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Chemo-enzymatic synthesis of conformationally constrained oligosaccharides †

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Received 24th July 2003, Accepted 18th September 2003
First published as an Advance Article on the web 14th October 2003

N-Acetyllactosamine derivative **4**, which has a methylene amide tether between C-6 and C-2′, was enzymatically glycosylated using rat liver α-2,6-sialyltransferase (ST6GalI) or recombinant human fucosyltansferase V (FucT-V) to give conformationally constrained trisaccharides **5** and **6**, respectively. The methylene amide linker of **4** was installed by a two-step procedure, which involved acylation of a C-6 amino function of a LacNAc derivative with chloroacetic anhydride followed by macrocyclization by nucleophilic displacement of the chloride by a C-2′ hydroxyl. The conformational properties of **4** were determined by a combination of NOE and *trans*-glycosidic heteronuclear coupling constant measurements and molecular mechanics simulations and these studies established that the glycosidic linkage of **4** is conformationally constrained and resides in only one of the several energy minima accessible to LacNAc. The apparent kinetic parameters of transfer to LacNAc and conformationally constrained saccharides **3** and **4** indicates that fucosyltransferase V recognize LacNAc in its A-conformer whereas α-2,6-sialyltransferase recongizes the B-conformer of LacNAc.

Introduction

The cornerstone of biological advances has been the understanding of the biological interactions of nucleic acids and proteins between either themselves or each other. However, in recent years, the recognition roles played by carbohydrates and glycoconjugates have received considerable attention. Carbohydrate recognition has been implicated in fertilization, embryogenesis, neuronal development, hormonal activities, cell proliferation and their organization into specific tissues.[1,2] As many of these processes involve the interaction between proteins and oligosaccharides, an understanding of the factors that control these interactions at the molecular level is of prime importance.

It is well established now that the majority of oligosaccharides have some degree of flexibility around their glycosidic linkages and as a result can adopt several distinct low energy minima on their conformational surface.[3–5] Furthermore, there is evidence to suggest that the conformational properties of a glycosidic linkage can be modulated by the macromolecular structure it is part of and in particular flanking oligosaccharide residues or attachment to proteins and lipids may result in conformational changes.[4,6] In this respect, the examination of structural properties of a wide range of glycoproteins has revealed that glycosidic torsional angles may deviate as much as 20–30° from minimum energy conformations.[7] In the binding site of proteins, not only global but also secondary minima with very different conformations can be complexed.[4]

Little is known about the effect of inter-residual flexibility of glycosyl acceptors on kinetic parameters of glycosyl transfer. Furthermore, structural studies have provided little information about the conformation of a glycosyl acceptor that is recognized by a glycosyltransferase. To address these issues, we recently reported the chemical synthesis of conformationally constrained *N*-acetyl lactosamine (LacNAc) derivatives **1**–**3** and determined apparent kinetic parameters of sialylation by rat liver α(2–6)-sialyltransferase (Fig. 1).[8] The conformational properties of **1**–**3** were determined by a combination of molecular mechanics calculations and NMR spectroscopy. It was found that compound **3** adopts a low energy conformations that are centered about the A- and B-conformers of LacNAc whereas **1** and **2** adopt conformational spaces outside these regions (Fig. 1). Compound **3** was by far the best acceptor tested indicating that the enzyme recognizes LacNAc in one of the two low energy conformations.

In order to determine in more detail the conformational requirements of glycosyltransferases, conformational constrained analogs of LacNAc are required that are more rigid than **3** but still can adopt a low energy conformation. Furthermore, it is important to investigate whether such derivatives can be used as substrates for the enzymatic synthesis of more complex conformationally constrained oligosaccharides. It is to be expected that the resulting compounds will be important probes to determine in which conformation lectins or other glycosyltransferases recognize their substrates. In this respect, very few conformationally constrained saccharides have been reported,[8–16] which has made it difficult to establish the effect of conformationally flexibility on protein-carbohydrate recognition.

Here we report the chemical synthesis of a LacNAc derivative (**4**), which is preorganized by a methylene amide linker between the C-6 and C-2′. A combination of NOE and *trans*-glycosidic heteronuclear coupling constant measurements and molecular mechanics simulations established that the glycosidic linkage of compound **4** is highly conformationally constrained and resides in the B-conformer on the energy map of LacNAc. The apparent kinetic parameters for the α-(2,6)-sialyltrans-

