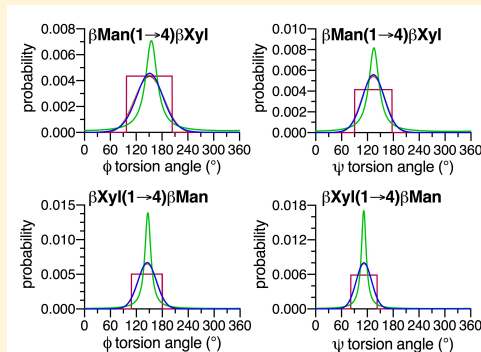


Synthesis and O-Glycosidic Linkage Conformational Analysis of ^{13}C -Labeled Oligosaccharide Fragments of an Antifreeze GlycolipidWenhui Zhang,[†] Reagan Meredith,[†] Mi-Kyung Yoon,[†] Xiacong Wang,[§] Robert J. Woods,[§] Ian Carmichael,[‡] and Anthony S. Serianni^{*,†,§}[†]Department of Chemistry and Biochemistry and [‡]Radiation Laboratory, University of Notre Dame, Notre Dame, Indiana 46556-5670 United States[§]Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602, United States

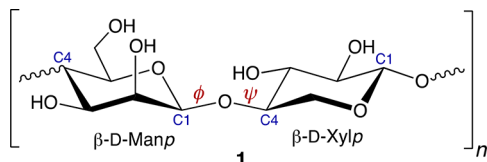
Supporting Information

ABSTRACT: NMR studies of two ^{13}C -labeled disaccharides and a tetrasaccharide were undertaken that comprise the backbone of a novel thermal hysteresis glycolipid containing a linear glycan sequence of alternating $[\beta\text{Xylp}-(1\rightarrow4)-\beta\text{Manp}-(1\rightarrow4)]_n$ dimers. Experimental transglycoside NMR J -couplings, parameterized equations obtained from density functional theory (DFT) calculations, and an in-house circular statistics package (MA'AT) were used to derive conformational models of linkage torsion angles ϕ and ψ in solution, which were compared to those obtained from molecular dynamics simulations. Modeling using different probability distribution functions showed that MA'AT models of ϕ in $\beta\text{Man}(1\rightarrow4)\beta\text{Xyl}$ and $\beta\text{Xyl}(1\rightarrow4)\beta\text{Man}$ linkages are very similar in the disaccharide building blocks, whereas MA'AT models of ψ differ. This pattern is conserved in the tetrasaccharide, showing that linkage context does not influence linkage geometry in this linear system. Good agreement was observed between the MA'AT and MD models of ψ with respect to mean values and circular standard deviations. Significant differences were observed for ϕ , indicating that revision of the force-field employed by GLYCAM is probably needed. Incorporation of the experimental models of ϕ and ψ into the backbone of an octasaccharide fragment leads to a helical amphipathic topography that may affect the thermal hysteresis properties of the glycolipid.



INTRODUCTION

Recent work by Duman and co-workers has revealed the presence of a novel glycolipid in the Alaskan beetle, *Upis ceramoides*, that possesses potent thermal hysteresis (TH) properties.¹ This antifreeze glycolipid (AFGL) is composed of alternating βXylp and βManp residues connected by (1 $\rightarrow$ 4) O-glycosidic linkages (1), although branching and covalent modification (e.g., fatty acylation) of this core structure is suspected but not yet demonstrated. Structural characterization by NMR has proven difficult because of limited sample quantities, but Crich and Rahaman² have confirmed the core backbone structure through chemical synthesis of smaller oligosaccharide fragments.



Since the first report of the AFGL, efforts have been made to prepare synthetic analogues in order to identify smaller chemically accessible fragments possessing TH properties. Presumably the conformational properties of the AFGL,

superimposed on the covalent structure of its constituent monosaccharides, confer TH activity, possibly through selective interactions with ice to inhibit its growth in vivo.

A key conformational feature of oligosaccharides is encoded in the linkages between their constituent monosaccharides. In 1, all linkages are β -(1 $\rightarrow$ 4) and are composed of two exocyclic C–O bonds, denoted ϕ (phi) and ψ (psi), both of which are rotatable in solution. The conformational preference of ϕ is determined largely by stereoelectronic (*exo*-anomeric) and steric factors, whereas that of ψ is largely determined by sterics.^{3–8} Methods to determine the conformational preferences of ϕ and ψ in solution by NMR have focused heavily on analyses of inter-residue ROEs or NOEs and more recently on residual dipolar couplings (RDCs).^{9–11} These parameters, however, are low in abundance and/or average nonlinearly in the presence of conformational exchange and thus do not allow explicit determinations of a preferred linkage conformation. An alternative method to determine ϕ and ψ involves measurements and analyses of NMR J -couplings across O-glycosidic linkages in suitably ^{13}C -labeled samples.^{12–18} Multiple J -

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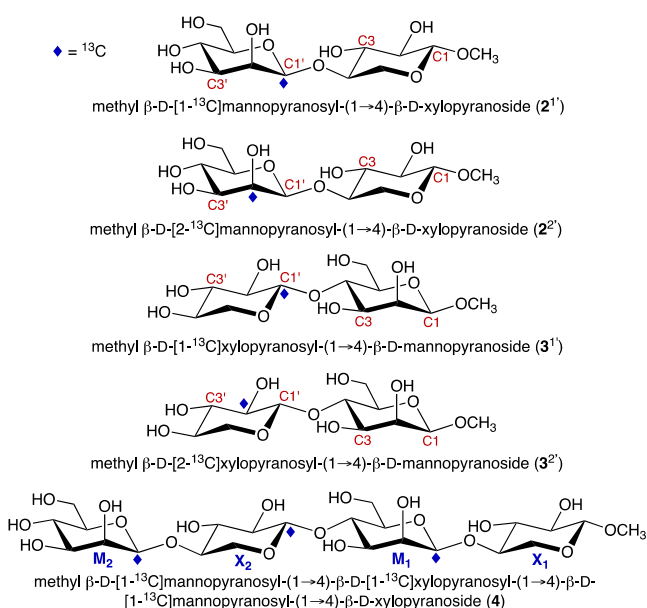
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couplings sensitive to either ϕ or ψ are measured, and the redundant information is used to calculate conformational models for each torsion angle independent of informational bias provided by theoretical methods such as potential energy calculations or molecular dynamics simulations.

In this work, we posed two questions: (1) What are the preferred conformations about ϕ and ψ in the two different β -(1 $\rightarrow$ 4) O-glycosidic linkages in **1**, and (2) how sensitive are these conformational preferences to structural context; that is, do the conformations of the individual linkages change when inserted into a larger structure? To answer these questions, four singly ^{13}C -labeled disaccharides, **2'**, **2''**, **3'**, and **3''** (superscripts denote the labeled carbon), and a triply ^{13}C -labeled tetrasaccharide **4** were prepared to enable accurate measurements of multiple J_{CH} and J_{CC} values across the “isolated” linkages in **2** and **3**, and across the “in-context” linkages in **4** (Scheme 1). We show that (a) context plays a

Scheme 1. Singly ^{13}C -Labeled Disaccharides **2'**, **2''**, **3'**, and **3''** and Triply ^{13}C -Labeled Tetrasaccharide **4**^a



^aSuperscripts denote the labeled carbons in the disaccharides.

minor role in determining linkage conformation in **4** and that (b) the two different types of β -(1 $\rightarrow$ 4) linkages in **4** are conformationally distinct.

RESULTS AND DISCUSSION

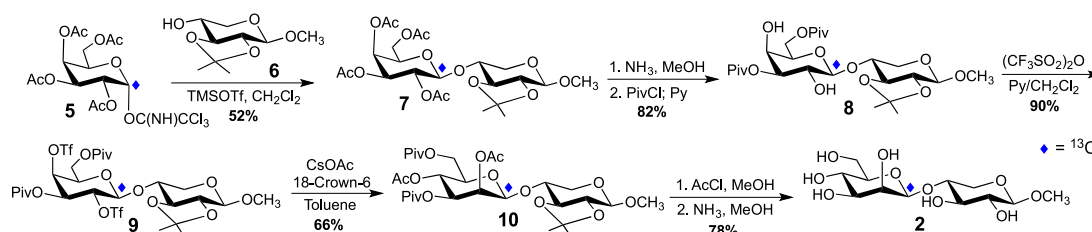
Synthesis of Disaccharides **2 and **3** and Tetrasaccharide **4**.** The backbone of AFGL **1** is composed of

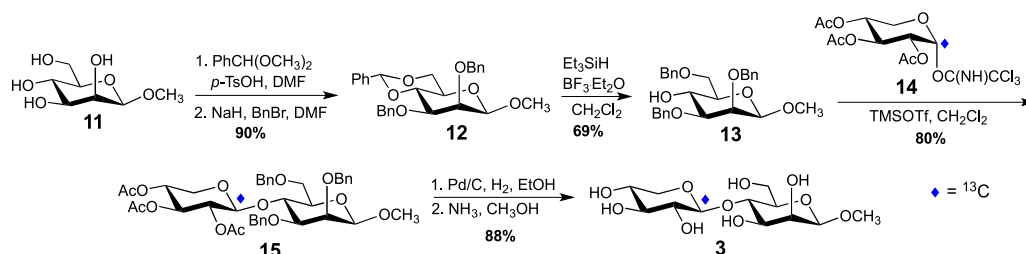
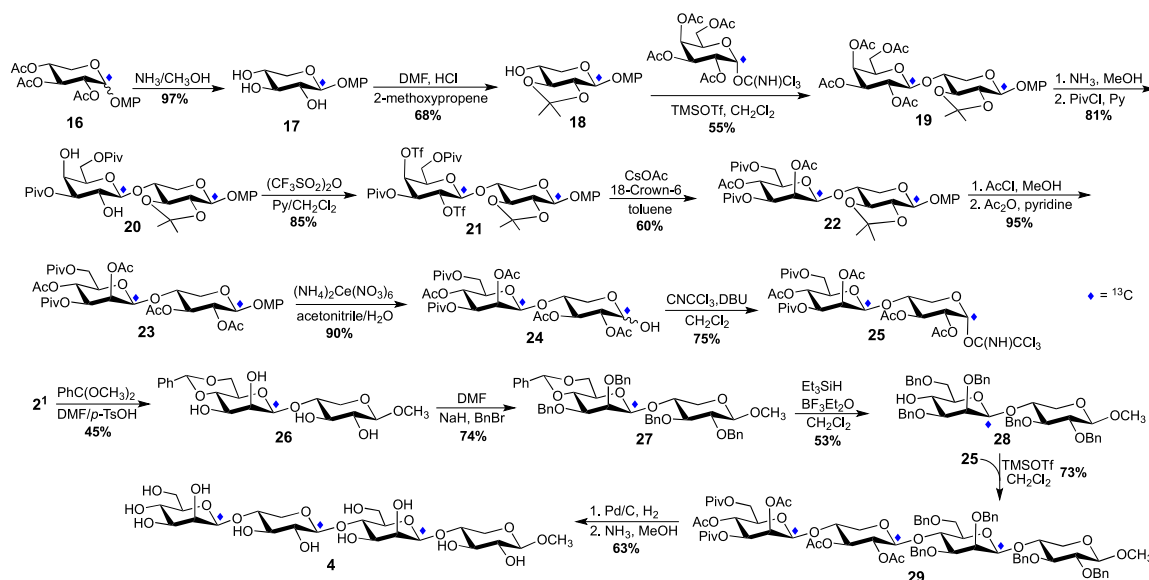
alternating β Xylp and β Manp residues connected by (1 $\rightarrow$ 4) O-glycosidic linkages. β -(1 $\rightarrow$ 4) Linkages involving Manp residues in **2** and **4** have been challenging to install into oligosaccharides by chemical glycosylation. Previous efforts have been reported by Crich² and Ito.¹⁹ Ito's approach involves 2-naphthylmethyl (NAP)-ether-mediated intramolecular aglycon delivery (IAD)²⁰ to introduce the β Manp linkage stereoselectively. In Crich's work, a thiomannopyranoside was used as the donor, and the β Manp linkage was installed via preactivation of the donor at a low temperature with 1-benzenesulfonyl piperidine (BSP), trifluoromethanesulfonic anhydride (TF_2O), and the hindered base, 2,4,6-tri-*tert*-butylpyrimidine (TTBP). In the present work, a double-inversion method^{21,22} was used to introduce β Manp linkages into disaccharide **2** and tetrasaccharide **4** stereospecifically. To prepare **2**, 2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl trichloroacetimidate (**5**)²³ and methyl 2,3-O-isopropylidene- β -D-xylopyranoside (**6**) were condensed to give protected disaccharide **7** containing a β -(1 $\rightarrow$ 4) linkage (Scheme 2). In this reaction, the highly preferred β -linkage stereochemistry is dictated by neighboring group participation by the O-acetyl group at C2 of **5**.^{24–26} After deacetylation, the O3H and O6H hydroxyls in the Gal residue were selectively protected with pivaloyl groups,²⁷ followed by triflate activation at the O2H and O4H hydroxyls to give disaccharide **9**. Double inversion at C2 and C4 with CsOAc and 18-crown-6 converted the β Gal residue into the desired β Man residue in good yield (66%),^{21,22} as determined by changes in intraring $^3J_{\text{HH}}$ values measured from ^1H NMR spectra ($^3J_{\text{H1,H2}}$, 7.9 $\rightarrow$ 1.0 Hz; $^3J_{\text{H2,H3}}$, 10.2 $\rightarrow$ 3.4 Hz; $^3J_{\text{H3,H4}}$, 2.9 $\rightarrow$ 9.9 Hz; $^3J_{\text{H4,H5}}$, 0.7 $\rightarrow$ 10.0 Hz). After deprotection and chromatography, disaccharide **2** was obtained in high purity (>95%). The overall yield for the conversion of **5** to **2** (Scheme 2) was $\sim$ 20%. ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of **2** (Figures S1 and S2, Supporting Information) were assigned with assistance from 2D gCOSY and gHSQC spectra. ^1H and $^{13}\text{C}\{^1\text{H}\}$ chemical shifts and J_{HH} values for **2** are given in Tables 1 and 2, respectively.

Disaccharide **3** was prepared from methyl β -D-mannopyranoside (**11**)²⁸ by introducing a 4,6-O-benzylidene group at O4H and O6H, and O-benzyl groups at O2H and O3H, to give intermediate **12** (Scheme 3). Regioselective reductive cleavage of the 4,6-O-benzylidene acetal gave acceptor **13** containing a free O4H.²⁹ Coupling of **13** with donor **14**, followed by deprotection, gave methyl β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-mannopyranoside (**3**). ^1H and ^{13}C chemical shifts, and J_{HH} values, for **3** are given in Tables 1 and 2, respectively (see Figures S3 and S4, Supporting Information, for ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra).

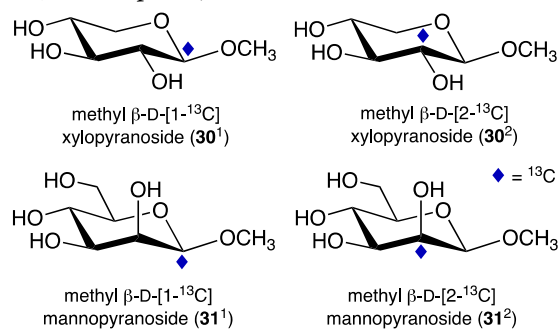
Tetrasaccharide **4** was prepared from disaccharides **25** and **28**, each of which contain a β Manp-(1 $\rightarrow$ 4) linkage (Scheme 4). Acceptor **18** was obtained in two steps from 4-

Scheme 2. Synthetic Route To Prepare β Man-(1 $\rightarrow$ 4)- β XylOCH₃ (**2**) Selectively Labeled with ^{13}C at C1 of the β Man Residue



Scheme 3. Synthetic Route To Prepare β Xyl-(1 $\rightarrow$ 4)- β ManOCH₃ (3) Selectively Labeled with ¹³C in the β Xyl ResidueScheme 4. Synthetic Route To Prepare Tetrasaccharide 4 Containing Selective ¹³C-Labeling at Three Anomeric Carbons

methoxyphenyl 2,3,4-tri-*O*-acetyl- α/β -D-xylopyranoside (16), and the β Manp-(1 $\rightarrow$ 4) linkage in disaccharide 22 was installed by the double-inversion method used to prepare 2 (Scheme 2). After removal of the 2,3-*O*-isopropylidene group in 22, the O2H and O3H hydroxyls were *O*-acetylated, the 4-methoxyphenyl group was removed by treatment with ammonium cerium(IV) nitrate to give 24, and the latter was converted to disaccharide 25. Following the synthesis of 13 from 11 (Scheme 3), disaccharide 28 was obtained from 2. Coupling of donor 25 and acceptor 28 afforded tetrasaccharide 29. The target tetrasaccharide 4 was obtained in high purity (>95%) after deprotection and chromatography. ¹H and ¹³C chemical shifts, and *J*_{HH} values, for 4 are given in Tables 1 and 2 (see Figures S5 and S6, Supporting Information, for ¹H and ¹³C{¹H} NMR spectra).



¹H and ¹³C Chemical Shifts and ¹H-¹H, ¹³C-¹H, and ¹³C-¹³C Spin-Couplings in 2-4. Access to ¹H and ¹³C NMR spectra of 2-4 obtained under identical solution

conditions allows an assessment of the effect of *O*-glycosylation on ¹H and ¹³C chemical shifts and a means of confirming their structures. This comparison assumes that the conformations of the β Xylp and β Manp rings in 2-4 are identical, which can be tested by inspection of intraring ³*J*_{HH} values in 2-4, and in the ¹³C-labeled methyl β -D-xylopyranosides (30) and methyl β -D-mannopyranosides (31). *J*_{HH} data in Table 2 show that (a) β Manp residues in 2-4 and 31 give essentially identical intraring ³*J*_{HH} values, that (b) β Xylp residues in 2-4 and 30 give essentially identical intraring ³*J*_{HH} values, and that (c) qualitative analyses of intraring ³*J*_{HH} values in both rings indicate that the ⁴C₁ conformer is highly preferred in aqueous (²H₂O) solution, as expected.

