novel technique[6] that is based on the transferred NOE (trNOE) effect[5] and allows the parallel screening of complex compound mixtures for binding activity with proteins. Very recently, a major improvement was achieved by introducing STD-NMR (Saturation Transfer Difference NMR) for the screening of compound libraries.[7] This technique is based on saturation transfer from a protein to a ligand that binds to a protein. In the following we refer to these new NMR-screening protocols as Bio-Affinity NMR.[6c]

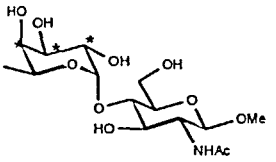
For the investigation of fucose-binding proteins we synthesized four disaccharides **1** - **4** that contain 2,3,4-trideuterio-α-L-fucose. The non-deuterated counterparts were also synthesized. We demonstrate the spectral simplifications that result from selective deuteration, and show an application of Bio-Affinity NMR to detect binding activity of the synthetic disaccharides with a fucose-binding lectin.

RESULTS AND DISCUSSION

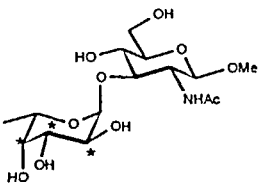
Synthesis of deuterated disaccharides 1 to 4. All compounds were synthesized according to published procedures. The synthetic routes will be described only briefly.



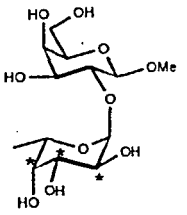
α-L-Fuc-(1→6)-β-D-GlcNAc-OMe **1**



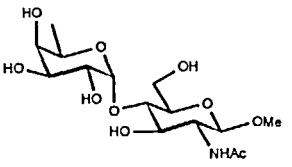
α-L-Fuc-(1→4)-β-D-GlcNAc-OMe **2**



α-L-Fuc-(1→3)-β-D-GlcNAc-OMe **3**



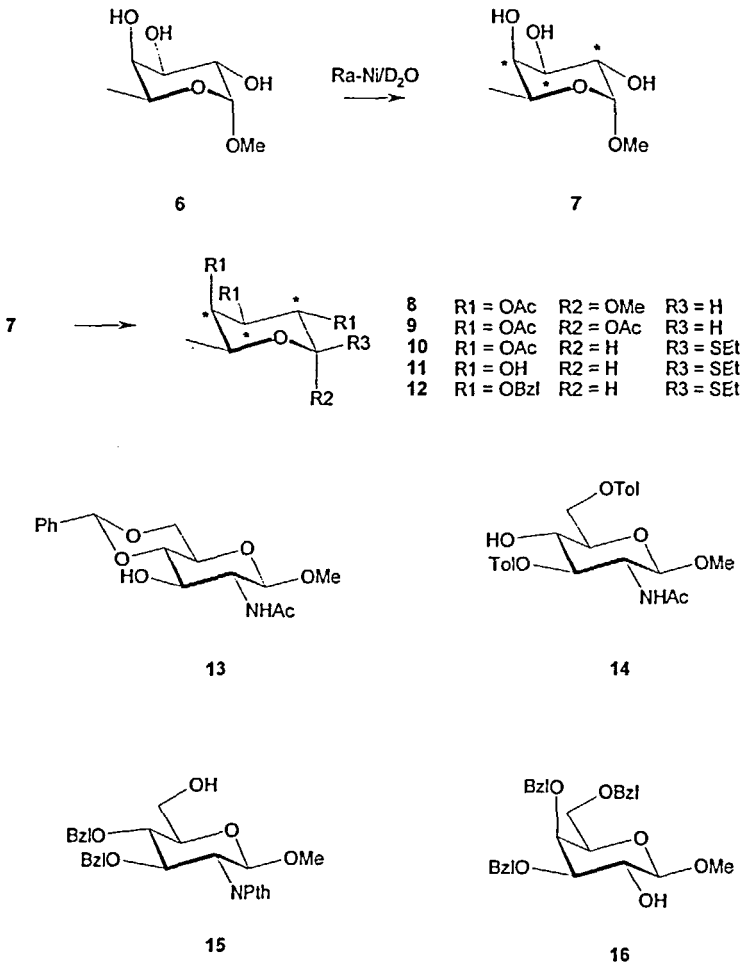
α-L-Fuc-(1→2)-β-D-Gal-OMe **4**



α-D-Fuc-(1→4)-β-D-GlcNAc-OMe **5**

Scheme 1. Compounds **1** to **5**. Asterisks indicate deuterated C-atoms.