† Electronic supplementary information (ESI) available: Region of 500 MHz $^{1}H\{^{13}C\}$-HSQC spectrum of compound **4** in D_2O at 25 °C. 600 MHz proton spectra of compound **4** in water and in 21% pyridine. Regions of 800 MHz ROESY spectrum of compound **4** in 10% DMSO–pyridine, showing some observed NOEs. Characteristics of the conformational families that can be adopted by compound **4**. Traces of the 2D HSQMBC spectrum for the determination of $^{3}J_{C1',H4}$ for compound **4**. Nomenclature and detailed geometrical description of the low energy conformation of compound **4**. ^{1}D NMR spectra for compounds **4**–**6**. Procedures for enzyme kinetics. See http://www.rsc.org/suppdata/ob/b3/b308559g/

DOI: 10.1039/b308559g

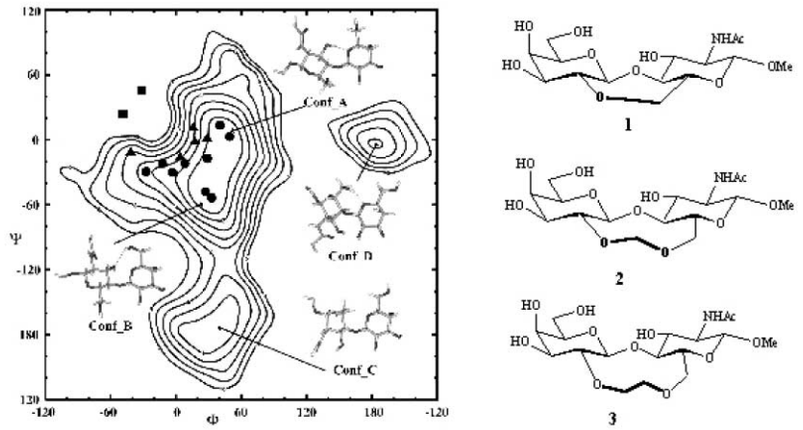


Fig. 1 Energy map of LacNAc glycosidic linkage[17] calculated with MM3. Conformations accessible to compounds **1**–**3** have been superimposed as ■ = **1**, ▲ = **2**, ● = **3**[8]

ferase and fucosyltransferase V catalyzed transformations indicate that the two enzymes respond differently to imposed conformational constraints of the acceptor and it appears that the fuocylstransferase recognizes LacNAc in the A conformation whereas the sialyltransferase prefers the B-conformer. Compound **4** was used as a substrate in the enzymatic synthesis of trisaccharides **5** and **6** (Fig. 2) These two compounds are analogs of 6′-sialyllactosamine (αNeu5Ac-(2–6)βGal(1–4)-βGlcNAc-OMe) and Lewis^x (βGal(1–4)[αFuc-(1–3)]βGlcNAc-OMe), which are involved in a wide range of biological processes and for example 6′-sialyllactosamine is a ligand for influenza virus[18] whereas Lewis^x is a tumor-associated antigen.[19]

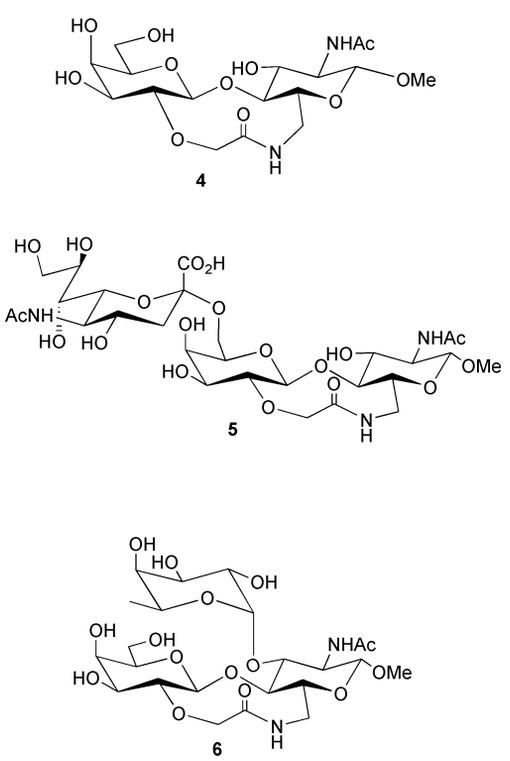
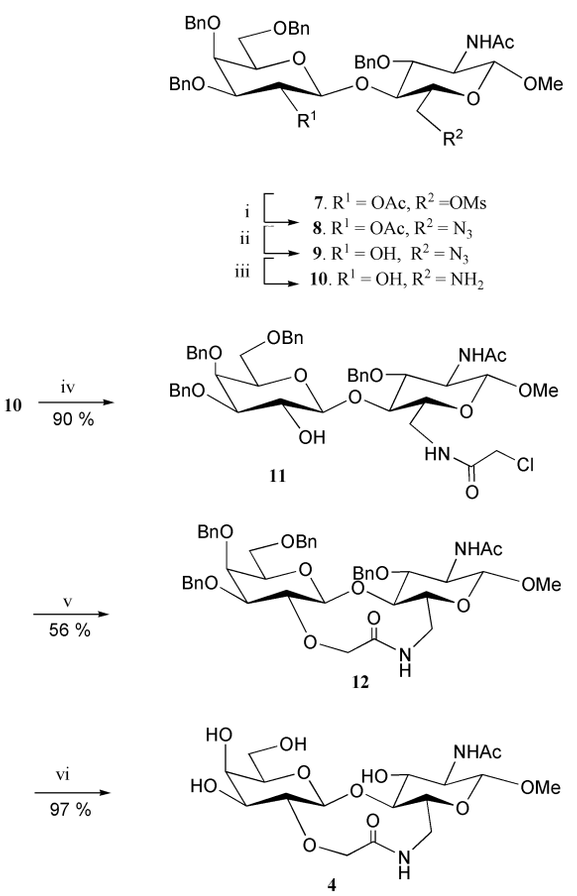