The effect of *O*-glycosylation on ¹H chemical shifts in 2-4 was determined using the terminal β Manp residues in 2 and 4 (Man₂) and the β Xylp residue in 3 as references, since they are unsubstituted at O4. Since OCH₃ and OR groups (R = sugar residue) appended to C1 are not structurally equivalent, comparisons were made only for δ_{H_3} , δ_{H_4} , and δ_{H_5} . For example, δ_{H_3} in the β Xylp residue of 2 (3.580 ppm) and those in the two β Xylp residues of 4 (3.583 and 3.590 ppm) average to 3.584 ppm, from which was subtracted 3.425 ppm in the β Xylp residue of 3 to give a chemical shift difference ($\Delta\delta_{\text{H}_3} = \delta_{\text{glycosylated}} - \delta_{\text{unglycosylated}}$) of +0.16 ppm, with the (+) sign signifying a downfield shift upon *O*-glycosylation. The value of $\Delta\delta_{\text{H}_3}$ for β Manp (3.701 and 3.700 ppm for the β Manp residues in 3 and 4, respectively; 3.612 and 3.612 ppm for the β Manp residues in 2 and 4, respectively) was found to be +0.09 ppm. Similar treatments of δ_{H_4} and δ_{H_5} gave the following $\Delta\delta_{\text{H}_4}$ and

Table 1. ^1H and ^{13}C Chemical Shifts^a in Disaccharides 2 and 3 and Tetrasaccharide 4

residue	nucleus							
	H1	H2	H3	H4	H5 _{eq}	H5 _{ax}	H6	H6'
Xyl (2)	4.317	3.267	3.580	3.828	4.069	3.357		
Man (2)	4.762	3.955	3.612	3.540		3.365	3.908	3.706
Xyl (3)	4.382	3.267	3.425	3.611	3.971	3.301		
Man (3)	4.572	4.008	3.701	3.730		3.490	3.984	3.797
Xyl ₁ (4)	4.316	3.267	3.583	3.827	4.070	3.356		
Man ₁ (4)	4.779	3.997	3.700	3.736		3.500	3.981	3.788
Xyl ₂ (4)	4.400	3.308	3.590	3.851	4.096	3.351		
Man ₂ (4)	4.761	3.953	3.612	3.539		3.366	3.910	3.707
	C1	C2	C3	C4	C5	C6	OMe	
Xyl (2)	106.49	75.36	76.60	78.92	65.53		59.90	
Man (2)	101.04	73.45	75.54	69.44	79.01	63.72		
Xyl (3)	106.14	75.82	78.31	71.85	67.88			
Man (3)	103.58	72.60	74.24	79.25	77.72	63.04	59.53	
Xyl ₁ (4)	106.49	75.33	76.56	78.84	65.48		59.89	
Man ₁ (4)	100.86	73.12	74.13	78.96	77.81	62.97		
Xyl ₂ (4)	105.94	75.52	76.52	78.82	65.61			
Man ₂ (4)	100.99	73.41	75.52	69.44	79.00	63.72		

^aIn ppm ± 0.001 (^1H) or ± 0.01 ppm (^{13}C); in $^2\text{H}_2\text{O}$; 22 °C; referenced internally to DSS. In Man residues, H6' is defined as the more shielded H6 hydrogen.

Table 2. ^1H – ^1H Spin-Couplings^a in Disaccharides 2 and 3, Tetrasaccharide 4, and Monosaccharides 30 and 31

residue	coupled nuclei							
	H1–H2	H2–H3	H3–H4	H4–H5 _{eq}	H4–H5 _{ax}	H5 _{eq} –H5 _{ax}	H5–H6	H5–H6'
Xyl (2)	7.8	9.4	8.9	5.4	10.3	(–)11.8		
Man (2)	1.0	3.2	9.6		9.7		2.3	6.8
Xyl (3)	7.9	9.4	9.1	5.5	10.6	(–)11.6		
Man (3)	1.0	2.9	~9.4		9.3		2.3	5.7
Xyl ₁ (4)	7.8	9.4	9.0	5.3	10.4	(–)11.8		
Man ₁ (4)	1.0	3.2	9.5		9.4		2.2	5.9
Xyl ₂ (4)	7.9	9.5	9.0	5.4	10.3	(–)11.7		
Man ₂ (4)	1.0	3.2	9.6		9.7		2.3	6.8
Xyl (30) ^b	7.8	9.3	9.1	5.5	10.5	(–)11.6		
Man (31) ^b	0.9	3.2	9.6		9.7		2.4	6.5

^aIn Hz ± 0.1 Hz, in $^2\text{H}_2\text{O}$, 22 °C. In Man residues, H6' is defined as the more shielded H6 hydrogen. In Xyl residues, H5_{eq} = H5 and H5_{ax} = H5', where H5' is the more shielded H5 hydrogen. Signs of $^2J_{\text{HH}}$ values (shown in parentheses) are assumed to be negative. ^bData for monosaccharides 30 and 31 were taken from ref 28.

$\Delta\delta_{\text{H5}}$ values: βManp $\Delta\delta_{\text{H4}}$, +0.19 ppm; βXylp $\Delta\delta_{\text{H4}}$, +0.22 ppm; βManp $\Delta\delta_{\text{H5}}$, +0.13 ppm; βXylp $\Delta\delta_{\text{H5}}$, +0.05 ppm; βXylp $\Delta\delta_{\text{H5eq}}$, +0.11 ppm). These results show that glycosylation at O4 causes downfield shifts in the H3, H4, and H5 signals of 0.05–0.22 ppm in βXylp and βManp rings. These downfield shifts are almost exclusively associated with axial hydrogens, except for H5_{eq} in the βXylp rings. In the latter rings, the downfield shift associated with H5_{eq} (+0.11 ppm) is considerably larger than that for H5_{ax} (+0.05 ppm), suggesting that the sensitivity of ^1H chemical shift to O-glycosylation depends on hydrogen disposition (axial vs equatorial). The largest downfield shifts were observed at H4, the site of O-glycosylation, and are very similar ($\sim +0.21$ ppm) in the βManp and βXylp rings, but the shifts at adjacent sites can have comparable magnitudes (e.g., $\Delta\delta_{\text{H3}}$ in βXylp is +0.16 ppm). The magnitudes of the downfield shifts observed at adjacent sites also appear to depend more strongly on ring substitution and configuration.

A similar treatment of δ_{C3} , δ_{C4} , and δ_{C5} values (Table 1) gave the following results: βManp $\Delta\delta_{\text{C3}}$, –1.3 ppm; βXylp $\Delta\delta_{\text{C3}}$, –1.8 ppm; βManp $\Delta\delta_{\text{C4}}$, +9.7 ppm; βXylp $\Delta\delta_{\text{C4}}$, +7.0 ppm; βManp $\Delta\delta_{\text{C5}}$, –1.2 ppm; βXylp $\Delta\delta_{\text{C5}}$, –2.3 ppm. Downfield shifts of ~ 8 ppm are observed for the directly substituted carbon (C4), whereas smaller upfield shifts ranging from 1.2 to 2.3 ppm are observed at carbons flanking the site of substitution.

Intraring $^3J_{\text{H1,H2}}$, $^3J_{\text{H2,H3}}$, and $^3J_{\text{H3,H4}}$ values in βManp and βXylp residues in 2–4, 30, and 31 are virtually identical (Table 2), indicating that ring conformation is unaffected by context (terminal vs internal residue). $^3J_{\text{H5,H6'}}$ values in internal βManp residues (i.e., those serving as acceptors) are smaller (5.7–5.9 Hz) than observed in the terminal (i.e., those serving only as donors) βManp residues (6.5–6.8 Hz). These findings suggest that the conformational preferences of the exocyclic hydroxymethyl group may be affected by local structure (e.g., proximity to an internal glycosidic linkage), and aqueous MD simulations support this conclusion (larger *gt/gg* ratio (~ 1.4) for terminal βManp than for internal βManp residues (~ 0.6)).

Table 3. Intra-Residue ^{13}C – ^1H and ^{13}C – ^{13}C Spin-Couplings^a in 2, 3, 30, and 31

residue/coupled carbon	coupled hydrogen					
	H1'	H2'	H3'	H4'	HS' _{ax}	HS' _{eq}
Man C1' (2)	160.3	(+1.7	0	~0	2.2	
Man C2' (2)	(+6.7	148.4	(+1.5	~0	0	
Xyl C1' (3)	162.5	(–)6.3	1.0	~0	2.9	10.6
Xyl C2' (3)	~0	144.7	~(–)4.1	~0	0	0
Xyl C1 (30)	161.7	(–)6.1	1.2	0	2.9	10.3
Xyl C2 (30)	~0	144.7	4.4	0.7		
Man C1 (31) ^b	159.5	~(+1.5	0	0	~2.2	
Man C2 (31) ^b	(+7.1	147.9	(+1.5	0	0	

residue/coupled carbon	coupled carbon					
	C1'	C2'	C3'	C4'	C5'	C6'
Man C1' (2)		43.8	(+4.1 ^c	~0	~0	4.0
Man C2' (2)	43.8		38.3	~0	~0	0
Xyl C1' (3)		46.8	(+4.6 ^c	~0	~0	
Xyl C2' (3)	46.8		38.7	(+2.7 ^c	~0	
Xyl C1 (30)		46.7	(+4.2 ^c	0	~0	
Xyl C2 (30)	46.7		38.8	(+2.7 ^c	~0	
Man C1 (31) ^b		43.8	(+3.9 ^c	0	0	4.0
Man C2 (31) ^b	43.8		38.2	(±)0.3	0	0

^aIn Hz ± 0.1 Hz, in $^2\text{H}_2\text{O}$, 22 °C; an entry of ~0 denotes $J < 0.5$ Hz; signs of geminal couplings are shown in parentheses. ^bData for 31 (or the corresponding ethyl glycosides) were taken from ref 27 (J_{CH}) and ref 30 (J_{CC}). ^cThe (+) signs of $^2J_{\text{CCC}}$ values in βXylp and βManp rings were determined by analogy to $^2J_{\text{C1,C3}}$ and $^2J_{\text{C2,C4}}$ values in methyl β -D-glucopyranoside, both of which have (+) signs based on experimental measurements and/or use of the projection resultant method (ref 31).

(Figure S19, Supporting Information). In contrast, $^3J_{\text{H5,H6}}$ and $^2J_{\text{H6,H6'}}$ values in βManp rings, which are also influenced by hydroxymethyl conformation, are unaffected by context, being 2.2–2.4 Hz and –12.2 to –12.3 Hz, respectively, in all cases (Table 2).

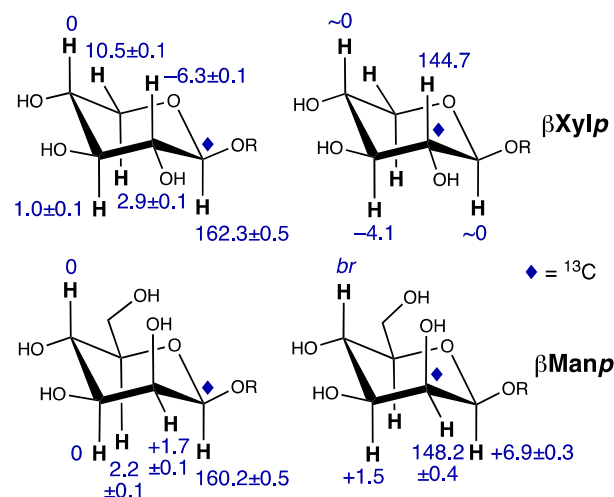
The introduction of ^{13}C enrichment at C1 and C2 of 2–4 and 30–31 enabled measurements of multiple ^{13}C – ^1H and ^{13}C – ^{13}C spin-couplings within (intra-residue) or between (inter-residue) residues. Intra-residue J_{CH} and J_{CC} values for βManp and βXylp residues are virtually identical, confirming that both rings adopt conformations that are essentially unaffected by context (Tables 3 and 4). However, different patterns of J_{CH} and J_{CC} values are observed in these rings (Schemes 5 and 6). For example, $^2J_{\text{C2,H1}}$ and $^2J_{\text{C2,H3}}$ values are ~0 Hz and –4.1 Hz, respectively, in βXylp rings and +6.9 Hz and +1.5 Hz, respectively, in βManp rings. This behavior

Table 4. Intra-Residue ^{13}C – ^1H and ^{13}C – ^{13}C Spin-Couplings^a in 4

residue/coupled carbon	coupled hydrogen					
	H1	H2	H3	H4	HS _{ax}	HS _{eq}
Man ₁ C1	160.6	(+1.7	~0	~0	2.2	
Man ₂ C1	160.3	(+1.7	~0	~0	2.1	
Xyl ₂ C1	162.7	(–)6.3	1.0	~0	3.0	10.4

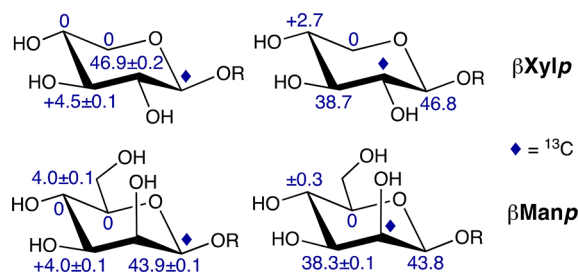
residue/coupled carbon	coupled carbon				
	C2	C3	C4	C5	C6
Man ₁ C1	44.0	(+3.9	~0	~0	3.9
Man ₂ C1	43.8	(+4.1	~0	~0	4.0
Xyl ₂ C1	47.1	(+4.4	~0	~0	

^aIn Hz ± 0.1 Hz, in $^2\text{H}_2\text{O}$, 22 °C. Signs of geminal couplings are shown in parentheses.

Scheme 5. J_{CH} Values (in Hz) in βXylp and βManp Rings Labeled with ^{13}C at C1 and C2^a

^aThe signs of $^1J_{\text{CH}}$ and $^3J_{\text{CH}}$ values are assumed to be positive; the signs of $^2J_{\text{CH}}$ values are shown. A broadened signal is denoted as br ($J_{\text{CH}} < 0.5$ Hz).

confirms preferred $^4\text{C}_1$ ring conformations for βManp rings based on the projection rule, but cannot be used to distinguish between $^4\text{C}_1$ and $^1\text{C}_4$ forms of βXylp rings (projections for each $^2J_{\text{CCH}}$ are identical in both ring conformations).³² In contrast, $^2J_{\text{C1,H2}}$ values can be used to confirm the preferred $^4\text{C}_1$ form for βXylp rings, but not for βManp rings. However, in both βManp and βXylp rings, $^3J_{\text{C1,H3}}$ values are <1.2 Hz, indicating that gauche arrangements between C1 and H3 are highly preferred, as found in $^4\text{C}_1$ forms; the small difference between $^3J_{\text{C1,H3}}$ values in βManp and βXylp rings are caused by the different configurations at C2 (pathway differences) and/or small

Scheme 6. J_{CC} Values (in Hz) in β Xylp and β Manp Rings Labeled with ^{13}C at C1 and C2^a

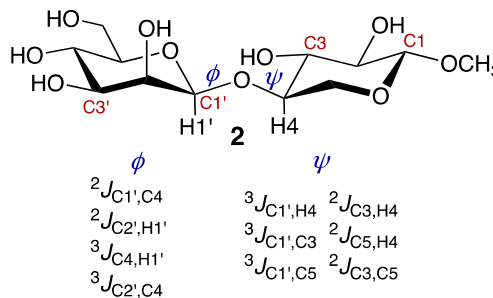
^aThe signs of $^1J_{CC}$ and $^3J_{CC}$ values are assumed to be positive; the signs of $^2J_{CC}$ values are shown.

differences in C1–C2–C3–H3 torsion angles. Very small $^3J_{C2,H4}$ values in both rings also support highly preferred 4C_1 ring forms. Prior work has shown that H5S of β Xylp rings is coupled to C1 by ~ 2.9 Hz.³³ Since H5S is H5_{ax} in 4C_1 forms, these data confirm that the β Xylp rings highly prefer 4C_1 forms. J_{CH} values involving C3–C5 might provide additional information on possible ring distortions in internal β Manp residues suggested from J_{HH} data (see above), but were not measured in this work.

$^1J_{C1,C2}$ values in β Manp rings (43.9 ± 0.1 Hz) are ~ 3 Hz smaller than corresponding values in β Xylp rings (46.9 ± 0.2 Hz) (Tables 3 and 4). This result is consistent with that made in the corresponding reducing sugars, where the difference is 3.2 Hz.³⁴ Since the O1–C1–C2–O2 torsion angles are the same in these rings ($\pm \sim 60^\circ$), this factor, which is known to affect $^1J_{CC}$ magnitudes, is probably not responsible for the difference. The smaller $^1J_{C1,C2}$ in the β Manp ring may be caused by a longer C1–C2 (longer bond = less *s*-character) brought about by the steric clustering of O1, O2, and O5. In addition, the C2–O2 bond torsion, which determines the disposition of the O2 lone-pair orbitals relative to the C1–C2 bond and thus affects $r_{C1,C2}$,¹⁶ may also differ between the two rings and contribute to the observed difference. The C1–O1 bond torsion exerts a similar lone-pair effect on $r_{C1,C2}$, but the behavior of this torsion is likely to be similar in both rings because of the *exo*-anomeric effect (C2 *anti* to the aglycone carbon).^{3,5–8} These lone-pair effects on $^1J_{CC}$ values are related to those observed previously for $^1J_{CH}$ values.³⁵ $^1J_{C2,C3}$ values in β Manp (38.3 Hz) and β Xylp (38.7 Hz) rings are similar in magnitude and 5.5 and 8.2 Hz smaller, respectively, than $^1J_{C1,C2}$ values (fewer oxygen substituents are appended to the coupled carbons in the former).

$^2J_{C1,C3}$ values in β Manp and β Xylp rings range from +3.9 to +4.6 Hz and have positive signs, as expected since O1 and O3 in both rings are equatorial (Tables 3 and 4). The effects of C2 configuration and C2–O2 bond conformation on $^2J_{C1,C3}$ may be partly responsible for the 0.7 Hz range in values.¹⁶ $^2J_{C2,C4}$ values, on the other hand, are very different in β Manp (< 0.3 Hz) and β Xylp rings (+2.7 Hz) due mainly to differences in the relative orientations of the hydroxyl groups appended to the coupled carbons (for β Manp, the axial–equatorial arrangement gives very small or zero couplings, whereas the equatorial–equatorial arrangement in the β Xylp ring leads to larger couplings having positive signs¹⁶).