Following the protocol of Stuart and Koch[8] we first synthesized methyl 2,3,4-tri-deuterio-α-L-fucopyranoside **7** from methyl α-L-fucopyranoside **6** using Raney-Nickel in the presence of deuterium oxide. Reaction progress was monitored using ^{1}H NMR spectroscopy. After complete deuteration the crude product was acetylated and purified by HPLC. The L-fuco configuration of the product **8** was verified by comparing ^{13}C and ^{1}H chemical shifts to the corresponding non-deuterated derivative. Especially, the ^{1}H chemical shifts of the anomeric proton H1 and the proton attached to C5 are sensitive to changes in configurations of the deuterated carbon atoms C2, C3 and C4. For the subsequent glycosylation reactions the deuterated compound **8** had to be converted into the glycosyl donor **12**.[9] This was achieved by cleaving the methyl glycoside **8** with sulfuric acid/acetic acid in a first step. Treatment with boron trifluoride and ethanethiol in dichloromethane then yielded the thioglycoside **10**. Acetates were removed with sodium methoxide in methanol, and, finally, the per-benzylated product **12** was formed by treating with benzyl bromide in DMF. All reactions proceeded with good yields. The glycosyl acceptors **13** - **16** were synthesized according to the literature.[10] Coupling with the glycosyl donor **12** was achieved by utilizing halide ion assistance and activation with copper dibromide to give the fully protected disaccharide derivatives **17**



Scheme 2. Synthetic building blocks.

to **20**. Protecting groups were successfully removed applying standard procedures as described in the experimental section to give the target compounds **1** to **4**. 2D-TOCSY, 2D-HMQC and 2D-HMBC experiments furnished a full assignment of all ^{1}H and ^{13}C resonances. The non-deuterated compounds **1** to **4** were also synthesized according to literature procedures.

NMR experiments. To demonstrate the benefits of the deuterated compounds for NMR spectroscopic analyses we present NMR data for the disaccharides α-L-Fuc-(1→6)-β-D-GlcNAc-OMe **1** and α-L-Fuc-(1→4)-β-D-GlcNAc-OMe **2**. A detailed NMR

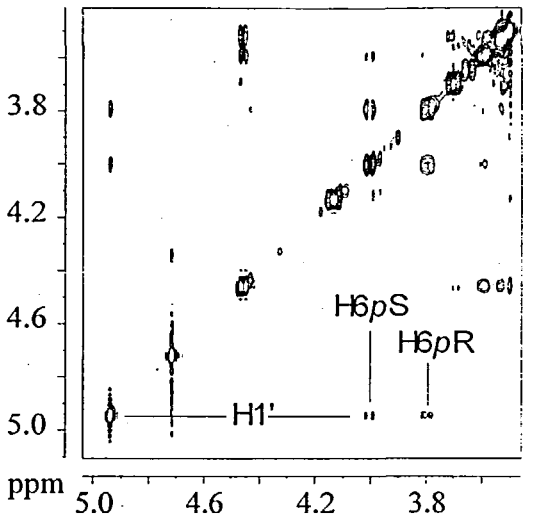


Figure 1. 2D-NOESY spectrum of disaccharide **1**. It is seen that the inter-glycosidic NOEs between H1'. and protons H6-*pro*-R and H6-*pro*-S are easily measured without overlap from other resonances.