Fig. 2 Preorganized compounds **4**–**6**.

Results and discussion

Synthesis

It was envisaged that compound **4**, which is modified by the methylene amide linker, should be more rigid than the corresponding ethylene analogue **3**, which is conformationally constraint by a more flexible ethylene tether. The methylene amide linker of **4** was installed by a two-step procedure, which involved acylation of the C-6 amino function of a LacNAc derivative **10** with chloroacetic anhydride followed by macrocylization by nucleophilic displacement of the chloride by a C-2′ hydroxyl (Scheme 1). Enzymatic sialylation or fucosylation of **4** using rat liver α-2,6-sialyltransferase (ST6GalI) or recombinant α-1,3-human fucosyltansferase V (FucT-V) provided preorganized **5** and **6**, respectively. Previous studies have shown that these transferases allow modifications of C-6 and C-2′ hydroxyls of LacNAc and therefore these positions are appropriate for installing a linker.[20,21]



Scheme 1 *Reagents and conditions*: (i) NaN_3, DMF; (ii) NaOMe, MeOH; (iii) $HS(CH_2)_3SH$, H_2O–pyridine; (iv) $(ClCH_2CO)_2O$, MeOH–DCM; (v) NaH, DMF; (vi) Pd/C, H_2.

Key intermediated **10** could easily be prepared starting from previously reported LacNAc derivative **7**,[8] which has a methanesulfonyl ester at C-6 and an acetyl ester at C-2′

Table 1 Comparison of experimental[a] and theoretical NMR values of optimized LacNAc and LacNAc-derivative **4**

	Φ_H/°	$J_{H1',C4}$/Hz	Ψ_H/°	$J_{H4,C1'}$/Hz	H1′–H4/Å	H1′–H6R/Å	H1′–H6S/Å	H1′–H3/Å
LacNAc-OMe								
conf_A	45	2.9	−5	5.5	2.2	2.8	3.2	
conf_B	28	4.4	−54	2.1	2.4	4.1	4.5	
conf_C	43	3.1	−174	6.7	3.7	5.2	4.1	
conf_D	176	6.8	−2.6	5.6	3.6	4.5	5.4	
av. Conf		3.7		3.9	2.3	3.1	3.5	
Exp.	35	3.8[b]	−25	4.6[b]	2.4	2.9	3.0	
Compound **4**								
Trans_ap	47	2.8	−19	5.0	2.3			4.7
Trans_p	43	3.1	−23	4.8	2.3			4.3
Trans_p_2	54	2.1	−5	5.6	2.4			4.4
Cis_p	28	4.4	−6	5.5	2.1			4.4
Cis_ap	−1	5.6	−32	4.1	2.1			3.8
Trans_p[c]	27	4.5	−63	1.4	2.4			3.1
Exp.[b]	28	4.4	−61	1.5	2.3			3.0

[a] Exp. *J* values ±15%; exp. NOE values ±0.3 Å. [b] Obtained in 21% pyridine–D_2O. [c] Distance optimized.

(Scheme 1). The key disaccharide **7**[8] was converted into the azido derivative **8** by displacement of the methanesulfonyl ester with sodium azide in DMF. After purification by silica gel column chromatography, **8** was isolated in a yield of 98%. Deacetylation of **8** under standard conditions gave the desired alcohol **9** in almost quantitative yield. Reduction of the azido group of **9** with 1,3-propanedithiol in a mixture of pyridine and water[22] gave the desired free amine **10**. The methylene amide bridge of compound **12** was installed by a two-step procedure, whereby **10** was first N-acylated with chloroacetic chloride to provide **11**, which was cyclized by base mediated ether formation. Thus, compound **10** was treated with chloroacetic anhydride in a mixture of DCM and MeOH to give **11** in a yield of 90%. A significant amount of acylation of the C2 hydroxyl was observed when the reaction was performed in the absence of methanol as a co-solvent. Treatment of **11** with NaH in DMF give a mixture of the required cyclized **12** and a dimerized side product. Fortunately, a significantly reduction of the amount of side product could be achieved by performing the reaction under dilute conditions. The two compounds could be separated by size exclusion chromatography over Sephadex LH-20 to give pure **12** in a yield of 56%. Catalytic hydrogenation of **12** over Pd/C gave the required derivative **4**. The presence of the tether in **4** was confirmed by a NOEs between (i) H2′ and H8R′; (ii) NH and H1′, H4 and H6S and by the relatively downfield chemical shift of the C2′ (δ 87.1), characteristic of hydroxyl alkylation.