Qualitative Treatment of Inter-Residue Spin-Couplings in 2–4. For *O*-glycosidic linkages composed of two C–O bonds, four *J*-couplings are sensitive to ϕ and six *J*-couplings are sensitive to ψ , respectively (Scheme 7).^{14,16,18,36}

Scheme 7. NMR Spin-Couplings Sensitive to the ϕ and ψ Torsion Angles Comprising the Internal *O*-Glycosidic Linkage in 2

Experimental measurements of J_{CC} values require ^{13}C -labeled samples, while J_{CH} values can be measured with natural abundance samples, although ^{13}C -labeling often simplifies these measurements and/or provides more accurate values. The simplest site-specific ^{13}C -labeling strategies for linkage conformational analysis involve the incorporation of ^{13}C at two sites proximal to the linkage.^{16,18} In this work, ^{13}C was incorporated at C1' and C2' of 2 and 3 (the least expensive double-labeling option), which allowed access to four of the five J_{CC} values ($^2J_{C3,C5}$ was inaccessible), and two ($^2J_{C2',H1'}$, $^3J_{C1',H4}$) of the five J_{CH} values. The remaining three J_{CH} values were measured at natural abundance (Table 5).^{18,37} Two singly labeled ^{13}C isotopomers of 2 and 3 were prepared (Scheme 1) to simplify spectral analysis. Representative ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of 2 and 3 are available in the Supporting Information (Figures S1–S4).

Tetrasaccharide 4 contains three internal *O*-glycosidic linkages, and each was studied with ^{13}C labels inserted at the participating anomeric carbons. A single triply labeled ^{13}C isotopomer was prepared (Scheme 1) to allow direct measurements of $^2J_{C1',C4}$, $^3J_{C1',H4}$, $^3J_{C1',C3}$, and $^3J_{C1',C5}$ values for each linkage. Measurements of $^3J_{C4,H1'}$, $^2J_{C3,H4}$, and $^2J_{C5,H4}$ values were made at natural abundance (Table 5).^{18,37}

Qualitative comparisons of *J*-coupling ensembles sensitive to *O*-glycosidic linkage torsion angles in structurally related oligosaccharides provide rapid assessments of whether these angles are similar or different.^{16,18} This comparison is contingent on the quantitative relationships between inter-residue *J*-coupling magnitudes and the torsion angles ϕ or ψ being similar in the structures being compared; this assumption is validated for 2 and 3 below (see **Parameterization of Spin-Coupling Equations for *O*-Glycosidic Linkages in 2^c and 3^c**). *J*-Coupling ensembles sensitive to ϕ in 2 and 3 are virtually identical (Table 5), indicating similar ϕ behaviors. In contrast, *J*-coupling ensembles sensitive to ψ differ appreciably, indicating different ψ behaviors. *J*-Coupling ensembles sensitive to ψ in 4 indicate that the differences in ψ observed between 2 and 3 are maintained in 4; that is, the two internal β Manp-(1 $\rightarrow$ 4)- β Xylp linkages in 4 mimic that in 2, while the internal β Xylp-(1 $\rightarrow$ 4)- β Manp linkage in 4 mimics that in 3. Qualitatively, it can be concluded that the ψ behaviors of the isolated linkages in disaccharides 2 and 3 are maintained when these linkages are embedded in the tetrasaccharide.

Parameterization of Spin-Coupling Equations for *O*-Glycosidic Linkages in 2^c and 3^c. The qualitative conclusions on the behavior of ϕ and ψ in 2–4 discussed above were validated by conducting density functional theory

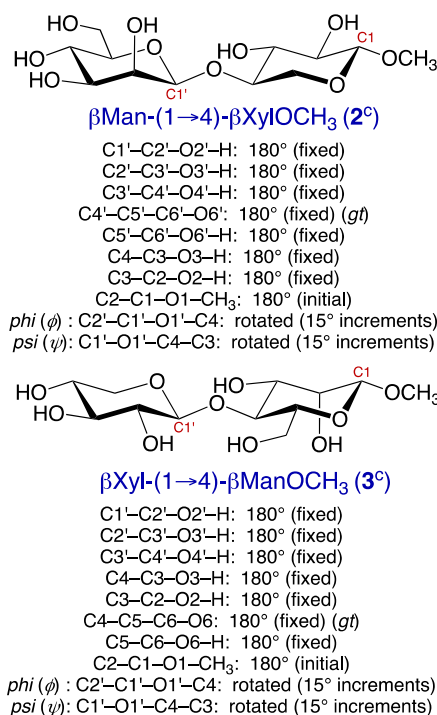
Table 5. Inter-Residue ^{13}C – ^1H and ^{13}C – ^{13}C Spin-Couplings^a in 2–4

donor residue/compound	ϕ -dependent J -coupling				Ψ -dependent J -coupling				
	$^2J_{\text{C1}',\text{C4}}$	$^3J_{\text{C4,H1}'}$	$^3J_{\text{C2}',\text{C4}}$	$^2J_{\text{C2}',\text{H1}'}$	$^3J_{\text{C1}',\text{H4}}$	$^3J_{\text{C1}',\text{C3}}$	$^3J_{\text{C1}',\text{C5}}$	$^2J_{\text{C3,H4}}$	$^2J_{\text{C5,H4}}$
Man (2)	(–)1.8	3.8	2.9	(+)6.7	4.4	1.7	0.9	(–)4.5	(–)2.8
Xyl (3)	(–)2.0	4.3	3.1	0	5.0	br	2.1	(–)5.2	(–)3.8
Man ₁ (4)	(–)1.6	n.o.		n.o.	4.4	1.6	br	(–)4.5	(–)2.9
Xyl ₂ (4)	(–)1.9	4.0		n.o.	5.1	br	1.9	(–)5.2	(–)3.8
Man ₂ (4)	(–)1.6	n.o.		n.o.	4.4	1.5	br	(–)4.4	(–)2.9

^aIn Hz $\pm$ 0.1 Hz, in $^2\text{H}_2\text{O}$, 22 °C. J -HMBC and HSQC-HECADE 2D NMR spectra were used to measure $^3J_{\text{C4,H1}'}$, $^2J_{\text{C3,H4}}$, and $^2J_{\text{C5,H4}}$ values; n.o. denotes values that could not be measured from 2D NMR spectra; br denotes broadened signal ($J < 0.5$ Hz). Signs of 2J values (shown in parentheses) were determined by analogy to related couplings in other β -(1→4)-linked disaccharides (ref 18), via experiment (ref 31), and/or through DFT calculations.

(DFT) calculations to derive quantitative relationships between inter-residue J -couplings and either ϕ or ψ in 2 and 3. DFT calculations were performed on model structures 2^c and 3^c (the superscripts “c” distinguish computed structures from experimental compounds), in which exocyclic C–O and C–C torsion angles were either fixed or set at initial values and allowed to optimize (Scheme 8). *O*-Glycosidic torsion angles ϕ

Scheme 8. Model Structures 2^c and 3^c Used in DFT Calculations, Showing Torsion Angle Constraints Applied during Geometry Optimizations and J -Coupling Calculations



(defined as C2'–C1'–O1'–C4) and ψ (defined as C1'–O1'–C4–C3) were each rotated in 15° increments through 360°, generating 576 optimized structures. (See Figure S7, Supporting Information, for DFT-derived potential energy surfaces for 2^c and 3^c.) J -Couplings were calculated in each structure, and hypersurface plots of the data are shown in the Supporting Information (Figures S8 and S9, Supporting Information). Trans-*O*-glycosidic vicinal $^3J_{\text{COCH}}$ values exhibit primary dependencies on either ϕ ($^3J_{\text{C4,H1}'}$) or ψ ($^3J_{\text{C1',H4}}$), and dynamic ranges of ~ 10 Hz. $^3J_{\text{C4,H1}'}$ is largely unaffected by ψ , and $^3J_{\text{C1',H4}}$ is largely unaffected by ϕ (Figures S8 and S9,

Supporting Information). Trans-*O*-glycosidic vicinal $^3J_{\text{CCOC}}$ values depend primarily on either ϕ ($^3J_{\text{C2',C4}}$) or ψ ($^3J_{\text{C1',C3}}$ and $^3J_{\text{C1',C5}}$). However, $^3J_{\text{CCOC}}$ values exhibited secondary dependencies on either ϕ or ψ ; for example, $^3J_{\text{C1',C5}}$ in 2^c depends primarily on ψ , but depends to a lesser degree on ϕ , especially at ψ values of 180–300° (Figure S8F, Supporting Information). Geminal $^2J_{\text{C1',C4}}$ values are negative in sign and depend primarily on ϕ , but they also show a substantial secondary dependence on ψ (Figures S8C and S9C, Supporting Information). In this work, equation parameterization for $^2J_{\text{C1',C4}}$ captured only its dependence on ϕ by linearly averaging the secondary effects of ψ .

J -Coupling data obtained from DFT calculations were processed using methods described previously^{18,37} and fit to an equation having the general form, $^nJ_{\text{ab}} = A + B \cos \theta + C \sin \theta + D \cos 2\theta + E \sin 2\theta$, where $^nJ_{\text{ab}}$ is the experimental J -coupling between atoms a and b, n is the number of covalent bonds between the coupled atoms, and θ is either ϕ (C2'–C1'–O1'–C4 torsion angle) or ψ (C1'–O1'–C4–C3 torsion angle) (Scheme 8). Conformations of exocyclic hydroxyl groups affect the magnitudes of $^2J_{\text{CCH}}$ and $^2J_{\text{CCC}}$ values when these groups are attached to the carbon bearing the coupled hydrogen (C–C–H pathway) or when they are attached to the central carbon in a C–C–C pathway, respectively.¹⁶ These two pathways are pertinent to *O*-glycosidic linkage conformation in 2–4, embodied in $^2J_{\text{C2',H1}'}$, $^2J_{\text{C3,H4}}$, $^2J_{\text{C5,H4}}$, and $^2J_{\text{C3,C5}}$ (Scheme 7). The remaining six J -couplings in Scheme 7 are relatively insensitive to exocyclic hydroxyl group conformation and were parameterized to give eqs 1a–6a for 2^c and eqs 1b–6b for 3^c. Equations for $^2J_{\text{C2',H1}'}$, $^2J_{\text{C3,H4}}$, $^2J_{\text{C5,H4}}$, and $^2J_{\text{C3,C5}}$ and their application to *O*-glycosidic linkage analysis will be described in a future report.

ϕ -Dependent J -couplings in 2^c and 3^c:

$$^2J_{\text{C1',C4}}(2^c) = -2.30 + 0.42 \cos \phi + 1.40 \sin \phi - 0.23 \cos 2\phi + 0.34 \sin 2\phi \quad \text{RMS 0.50 Hz} \quad (1a)$$

$$^2J_{\text{C1',C4}}(3^c) = -2.65 + 0.18 \cos \phi + 1.19 \sin \phi - 0.33 \cos 2\phi + 0.31 \sin 2\phi \quad \text{RMS 0.45 Hz} \quad (1b)$$

$$^3J_{\text{C2',C4}}(2^c) = 1.49 - 0.60 \cos \phi + 0.36 \sin \phi + 1.57 \cos 2\phi - 0.29 \sin 2\phi \quad \text{RMS 0.41 Hz} \quad (2a)$$

$$^3J_{\text{C2',C4}}(3^c) = 1.53 - 0.60 \cos \phi - 0.05 \sin \phi + 1.57 \cos 2\phi - 0.79 \sin 2\phi \quad \phi\text{RMS 0.37 Hz} \quad (2b)$$

$$^3J_{\text{C4,H1}'}(2^c) = 3.93 + 0.70 \cos \phi - 1.55 \sin \phi - 1.31 \cos 2\phi - 3.84 \sin 2\phi \quad \text{RMS 0.50 Hz} \quad (3a)$$

$$^3J_{C4,H1'}(3^\circ) = 3.69 + 0.77 \cos \phi - 1.82 \sin \phi - 1.19 \cos 2\phi - 3.60 \sin 2\phi \quad \text{RMS 0.61 Hz} \quad (3b)$$

ψ -Dependent J -couplings in 2° and 3° :

$$^3J_{C1',H4}(2^\circ) = 4.00 + 0.85 \cos \Psi - 1.90 \sin \Psi - 2.19 \cos 2\Psi - 3.58 \sin 2\Psi \quad \text{RMS 0.73 Hz} \quad (4a)$$

$$^3J_{C1',H4}(3^\circ) = 3.86 + 1.00 \cos \Psi - 1.80 \sin \Psi - 2.13 \cos 2\Psi - 3.51 \sin 2\Psi \quad \text{RMS 0.58 Hz} \quad (4b)$$

$$^3J_{C1',C3}(2^\circ) = 1.85 - 0.63 \cos \Psi - 0.14 \sin \Psi - 1.99 \cos 2\Psi - 0.16 \sin 2\Psi \quad \text{RMS 0.50 Hz} \quad (5a)$$

$$^3J_{C1',C3}(3^\circ) = 1.78 + 0.83 \cos \Psi - 0.20 \sin \Psi + 1.91 \cos 2\Psi - 0.22 \sin 2\Psi \quad \text{RMS 0.44 Hz} \quad (5b)$$

$$^3J_{C1',C5}(2^\circ) = 2.43 + 0.24 \cos \Psi - 0.91 \sin \Psi - 1.39 \cos 2\Psi + 2.50 \sin 2\Psi \quad \text{RMS 1.07 Hz} \quad (6a)$$

$$^3J_{C1',C5}(3^\circ) = 2.14 + 0.36 \cos \Psi + 0.84 \sin \Psi - 1.49 \cos 2\Psi + 2.12 \sin 2\Psi \quad \text{RMS 0.68 Hz} \quad (6b)$$

Statistical Modeling of O-Glycosidic Torsion Angles ϕ and ψ in 2 and 3. Parameterized eqs 1a/1b–6a/6b and experimental J -couplings (Table 5) were used to generate single-state models of the rotamer distributions about ϕ and ψ in 2 and 3 (Figure 1) using the MA'AT algorithm.¹⁸ These

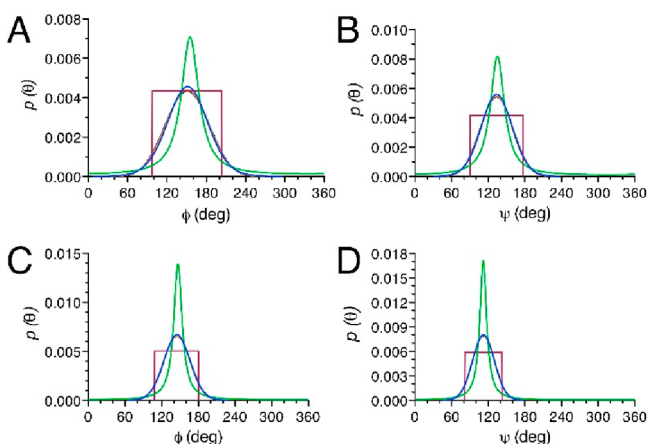


Figure 1. Different statistical models^{18,37} of ϕ and ψ in β Man-(1 $\rightarrow$ 4)- β XylOCH₃ (2) and β Xyl-(1 $\rightarrow$ 4)- β ManOCH₃ (3). (A) ϕ in 2. (B) ψ in 2. (C) ϕ in 3. (D) ψ in 3. Black, Cartwright's Power of Cosine. Orange, wrapped normal. Blue, von Mises. Green, wrapped Cauchy. Violet, uniform. The first three models gave similar probability distributions.

models yielded two parameters for each model, a mean and a circular standard deviation (CSD). Five probability distribution functions were used to fit the experimental J -couplings.¹⁸ Cartwright's Power of Cosine, Wrapped Normal, and von Mises models gave almost identical probability distributions (Figure 1) in each case. The mean positions, CSDs, and RMS errors of the models are shown in Table 6. The small RMS errors of 0.2–0.4 Hz indicate good fits of the experimental J -couplings to a single-state model.

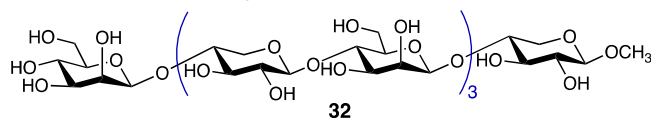
The uniqueness of the models was tested by visual inspection of the parameter space (Figures S10–S13, Supporting Information).^{18,38} Unique solutions were found

in nearly all model fittings of ϕ in 2 and 3. However, the parameter space of each model of ψ contained two or three minima, with the global minimum giving the smallest RMS error in most cases. The presence of two minima does not mean that two conformations are being sampled. Instead, this result indicates that two single-state solutions fit the criteria of the problem being solved. Use of additional J -coupling constraints such as $^2J_{C2',H1'}$ (for ϕ) and $^2J_{C3,H4}$, $^2J_{C5,H4}$, and $^2J_{C3,C5}$ (for ψ) is expected to reduce and/or eliminate local minima and allow more robust tests of the uniqueness of fit. Multi-state models of ϕ and ψ can also be investigated as more constraints become available.

The similar average mean values of ϕ in 2 ($151.8^\circ \pm 13.2^\circ$) and 3 ($145.4^\circ \pm 11.3^\circ$) show that the conformational properties of ϕ are essentially identical in 2 and 3 insofar as J -coupling ensembles are able to discriminate between different single-state models. However, the mean value of ψ in 2 differs significantly from that in 3 (22° difference; Table 6). This result is consistent with qualitative analyses of the J -coupling ensembles discussed above, and with results for other β -(1 $\rightarrow$ 4) linkages.¹⁸

Conformational Analysis of the O-Glycosidic Linkages in 4. Tetrasaccharide 4 contains two β Man-(1 $\rightarrow$ 4)- β Xyl linkages, and corresponding J -couplings sensitive to ψ in these linkages are virtually identical to those observed in 2 (Table 5). DFT-Parameterized ψ -sensitive J -coupling equations obtained from 2° and experimental J -couplings were used to generate single-state models of ψ in each β Man-(1 $\rightarrow$ 4)- β Xyl linkage in 4 (Figure 2; Table S1, Supporting Information). Virtually identical mean and CSD values of ψ were obtained for these linkages in 4 (135° ; 25°) and in 2 (134° ; 29°). DFT-Parameterized ψ -dependent J -coupling equations obtained from 3° and experimental J -couplings sensitive to ψ in the single β Xyl-(1 $\rightarrow$ 4)- β Man linkage in 4 yielded a statistical model with a mean and CSD (114° ; 18°) very similar to those determined in 3 (112° ; 20°) (Table 6; Table S1, Supporting Information). A comparison of statistical models of ψ in 2–4 shows that ψ values in 4 are identical to corresponding ψ values in 2 and 3; differences in ψ observed between 2 and 3 are maintained in the tetrasaccharide, supporting the qualitative conclusions drawn in Qualitative Treatment of Inter-Residue Spin-Couplings in 2–4.