and molecular modeling study of disaccharides **1** and **2** has been performed recently.[11] Inter-glycosidic NOEs mainly served as an experimental gauge for the conformational analysis. Unfortunately, the quantitative determination of one important inter-glycosidic NOE in **1** between H1' and H6-*pro*-R was complicated by an overlap of the resonance signal of H6-*pro*-R with those of H4' and H2'. Therefore, in our previous study line deconvolution procedures had to be applied. Fig. 1 shows a 2D-NOESY spectrum of deuterated α-L-Fuc-(1→6)-β-D-GlcNAc-OMe **1**. It is seen that both protons H6-*pro*-R and H6-*pro*-S are well separated from other signals and the inter-glycosidic NOEs to H1' are easily determined (Fig. 1).

For disaccharide **2** similar simplifications result. Especially, inter-glycosidic NOEs involving the protons H6-*pro*-R and H6-*pro*-S in **2** are easily measured whereas in the non-deuterated derivative H6-*pro*-R is overlapped with the resonance signals of protons H2', H3' and H4'. These examples demonstrate that the deuterated disaccharides **1** to **4** in conjunction with their non-deuterated counterparts will be useful tools for the NMR investigation of protein-carbohydrate complexes.

Bio-Affinity- and STD-NMR experiments. Prior to an investigation of protein-carbohydrate interactions by NMR, potential carbohydrate ligands have to be assayed for binding activity towards the protein(s) in question. Using classical assays such as ELISA or RIA, the compounds have to be tested individually, i.e., sequentially, for activity. Clearly, it would be of advantage to apply a parallel screening protocol instead. Bio-Affinity NMR[6,7] allows such a parallel and therefore rapid identification of biologically active compounds from mixtures. The observation of trNOEs[5] allows identification of compounds with binding activity. Small ligands (molecular weight less than approx. 2kD) that bind to a receptor protein undergo chemical exchange between the bound and the free form leading to the observation of strong negative trNOEs that are easily discriminated from small positive NOEs of ligands without binding activity. Alternatively, STD-NMR spectra[7] are used to identify bound ligands. Spectra are recorded with and without saturation of protein resonances. In the difference spectra only signals from bound ligands are visible, and thus allow their straightforward identification. This technique has the great advantage that it can be combined with any NMR pulse sequence.[7] In the 1D mode, the method is extremely fast and robust.[7]

Here, we assay the binding affinity of the synthetic deuterated as well as the non-deuterated disaccharides **1** to **4** towards a lectin, *Aleuria aurantia* agglutinin (AAA).[12] The non-deuterated disaccharides generate more signal overlap and thus provide a more challenging target for the new NMR screening techniques. Therefore, only these spectra will be discussed in the following. The results for the deuterated counterparts are identical. A ^{1}H NMR spectrum of a mixture of the non-deuterated disaccharides **1** to **4**, present in equimolar amounts, with the protein AAA is shown in Fig. 2a. The expansion of the anomeric region in the insert of Fig. 2a clearly shows that the anomeric protons of **3** and **4** are well separated. The resonance signals of the anomeric protons of **1** and **2** overlap leading to a broadened signal with twofold intensity. Compared to the spectra without AAA present (data not shown) a line broadening is detected for the anomeric signals indicating binding. A second compound mixture was prepared containing an additional reference compound, α-D-Fuc-(1→6)-β-D-GlcNAc-OMe **5**.[13] that contains D-fucose instead of L-fucose. The ^{1}H NMR spectrum of the mixture in the presence of AAA is shown in Fig. 2b.