Conformational analysis

The conformational properties of compounds **4** were determined by a combination of molecular mechanics simulations and NMR spectroscopy and the results compared with similar data for *N*-acetyllactosamine.

A complete conformational search of **4** was performed by systematically varying Φ and Ψ angles of the glycosidic linkages and the torsion angles of the methylene amide tether using the SEARCH procedure of the SYBYL software package (Tripos Inc., St Louis). Starting conformations were built by graphically editing the cyclic derivative of *N*-acetyllactosamine that were the subject of an earlier study[8] and conformational families of the cyclic derivative were obtained using a clustering procedure. Eight conformational families were identified and the characteristics of the five lowest energy ones are in Table 1. The tether of **4** can adopt different conformations that can be described by the conformations and orientations of the –CO–NH– segment. In particular, the amide bond can be *cis* or *trans*, and in each case, the NH vector, which is approximately parallel to C1′–H1′ and C4–H4, can point in the same direction as these two CH vectors (parallel orientation, *p*) or in the opposite direction (antiparallel orientation, *ap*). These five possible conformations, *i.e. Trans_ap, Trans_p, Trans_p_2, Cis_p* and *Cis_ap* correspond to the conformational families that can be adopted by compound **4** as described in Table 1. The *Trans_p_2* conformation displays a different orientation around O2′ as compared to *Trans_p* and is higher in energy. The lowest energy conformations of the two possible *trans* shapes (*Trans_ap, Trans_p*) have almost equal potential energies, whereas the *cis* conformations (*Cis_p* and *Cis_ap*) are calculated to have potential energies about 3 kcal mol^{-1} above the *trans* ones. (Table S2 and Fig. S6 of ESI †).

When analyzing the dihedral angles of the glycosidic linkage, it appears that each conformational family of the cyclic compound **4** can adopt only a narrow range of Φ and Ψ angles (Fig. 3). The possible conformations occupy a conformational plateau, with no significant energy barriers, that encompasses

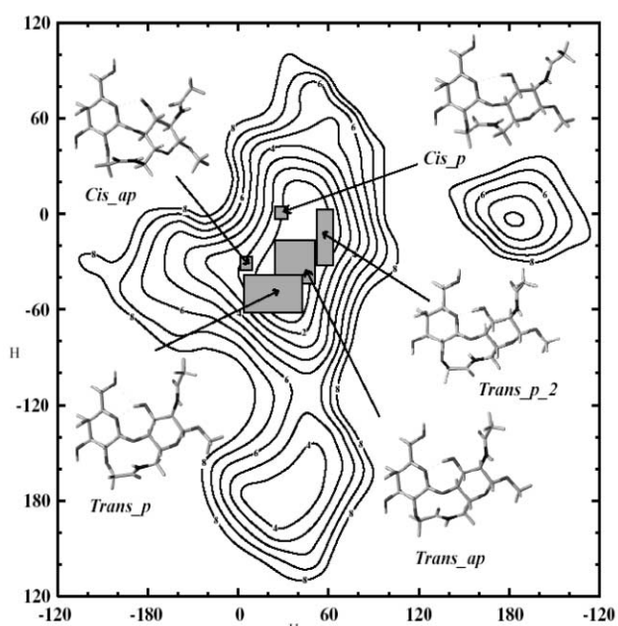


Fig. 3 Energy map of LacNAc glycosidic linkage[17] calculated with MM3. Conformations accessible to compound **4** have been superimposed

the two low energy conformations of LacNAc that have been previously described as conf_A ($\Phi = 40°$, $\Psi = 0°$) and conf_B ($\Phi = 40°$, $\Psi = -60°$).[8] The secondary energy minima corresponding to *trans* orientations of Φ or Ψ cannot be reached by compound **4**. The glycosidic linkage of the *Cis* families are limited to very narrow range of conformation at the limit of the low energy region. The *trans* families are somewhat more flexible, but *Trans_p*, the most populated family can adopt only conformations of the Conf_B region, whereas conformations of the *Trans_ap* family occupy a region in between Conf_A and Conf_B.