Only one or two ϕ -dependent J -couplings were measured for each internal O-glycosidic linkage in 4 (Table 5), precluding single-state statistical modeling.¹⁸ However, as a crude approximation, structure proximal to internal O-glycosidic linkages appears to affect ψ more significantly than ϕ ,¹⁸ and statistical modeling of ψ in 2–4 supports this contention. Given the behavior of ψ , that of ϕ in 4 is expected to resemble those found in 2 and 3, and this expectation is supported qualitatively by the nearly identical ϕ -dependent experimental J -couplings in 2–4.



Collectively, the above findings demonstrate that the internal β Man-(1 $\rightarrow$ 4)- β Xyl linkages in 4 mimic the related linkage in 2 ($\sim 152^\circ$ and $\sim 134^\circ$ for ϕ and ψ , respectively), and the internal β Xyl-(1 $\rightarrow$ 4)- β Man linkage in 4 mimics the related linkage in 3 ($\sim 145^\circ$ and $\sim 113^\circ$ for ϕ and ψ , respectively). Thus, the effect of context on glycosidic linkage conformation

Table 6. Statistical Model Parameters and RMS Errors for ϕ and ψ in Disaccharides 2 and 3 Obtained from MA'AT Analysis

statistical model	ϕ			ψ		
	mean $\pm$ SE ^a (deg)	CSD $\pm$ SE (deg)	RMSD (Hz)	mean $\pm$ SE (deg)	CSD $\pm$ SE (deg)	RMSD (Hz)
β Man-(1 $\rightarrow$ 4)- β XylOCH ₃ (2)						
Cartwright's Power of Cosine	151.0 $\pm$ 13.6	32.7 $\pm$ 8.7	0.30	133.6 $\pm$ 8.8	26.2 $\pm$ 4.5	0.39
wrapped normal	151.0 $\pm$ 13.5	32.5 $\pm$ 13.6	0.30	133.6 $\pm$ 8.8	26.2 $\pm$ 8.9	0.39
von Mises	151.4 $\pm$ 13.3	32.8 $\pm$ 16.3	0.30	133.7 $\pm$ 8.8	26.4 $\pm$ 9.4	0.39
wrapped Cauchy	155.2 $\pm$ 11.9	43.2 $\pm$ 21.9	0.36	134.9 $\pm$ 9.6	40.2 $\pm$ 14.6	0.40
uniform	150.4 $\pm$ 13.8	30.6 $\pm$ 10.6	0.30	133.6 $\pm$ 8.8	24.9 $\pm$ 7.6	0.38
average	151.8 $\pm$ 13.2	34.4 $\pm$ 14.2	0.31	133.9 $\pm$ 8.9	28.8 $\pm$ 9.0	0.39
β Xyl-(1 $\rightarrow$ 4)- β ManOCH ₃ (3)						
Cartwright's Power of Cosine	145.1 $\pm$ 11.3	21.8 $\pm$ 5.0	0.27	112.1 $\pm$ 6.8	18.5 $\pm$ 3.6	0.31
wrapped normal	145.1 $\pm$ 11.3	21.8 $\pm$ 12.4	0.27	112.1 $\pm$ 6.8	18.2 $\pm$ 10.9	0.31
von Mises	145.1 $\pm$ 11.3	21.9 $\pm$ 12.6	0.27	112.1 $\pm$ 6.8	18.2 $\pm$ 11.0	0.31
wrapped Cauchy	146.7 $\pm$ 11.2	30.8 $\pm$ 19.9	0.31	111.7 $\pm$ 7.1	27.7 $\pm$ 17.4	0.31
uniform	145.0 $\pm$ 11.3	21.1 $\pm$ 11.2	0.27	112.1 $\pm$ 6.8	17.7 $\pm$ 10.1	0.31
average	145.4 $\pm$ 11.3	22.7 $\pm$ 12.2	0.28	112.0 $\pm$ 6.9	20.0 $\pm$ 10.6	0.31

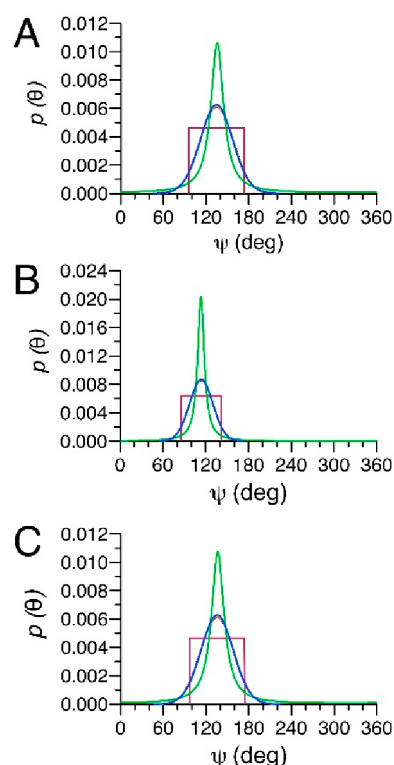
^aSE = standard errors.

Figure 2. Different statistical models of ψ in tetrasaccharide 4. (A) M_2 - X_2 linkage. (B) X_2 - M_1 linkage. (C) M_1 - X_1 linkage. (See Scheme 1 for residue definitions.) Black, Cartwright's Power of Cosine. Orange, wrapped normal. Blue, von Mises. Green, wrapped Cauchy. Violet, uniform. The first three models gave similar probability distributions.

in 4, if present, is too small to be detected by the J -coupling methodology.

The MA'AT-derived models obtained for the three internal O -glycosidic linkages in 4 were used to construct a structural model of $[\beta\text{Man}-(1\rightarrow4)-\beta\text{Xyl}]_4$ (32), assuming that context effects on linkage conformation are small. This treatment yielded a helical structure of 4. An angle of $\sim 50^\circ$ was found between Man_1 and Man_2 , which is somewhat smaller than the 70° angle reported by Ito and co-workers.¹⁹ A global minimum energy structure of 4 in H_2O was calculated by Ito and co-

workers¹⁹ assuming identical torsion angles of $\sim 98^\circ$ and 140° for the $\text{O}5'-\text{C}1'-\text{O}1'-\text{C}4$ (ϕ) and $\text{C}1'-\text{O}1'-\text{C}4-\text{C}5$ (ψ) torsion angles, respectively, in the two types of linkages. These torsion angles translate into $\sim 22^\circ$ and $\sim 20^\circ$ when phase shifts of -120° are applied to allow comparison to the MA'AT torsion angles ($\phi = \text{C}2'-\text{C}1'-\text{O}1'-\text{C}4$; $\psi = \text{C}1'-\text{O}1'-\text{C}4-\text{C}3$). The adjusted torsion angles reported by Ito and co-workers¹⁹ differ appreciably from those obtained by MA'AT analysis (Table 6; Table S1, Supporting Information) for reasons that are unclear.

DFT calculations were performed on model octasaccharide 32, in which ϕ and ψ for each O -glycosidic linkage were fixed at values determined in 2 or 3 (Table 6), while all other torsion angles were allowed to optimize. The optimized structure (Figure 3) showed putative inter-residue H-bonds across the

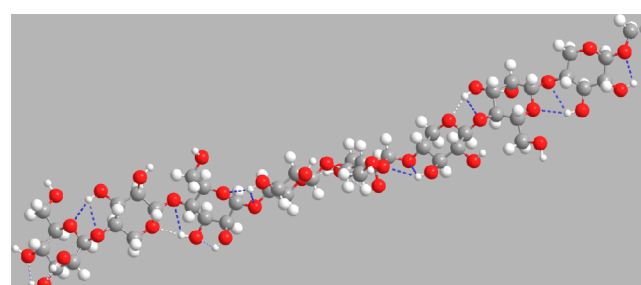


Figure 3. Geometry-optimized structure of octasaccharide 32 obtained from DFT calculations (Gaussian09), showing putative inter-residue H-bonds across the $\beta\text{Xyl}-(1\rightarrow4)-\beta\text{Man}$ and $\beta\text{Man}-(1\rightarrow4)-\beta\text{Xyl}$ linkages and an overall helical shape. Potential intra- and inter-residue H-bonds are also shown. See text for discussion.

$\beta\text{Xyl}-(1\rightarrow4)-\beta\text{Man}$ linkages ($\text{O}5'\cdots\text{H}-\text{O}3$; $r_{\text{O}5'-\text{O}3}$ 1.995–2.004 Å) and the $\beta\text{Man}-(1\rightarrow4)-\beta\text{Xyl}$ linkages ($\text{O}5'\cdots\text{H}-\text{O}3$; $r_{\text{O}5'-\text{O}3}$ 2.433–2.446 Å) that may influence linkage geometries and/or affect linkage flexibility.^{39,40} Putative H-bonds in 32 are also observed involving O -glycosidic linkage oxygens as acceptors, and between the contiguous $\text{O}2\text{H}$ and $\text{O}3\text{H}$ groups in βManp residues. In all cases, these H-bonds are predicted based on optimal internuclear distances between the donor and acceptor oxygens, and on the geometry of the H-bond interaction. Whether these putative H-bonds are sufficiently strong and/or persistent in aqueous solution to affect the

overall structure of **32** remains to be tested experimentally. Nonetheless, the preferred linkage geometries in **32** suggested from MA'AT analysis induce an overall helical shape to the molecule, in which a relatively continuous band of hydrophilic residues winds around the helical axis, creating regularly spaced hydrophilic patches on the helix surface. This structural property may contribute to the thermal hysteresis (TH) properties of antifreeze glycolipid **1** through differential binding to specific ice surfaces, although modeling of these putative interactions remains to be performed.

Behavior of ϕ and ψ in 2–4 and 32 Determined from Aqueous Molecular Dynamics (MD) Simulations. Mean and CSD values for ϕ and ψ in 2–4, obtained from aqueous 1- μ s MD simulations, are summarized in Table 7. MD

Table 7. Behavior of ϕ and ψ in 2–4 and 32 Determined from 1- μ s Aqueous Molecular Dynamics Simulations

compd	linkage	ϕ		ψ	
		mean (deg)	CSD (deg)	mean (deg)	CSD (deg)
2	β Man-(1 $\rightarrow$ 4)- β Xyl	162.3	20.1	138.9	32.2
3	β Xyl-(1 $\rightarrow$ 4)- β Man	161.1	17.8	115.8	22.9
4	β Man ₁ -(1 $\rightarrow$ 4)- β Xyl ₁	160.5	19.7	139.8	31.9
	β Xyl ₁ -(1 $\rightarrow$ 4)- β Man ₂	160.8	16.8	115.8	20.3
	β Man ₂ -(1 $\rightarrow$ 4)- β Xyl ₂	162.3	19.9	139.3	31.8
32	β Man ₁ -(1 $\rightarrow$ 4)- β Xyl ₁	162.3	19.6	136.6	34.2
	β Xyl ₁ -(1 $\rightarrow$ 4)- β Man ₂	159.1	17.1	115.0	24.6
	β Man ₂ -(1 $\rightarrow$ 4)- β Xyl ₂	162.2	20.6	139.5	30.0
	β Xyl ₂ -(1 $\rightarrow$ 4)- β Man ₃	160.9	16.7	115.6	19.5
	β Man ₃ -(1 $\rightarrow$ 4)- β Xyl ₃	162.3	20.1	139.7	31.3
	β Xyl ₃ -(1 $\rightarrow$ 4)- β Man ₄	160.8	18.0	115.8	17.0
	β Man ₄ -(1 $\rightarrow$ 4)- β Xyl ₄	162.3	19.7	137.8	32.6

histograms for **2** and **3** are shown in Figure 4, and for tetrasaccharide **4** in Figure 5. Mean values of ϕ in 2–3 ($\sim 160^\circ$) predicted by MD are slightly larger than MA'AT-determined mean values (Figure 4; Tables 6 and 7). Mean values of ψ compare favorably to corresponding MA'AT-determined mean values (Figure 4; Tables 6 and 7), giving ψ at $\sim 139^\circ$ for the β Man-(1 $\rightarrow$ 4)- β Xyl linkage and 116° for the β Xyl-(1 $\rightarrow$ 4)- β Man linkage. The similar ϕ values and different ψ values for the two different types of linkages in 2–4 obtained from the MD simulations replicate the differences obtained from MA'AT analyses of **2** and **3**. The MD results also show that context has little effect on linkage conformation in **4**, in agreement with the findings from MA'AT analyses.

Context effects were further investigate by conducting an aqueous 1 μ s MD simulation of octasaccharide **32**, which contains four repeating β Man-(1 $\rightarrow$ 4)- β Xyl disaccharide units (Figure S18, Supporting Information). No significant differences were observed in the behaviors of ϕ and ψ values for similar linkages in **32**; that is, ϕ and ψ are virtually identical in the four β Man- β Xyl linkages and in the three β Xyl- β Man linkages in **32** (Table 7). Furthermore, conformations of the two types of O-glycosidic linkages in **32** are similar to those of

the corresponding “isolated” linkages in disaccharides **2** and **3** and the corresponding “in-context” linkages in **4** (Tables 6 and 7).

CONCLUSIONS

The introduction of β Manp residues in the chemical syntheses of disaccharide **2** and tetrasaccharide **4** was accomplished by double inversion at C2 and C4 of surrogate β Galp residues. This approach gave **2** and **4** with β Man-(1 $\rightarrow$ 4) linkages in a high yield and devoid of byproducts containing α Man-(1 $\rightarrow$ 4) linkages. The terminal β Manp linkage in disaccharide **3** was derived straightforwardly from a β Manp acceptor prepared by Fischer glycosidation of free mannose. The chemical routes to prepare **2–4** were compatible with selective ^{13}C -labeling from the standpoint of simplicity, reliability, and yield, and the selection of the labeled sites was based on the desire to optimize the measurements of J_{CH} and J_{CC} values across their internal O-glycosidic linkages.

A qualitative inspection of the J -coupling ensembles that are sensitive to ϕ and ψ in 2–4 supports two structural conclusions: (1) The two different types of β -(1 $\rightarrow$ 4) O-glycosidic linkages in **2** and **3** adopt different conformations in aqueous solution, and (2) this difference is maintained when these linkages are embedded into tetrasaccharide **4**. One of the advantages of trans-O-glycosidic J -couplings to interrogate linkage geometry over other NMR constraints is that qualitative inspections of the data allow firm conclusions to be drawn with regard to whether linkages assume different geometries in different structural contexts. This analysis evolves from the linear averaging of these parameters in the presence of conformational exchange in the fast-exchange limit, and from relationships between J -coupling and molecular torsion angle for an isolated linkage that are transferable to the same linkage found in a different structural context.

More detailed conformational information can be extracted from J -coupling ensembles by applying circular statistics, leading to MD-like conformational models of ϕ and ψ in 2–4.¹⁸ In the present work, only three J -couplings were used to evaluate ϕ and ψ (total of six J -couplings), and in general, all exhibit a dominant dependence on only one of the two torsion angles. In this work, given the small number of J values used to determine ϕ and ψ , only single-state models of each torsion angle were permissible statistically. However, given the uncertainties associated with the DFT calculations and with equation parameterization, the RMS errors calculated for single-state fits were very low, suggesting that single-state models are reasonable approximations of solution behavior.

Conventional wisdom holds that β -(1 $\rightarrow$ 4) linkages are largely similar in conformation based on interpretations of ^1H – ^1H NOEs and other distance-dependent NMR parameters.^{41–43} The NOE approach, however, suffers from an inability to derive explicit conformational models exclusively from the experimental data and cannot interrogate the degree of flexibility of the linkage. The J -coupling approach has sufficient dynamic range and sensitivity to distinguish between subtle conformational differences in otherwise similar linkages. Collectively these small differences will propagate and impact the overall topology of larger molecules. Prior work on “isolated” β -(1 $\rightarrow$ 4) linkages has showed that linkage conformations can be classified based largely on the behavior of ψ .¹⁸ However, the expectation that these linkages can be classified into discrete or quantized groups is likely to be

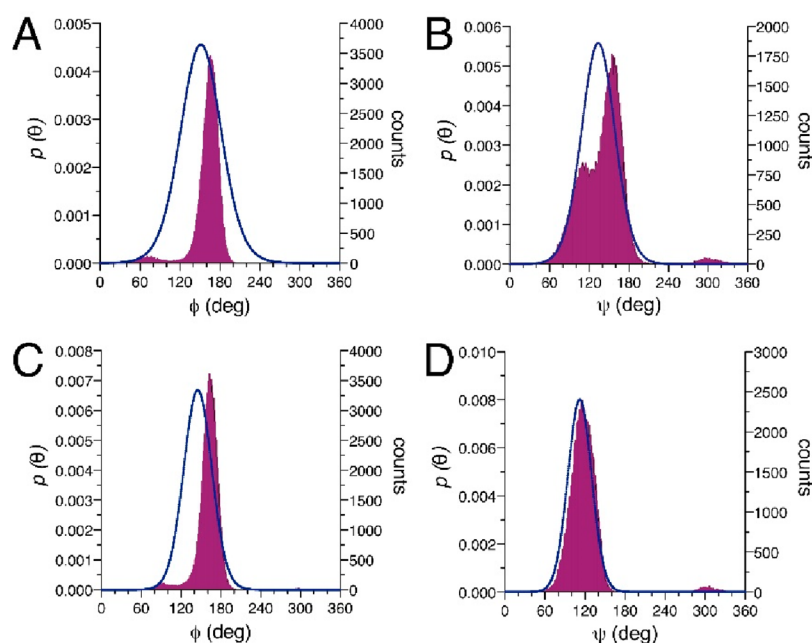


Figure 4. Aqueous 1- μs MD simulation histograms (red) for ϕ and ψ in $\beta\text{Man}-(1\rightarrow4)-\beta\text{XylOCH}_3$ (2) (A and B) and $\beta\text{Xyl}-(1\rightarrow4)-\beta\text{ManOCH}_3$ (3) (C and D) superimposed on statistical distributions of ϕ and ψ (von Mises) determined by NMR J -coupling analysis (MA'AT) (blue lines). Mean positions of ϕ (MD) are $+162.3^\circ$ and $+161.1^\circ$ for 2 and 3, respectively. Mean positions of ψ (MD) are $+138.9^\circ$ and $+115.8^\circ$ for 2 and 3, respectively. See Tables 6 and 7 for CSDs.