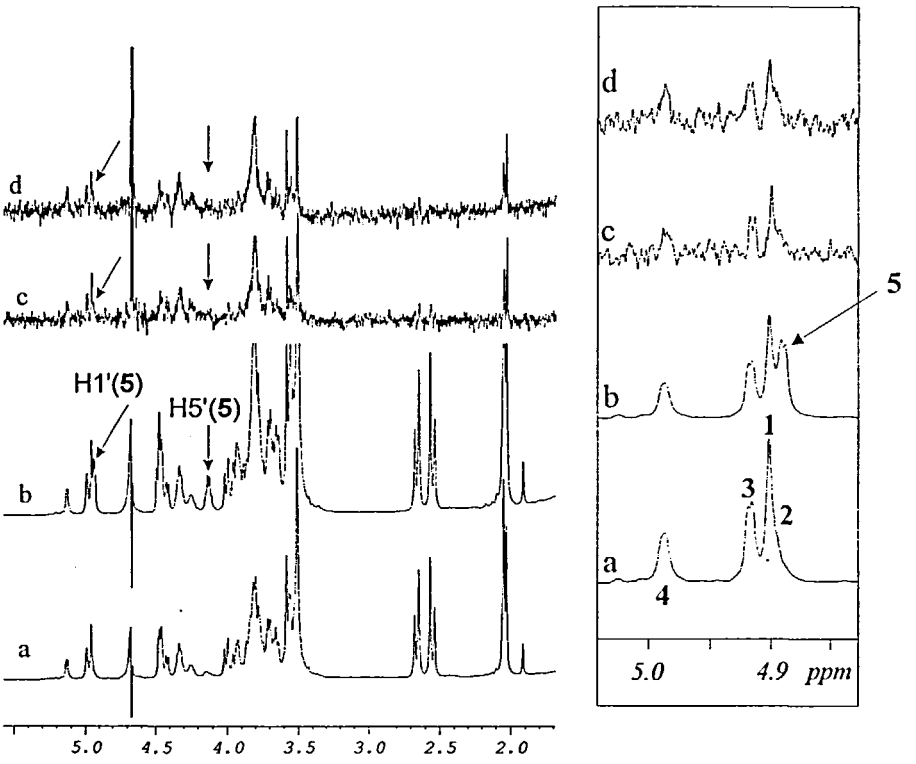


Figure 2. ^{1}H NMR spectra for the mixture of the deuterated disaccharides **1** to **4** (a), and for the disaccharides **1** to **5** (b) in the presence of AAA. Anomeric protons are labeled with the number of the corresponding disaccharide. Spectra (c) and (d) are 1D STD-NMR spectra that correspond to spectra (a) and (b), respectively. It is obvious that disaccharides **1** to **4** bind to AAA whereas disaccharide **5** shows no signals in the STD-NMR spectrum (d) and thus has no binding activity. The amount of protein (AAA) used was 770 μg (11 nmol). The disaccharides were present in 40 fold excess each. The measurement time for each experiment was less than five minutes. The insert is the expansion of the ^{1}H NMR and 1D STD-NMR spectra for the region of anomeric protons. The anomeric resonances are labeled with the corresponding compound numbers.

An expansion of the anomeric region is depicted in the insert in Fig. 2b. The extra anomeric signal of disaccharide **5** is clearly distinguished from the other anomeric resonances. Other prominent signals stemming from **5** are H5' and H6' (Fig. 2b). 1D STD-NMR spectra were obtained for both samples yielding the spectra in Figs. 2c and 2d.

As a result of saturation transfer to the bound carbohydrate ligands all signals from the disaccharides **1** to **4** are visible in the STD-NMR spectra (Fig. 2c). No signals of the reference disaccharide **5** are visible (Figs. 2d), clearly showing that it does not bind to AAA. Also, it is important to notice that signals resulting from the buffer (strong signals around 2.6 ppm in Figs. 2a and 2b) do not disturb the STD NMR spectra. Since the buffer has no binding activity towards AAA, no signals are left in the STD-NMR spectrum. This demonstrates how robust this new method is. To summarize, the STD-NMR experiments show that disaccharides **1** to **4**, but not **5** have binding activity towards AAA. This result was obtained with a total measurement time of less than five minutes, using only 21 nmol (1.5 mg) of AAA.