NMR data and comparison with molecular modeling

The ^{1}H and ^{13}C spectra of compound **4** were assigned by a combination of one-dimensional proton, and two-dimensional HSQC and COSY experiments. (Fig. S1 of ESI†) Due to signal overlap and strong coupling, most of the data for conformational analysis ($^3J_{CH}$ and NOEs) were obtained with a sample in 21% pyridine–D_2O and 10% DMSO–pyridine. (Fig. S2 of ESI†) Heteronuclear $^3J_{CH}$ coupling constants for LacNAc were also measured in a 30% pyridine–D_2O solution at 40 °C to perturb the chemical shifts of H1, H1′, H3 and H4. Earlier values reported[8] were underestimated due to overlap of H1 and H1′, and strong coupling between H3 and H4.

Assignment of pro-R and pro-S hydroxymethylene protons of the GlcNAc moiety of LacNAc and compound **4** was based on relative $^3J_{HH}$ coupling constants and H5–H6 NOEs. For LacNAc, it is assumed that predominantly gg and gt rotamers are populated,[23] and it is to be expected that H5–H6$_{pro\text{-}R}$ is larger due to the *trans* relationship in the gt conformer, $^3J_{H5,H6R}$ = 5.5 Hz, while $^3J_{H5,H6S}$ = 2.0 Hz. On the contrary, the tether of compound **4** restricts the rotamers to tg or possibly eclipsed forms, and therefore the H5–H6$_{pro\text{-}S}$ coupling is expected to be similar or larger than H5–H6$_{pro\text{-}R}$. Although the line widths were large for these signals, estimates of the couplings could be made and $^3J_{H5,H6R}$ = 5 Hz, while $^3J_{H5,H6S}$ = 10 Hz. A weak NOE between the assigned H6$_{pro\text{-}S}$ and H5, and the fact that no NOEs were observed between H1′ and H6$_{pro\text{-}R}$ or H1′ and H6$_{pro\text{-}S}$ is consistent with a tg rotamer.

trans-Glycosidic heteronuclear coupling constants, which are sensitive to orientations around glycosidic linkages, were measured using one- and two-dimensional HSQMBC experiments.[24] The resulting values are listed in Table 1 together with the calculated values for each low energy conformer using an empirical Karplus-type equation.[25] For LacNAc, the $^3J_{H1',C4}$ and $^3J_{H4,C1'}$ values of 3.8 and 4.6 Hz, respectively, support the mixture of conformers as determined by the modeling studies. Compound **4** provided a value of $^3J_{H1',C4}$ = 4.4 Hz corresponding to Φ of approximately 48°, and a small value of $^3J_{H4,C1'}$ = 1.5 Hz corresponding to Ψ of approximately −61°. Although these values were obtained in solvent systems other than pure water, it is reasonable to assume that the conformations are not greatly altered.[26]

Distance constraints were obtained from relative NOE intensities using the isolated spin-pair approximation.[27] A strong H1′–H4 NOE (2.3 Å) was obtained for compound **4**, which was expected for a compound restricted to *syn* conformations. Furthermore a medium strong H1′,H3 (3.0 Å) NOE was detected which is consistent with a Ψ value of −61° since that positions H3 closer to H1′. Additional qualitative NOE data were obtained in a solution of 10% dmso–pyridine that support the presence of the *trans_p* conformer. (Table S2 of ESI†) In particular, NOEs were observed between the NH and linker H8S′, NH and H4, H6S, H6R and H1′. The NOE between H2′ and H8R′ proposed for *trans-p* structure was also observed, whereas in the other solvents (21% pyridine–D_2O), it could not be measured due to signal overlap. Moreover, neither the NOE between H6S and H8S′ expected for *cis-ap* structure nor the one between H6R and H8R′ proposed for the *cis-p* structure was observed indicating that the *cis* conformations are not significantly populated in solution. An NOE between H3′ and H8S′ proposed for the *trans-ap* structure could not be confirmed because of a strong NOE between H8S′ and H8R′, and H8R′ is very close to H3′. The estimated $^3J_{H5,H6R}$ = 5 Hz, and $^3J_{H5,H6S}$ = 10 Hz couplings were consistent with a tg rotamer form, and the *Trans-p* structure.