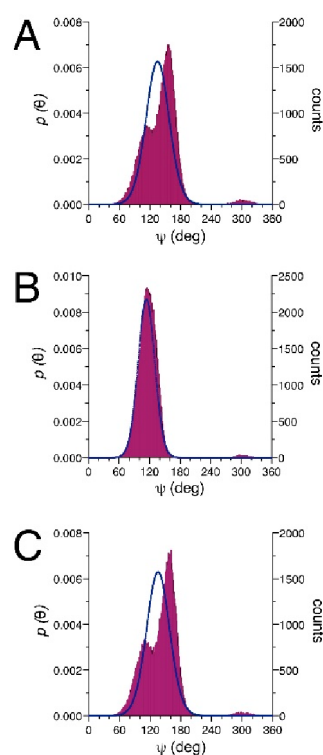


Figure 5. Statistical distributions of ψ (von Mises) determined by NMR J -coupling analysis (MA'AT) (blue lines) superimposed on histograms obtained from aqueous 1- μs MD simulations (red) for tetrasaccharide 4. (A) M_2-X_2 linkage. (B) X_2-M_1 linkage. (C) M_1-X_1 linkage. See Scheme 1 for residue definitions.

frustrated. As more structures are investigated, a continuum of torsion angles is likely to be observed, especially for ψ .

One of the attractive features of MA'AT analysis is that the conformational models so obtained are amenable to direct

comparison to those computed from MD simulation. Indeed, a major motivation for developing the MA'AT method is to provide an experimental means of validating MD predictions. In the present work, the comparisons revealed that models of ψ in 2–4 based on MA'AT analysis and MD simulation are in good agreement with respect to mean values and CSDs. However, the fine structure of the ψ distributions obtained from MD simulation of all $\beta\text{Man}-(1\rightarrow4)-\beta\text{Xyl}$ linkages in 2–4 is not reproduced in the MA'AT-derived models. However, the number of J -coupling constraints used in the current treatment precludes the testing of multi-state models, thus leaving open the possibility that the behavior predicted by MD is real. In contrast, ϕ distributions obtained from MA'AT and MD studies differ appreciably with respect to both mean positions and CSDs; similar behavior was observed in recent studies of a wide range of $\beta-(1\rightarrow4)$ -linkages.¹⁸ Larger CSDs were obtained from MA'AT analysis than from MD simulation, indicating greater librational motion than predicted by MD. These findings may require adjustments to the GLYCAM force field to bring it into better alignment with the experimental results.

MD simulations of the βXylp rings in 1–3 were found to be flawed in that $\sim 50\%$ of these rings were found in the ring-inverted 1C_4 chair form regardless of how the βXylp residue was linked to βManp residues (i.e., as donor or acceptor). As discussed herein, ${}^3J_{\text{HH}}$, ${}^2J_{\text{CH}}$, and ${}^3J_{\text{CH}}$ values indicate that the 4C_1 form (D-isomer) highly predominates in aqueous solution. In this work, these ring-inverted conformers were excluded when MD histograms were generated. This flaw was observed in recent work,¹⁸ and points to the need for MD force-field reparameterization. This problem raises obvious concerns about the reliability of MD to predict ring conformational equilibria in more complex cases, such as aldofuranosyl and idohexopyranosyl rings, where equilibria involving multiple conformers in comparable abundances may pertain.

Incorporation of β Man-(1 $\rightarrow$ 4)- β Xyl and β Xyl-(1 $\rightarrow$ 4)- β Man linkages into linear structures such as octasaccharide **32** does not appear to affect their intrinsic conformational preferences. This is not a surprising finding given the linear character of **4**, although context effects may become evident as oligosaccharide length increases. In cases where multiple O-glycosidic linkages exist within the same residue, local steric factors may prove significant which favor linkage geometries that differ appreciably from those observed in isolated disaccharides.

EXPERIMENTAL SECTION

Synthesis of Methyl β -D-Mannopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranoside **2 (Scheme 2).** 2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl trichloroacetimidate (**5**). D-Galactose (1.20 g, 6.67 mmol) was dissolved in pyridine (40 mL), and acetic anhydride (6.25 mL, 66.7 mmol) was added. The reaction mixture was stirred at rt overnight and concentrated in vacuo to afford D-galactopyranose pentaacetate. The pentaacetate was selectively deacetylated at C1 in THF (40 mL) containing benzylamine (0.87 mL, 8.00 mmol). After purification, the product tetraacetate was converted to the corresponding trichloroacetimidate with trichloroacetonitrile and 1,8-diazobicyclo [5.4.0]-undec-7-ene (DBU) as described by Schmidt and co-workers,²³ affording **5** as a yellow syrup (1.96 g, 4.00 mmol, 60%).

Methyl 2,3-O-Isopropylidene- β -D-xylopyranoside (6**).** To a sealed flask containing methyl β -D-xylopyranoside (1.00 g, 6.10 mmol), dry *N,N*-dimethylformamide (DMF) (10 mL), and methanol (0.20 mL) was added acetyl chloride (40 μ L), and the resulting solution was cooled in an ice bath. 2-Methoxypropene (1.40 mL) was added dropwise to the ice-cold reaction mixture over 10 min. The reaction mixture, which clarified over a short time period, was stirred for 2 h at rt. CH_2Cl_2 (100 mL) was added, and the solution was extracted with an aqueous NaHCO_3 solution (0.1 M, 50 mL) and water (50 mL). The NaHCO_3 solution and water extracts were back-extracted with CH_2Cl_2 (2 $\times$ 50 mL). The organic solutions were combined, dried over anhydrous Na_2SO_4 , and concentrated at 30 $^\circ\text{C}$ in vacuo, and the residue was purified by flash chromatography on a silica gel column (2.5 cm $\times$ 30 cm) (eluent: hexanes/ethyl acetate, 1.5:1) to afford **6** as a white solid (0.95 g, 4.66 mmol, 76%).⁴⁴ ^1H NMR (600 MHz, CDCl_3): δ 4.53 (d, J = 7.5 Hz, H-1, 1H), 4.06 (dd, J = 11.7, 5.4 Hz, H-5a, 1H), 4.03–3.99 (m, H-4, 1H), 3.54 (s, OCH_3 , 3H), 3.52 (t, J = 9.4 Hz, H-3, 1H), 3.32 (dd, J = 9.5, 7.5 Hz, H-2, 1H), 3.24 (dd, J = 11.7, 8.3 Hz, H-5b, 1H), 2.88 (d, J = 3.9 Hz, OH-4, 1H), 1.46, 1.45 (s, CMe_2 , 6H). ^{13}C NMR (150 MHz, CDCl_3): δ 111.8 (CMe_2), 102.9 (C-1), 81.2, 76.6, 69.3, 67.4, 56.8 (OCH_3), 26.9 (CMe_2), 26.7 (CMe_2).

Methyl 2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-xylopyranoside (7**).** Donor **5** (1.30 g, 2.65 mmol) and acceptor **6** (0.50 g, 2.45 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL) after drying under a high vacuum, and the solution was treated with molecular sieves (4 $\text{\AA}$) (1.0 g). A catalytic amount of trimethylsilyltriflate (15 μ L, 0.08 mmol) was added under N_2 at 0 $^\circ\text{C}$. After the mixture stirred for 2 h at rt, additional trimethylsilyltriflate (15 μ L, 0.08 mmol) was added, and the reaction mixture was stirred at rt overnight. The reaction mixture was quenched with the addition of triethylamine (30 μ L), and the molecular sieves were removed by filtration. The solution was concentrated and purified by flash chromatography on a silica gel column (2.5 cm $\times$ 40 cm) (eluent: hexanes/ethyl acetate, 2:1) to afford **7** (0.68 g, white foam with minor impurities, 1.27 mmol, ~52%). ^1H NMR (600 MHz, CDCl_3): δ 5.36 (dd, J = 3.5, 1.2 Hz, H-4^{Gal}, 1H), 5.18 (dd, J = 10.4, 8.1 Hz, H-2^{Gal}, 1H), 5.00 (dd, J = 10.4, 3.5 Hz, H-3^{Gal}, 1H), 4.63 (d, J = 8.1 Hz, H-1^{Gal}, 1H), 4.53 (d, J = 7.4 Hz, H-1^{Xyl}, 1H), 4.16 (dd, J = 11.0, 6.0 Hz, H-6a^{Gal}, 1H), 4.12–3.98 (m, H-6b^{Gal}, H-5a^{Xyl}, H-4^{Xyl}, 3H), 3.92–3.90 (m, H-5^{Gal}, 1H), 3.67 (dd, J = 9.7, 8.5 Hz, H-3^{Xyl}, 1H), 3.49 (s, OCH_3 , 3H), 3.33–3.30 (m, H-5b^{Xyl}, H-2^{Xyl}, 2H), 2.12, 2.04, 2.02, 1.96 (s, 4COCH_3 , 12H), 1.43, 1.42 (s, CMe_2 , 6H). ^{13}C NMR (150 MHz, CDCl_3): δ 170.4, 170.3, 170.2, 169.4 (4COCH_3), 111.9 (CMe_2), 102.5 (C-1^{Xyl}), 100.0 (C-

1^{Gal}), 78.8 (C-3^{Xyl}), 76.8 (C-4^{Xyl}), 76.7 (C-2^{Xyl}), 71.2 (C-3^{Gal}), 71.0 (C-5^{Gal}), 68.9 (C-2^{Gal}), 67.0 (C-4^{Gal}), 65.1 (C-5^{Xyl}), 61.0 (C-6^{Gal}), 56.6 (OCH_3), 26.8, 26.7 (CMe_2), 20.8, 20.8, 20.7, 20.7 (4COCH_3). HRMS (ESI-TOF) m/z [$\text{M} + \text{Na}$]⁺: calcd for $\text{C}_{23}\text{H}_{34}\text{O}_{14}\text{Na}$, 557.1841; found, 557.1847.

Methyl 4-O-(3,6-Di-O-pivaloyl- β -D-galactopyranosyl)-2,3-O-isopropylidene- β -D-xylopyranoside (8**).** Compound **7** (1.0 g, 1.87 mmol) was dissolved in methanol (20 mL), and the solution was saturated with NH_3 (g). The reaction mixture was stirred at rt overnight and then concentrated and purified on a silica gel column (1.5 cm $\times$ 30 cm; eluted with methanol/ethyl acetate (1:6)) to obtain methyl β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-xylopyranoside (0.64 g, 1.75 mmol). The latter compound was dissolved in dry pyridine (10 mL) at -20 $^\circ\text{C}$, and pivaloyl chloride (0.45 mL, 3.68 mmol) was added dropwise. The reaction mixture was stirred at rt overnight, diluted with CH_2Cl_2 (30 mL), and washed successively with aqueous HCl (1 N) and distilled water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under a vacuum, and the residue was applied to a silica gel column (2.5 cm $\times$ 30 cm; eluted with hexane/ethyl acetate (2:1)) to afford **8** as a white foam (0.77 g, 1.44 mmol, 82%). ^1H NMR (600 MHz, CDCl_3): δ 4.77 (dd, J = 10.1, 3.3 Hz, H-3^{Gal}, 1H), 4.47 (d, J = 7.4 Hz, H-1^{Xyl}, 1H), 4.40 (d, J = 7.9 Hz, H-1^{Gal}, 1H), 4.22–4.19 (m, H-6a^{Gal}, H-6b^{Gal}, 2H), 4.06 (ddd, J = 8.9, 6.9, 5.1 Hz, H-4^{Xyl}, 1H), 4.00 (dd, J = 12.1, 5.1 Hz, H-5a^{Xyl}, 1H), 3.91 (dd, J = 3.4, 1.0 Hz, H-4^{Gal}, 1H), 3.86 (dd, J = 10.1, 7.9 Hz, H-2^{Gal}, 1H), 3.71–3.69 (m, H-5^{Gal}, 1H), 3.60 (dd, J = 9.7, 8.9 Hz, H-3^{Xyl}, 1H), 3.41 (s, OCH_3 , 3H), 3.32 (dd, J = 12.2, 6.9 Hz, H-5b^{Xyl}, 1H), 3.26 (dd, J = 9.8, 7.4 Hz, H-2^{Xyl}, 1H), 1.36, 1.35 (s, CMe_2 , 6H), 1.15, 1.10 (s, 2piv, 18H). ^{13}C NMR (150 MHz, CDCl_3): δ 178.2, 178.0 (2piv), 111.7 (CMe_2), 102.3 (C-1^{Xyl}), 101.7 (C-1^{Gal}), 78.8 (C-3^{Xyl}), 76.5 (C-2^{Xyl}), 74.7 (C-3^{Gal}), 74.3 (C-4^{Xyl}), 72.6 (C-5^{Gal}), 68.3 (C-2^{Gal}), 66.9 (C-4^{Gal}), 65.2 (C-5^{Xyl}), 62.2 (C-6^{Gal}), 56.3 (OCH_3), 38.9, 38.7 (2piv), 27.1, 27.1 (2piv), 26.7, 26.5 (CMe_2). HRMS (ESI-TOF) m/z [$\text{M} + \text{Na}$]⁺: calcd for $\text{C}_{25}\text{H}_{42}\text{O}_{12}\text{Na}$, 557.2568; found, 557.2569.

Methyl 4-O-(2,4-Di-O-trifluoromethanesulfonyl-3,6-di-O-pivaloyl- β -D-galactopyranosyl)-2,3-O-isopropylidene- β -D-xylopyranoside (9**).** Compound **8** (0.55 g, 1.03 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and dry pyridine (10 mL), and trifluoromethanesulfonic anhydride (0.69 mL, 4.12 mmol) was added slowly at -20 $^\circ\text{C}$. The mixture was stirred overnight at rt, diluted with CH_2Cl_2 (30 mL), and washed with distilled water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo, and the residue was applied to a silica gel column (2.5 cm $\times$ 30 cm; eluted with hexane/ethyl acetate (6:1)) to give product **9** as a white foam (0.74 g, 0.93 mmol, 90%). ^1H NMR (600 MHz, CDCl_3): δ 5.27 (dd, J = 2.9, 0.7 Hz, H-4^{Gal}, 1H), 5.22 (dd, J = 10.2, 2.9 Hz, H-3^{Gal}, 1H), 4.93 (dd, J = 10.2, 7.9 Hz, H-2^{Gal}, 1H), 4.85 (d, J = 7.9 Hz, H-1^{Gal}, 1H), 4.58 (d, J = 7.3 Hz, H-1^{Xyl}, 1H), 4.47 (dd, J = 11.2, 5.7 Hz, H-6a^{Gal}, 1H), 4.10–4.01 (m, H-5^{Gal}, H-5a^{Xyl}, H-4^{Xyl}, 3H), 3.85 (dd, J = 11.4, 9.4 Hz, H-6b^{Gal}, 1H), 3.60 (dd, J = 9.8, 8.5 Hz, H-3^{Xyl}, 1H), 3.49 (s, OCH_3 , 3H), 3.41 (dd, J = 12.2, 6.3 Hz, H-5b^{Xyl}, 1H), 3.33 (dd, J = 9.8, 7.3 Hz, H-2^{Xyl}, 1H), 1.46, 1.43 (s, CMe_2 , 6H), 1.26, 1.18 (s, 2piv, 18H). ^{13}C NMR (150 MHz, CDCl_3): δ 178.1, 177.6 (2piv), 112.2 (CMe_2), 102.4 (C-1^{Xyl}), 98.8 (C-1^{Gal}), 80.7 (C-4^{Gal}), 80.3 (C-2^{Gal}), 78.9 (C-3^{Xyl}), 78.0 (C-4^{Xyl}), 76.8 (C-2^{Xyl}), 70.3 (C-5^{Gal}), 70.2 (C-3^{Gal}), 64.3 (C-5^{Xyl}), 59.4 (C-6^{Gal}), 56.5 (OCH_3), 39.4, 38.9 (2piv), 27.1, 27.0 (2piv), 26.9, 26.8 (CMe_2).

Methyl 4-O-(2,4-Di-O-acetyl-3,6-di-O-pivaloyl- β -D-mannopyranosyl)-2,3-O-isopropylidene- β -D-xylopyranoside (10**).** A mixture of compound **9** (638 mg, 0.80 mmol), cesium acetate (614 mg, 3.20 mmol), and 18-crown-6 (845 mg, 3.20 mmol) was dried under a vacuum, and dry toluene (15 mL) was added. The reaction mixture was stirred for 2 h at 90 $^\circ\text{C}$. The reaction solution was then diluted with CH_2Cl_2 (30 mL), and washed with aqueous NaHCO_3 solution (1 N), followed by distilled water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under a vacuum, and the residue was applied to a silica gel column (1.5 cm $\times$ 40 cm; eluted with hexane/ethyl acetate (3:1)) to obtain product **10** as a white foam (330 mg, 0.53 mmol, 66%). ^1H NMR (600 MHz, CDCl_3): δ 5.44 (dd,

$J = 3.4, 1.1 \text{ Hz}$, H-2^{Man}, 1H), 5.29 (t, $J = 9.9 \text{ Hz}$, H-4^{Man}, 1H), 4.79 (dd, $J = 10.0, 3.4 \text{ Hz}$, H-3^{Man}, 1H), 4.83 (d, $J = 1.1 \text{ Hz}$, H-1^{Man}, 1H), 4.49 (d, $J = 7.4 \text{ Hz}$, H-1^{Xyl}, 1H), 4.24 (dd, $J = 12.1, 2.8 \text{ Hz}$, H-6a^{Man}, 1H), 4.15 (dd, $J = 12.1, 5.2 \text{ Hz}$, H-6b^{Man}, 1H), 4.00–3.96 (m, H-5a^{Xyl}, H-4^{Xyl}, 2H), 3.68–3.64 (m, H-5^{Man}, H-3^{Xyl}, 2H), 3.46 (s, OCH₃, 3H), 3.31–3.27 (m, H-5b^{Xyl}, 1H), 3.25 (dd, $J = 9.8, 7.4 \text{ Hz}$, H-2^{Xyl}, 1H), 2.08, 1.97 (s, 2COCH₃, 6H), 1.39, 1.38 (s, CMe₂, 6H), 1.19, 1.07 (s, 2piv, 18H). ¹³C NMR (150 MHz, CDCl₃): δ 178.0, 177.3 (2piv), 170.1, 169.3 (2COCH₃), 112.8 (CMe₂), 102.4 (C-1^{Xyl}), 96.9 (C-1^{Man}), 78.8 (C-3^{Xyl}), 76.7 (C-2^{Xyl}), 76.0 (C-4^{Xyl}), 72.6 (C-5^{Man}), 71.0 (C-3^{Man}), 68.6 (C-2^{Man}), 66.0 (C-4^{Man}), 65.0 (C-5^{Xyl}), 62.2 (C-6^{Man}), 56.4 (OCH₃), 38.9, 38.8 (2piv), 27.2, 26.8 (2piv), 26.8, 26.6 (CMe₂), 20.8, 20.7 (2COCH₃). HRMS (ESI-TOF) m/z [M + Na]⁺: calcd for C₂₉H₄₆O₁₄Na, 641.2785; found, 641.2786.