Additionally, trNOESY spectra were recorded. A trNOESY spectrum of the mixture of disaccharides **1** to **5** in the presence of AAA is shown in Fig. 3. All disaccharides give rise to small positive NOEs when no receptor protein is present. The trNOESY spectrum clearly shows that binding of ligands occurs, since negative trNOEs are observed (no contour lines in Fig. 3). With the knowledge about the chemical shifts of all synthetic disaccharides, an assignment of the components leading to trNOEs is straightforward. The only component that gives rise to positive NOEs is disaccharide **5** indicating that it is not bound by AAA (black contour lines in Fig. 3). The total measurement time of the spectrum was ca. 3.5 h. The spectrum confirms the results from the 1D STD-NMR spectra and further verifies the identity of the bound disaccharides by their typical trNOESY cross peak patterns.

In summary, Bio-Affinity NMR based on STD-NMR and trNOEs allowed us to perform a fast and parallel assay for binding activity of a mixture of five structurally closely related disaccharides. It should be emphasized that three of the compounds (**1** to **3**) are regioisomers, and that compound **1** and **5** differ only by the chirality of the fucose residue. It would be difficult if not impossible to identify the bioactive components directly from the mixture with other screening methods.

EXPERIMENTAL

General Methods. All reactions were monitored by TLC on silica gel-coated aluminium foil (Merck, silica gel 60 F_{254}). Detection was achieved by UV absorption

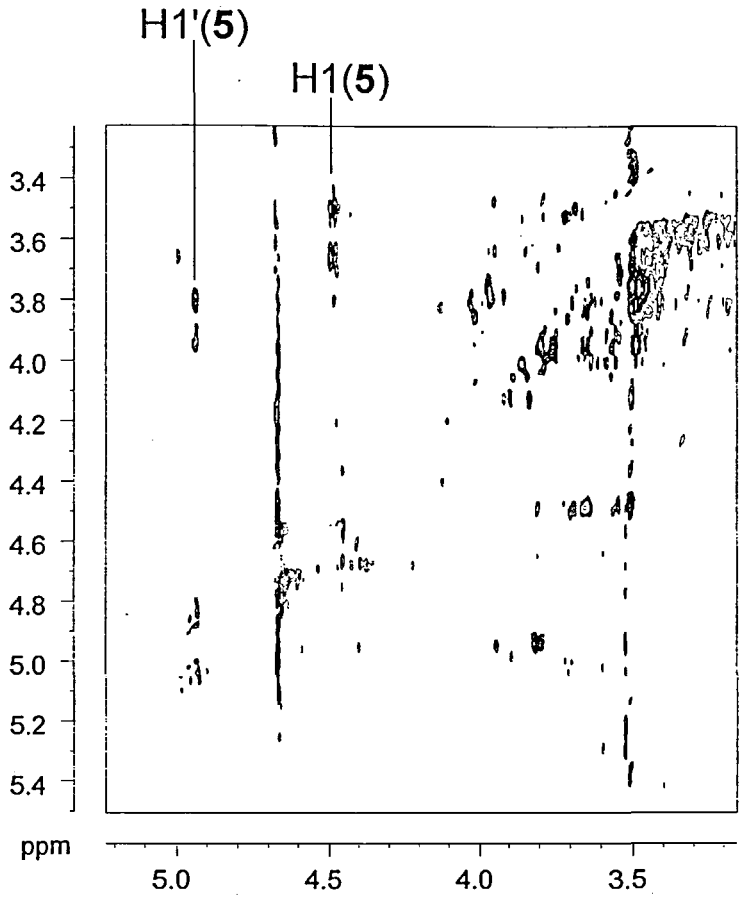


Figure 3. 2D-trNOESY spectrum of the mixture of disaccharides **1** to **5** in the presence of AAA. The mixing time was 150 ms. Only positive contour lines are shown. Disaccharides **1** to **4** show negative trNOEs (no contours) and therefore have binding activity for AAA, whereas disaccharide **5** shows positive NOEs (black contours) indicating that it is not bound by the protein.

and/or charring with 5% H_2SO_4 in ethanol. Optical rotations were determined on a Perkin 243 polarimeter. Preparative scale silica gel separations were performed on silica gel 60 (230 - 400 mesh, Merck). HPLC separations were performed using a Merck-Hitachi system. Reactions with dried solvents were performed under a nitrogen atmosphere. Glycosylation reactions were conducted in the dark. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 500 spectrometer operating at 500.13 MHz for

^{1}H and 125.77 MHz for ^{13}C. Solutions in organic solvents were referenced to internal tetramethylsilane, and solutions in D_2O to internal acetone (δ = 2.225 ppm). Chemical shifts and coupling constants are first order values.