The experimental and computer modeling data indicate that the conformation of compound **4** is consistent with the *Trans-p* conformational family. This locates the amide proton on the side of H1′ and H4, and the carbonyl oxygen near H6R and H5. However, the calculated Φ and Ψ angles of these conformers are not in perfect agreement with experimental data and therefore a final geometry optimization was performed on the lowest energy conformation of the *Trans_p* family, with the inclusion of a distance constraint between H1′ and H3, since this proton pair distance is more dependant on the glycosidic linkage conformation than H1′/H4.[28] The refined structure provided a structure that has Φ angle and $^3J_{H1',C4}$ value very close to the experimentally observed value (Table 1). The resulting conformation is displayed in Fig. 4. The glycosidic linkage adopts a conformation with ($\Phi_H = 27°$ and $\Psi_H = -64°$), which corresponds to the low energy conformation Conf_B of LacNAc. The calculated heteronuclear coupling constants are in agreement with the experimentally determined ones (Table 1). Comparison of the several observed strong NOEs with the theoretical distances indicate that the conformation displayed in Fig. 4 is the major one in solution. Some fluctuations may occur around this equilibrium value, especially in the tethering bridge. Furthermore, it can be concluded that **4** adopts a low minimum energy conformation on the energy map of LacNAc and furthermore, it is significantly more rigid that the corresponding ethylene tethered analogue **3**.

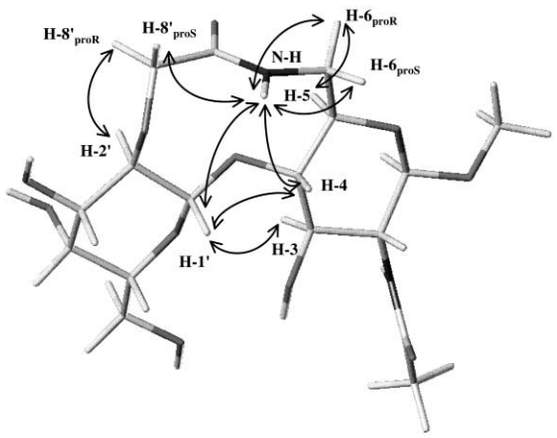


Fig. 4 Low energy conformation of optimized compound **4**. The structure has been colored according to atom types. Arrows indicate predicted NOEs.

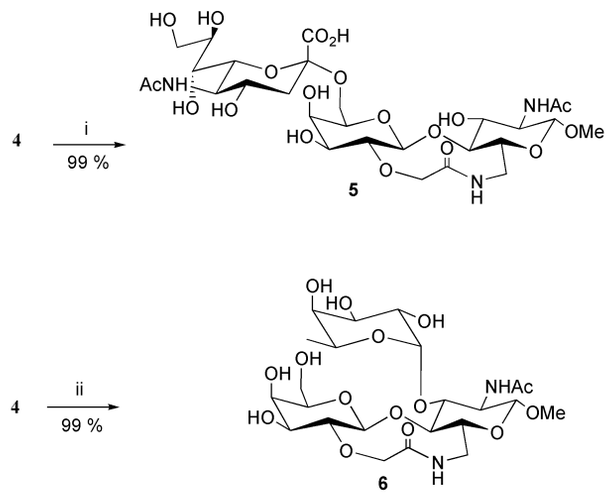
Enzymatic glycosylations

After having established that compound **4** can adopt a low energy conformation on the energy surface of LacNAc, attention was focused on the enzymatic glycosylation by α-2,6-sialyltransferase and α-1,3-fucosyltransferase V (Scheme 2). The transfer of sialic acid was accomplished utilizing an excess of CMP-NeuAc as glycosyl donor in the presence of rat liver α-2,6-sialyltransferase as the catalyst and calf alkaline phosphatase to ensure low levels of CMP released during the reaction which is known to inhibit the enzyme.[29] The reaction was carried out for two days after which the crude reaction mixture was purified by ion exchange chromatography (Dowex 1 × 8–200, PO_4^{2-}, 100–200 mesh) followed by biogel P2 size exclusion column chromatography to give **5** in quantitative yield.

In order to obtain fucosylated derivative **6**, compound **4** was treated with an excess of GDP-fucose in the presence of

Table 2 Apparent kinetic parameters for the transfer of *N*-acetylneuraminic acid by α-2,6-sialyltransferase and relative rates of transfer of fucose by fucosyltransferase V.

	α-2,6-ST			FucT V
	K_m/mM	Rel. V_{max}	(Rel. V_{max}/K_m)/mM^{-1}	Rel. rate transfer (%)
LacNAc-OMe	1.7 ± 0.2	1.0	0.6	100
3	1.1 ± 0.1	1.3	1.2	39
4	1.0 ± 0.1	1.1	1.1	9



Scheme 2 *Reagents and conditions*: enzyme reactions; (i) α-2,6-sialyltransferase, CMP-NeuAc; (ii) α-1,3-fucosyltransferase V, GDP-fucose

recombinant human α-1,3-fucosyltransferase V and alkaline phosphatase to ensure a low concentration of GDP. In this case, the rate of transfer was significantly slower, however, after a reaction time of 5 days, MALDI-TOF mass spectrometry indicated completion of the reaction. The crude reaction mixture was purified by ion exchange column chromatography followed by biogel P2 size exclusion column chromatography to give **6** in an excellent yield of 99%.