Methyl 4-O- β -D-Mannopyranosyl- β -D-xylopyranoside (2). Compound **10** (330 mg, 0.53 mmol) was dissolved in anhydrous methanol (10 mL), and acetyl chloride (10 μ L) was added at 0 °C. After stirring for 2 h at rt, the reaction solution was saturated with NH₃ (g) and stirred for 3 days at 50 °C. The reaction mixture was concentrated at 30 °C in vacuo, the residue was dissolved in ~0.5 mL of distilled water, and the solution was applied to a column (2.5 cm $\times$ 100 cm) containing Dowex 50 $\times$ 8 (200–400 mesh) ion-exchange resin in the Ca²⁺ form.⁴⁵ The column was eluted with distilled, decarbonated water at ~1.5 mL/min, and fractions (~60 mL) containing pure product were collected and concentrated at 30 °C in vacuo to give **2** as a white solid (135 mg, 0.41 mmol, 78%). Detailed characterization of **2** by ¹H and ¹³C NMR is found in the text. Mp: 210–216 °C. HRMS (ESI-TOF) m/z [M + Na]⁺: calcd for C₁₂H₂₂O₁₀Na, 349.1105; found, 349.1102.

Synthesis of Methyl β -Xylopyranosyl-(1 $\rightarrow$ 4)- β -D-mannopyranoside 3 (Scheme 3). Methyl 2,3-Di-O-benzyl-4,6-O-benzylidene- β -D-mannopyranoside (**12**). Methyl β -D-mannopyranoside (**11**) (2.00 g, 10.3 mmol) was dissolved in dry *N,N*-dimethylformamide (DMF) (30 mL), and benzaldehyde dimethylacetal (1.78 mL, 11.9 mmol) and a catalytic amount of *p*-toluenesulfonic acid were added. The reaction mixture was stirred at rt overnight and neutralized by adding one drop of triethylamine. The DMF was removed in vacuo on a rotovap, the syrup was dissolved in CH₂Cl₂, and the resulting solution was washed with water. The organic phase was dried over Na₂SO₄ and concentrated in vacuo to a syrup. The methyl 4,6-O-benzylidene- β -D-mannopyranoside product was isolated by crystallization from hexane/ethyl acetate (1.80 g, 6.3 mmol, 61%). A portion of the 4,6-O-benzylidene derivative (1.20 g, 4.25 mmol) was dissolved in DMF (30 mL), and NaH (90%, 0.85 g, 21.3 mmol) was added to the solution. After the mixture stirred at rt for 1 h, benzyl bromide (2.03 mL, 17.0 mmol) was added dropwise at 0 °C and the mixture was stirred at rt for 20 h. The mixture was then diluted with CH₂Cl₂ (50 mL) and washed with water. The organic phase was dried over anhydrous Na₂SO₄ and concentrated in vacuo to dryness, and the residue purified by flash chromatography on silica gel (2.5 cm $\times$ 40 cm, eluted with hexane/ethyl acetate (4:1)) to afford **12** as white crystals (1.76 g, 3.83 mmol, 90%). ¹H NMR (600 MHz, CDCl₃): δ 7.51–7.25 (m, 2PhCH₂, PhCH, 15H), 5.65 (s, PhCH, 1H), 4.99 (d, $J = 12.2 \text{ Hz}$, PhCH₂, 1H), 4.87 (d, $J = 12.2 \text{ Hz}$, PhCH₂, 1H), 4.70 (d, $J = 12.2 \text{ Hz}$, PhCH₂, 1H), 4.59 (d, $J = 12.2 \text{ Hz}$, PhCH₂, 1H), 4.40 (d, $J = 1.0 \text{ Hz}$, H-1, 1H), 4.35 (dd, $J = 10.4, 4.8 \text{ Hz}$, H-6a, 1H), 4.23 (t, $J = 9.6 \text{ Hz}$, H-4, 1H), 3.96 (t, $J = 10.3 \text{ Hz}$, H-6b, 1H), 3.95 (dd, $J = 3.2, 1.0 \text{ Hz}$, H-2, 1H), 3.61 (dd, $J = 9.9, 3.3 \text{ Hz}$, H-3, 1H), 3.56 (s, OCH₃, 3H), 3.35 (ddd, $J = 10.1, 9.3, 4.9 \text{ Hz}$, H-5, 1H). ¹³C NMR (150 MHz, CDCl₃): δ 138.6, 138.5, 137.8, 129.0–126.2 (2PhCH₂, PhCH), 103.6, 101.6, 78.8, 78.0, 76.0, 75.0, 72.5, 68.8, 67.8, 57.6 (OCH₃).

Methyl 2,3,6-Tri-O-benzyl- β -D-mannopyranoside (13). Compound **12** (2.10 g, 4.52 mmol) was dissolved in anhydrous CH₂Cl₂ (40 mL), and triethylsilane (8.70 mL, 54.2 mmol) and BF₃·Et₂O (1.15 mL, 9.04 mmol) were added at 0 °C. The reaction mixture was stirred at rt for 5 h. The mixture was then diluted with CH₂Cl₂ (50 mL), and the resulting solution was washed with aqueous NaHCO₃ solution (1 N) (20 mL), followed by distilled water (50 mL). The organic phase was dried over Na₂SO₄ and concentrated at 30 °C in

vacuo to dryness, and the residue was purified on a silica gel column (2.5 cm $\times$ 50 cm; eluent: hexanes/ethyl acetate, 3:1) to afford **13** as white crystals (1.45 g, 3.12 mmol, 69%).⁴⁶ ¹H NMR (600 MHz, CDCl₃): δ 7.46–7.25 (m, 3PhCH₂, 15H), 4.98 (d, $J = 12.4 \text{ Hz}$, PhCH₂, 1H), 4.76 (d, $J = 12.4 \text{ Hz}$, PhCH₂, 1H), 4.62 (s, PhCH₂, 2H), 4.49 (d, $J = 11.9 \text{ Hz}$, PhCH₂, 1H), 4.36 (d, $J = 11.9 \text{ Hz}$, PhCH₂, 1H), 4.34 (d, $J = 0.9 \text{ Hz}$, H-1, 1H), 3.98 (dt, $J = 1.9, 9.6 \text{ Hz}$, H-4, 1H), 3.91–3.88 (m, H-6a, H-2, 2H), 3.80 (dd, $J = 10.4, 6.1 \text{ Hz}$, H-6b, 1H), 3.56 (s, OCH₃, 3H), 3.46 (ddd, $J = 9.6, 6.1, 3.7 \text{ Hz}$, H-5, 1H), 3.32 (dd, $J = 9.5, 3.1 \text{ Hz}$, H-3, 1H). ¹³C NMR (150 MHz, CDCl₃): δ 138.9, 138.3, 138.0, 128.7–127.6 (3PhCH₂), 103.0 (C-1), 81.6 (C-3), 75.4 (C-5), 74.3, 73.9, 71.4 (3PhCH₂), 73.5 (C-2), 71.1 (C-6), 68.5 (C-4), 57.4 (OCH₃).

2,3,4-Tri-O-acetyl- α -D-xylopyranosyl Trichloroacetimidate (14). D-Xylose (0.60 g, 4.00 mmol) was dissolved in pyridine (15 mL), and acetic anhydride (2.25 mL, 24.0 mmol) was added. The reaction mixture was stirred at rt overnight and then concentrated at 30 °C in vacuo to afford D-xylopyranose tetraacetate. The tetraacetate was selectively deacetylated at C1 with benzylamine (0.52 mL, 4.80 mmol) in THF (20 mL). After purification, the triacetate product was converted to the corresponding trichloroacetimidate with trichloroacetonitrile and 1,8-diazobicyclo [5.4.0]undec-7-ene (DBU) as described by Schmidt and co-workers,²³ affording **14** as a white foam (0.94 g, 2.24 mmol, 56%). ¹H NMR (600 MHz, CDCl₃): δ 8.67 (s, NH, 1H), 6.48 (d, $J = 3.7 \text{ Hz}$, H-1, 1H), 5.56 (t, $J = 9.9 \text{ Hz}$, H-3, 1H), 5.07 (ddd, $J = 10.9, 9.6, 5.9 \text{ Hz}$, H-4, 1H), 5.06 (dd, $J = 10.1, 3.7 \text{ Hz}$, H-2, 1H), 3.98 (dd, $J = 11.1, 5.9 \text{ Hz}$, H-5a, 1H), 3.81 (t, $J = 11.6 \text{ Hz}$, H-5b, 1H), 2.05, 2.05, 2.01 (s, 3COCH₃, 9H). ¹³C NMR (150 MHz, CDCl₃): δ 170.1, 170.1, 170.0 (3COCH₃), 161.1 (CNHCCl₃), 93.3 (C-1), 70.1 (C-2), 69.5 (C-3), 68.7 (C-4), 60.9 (C-5), 20.9, 20.8, 20.6 (3COCH₃).

Methyl 2,3,4-Tri-O-acetyl- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzyl- β -D-mannopyranoside (15). Donor **14** (0.96 g, 2.28 mmol) and acceptor **13** (0.96 g, 2.07 mmol) were dissolved in anhydrous CH₂Cl₂ (20 mL) after drying over a high vacuum, and the solution was treated with molecular sieves (4 Å, 1.0 g). A catalytic amount of trimethylsilyltriflate (25 μ L, 0.13 mmol) was added at 0 °C. The reaction mixture was stirred at rt overnight and then neutralized with the addition of triethylamine (25 μ L), and the molecular sieves were removed by filtration. The solution was concentrated, and the residue was purified by flash chromatography on a silica gel column (1.5 cm $\times$ 50 cm; eluent: hexanes/ethyl acetate, 3:1) to afford **15** as a white foam (1.20 g, 1.66 mmol, 80%). ¹H NMR (600 MHz, CDCl₃): δ 7.40–7.20 (m, 3PhCH₂, 15H), 5.05 (t, $J = 9.1 \text{ Hz}$, H-3^{Xyl}, 1H), 4.93 (d, $J = 12.0 \text{ Hz}$, PhCH₂, 1H), 4.92–4.88 (m, H-4^{Xyl}, 1H), 4.87 (dd, $J = 9.3, 7.5 \text{ Hz}$, H-2^{Xyl}, 1H), 4.82 (d, $J = 12.3 \text{ Hz}$, PhCH₂, 1H), 4.74 (d, $J = 11.9 \text{ Hz}$, PhCH₂, 1H), 4.61 (d, $J = 7.5 \text{ Hz}$, H-1^{Xyl}, 1H), 4.58 (d, $J = 11.8 \text{ Hz}$, PhCH₂, 1H), 4.54 (d, $J = 11.8 \text{ Hz}$, PhCH₂, 1H), 4.53 (d, $J = 11.8 \text{ Hz}$, PhCH₂, 1H), 4.28 (d, $J = 0.9 \text{ Hz}$, H-1^{Man}, 1H), 4.17 (t, $J = 9.2 \text{ Hz}$, H-4^{Man}, 1H), 3.92 (dd, $J = 11.8, 5.5 \text{ Hz}$, H-5a^{Xyl}, 1H), 3.86 (dd, $J = 3.1, 0.7 \text{ Hz}$, H-2^{Man}, 1H), 3.79–3.77 (m, H-6a^{Man}, H-6b^{Man}, 2H), 3.52 (s, OCH₃, 3H), 3.44 (dd, $J = 9.1, 3.2 \text{ Hz}$, H-3^{Man}, 1H), 3.38 (dt, $J = 9.3, 3.5 \text{ Hz}$, H-5^{Man}, 1H), 2.96 (dd, $J = 11.9, 9.8 \text{ Hz}$, H-5b^{Xyl}, 1H), 2.06, 2.02, 2.01, 1.96 (s, 3COCH₃, 9H). ¹³C NMR (150 MHz, CDCl₃): δ 170.3, 170.1, 169.7 (3COCH₃), 138.9, 138.6, 138.5, 128.5–127.5 (3PhCH₂), 102.8 (C-1^{Man}), 100.8 (C-1^{Xyl}), 80.2 (C-3^{Man}), 75.9 (C-5^{Man}), 75.1 (C-4^{Man}), 74.2 (C-2^{Man}), 74.0, 73.8, 72.2 (3PhCH₂), 72.4 (C-3^{Xyl}), 72.0 (C-2^{Xyl}), 69.2 (C-4^{Xyl}), 68.8 (C-6^{Man}), 62.4 (C-5^{Xyl}), 57.3 (OCH₃), 20.9, 20.9, 20.8 (3COCH₃).

Methyl β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-mannopyranoside (3). Compound **15** (0.76 g, 1.05 mmol) was dissolved in ethanol (20 mL) and treated with Pd/C (10%, 200 mg) and H₂ overnight. The Pd/C was removed by filtration, and the filtrate was concentrated at 30 °C in vacuo. The residue was dissolved in methanol (20 mL) saturated with NH₃ (g). After 15 h, the reaction mixture was concentrated at 30 °C in vacuo. The residue was dissolved in ~1 mL of distilled water, and the solution was applied to a column (2.5 cm $\times$ 100 cm) containing Dowex 50 $\times$ 8 (200–400 mesh) ion-exchange resin in the Ca²⁺ form.⁴⁵ The column was eluted with distilled, decarbonated water at ~1.5 mL/min, and fractions (~10 mL) were

collected and assayed by TLC. Fractions containing product were pooled and concentrated at 30 °C in vacuo to give **3** as a white solid (0.30 g, 0.92 mmol, 88%). Detailed characterization of **3** by ^1H and ^{13}C NMR is found in the text. Mp: 175–182 °C. HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$: calcd for $\text{C}_{12}\text{H}_{22}\text{O}_{10}\text{Na}$, 349.1105; found, 349.1127.

Synthesis of ^{13}C -Labeled Methyl β -D-Mannopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-mannopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranoside **4 (Scheme 4).** 4-Methoxyphenyl 2,3,4-Tri-O-acetyl- α -D-[1- ^{13}C]xylopyranoside (**16**). Per-O-acetylated D-[1- ^{13}C]xylopyranose (4.00 g, 12.54 mmol) was dried in vacuo and dissolved in anhydrous CH_2Cl_2 (30 mL). 4-Methoxyphenol (4.96 g, 40 mmol) and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (1.0 mL) were added to the solution. The reaction mixture was stirred at rt overnight, and then triethylamine (1.0 mL) was added to neutralize it. The solution was concentrated to dryness at 30 °C in vacuo, and the residue was purified by flash chromatography on a Grace REVELERIS X2 flash chromatography system using a silica gel flash cartridge (40 g) to afford **16** as white crystals (3.77 g, 9.87 mmol, 79%). The same purification procedure was applied to the following steps using silica gel flash cartridges of various sizes (4–80 g) and variable ratios of hexanes/ethyl acetate as the solvent. Product **16** was used directly to prepare **18** without characterization by NMR and MS (see below).

4-Methoxyphenyl 2,3-O-Isopropylidene- β -D-[1- ^{13}C]xylopyranoside (18**).** Compound **16** (4.00 g, 10.5 mmol) was dissolved in methanol (50 mL) saturated with NH_3 , and the solution was stirred at rt for 16 h to obtain product **17**. Then, to a sealed flask containing compound **17** (2.60 g, 10.1 mmol), dry N,N -dimethylformamide (DMF) (40 mL), and methanol (0.40 mL) was added acetyl chloride (200 μL). The reaction vessel was cooled in an ice bath, and 2-methoxypropene (2.50 mL, 26.0 mmol) was added dropwise to the ice-cold reaction mixture over 10 min. The reaction mixture, which was initially cloudy but soon cleared, was stirred for 4 h at rt. CH_2Cl_2 (200 mL) was added, and the solution was extracted successively with an aqueous NaHCO_3 solution (0.1 M, 100 mL) and distilled water (100 mL). The NaHCO_3 and water washes were back-extracted with CH_2Cl_2 twice (100 mL each). The organic solutions were combined, dried over anhydrous Na_2SO_4 , and concentrated at 30 °C in vacuo to a syrup, which was purified by flash chromatography to afford the β anomer **18** as a white solid (2.04 g, 6.90 mmol, 68%). ^1H NMR (600 MHz, CDCl_3): δ 7.04–7.01, 6.84–6.81 (m, PhOCH_3 , 4H), 5.16 (dd, J = 165.7, 7.0 Hz, H-1, 1H), 4.15–4.07 (m, H-4, H-5a, 2H), 3.77 (s, PhOCH_3 , 3H), 3.65–3.58 (m, H-2, H-3, 2H), 3.39–3.35 (m, H-5b, 1H), 2.95 (d, J = 4.0 Hz, OH-4, 1H), 1.51, 1.49 (s, CMe_2 , 6H). ^{13}C NMR (150 MHz, CDCl_3): δ 155.5, 150.6, 118.6, 118.6, 114.7 (PhOCH_3), 112.1 (CMe_2), 100.7 (C-1), 81.1 (C-3), 76.4 (C-2), 69.3 (C-4), 67.6 (C-5), 55.8 (PhOCH_3), 27.0 (CMe_2), 26.7 (CMe_2). HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$: calcd for $^{13}\text{C}_{14}\text{H}_{20}\text{O}_8\text{Na}$, 320.1186; found, 320.1185.