Glycosylation reactions.[14] Glycosyl donor **12** (700 mg, 1.46 mmol) and glycosyl acceptor **14** (1.0 g, 2.12 mmol) were dissolved in 50 mL dichloromethane/dimethylformamide (1:1, v/v). 3-Å molecular sieves (1 g) were added, and the mixture was stirred for 0.5 h at rt. Tetrabutylammonium bromide (1.5 g, 4.66 mmol) and copper dibromide (1 g, 4.48 mmol) were added, and stirring was continued for 36 h at rt. The reaction mixture was filtered over a layer of Celite, and the residue was washed twice with dichloromethane (50 mL). Solvents were removed in vacuo, and the syrup was dissolved in dichloromethane (50 mL) and washed with saturated $NaHCO_3$ solution and saturated NaCl solution. The organic phase was dried over $MgSO_4$, and rotary evaporation furnished crude disaccharide **18**. Purification over a silica gel column (hexane/ethylacetate 5:1, v/v) yielded pure **18**; yield 560 mg (0.63 mmol, 43%). The reactions with the other acceptors **13**, **15**, and **16** followed the same protocol and delivered the protected disaccharides **17**, **19**, and **20**, respectively, in yields between 40 and 80 % and elemental analysis. All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy.

Removal of protecting groups. Protecting groups were removed by applying standard procedures. For **17**, a deacylation step had to be performed prior to removal of the benzyl ether groups, using sodium methoxide in methanol. Purification of the intermediate product was not necessary. For the (1→6) linked disaccharide **19** the phthalimido group had to be removed first using hydrazine hydrate in ethanol. The intermediate product was purified via HPLC chromatography. Removal of benzyl ethers in all cases was achieved by hydrogenolysis at rt using 10% Pd/C in dry methanol. The reaction products were filtered, and concentrated in vacuo. All compounds were passed over a Biogel P2 column.

Methyl-2-acetamido-2-deoxy-6-*O*-([2′,3′,4′-$^2H_3$]-α-L-fucopyranosyl)-β-D-glucopyranoside (1). $[\alpha]_D^{22}$ –79.4 (*c* 0.53, water). ^{1}H NMR (500 MHz, D_2O): δ = 1.20 (d, $J_{5',6'}$ = 6.6 Hz, 3 H; H-6′), 2.01 (s, 3 H, NHAc), 3.47 (s, 3 H, OMe), 3.49 – 3.52 (m, 2 H; H-3, H-4), 3.55 – 3.60 (m, 1 H; H-5), 3.68 (br. t, J = 9.0 Hz, 1 H; H-2), 3.77 (br. dd, $J_{5,6}$

$_{pro\text{-}R}$ = 5.9 Hz, $J_{6\text{-}pro\text{-}R,6\text{-}pro\text{-}S}$ = 12.1 Hz, 1 H; H-6-pro-R), 3.91 (d, $J_{6\text{-}pro\text{-}R,6\text{-}pro\text{-}S}$ = 12.1 Hz, 1 H; H-6-pro-S), 4.11 (q, $J_{5',6'}$ = 6.6 Hz, 1 H; H-5′), 4.43 (d, $J_{1,2}$ = 8.5 Hz, 1 H; H-1), 4.90 (s, 1 H; H-1′). ^{13}C NMR (125 MHz, D_2O): δ = 19.2 (C-6′), 26.0 ($NCOCH_3$), 59.3 (C-2), 60.9 (OMe), 70.5 (C-5′), 71.1 (C-6), 73.7, 77.7 (C-3, C-4), 78.8 (C-5), 103.2 (C-1′), 105.9 (C-1), 178.6 ($NCOCH_3$).