The presence of the linker in compounds **5** and **6** was confirmed by the shifts of H8R′ and H8S′ and a large coupling constant $J_{8R',8S'}$ that were also observed in compound **4** (4.56, 3.79 ppm, $J_{8R',8S'}$ 16.5 Hz), for compound **5** (4.52, 3.79 ppm, $J_{8R',8S'}$ 16.6 Hz) and for compound **6** (4.47, 3.74 ppm, $J_{8R',8S'}$ 17.6 Hz). The presence the α-sialyl linkage of compound **5** was confirmed by a typical shift of H4″ (δ 3.57 ppm) and the difference in chemical shift of H9a and H9b (H9″a – H9″b = 0.23 ppm).[30] The typical shift and coupling constant of the anomeric proton of the α-fucosyl linkage (H1″ δ 5.04 ppm, $J_{1'',2''}$ 5.1 Hz) confirmed its presence in compound **6**.

Based on the observation that compound **4** is an appropriate substrate for glycosyl transferases, apparent kinetic parameters for α-(2,6)-sialyltransferase catalyzed transfer of CMP-(^{14}C)Neu5Ac to LacNAc and conformationally constraint compounds **3** and **4** were determined using a reported assay[20] (Table 2). The K_m for **1** was in close agreement with previous data and its V_{max} was set at 1. As can be seen in Table 2, very similar K_m and relative V_{max} values were obtained for each compound. Compound **4** is contained in the B-conformer on the energy surface of LacNAc whereas the previously studied **3** can adopt the A- and the B-conformer. When a transferase prefers the A-conformer, it is to be expected that only **3** gives favorable kinetic parameters. On the other hand, when the B-conformer is recognized both compounds should display good transformations. Thus, the results indicate that α-(2,6)-sialyltransferase recognizes LacNAc in the B-conformation.

In the case of fucosyltransferase V, only relative rates could be measured due to high Michaelis–Menten constants. It is, however, apparent, that this transferase responds differently to the imposed constraints and in this case the ethylene bridged derivative **3** was the substrate that was employed more efficiently. Thus, this finding indicates that the fucosyltransferase recognize LacNAc in its A-conformer. Further studies with a wider range of glycosyltransferases and modified LacNAc acceptors will be reported elsewere to support the notion that different transferases may recognize LacNAc in different conformations.

Conclusion

Compound **4**, which is preorganized by a methylene amide linker between the C-6 and C-2′, was obtained by first synthesizing a selectively protected 6-amino LacNAc derivative, which was N-acylated with chloroacetic anhydride followed by macro-cyclization and finally deprotection. A combination of NOE and *trans*-glycosidic heteronuclear coupling constant measurements and molecular mechanics simulations established that the glycosidic linkage of compound **4** is highly conformationally constrained and resides in the B-conformer on the energy map of LacNAc. Previously, we synthesized a LacNAc derivative (**3**) that is conformationally constraint by an ethylene tether and this compound could adopt both A and B conformations. Thus, LacNAc derivative **4** is more rigid than the ethylene analogue and provides an important complementary derivative to study conformational requirements of a range of transferases and lectins. Compound **4** proved to be a substrate for rat liver α-2,6-sialyltransferase and fucosyltransferase V and this finding was exploited for the preparative enzymatic synthesis of conformationally constrained sialyllactosamine **5** and Lewis^x **6**, respectively. Although several studies have shown that glycosyltransferases can accept small changes in their glycosyl acceptors,[8] it is shown here for the first time that these enzymes can also be employed for the chemoenzymatic synthesis of oligosaccharides that are locked in a particular conformation. The apparent kinetic parameters for the α-(2,6)-sialyltransferase and fucosyltransferase V catalyzed transformations indicate that the two enzymes respond differently to imposed conformational constraints of the acceptor opening an exciting route for the design of selective glycosyltransferase substrates and inhibitors.

Experimental

General remarks

Chemicals were purchased from Aldrich and Fluka and used without further purification. Molecular sieves were activated at 350 °C for 3 h *in vacuo*. Dichloromethane was distilled from CaH_2 and stored over 4Å molecular sieves. All the reactions were performed under anhydrous conditions and monitored by TLC on Kieselgel 60 F_{254} (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol. Flash chromatography was performed on silica gel (Merck, mesh 70–230). Extracts were concentrated under reduced pressure at <40 °C (bath). ^{1}H NMR (1D, 2D) and ^{13}C NMR spectra were recorded on a Varian Merc300 spectrometer and Varian 500, 600 and 800 MHz spectrometers equipped with Sun workstations. For ^{1}H and ^{13}C NMR spectra recorded in $CDCl_3$, chemical shifts (δ) are given in ppm relative to solvent peaks (^{1}H, δ 7.26; ^{13}C, δ 77.3) as internal standard for

protected compounds and using NAc proton (2.025 ppm) and OMe carbon (59.82 ppm) as internal standard for deprotected molecules. Negative ion matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectra were recorded using an HP-MALDI instrument using gentisic acid matrix. Optical rotations were measured on a Jasco P-1020 polarimeter, and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^3 g^{-1} at 26 °C, 50 mm cell.