4-Methoxyphenyl 2,3,4,6-Tetra-O-acetyl- β -D-[1- ^{13}C]galactopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-[1- ^{13}C]xylopyranoside (19**).** Glycoside **18** (2.04 g, 6.90 mmol) and 2,3,4,6-tetra-O-acetyl- α -D-[1- ^{13}C]galactopyranosyl trichloroacetimidate (3.40 g, 6.91 mmol) (prepared from D-[1- ^{13}C]galactose)⁴⁷ were dissolved in anhydrous CH_2Cl_2 (40 mL) after drying over a high vacuum, and the solution was treated with 4 Å molecular sieves (3.0 g). Trimethylsilyltriflate (100 μL , 0.53 mmol) was added under N_2 at 0 °C, and the reaction mixture was stirred at rt overnight. The reaction mixture was quenched with the addition of triethylamine (100 μL), and the molecular sieves were removed by filtration. The solution was concentrated at 30 °C in vacuo, and the residue was purified by flash chromatography to afford disaccharide **19** as a white powder (2.40 g, 3.82 mmol, 55%). ^1H NMR (600 MHz, CDCl_3): δ 7.03–7.01, 6.84–6.81 (m, PhOCH_3 , 4H), 5.40 (dd, J = 3.5, 1.2 Hz, H-4^{Gal}, 1H), 5.24 (ddd, J = 10.4, 7.9, 6.5 Hz, H-2^{Gal}, 1H), 5.23 (dd, J = 167.4, 7.2 Hz, H-1^{Xyl}, 1H), 5.04 (ddd, J = 10.4, 3.4, 1.2 Hz, H-3^{Gal}, 1H), 4.66 (dd, J = 159.8, 8.0 Hz, H-1^{Gal}, 1H), 4.21–4.08 (m, H-6a^{Gal}, H-6b^{Gal}, H-5a^{Xyl}, H-4^{Xyl}, 4H), 3.96–3.92 (m, H-5^{Gal}, 1H), 3.83 (m, H-3^{Xyl}, 1H), 3.77 (s, PhOCH_3 , 3H), 3.66–3.62 (m, H-2^{Xyl}, 1H), 3.53–3.49 (m, H-5b^{Xyl}, 1H), 2.16, 2.07, 2.05, 1.99 (s, 4 COCH_3 , 12H), 1.51, 1.49 (s, CMe_2 , 6H). ^{13}C NMR (150 MHz, CDCl_3): δ 170.5, 170.5, 170.4, 169.5

(4 COCH_3), 155.6, 150.6, 118.6, 118.6, 114.7 (PhOCH_3), 112.5 (CMe_2), 100.4 (C-1^{Xyl}), 100.1 (C-1^{Gal}), 78.8 (C-3^{Xyl}), 77.0 (C-4^{Xyl}), 76.6 (C-2^{Xyl}), 71.2 (C-3^{Gal}), 71.1 (C-5^{Gal}), 68.9 (C-2^{Gal}), 67.0 (C-4^{Gal}), 65.4 (C-5^{Xyl}), 61.0 (C-6^{Gal}), 55.8 (PhOCH_3), 27.1, 26.9 (CMe_2), 21.0, 20.9, 20.9, 20.8 (4 COCH_3).

4-Methoxyphenyl 3,6-Di-O-pivaloyl- β -D-[1- ^{13}C]galactopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-[1- ^{13}C]xylopyranoside (20**).** Disaccharide **19** (2.40 g, 3.82 mmol) was dissolved in methanol (40 mL), and the solution was saturated with NH_3 (g). After stirring at rt overnight, the reaction mixture was concentrated at 30 °C in vacuo and purified by chromatography. The product (1.67 g, 3.63 mmol) was dissolved in dry pyridine (10 mL), and pivaloyl chloride (1.06 mL, 8.71 mmol) was added dropwise to the solution at –20 °C. The reaction mixture was stirred overnight at rt, diluted with CH_2Cl_2 (30 mL), and washed successively with aqueous HCl (1 N) and distilled water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated at 30 °C in vacuo, and the residue was purified by flash chromatography to afford **20** as a white foam (1.95 g, 3.10 mmol, 81%). ^1H NMR (600 MHz, CDCl_3): δ 6.95–6.92, 6.75–6.72 (m, PhOCH_3 , 4H), 5.16 (dd, J = 167.4, 7.3 Hz, H-1^{Xyl}, 1H), 4.80 (dd, J = 10.2, 3.1 Hz, H-3^{Gal}, 1H), 4.44 (dd, J = 159.7, 8.0 Hz, H-1^{Gal}, 1H), 4.27–4.21 (m, H-6a^{Gal}, H-6b^{Gal}, 2H), 4.17–4.07 (m, H-4^{Xyl}, H-5a^{Xyl}, 2H), 3.94 (dd, J = 3.3, 0.7 Hz, H-4^{Gal}, 1H), 3.89 (ddd, J = 10.1, 7.9, 5.9 Hz, H-2^{Gal}, 1H), 3.78–3.72 (m, H-3^{Xyl}, H-5^{Gal}, 2H), 3.67 (s, PhOCH_3 , 3H), 3.57 (ddd, J = 10.0, 7.1, 5.0 Hz, H-2^{Xyl}, 1H), 3.50 (ddd, J = 12.3, 5.5, 4.5 Hz, H-5b^{Xyl}, 1H), 1.41, 1.40 (s, CMe_2 , 6H), 1.17, 1.12 (s, 2piv, 18H). ^{13}C NMR (150 MHz, CDCl_3): δ 178.2, 178.0 (2piv), 155.2, 150.3, 118.4, 118.3, 114.4 (PhOCH_3), 112.1 (CMe_2), 101.8 (C-1^{Gal}), 100.2 (C-1^{Xyl}), 78.6 (C-3^{Xyl}), 76.3 (C-2^{Xyl}), 74.8 (C-3^{Gal}), 74.7 (C-4^{Xyl}), 72.6 (C-5^{Gal}), 68.3 (C-2^{Gal}), 66.9 (C-4^{Gal}), 66.4 (C-5^{Xyl}), 62.1 (C-6^{Gal}), 55.5 (PhOCH_3), 38.9, 38.7 (2piv), 27.1, 27.1 (2piv), 26.8, 26.6 (CMe_2). HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$: calcd for $^{13}\text{C}_{29}\text{H}_{46}\text{O}_{13}\text{Na}$, 651.2566; found, 651.2571.

4-Methoxyphenyl 2,4-Di-O-trifluoromethanesulfonyl-3,6-di-O-pivaloyl- β -D-[1- ^{13}C]galactopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-[1- ^{13}C]xylopyranoside (21**).** Disaccharide **20** (0.50 g, 0.79 mmol) was dissolved in a mixed solvent (4 mL of dry CH_2Cl_2 and 20 mL of dry pyridine), and trifluoromethanesulfonic anhydride (0.47 mL, 2.80 mmol) was added slowly at –20 °C. The mixture was stirred at rt for 3 h, diluted with CH_2Cl_2 (30 mL), and washed successively with saturated aqueous NaHCO_3 solution and distilled water. The organic phase was dried over anhydrous Na_2SO_4 and concentrated at 30 °C in vacuo, and the residue was purified by flash chromatography to give **21** as a white foam (0.60 g, 0.67 mmol, 85%). HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$: calcd for $^{13}\text{C}_{29}\text{H}_{44}\text{F}_6\text{O}_{17}\text{S}_2\text{Na}$, 915.1889; found, 915.1880.

4-Methoxyphenyl 2,4-Di-O-acetyl-3,6-di-O-pivaloyl- β -D-[1- ^{13}C]mannopyranosyl-(1 $\rightarrow$ 4)-2,3-O-isopropylidene- β -D-[1- ^{13}C]xylopyranoside (22**).** A mixture of **21** (600 mg, 0.67 mmol), cesium acetate (645 mg, 3.36 mmol), and 18-crown-6 (875, 3.36 mmol) was dried under a vacuum and then dissolved in dry toluene (18 mL). The reaction mixture was stirred for 2.5 h at 90 °C. The reaction solution was diluted with ethyl acetate (30 mL) and washed with aqueous NaHCO_3 solution (1 N), followed by distilled water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated at 30 °C in vacuo, and the residue was purified by flash chromatography to give **22** as a white foam (280 mg, 0.40 mmol, 60%). ^1H NMR (600 MHz, CDCl_3): δ 7.00–6.97, 6.81–6.78 (m, PhOCH_3 , 4H), 5.49–5.47 (m, H-2^{Man}, 1H), 5.34 (t, J = 9.9 Hz, H-4^{Man}, 1H), 5.19 (dd, J = 167.3, 7.2 Hz, H-1^{Xyl}, 1H), 5.01 (dd, J = 10.0, 3.4 Hz, H-3^{Man}, 1H), 4.86 (dd, J = 157.2, 1.1 Hz, H-1^{Man}, 1H), 4.28 (dd, J = 12.0, 2.8 Hz, H-6a^{Man}, 1H), 4.19 (dd, J = 12.0, 4.9 Hz, H-6b^{Man}, 1H), 4.11–4.06 (m, H-5a^{Xyl}, H-4^{Xyl}, 2H), 3.83–3.79 (m, H-3^{Xyl}, 1H), 3.74 (s, PhOCH_3 , 3H), 3.71–3.67 (m, H-5^{Man}, 1H), 3.61–3.57 (m, H-2^{Xyl}, 1H), 3.50–3.45 (m, H-5b^{Xyl}, 1H), 2.12, 2.00 (s, 2 COCH_3 , 6H), 1.46, 1.44 (s, CMe_2 , 6H), 1.22, 1.11 (s, 2piv, 18H). ^{13}C NMR (150 MHz, CDCl_3): δ 178.1, 177.3 (2piv), 170.2, 169.4 (2 COCH_3), 155.4, 150.5, 118.5, 114.6 (PhOCH_3), 112.3 (CMe_2), 100.3 (C-1^{Xyl}), 97.0 (C-1^{Man}), 78.7 (C-3^{Xyl}), 76.6 (C-2^{Xyl}), 76.2 (C-4^{Xyl}), 72.7 (C-5^{Man}), 71.1 (C-3^{Man}), 68.7

(C-2^{Man}), 66.0 (C-4^{Man}), 65.2 (C-5^{Xyl}), 62.2 (C-6^{Man}), 55.7 (PhOCH₃), 39.0, 38.87 (2piv), 27.2, 26.9 (2piv), 27.0, 26.8 (CMe₂), 20.8, 20.8 (2COCH₃).

4-Methoxyphenyl 2,4-Di-O-acetyl-3,6-di-O-pivaloyl-β-D-[1-¹³C]-mannopyranosyl-(1→4)-2,3-di-O-acetyl-β-D-[1-¹³C]xylopyranoside (23). Compound 22 (160 mg, 0.22 mmol) was dissolved into dry methanol (10 mL), and acetyl chloride (10 μL) was added at 0 °C. The reaction mixture was stirred at 0 °C, and the progress of the reaction was monitored by TLC. After 50 min, triethylamine (20 μL) was added to neutralize the solution. The product was not isolated but was dried under a vacuum and dissolved in dry pyridine (15 mL). Acetic anhydride (0.25 mL) was added, and the solution was stirred at rt overnight. The reaction solution was diluted with ethyl acetate (30 mL) and washed twice with distilled water (20 mL each). The organic layer was dried over anhydrous Na₂SO₄ and concentrated at 30 °C in vacuo, and the residue was purified by flash chromatography to give 23 as a white foam (~130 mg, ~0.17 mmol, ~77%). This foam contained a minor impurity that could not be removed by chromatography on silica gel. This impurity was, however, removed in subsequent steps to prepare 27. ¹H NMR (600 MHz, CDCl₃): δ 3.90–6.85, 6.79–6.74 (m, PhOCH₃, 4H), 5.39–5.38 (m, H-2^{Man}, 1H), 5.23 (t, J = 10.0 Hz, H-4^{Man}, 1H), 5.12 (t, J = 9.0 Hz, H-3^{Xyl}, 1H), 5.04 (dd, J = 10.0, 7.3 Hz, H-2^{Xyl}, 1H), 4.94 (dd, J = 10.0, 3.5 Hz, H-3^{Man}, 1H), 4.85 (dd, J = 163.3, 7.2 Hz, H-1^{Xyl}, 1H), 4.69 (dd, J = 158.3, 1.0 Hz, H-1^{Man}, 1H), 4.25 (dd, J = 12.3, 6.1 Hz, H-6a^{Man}, 1H), 4.12 (dd, J = 12.3, 2.1 Hz, H-6b^{Man}, 1H), 4.05–4.01 (m, H-5a^{Xyl}, 1H), 3.94–3.89 (m, H-4^{Xyl}, 1H), 3.71 (s, PhOCH₃, 3H), 3.69–3.64 (m, H-5^{Man}, 1H), 3.40–3.36 (m, H-5b^{Xyl}, 1H), 2.07, 2.03, 2.01, 1.97 (s, 4COCH₃, 12H), 1.21, 1.106 (s, 2piv, 18H). ¹³C NMR (150 MHz, CDCl₃): δ 178.0, 177.3 (2piv), 170.1, 170.0, 169.5, 169.4 (2COCH₃), 155.6, 150.9, 118.4, 118.4, 114.6 (PhOCH₃), 100.5, 96.4, 73.6, 72.4, 71.7, 71.0, 70.7, 68.4, 65.8, 62.8, 62.2, 55.6 (PhOCH₃), 38.9, 38.8 (2piv), 27.1, 26.8 (2piv), 20.8, 20.7, 20.7, 20.5 (4COCH₃).

2,4-Di-O-acetyl-3,6-di-O-pivaloyl-β-D-[1-¹³C]mannopyranosyl-(1→4)-2,3-di-O-acetyl-α/β-D-[1-¹³C]xylopyranose (24). Compound 23 (160 mg, 0.21 mmol) was dissolved into acetonitrile/H₂O (4:1, 10 mL), and ammonium cerium(IV) nitrate (0.66 g, 1.20 mmol) was added. The reaction mixture was stirred at rt for 30 min. The solution was then diluted with CH₂Cl₂ (20 mL) and washed with distilled water (10 mL). The organic solution was dried over anhydrous Na₂SO₄ and concentrated to a syrup at 30 °C in vacuo, and the residue was purified by flash chromatography to afford product 24 as a syrup (136 mg, 0.20 mmol, 90%). Product 24 was used directly to prepare 25 without characterization by NMR. HRMS (ESI-TOF) *m/z* [M + Na]⁺: calcd for ¹³C₂₇H₄₄O₁₆Na, 673.2595; found, 673.2602.

2,4-Di-O-acetyl-3,6-di-O-pivaloyl-β-D-[1-¹³C]mannopyranosyl-(1→4)-2,3-di-O-acetyl-α-D-[1-¹³C]xylopyranosyl trichloroacetimidate (25). Compound 24 (130 mg, 0.20 mmol) was dissolved in CH₂Cl₂ (10 mL), and trichloroacetonitrile (0.15 mL, 1.50 mmol) and a catalytic amount of 1,8-diazobicyclo [5.4.0]-undec-7-ene (DBU) were added. After stirring at rt for 1.5 h, the reaction mixture was concentrated to a syrup at 30 °C in vacuo, and the syrup was purified by flash chromatography to afford pure 25 as a white foam (120 mg, 0.15 mmol, 75%). Product 25 was used directly to prepare 29 without characterization by NMR and MS (see below).

Methyl 4,6-O-Benzylidene-β-D-[1-¹³C]mannopyranosyl-(1→4)-β-D-xylopyranoside (26). Compound 2¹ (340 mg, 1.04 mmol) was dissolved in dry DMF (20 mL), and benzaldehyde dimethylacetal (0.54 mL, 3.6 mmol) and *p*-toluenesulfonic acid (4 mg) were added. The reaction mixture was stirred at 50 °C overnight and neutralized with the addition of one drop of triethylamine. The DMF was removed by evaporation in vacuo at 60 °C, the syrup was dissolved in CH₂Cl₂ (20 mL), and the organic solution was washed with distilled water (10 mL). The organic phase was dried over Na₂SO₄ and concentrated at 30 °C in vacuo to dryness, and the residue was purified by flash chromatography to afford 26 as a white solid (190 mg, 0.47 mmol, 45%). Product 26 was used directly to prepare 27 without characterization by NMR and MS.

Methyl 2,3-Di-O-benzyl-4,6-O-benzylidene-β-D-[1-¹³C]-mannopyranosyl-(1→4)-2,3-di-O-benzyl-β-D-xylopyranoside (27).

Compound 26 (110 mg, 0.27 mmol) was dissolved in DMF (10 mL), and NaH (60%, 110 mg) was added to the solution. After stirring at 0 °C for 0.5 h, benzyl bromide (0.32 mL, 2.70 mmol) was added dropwise at 0 °C and the mixture was stirred at rt for 20 h. The mixture was diluted with CH₂Cl₂ (20 mL) and washed with distilled water (15 mL). The organic phase was dried over Na₂SO₄ and evaporated at 30 °C in vacuo to dryness, and the residue was purified by flash chromatography to give 27 as a white solid (150 mg, 0.20 mmol, 74%). ¹H NMR (600 MHz, CDCl₃): δ 7.62–7.32 (m, PhCH₂, PhCH, 25H), 5.68 (s, PhCH, 1H), 5.10 (d, J = 10.9 Hz, PhCH₂, 1H), 4.98 (s, PhCH₂, 2H), 4.92 (d, J = 10.9 Hz, PhCH₂, 1H), 4.86–4.80 (m, PhCH₂, 3H), 4.72 (d, J = 12.20 Hz, PhCH₂, 1H), 4.65 (dd, J = 158.2, 1.0 Hz, H-1^{Man}, 1H), 4.40 (d, J = 7.4 Hz, H-1^{Xyl}, 1H), 4.36–4.28 (m, 2H), 4.06–3.95 (m, 3H), 3.88 (t, J = 10.4 Hz, 1H), 3.72–3.66 (m, 2H), 3.63 (s, OCH₃, 3H), 3.46 (dd, J = 8.9, 7.4 Hz, 1H), 3.40–3.28 (m, 2H). ¹³C NMR (150 MHz, CDCl₃): δ 139.0, 138.7, 138.6, 138.5, 137.7, 129.0–126.0 (PhCH₂, PhCH), 105.2, 101.6, 100.6, 82.3, 81.3, 78.80, 78.1, 76.8, 76.8, 75.1, 75.0, 75.0, 72.6, 68.7, 67.8, 57.1 (OCH₃). HRMS (ESI-TOF) *m/z* [M + Na]⁺: calcd for ¹³C₄₆H₅₀O₁₀Na, 798.3330; found, 798.3316.