Anal. Calcd for $C_{15}H_{24}D_3NO_{10}$: C, 46.87; H, 6.29; N, 3.64. Found: C, 46.93; H, 6.42; N, 3.76.

Methyl-2-acetamido-2-deoxy-4-*O*-([2′,3′,4′-$^2H_3$]-α-L-fucopyranosyl)-β-D-glucopyranoside (2). $[\alpha]_D^{22}$ –136.7 (*c* 0.88, water). ^{1}H NMR (500 MHz, D_2O, 300 K): δ = 1.10 (d, $J_{5',6'}$ = 6.6 Hz, 3 H; H-6′), 1.99 (s, 3 H; NHAc), 3.45 (s, 3 H; OMe), 3.48 – 3.53 (m, 2 H; H-4; H-5), 3.56 – 3.60 (m, 1 H; H-3), 3.66 (dd, $J_{1,2}$ = 8.4 Hz, 1 H; H-2), 3.77 – 3.82 (m, 1 H; H-6-pro-R), 3.98 (dd, $J_{6\text{-}pro\text{-}R,\ 6\text{-}pro\text{-}S}$ = 12.2 Hz, 1 H; H-6-pro-S), 4.29 (q, $J_{5',6'}$ = 6.6 Hz, 1 H; H-5′), 4.39 (d, $J_{1,2}$ = 8.4 Hz, 1 H; H-1), 4.89 (s, 1 H, H-1′). ^{13}C NMR (125 MHz, D_2O, 300 K): δ = 19.0 (C-6′), 26.0 ($NCOCH_3$), 59.8 (C-2), 60.9 (OMe), 63.8 (C-6), 70.8 (C-5′), 76.6 (C-3), 79.1, 81.3 (C-4, C-5), 103.5 (C-1′), 105.7 (C-1), 178.5 ($NCOCH_3$).

Anal. Calcd for $C_{15}H_{24}D_3NO_{10}$: C, 46.87; H, 6.29; N, 3.64. Found: C, 46.72; H, 6.51; N, 3.79.

Methyl-2-acetamido-2-deoxy-3-*O*-([2′,3′,4′-$^2H_3$]-α-L-fucopyranosyl)-β-D-glucopyranoside (3). $[\alpha]_D^{22}$ –124.78 (*c* 0.67, water). ^{1}H NMR (500 MHz, D_2O, 300 K): δ = 1.13 (d, $J_{5',6'}$ = 6.6 Hz, 3 H; H-6′), 1.99 (s, 3 H; NHAc), 3.43 – 3.53 (m, superimposed by singlet at 3.48, 5 H; H-4; H-5; OMe), 3.61 (dd, $J_{2,3}$ = 10.2 Hz, $J_{3,4}$ = 8.4 Hz, 1 H; H-3), 3.73 (dd, $J_{5,6\text{-}pro\text{-}R}$ = 5.5 Hz, $J_{6\text{-}pro\text{-}R,6\text{-}pro\text{-}S}$ = 12.2 Hz, 1 H; H-6-pro-R), 3.79 (dd, $J_{1,2}$ = 8.5 Hz, $J_{2,3}$ = 10.2 Hz, 1 H; H-2), 3.91 (dd, $J_{5,6\text{-}pro\text{-}S}$ = 2.0 Hz, $J_{6\text{-}pro\text{-}R,6\text{-}pro\text{-}S}$ = 12.2 Hz, 1 H; H-6-pro-S), 4.26 (q, $J_{5',6'}$ = 6.6 Hz, 1 H; H-5′), 4.43 (d, $J_{1,2}$ = 8,59 Hz, 1 H; H-1), 4.92 (s, 1 H, H-1′). ^{13}C NMR (125 MHz, D_2O, 300 K): δ = 16.4 (C-6′), 23.5 ($NCOCH_3$), 56.4 (C-2), 58.4 (OMe), 62.0 (C-6), 68.1 (C-5′), 69.9 (C-4), 77.1 (C-5), 81.8 (C-3), 101.2 (C-1′), 103.0 (C-1), 176.0 ($NCOCH_3$).