Methyl 2,3,6-Tribenzyl-β-D-[1-¹³C]mannopyranosyl-(1→4)-2,3-di-O-benzyl-β-D-xylopyranoside (28). Compound 27 (150 mg, 0.20 mmol) was dissolved in anhydrous CH₂Cl₂ (5.0 mL), and triethylsilane (0.38 mL, 2.40 mmol) and BF₃·Et₂O (0.05 mL, 0.40 mmol) were added at 0 °C. The reaction mixture was stirred at rt for 4 h. The mixture was then diluted with CH₂Cl₂ (20 mL) and washed with aqueous NaHCO₃ solution (1 N) (10 mL), followed by distilled water (10 mL). The organic phase was dried over Na₂SO₄ and evaporated at 30 °C in vacuo to dryness, and the residue was purified by flash chromatography to give 28 as a white solid (80 mg, 0.10 mmol, 53%). ¹H NMR (600 MHz, CDCl₃): δ 7.40–7.20 (m, PhCH₂, 25H), 5.02 (d, J = 11.2 Hz, PhCH₂, 1H), 4.91 (d, J = 12.0 Hz, PhCH₂, 1H), 4.84 (d, J = 11.1 Hz, PhCH₂, 1H), 4.80 (d, J = 12.0 Hz, PhCH₂, 1H), 4.75 (d, J = 11.1 Hz, PhCH₂, 1H), 4.74 (d, J = 11.1 Hz, PhCH₂, 1H), 4.59 (d, J = 12.0 Hz, PhCH₂, 1H), 4.57 (dd, J = 153.9, 0.9 Hz, H-1^{Man}, 1H), 4.54 (s, PhCH₂, 2H), 4.34 (d, J = 7.2 Hz, H-1^{Xyl}, 1H), 4.06 (t, J = 9.4 Hz, H-4^{Man}, 1H), 4.03 (m, H-4^{Xyl}, 1H), 3.98 (dd, J = 11.7, 5.1 Hz, H-5a^{Xyl}, 1H), 3.88 (m, H-2^{Man}, 1H), 3.80 (dd, J = 10.4, 4.4 Hz, H-6a^{Man}, 1H), 3.73 (dd, J = 10.4, 5.6 Hz, H-6b^{Man}, 1H), 3.65 (t, J = 8.4 Hz, H-3^{Xyl}, 1H), 3.56 (s, OCH₃, 3H), 3.41 (m, H-5^{Man}, 1H), 3.39 (dd, J = 8.7, 7.2 Hz, H-2^{Xyl}, 1H), 3.35 (dd, J = 9.4, 2.9 Hz, H-3^{Man}, 1H), 3.31 (dd, J = 11.7, 9.3 Hz, H-5b^{Xyl}, 1H). ¹³C NMR (150 MHz, CDCl₃): δ 139.1, 138.8, 138.7, 138.2, 138.1, 128.0–127.5 (PhCH₂), 105.1, 99.8, 82.0, 81.7, 81.2, 76.0, 75.3, 74.9, 74.8, 74.4, 74.4, 73.9, 71.7, 71.1, 68.8, 66.5, 57.1 (OCH₃). HRMS (ESI-TOF) *m/z* [M + Na]⁺: calcd for ¹³C₄₆H₅₂O₁₀Na, 800.3486; found, 800.3494.

Methyl 2,4-Di-O-acetyl-3,6-di-O-pivaloyl-β-D-[1-¹³C]-mannopyranosyl-(1→4)-2,3-di-O-acetyl-β-D-[1-¹³C]xylopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-xylopyranoside (29). Anhydrous CH₂Cl₂ (10.0 mL) was added to a flask containing dry 25 (80 mg, 0.101 mmol), dry 28 (60 mg, 0.078 mmol), and molecular sieves (4 Å) (0.5 g). To this mixture was added trimethylsilyltriflate (10 μL) under N₂ at –50 °C. The reaction mixture was stirred at rt overnight and quenched with the addition of triethylamine (10 μL). The molecular sieves were removed by filtration, and the solution was concentrated at 30 °C in vacuo to a syrup. The syrup was purified by flash chromatography to afford 29 as a white foam (80 mg, 0.057 mmol, 73%). ¹H NMR (600 MHz, CDCl₃): δ 7.40–7.12 (m, PhCH₂, 25H), 5.39 (m, H-2^{Man2}, 1H), 5.27 (t, J = 9.9 Hz, H-4^{Man2}, 1H), 5.03 (d, J = 11.4 Hz, PhCH₂, 1H), 4.96 (dd, J = 10.0, 3.4 Hz, H-3^{Man2}, 1H), 4.95 (t, J = 9.0 Hz, H-3^{Xyl2}, 1H), 4.87–4.76 (m, PhCH₂, H-2^{Xyl2}, 4H), 4.73–4.65 (m, PhCH₂, 4H), 4.58 (d, J = 11.0 Hz, PhCH₂, 1H), 4.57 (d, J = 158.2 Hz, H-1^{Man2}, 1H), 4.53 (dd, J = 163.9, 7.4 Hz, H-1^{Xyl2}, 1H), 4.46 (d, J = 154.6 Hz, H-1^{Man1}, 1H), 4.39 (d, J = 11.8 Hz, PhCH₂, 1H), 4.31–4.27 (m, H-1^{Xyl1}, H-6a^{Man2}, 2H), 4.24 (dt, J = 9.3, 4.6 Hz, H-4^{Man1}, 1H), 4.13 (dd, J = 11.7, 2.1 Hz, H-6b^{Man2}, 1H), 3.98 (m, H-4^{Xyl1}, 1H), 3.92 (dd, J = 11.8, 5.2 Hz, H-5a^{Xyl1}, 1H), 3.84–3.76 (m, H-2^{Man1}, H-4^{Xyl2}, H-5a^{Xyl2}, 3H), 3.72–3.62 (m, H-6a^{Man1}, H-6b^{Man1}, H-5^{Man2}, 3H), 3.60 (t, J = 8.5 Hz, H-3^{Xyl1}, 1H), 3.54 (s, OCH₃, 3H), 3.41 (dd, J =

9.5, 3.3 Hz, H-3^{Man1}, 1H), 3.36 (dd, $J = 8.9$, 7.3 Hz, H-2^{Xyl1}, 1H), 3.28–3.21 (m, H-5^{Man1}, H-5b^{Xyl1}, 2H), 2.99–2.93 (m, H-5b^{Xyl2}, 1H), 2.13, 2.04, 2.02, 1.93 (s, 4COCH₃, 12H), 1.23, 1.13 (s, 2piv, 18H). ¹³C NMR (150 MHz, CDCl₃): δ 178.1, 177.4 (2 piv), 170.1, 170.0, 169.7, 169.5 (4COCH₃), 139.1, 138.8, 138.8, 138.6, 138.4, 128.5–127.3 (SPhCH₂), 105.1 (C-1^{Xyl1}), 100.9 (C-1^{Xyl2}), 99.7 (C-1^{Man1}), 96.5 (C-1^{Man2}), 82.1 (C-3^{Xyl1}), 81.3 (C-2^{Xyl1}), 80.3 (C-3^{Man1}), 76.3 (C-5^{Man1}), 76.2 (C-4^{Xyl1}), 75.4 (C-2^{Xyl2}), 75.0, 74.7, 74.7, 73.8, 72.4 (SPhCH₂), 74.3 (C-4^{Man1}), 74.0 (C-4^{Xyl2}), 72.5 (C-3^{Xyl2}), 72.5 (C-5^{Man2}), 71.9 (C-2^{Man1}), 71.0 (C-3^{Man2}), 68.4 (C-2^{Man2}), 68.4 (C-6^{Man1}), 65.9 (C-4^{Man2}), 63.0 (C-5^{Xyl2}), 62.9 (C-5^{Xyl1}), 62.3 (C-6^{Man2}), 57.1 (OCH₃), 39.0, 38.9 (2piv), 27.2, 26.9 (2piv), 21.0, 20.9, 20.8, 20.6 (4COCH₃).

Methyl β -D-[1-¹³C]Mannopyranosyl-(1 $\rightarrow$ 4)- β -D-[1-¹³C]-xylopyranosyl-(1 $\rightarrow$ 4)- β -D-[1-¹³C]mannopyranosyl-(1 $\rightarrow$ 4)- β -D-xylopyranoside (4). Compound **29** (80 mg, 0.057 mmol) was dissolved in dry methanol (15 mL), and the solution was saturated with NH₃ (g). The reaction mixture was stirred at 50 °C for 2 days. The solution was then concentrated at 30 °C in vacuo to dryness, the residue was dissolved in dry methanol (10 mL), and Pd/C (10%, 80 mg) was added. The reaction mixture was stirred under H₂ at rt for 2 days. The Pd/C catalyst was then removed by vacuum filtration, and the filtrate was concentrated at 30 °C in vacuo to a syrup. The syrup was dissolved in ~0.2 mL of distilled water, and the solution was applied to a column (2.5 cm $\times$ 100 cm) containing Biogel P2 gel-filtration resin (45–90 μ m). The column was eluted with distilled, decarbonated water at ~1.5 mL/min, and fractions (~10 mL) were collected and assayed by HPLC. Fractions containing product were pooled and concentrated at 30 °C in vacuo to give **4** as a syrup (23 mg, 0.037 mmol, 65%). Detailed characterization of **4** by ¹H and ¹³C NMR is found in the text. HRMS (ESI-TOF) m/z [M + Na]⁺: calcd for ¹³C₃C₂₀H₄₀O₁₉Na, 646.2157; found, 646.2173.

NMR and Mass Spectrometry. High-resolution 1D ¹H and ¹³C{¹H} NMR spectra were obtained using 5-mm NMR tubes on a 600-MHz FT-NMR spectrometer equipped with a 5-mm ¹H-¹⁹F/¹⁵N-³¹P AutoX dual broadband probe. NMR spectra of intermediates were collected in CDCl₃ at 22 °C. ¹H NMR spectra were typically collected with a ~6000 Hz spectral window and a ~4.0 s recycle time. ¹³C{¹H} NMR spectra (150 MHz) were collected with ~30 000 Hz spectral windows and ~3.0 s recycle times. 2D ¹H-¹H gCOSY and ¹³C-¹H gHSQC spectra were used to confirm ¹H and ¹³C chemical shift assignments of synthetic intermediates. ¹H and ¹³C chemical shifts were referenced internally to chloroform (see the Supporting Information for ¹H and ¹³C{¹H} NMR spectra of synthetic intermediates).

NMR spectra of final products **2–4** were collected in ²H₂O at 22 °C. ¹H NMR spectra were typically collected with a ~5000 Hz spectral window and a ~4.0 s recycle time. ¹H NMR FIDs were processed to optimize spectral S/N, and final spectra had digital resolutions of ~0.02 Hz/pt. ¹³C{¹H} NMR spectra were collected with ~15 000 Hz spectral windows and ~4.5 s recycle times and had digital resolutions of ~0.05 Hz/pt. ¹H and ¹³C chemical shifts (in ppm) were referenced externally to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). 2D ¹H-¹H gCOSY and ¹³C-¹H gHSQC spectra were used to confirm the ¹H and ¹³C chemical shift assignments for **2–4**. 2D ¹³C-¹H HSQC-HECADE spectra⁴⁸ were recorded to measure long-range intra-residue ²J_{CH} couplings using a DIPSI-2 spin-lock of 60–90 ms and a scaling factor of 10. For long-range ²J_{CH} couplings across O-glycosidic linkages, 2D ¹³C-¹H J-HMBC spectra³⁷ were employed with scaling factors of 7–23, and a 2-fold low-pass J-filter was applied to suppress ¹J_{CH} values.

Non-first-order contributions to homonuclear trans-glycosidic J_{CC} values measured directly from signal splittings in 1D ¹³C{¹H} NMR spectra (150 MHz) of **2–4** are expected to be small for compounds ¹³C-labeled at C1', since the differences in chemical shifts between the C1' signals and those of the natural abundance aglycone carbons (secondary OH carbons) are greater than 10 $\times$ the observed J_{CC} values. For compounds **2** and **3** that were ¹³C-labeled at C2', the differences in chemical shifts between C2' and C4 are greater than 3 ppm (see Figures S2 and S4, Supporting Information); at 150 MHz,

this difference translates into >450 Hz, which is more than 10 $\times$ the observed ³J_{C2',C4} values of ~3 Hz (Table S). For measurements of trans-glycosidic heteronuclear ³J_{COCH} values, attention was paid to non-first-order effects in 1D ¹H spectra that might affect their measurements directly from signal splittings. For **2**, the ¹H spectrum is essentially first-order at 600-MHz (see Figure S1, Supporting Information), allowing measurement of ³J_{C1',H4} directly from the H4 signal. For **3**, however, the H3 and H4 signals are close at 600 MHz, and spectral simulation (TopSpin) was used to obtain an accurate ³J_{C1',H4} value. Higher field ¹H NMR spectra (800 MHz) were also obtained and simulated to confirm the value of ³J_{C1',H4} in **3**. Similar behaviors were observed in **4** for the corresponding linkages, and similar approaches were applied to obtain accurate ³J_{C1',H4} values. ³J_{C4,H1'} values were obtained from 2D NMR spectra of compounds at natural abundance (see above). In all cases, non-first-order effects are small given the isolated anomeric proton signals and the lack of signal overlap between the H2' and H3' signals in the compounds studied.

High-resolution mass spectra (HRMS) were obtained on a BRUKER micrOTOF-Q II instrument with an ESI source. The dry heater was set at 180 °C, and the nebulizer was set at 0.4 bar. The capillary voltage was 4.5 kV, and the end plate offset was -0.5 kV. Full MS scans were collected over a range of 50–1650 m/z .

CALCULATIONS

Selection and Geometric Optimization of Model Compounds. Structures **2^c** and **3^c** (Scheme 8) were chosen for theoretical studies of J-couplings. Density functional theory (DFT) calculations were conducted within Gaussian09⁴⁹ using the B3LYP functional^{50,51} and 6-31G* basis set⁵² for geometric optimization. Initial torsion angle restrictions in **2^c** were as follows: the C3–C2–O2–H, C4–C3–O3–H, C1'–C2'–O2'–H, C2'–C3'–O3'–H, C3'–C4'–O4'–H, C4'–C5'–C6'–O6', and C5'–C6'–O6'–H torsion angles were fixed at 180°. Initial torsion angle constraints in **3^c** were as follows: the C3–C2–O2–H, C4–C3–O3–H, C4–C5–C6–O6, C5–C6–O6–H, C1'–C2'–O2'–H, C2'–C3'–O3'–H, and C3'–C4'–O4'–H torsion angles were fixed at 180°. In both structures, the C2–C1–O1–CH₃ torsion angle was initially set at 180° and allowed to optimize during the calculations. These torsional restrictions were implemented to simplify the optimizations in order to obtain heavy atom geometric information to parameterize J-coupling equations. The limited energetic landscapes that result from this treatment may not be emblematic of the total landscapes. Therefore, preferred geometries about ϕ and ψ in **2** and **3** obtained from the derived potential energy surfaces are susceptible to error, although the data can distinguish general regions of ϕ/ψ space that are more likely to be occupied than others. The internal β -(1 $\rightarrow$ 4) linkages in **2^c** and **3^c** are characterized by two torsion angles, C2'–C1'–O1'–C4 (ϕ) and C1'–O1'–C4–C3 (ψ), each of which were rotated systematically in 15° increments through 360° to give a 24 $\times$ 24 matrix of optimized structures. All remaining geometric parameters were optimized except those identified above. The calculations included the effects of solvent water, which were treated using the Self-Consistent Reaction Field (SCRF)⁵³ and the Integral Equation Formalism (polarizable continuum) model (IEFPCM)⁵⁴ as implemented in Gaussian09.

Theoretical Calculations of ¹H-¹H, ¹³C-¹H, and ¹³C-¹³C Spin-Coupling Constants. J_{HH}, J_{CH}, and J_{CC} values were calculated in **2^c** and **3^c** using Gaussian09⁴⁹ and DFT (B3LYP).^{50,51} The Fermi contact,^{55–57} diamagnetic and paramagnetic spin-orbit, and spin-dipole terms were recovered using a [5s2p1d13s1p] basis set,⁵⁸ and raw (unscaled) calculated J-couplings are reported. All DFT

calculations included the effects of solvent water, which were treated using the Self-Consistent Reaction Field (SCRF)⁵³ and the Integral Equation Formalism (polarizable continuum) model (IEFPCM)⁵⁴ as implemented in *Gaussian09*.

Parameterization of J -Coupling Equations. All geometrically optimized conformers of 2^c and 3^c were inspected to ensure that structurally distorted structures were not used in equation parameterization, including the use of a 10 kcal/mol energy cutoff as described previously.¹⁸ Equations relating DFT-calculated J -couplings to either ϕ or ψ were parameterized using the *scipy* and *numpy* packages in *Python*.⁵⁹ The goodness-of-fit of each equation is reported as a root mean squared (RMS) deviation.

Conformational Modeling of ϕ and ψ . O-Glycosidic torsion angles, ϕ and ψ , were modeled as different single-state distributions, using an in-house statistical software package, MA'AT.¹⁸ The allowed probability distributions included Cartwright's Power of Cosine, wrapped Normal, von Mises, wrapped Cauchy, and uniform.^{60–64} Each distribution contained two fitting parameters, the mean position and a circular standard deviation (CSD) of ϕ or ψ . Monte Carlo methods were used to generate model parameters, and least-squares methods were used to minimize the RMS deviation between the experimental and predicted J -couplings.

Molecular Dynamics Simulations of 2–4 and 32. Initial structures of 2–4 and 32 were built using the Carbohydrate Builder module available at the GLYCAM Web site (<http://www.glycam.org>). The GLYCAM06⁶⁵ (version j) force field was employed in all simulations. The saccharides were solvated with TIP3P⁶⁶ water using a 12 Å buffer in a cubic box, using the LEaP module in the AMBER14 software package.⁶⁷ Energy minimizations for the solvated disaccharides were performed separately under constant volume (500 steps steepest descent, followed by 24 500 steps of conjugate-gradient minimization). Each system was subsequently heated to 300 K over a period of 50 ps, followed by equilibration at 300 K for a further 0.5 ns using the nPT condition, with the Berendsen thermostat⁶⁸ for temperature control. All covalent bonds involving hydrogen atoms were constrained using the SHAKE algorithm,⁶⁹ allowing a simulation time step of 2 fs throughout the simulation. After equilibration, production simulations were carried out with the GPU implementation⁷⁰ of the PMEMD.MPI module, and trajectory frames were collected every 1 ps for a total of 1 μ s. 1–4 Nonbonded interactions were not scaled,⁷¹ and a nonbonded cutoff of 8 Å was applied to van der Waals interactions, with long-range electrostatics treated with the particle mesh Ewald approximation. Output from each MD simulation was imported into *Prism*⁷² for visualization.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.8b01411.

600 MHz ^1H NMR spectra of 2; 150 MHz $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of 2; 600 MHz ^1H NMR spectra of 3¹; 150 MHz $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of 3¹; 600 MHz ^1H NMR spectra of 4; 150 MHz $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of 4; ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of synthetic intermediates 6–10, 13, 15, 18–20, 22–23, and 27–29; potential energy contour maps for 2^c and 3^c ; hypersurfaces for ϕ - and ψ -dependent J -couplings in 2^c

and 3^c ; parameter space for single-state models of ϕ and ψ in 2; parameter space for single-state models of ϕ and ψ in 3; parameter space for single-state models of ψ for the βMan_1 -(1 $\rightarrow$ 4)- βXyl_1 , βMan_2 -(1 $\rightarrow$ 4)- βXyl_2 , and βXyl_2 -(1 $\rightarrow$ 4)- βMan_1 linkages in 4; aqueous 1- μ s MD simulation histograms for ϕ and ψ in 4 and 32; aqueous 1- μ s MD simulation histograms for hydroxymethyl groups in 2–4; statistical model parameters and RMS errors for ψ in 4; Cartesian coordinates for 2^c , 3^c , 4^c , and 32^c (PDF)

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