Anal. Calcd for $C_{15}H_{24}D_3NO_{10}$: C, 46.87; H, 6.29; N, 3.64. Found: C, 46.69; H, 6.47; N, 3.71.

Methyl-2-*O*-([2′,3′,4′-$^2H_3$]-α-L-fucopyranosyl)-β-D-galactopyranoside (4). $[\alpha]_D^{22}$ –83.85 (*c* 0.78, water). ^1H NMR (500 MHz, D_2O, 300 K): δ = 1.18 (d, $J_{5',6'}$ = 6.6 Hz, 3 H; H-6′), 3.49 (dd, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.3 Hz, 1 H; H-2), 3.58 (s, 3 H; OMe), 3.62 – 3.67 (m, 1 H; H-5), 3.70 – 3.84 (m, 3 H; H-3; H-6-pro-R; H-6-pro-S), 3.88 (br. d, $J_{3,4}$ = 3.3 Hz, 1 H; H-4), 4.22 (q, $J_{5',6'}$ = 6.6 Hz, 1 H; H-5′), 4.38 (d, $J_{1,2}$ = 7.8 Hz, 1 H; H-1), 5.09 (s, 1 H; H-1′). ^{13}C NMR (125 MHz, D_2O, 300 K): δ = 16.3 (C-6′), 58.3 (OMe), 62.1 (C-6), 68.0 (C-5′), 70.0 (C-4), 74.4 (C-3), 76.1 (C-5), 79.4 (C-2), 101.2 (C-1′), 103.9 (C-1).

Anal. Calcd for $C_{13}H_{21}D_3O_{10}$: C, 45.48; H, 6.17. Found: C, 45.65; H, 6.32.

NMR experiments. All spectra were recorded without sample spinning. Data acquisition and processing were performed with XWINNMR software (Bruker) running on Silicon Graphics O_2 workstations. For Bio-Affinity NMR and STD-NMR, a sample was prepared containing 163 μg (428 nmol) of disaccharides **1**, **2** and **3** each, and 146 μg (428 nmol) of **4**. The amount of *Aleuria aurantia* agglutinin (AAA) was 0.77 mg (11 nmol) dissolved in 560 μL D_2O. All experiments were performed with this sample and repeated after addition of 163 μg (428 mmol) of **5**. The 2D trNOESY experiment was performed applying a pulse sequence with a spin lock filter[15] to suppress protein resonances. The spectral width was set to 10 ppm. 512 increments were recorded in t_1 and 2 K data points in t_2. After 16 dummy scans, 16 scans were performed per increment. The residual HDO signal was suppressed by low power presaturation during the relaxation delay and the mixing time. A spin lock field of 2.5 kHz with a length of 20 ms after the first 90° pulse was used to suppress protein signals. A relaxation time of 1 s and a mixing time of 150 ms was used. A gradient pulse was applied during the mixing time. The total experiment time was ca. 3.5 h. Prior to Fourier transformation, zero filling in t_1 and multiplication with squared cosine functions in each dimension were applied to yield matrices of 1 K by 2 K data points. For 2D-NOESY spectra, the experimental conditions were the same except for the mixing time which was set to 900 ms. STD-NMR spectra were performed applying a cascade of 40 gaussian shaped pulses (50 ms, 63 dB) at 7 ppm (on-resonance) and 40 ppm (off-resonance). 16 K data points were accumulated and 16 transients. Subtraction, zero-filling to 32 K data points

and Fourier transformation delivered the difference spectra. Total measurement time for one spectrum is ca. 2 min.

ACKNOWLEDGMENTS

This work was financially supported by grants from the Deutsche Forschungsgemeinschaft DFG (SFB470, project B3), from the Fonds der Chemischen Industrie (VCI), and from the Bundesministerium für Bildung und Forschung BMBF (BMBF 0311361).

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