Recombinant human α-1,3-fucosyltransferase V, rat liver α-2,6-sialyltransferase, CTP, CMP-Neu5Ac and calf alkaline phosphatase were purchased from Calbiochem. ACS liquid scintillation cocktail was obtained from Fisher Scientific.

Methyl 2-acetamido-6-azido-3-*O*-benzyl-2,6-dideoxy-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside (8)

Sodium azide (130 mg, 2.0 mmol) was added to a stirred solution of **7** (438.7 mg, 0.5 mmol) in dry *N*,*N*-dimethylformamide (20 mL). After stirring for 2 h at 90°C, TLC (chloroform–methanol, 9 : 1, v/v) indicated completion of the reaction. The reaction mixture was diluted with ethyl acetate (100 mL) and successively washed with water (2 × 20 mL) and brine (20 mL), followed by drying over $MgSO_4$. After evaporation of the solvent, the residue was purified by flash silica gel chromatography (hexane–acetone, gradient 3 : 1 to 1 : 1, v/v) to afford compound **8** (403.9 mg, 98%) as a white foam. $[\alpha]_D$ = 3.8 (*c* 5.03, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40–7.10 (m, 20H, arom), 6.04 (d, 1H, $J_{NH,2}$ 8.5 Hz, N*H*COCH$_3$), 5.33 (dd, 1H, $J_{2',3'}$ 10.2 Hz, H-2′), 4.93, 4.58 (AB q, 2H, J_{AB} 11.3 Hz, OC*H*$_2$Ph), 4.70, 4.64 (AB q, 2H, J_{AB} 11.8 Hz, OC*H*$_2$Ph), 4.60, 4.52 (AB q, 2H, J_{AB} 12.1 Hz OC*H*$_2$Ph), 4.56 (d, 1H, $J_{1,2}$ 9.1 Hz, H-1), 4.40, 4.33 (AB q, 2H, J_{AB} 12.9 Hz, OC*H*$_2$Ph), 4.36 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1′), 3.97 (d, 1H, $J_{3',4'}$ 2.8 Hz, H-4′), 3.92 (m, 1H, H-2), 3.85 (m, 1H, H-3), 3.74 (t, 1H, $J_{3,4} = J_{4,5}$ 5.5 Hz, H-4), 3.70–3.40 (m, 4H, H-6a, H-6b, H-6a′, H-6b′), 3.67 (m, 1H, H-5), 3.52 (m, 1H, H-5′), 3.46 (m, 1H, H-3′), 3.44 (s, 3H, OCH$_3$), 1.99 (s, 3H, C*H*$_3$C(O)NH), 1.85 (s, 3H, CH$_3$C(O)O). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.03, 169.78, 138.18, 137.62, 137.57, 128.29, 128.08, 128.07, 128.00, 127.74, 127.72, 127.67, 127.53, 127.47, 127.28, 101.20, 100.48, 79.86, 76.58, 75.47, 74.58, 74.08, 73.67, 73.49, 72.92, 72.47, 72.02, 71.74, 68.02, 56.44, 51.76, 51.71, 23.33. 21.06. MALDI-TOF: *m*/*z* 847.6 [M + Na]$^+$. Anal. Calc. (%) for $C_{45}H_{52}N_4O_{11}$: C, 65.52; H, 6.35; N, 6.79; O, 21.33. Found: C, 65.22; H, 6.40; N, 6.84.

Methyl 2-acetamido-6-azido-3-*O*-benzyl-2-dideoxy-4-*O*-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside (9)

Sodium methoxide (10.8 mg, 0.2 mmol) was added to the stirred solution of **8** (256.2 mg, 0.31 mmol) in methanol (20 mL). The mixture was left stirring at room temperature for 48 h. TLC (hexane–acetone, 2 : 1, v/v) indicated completion of the reaction. The mixture was neutralized with Dowex 50H$^+$ WX4–200 ion exchange resin until pH = 7, filtered and concentrated *in vacuo*. The residue was purified by flash silica gel chromatography (gradient hexane–acetone, 3 : 1 to 1 : 1, v/v) to afford compound **9** (237.7 mg, 98%) as a white foam. $[\alpha]_D$ = 10.5 (*c* 5.04, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40–7.10 (m, 20H, arom), 5.65 (d, 1H, $J_{NH,2}$ 7.9 Hz, N*H*COCH$_3$), 4.80, 4.62 (AB q, 2H, J_{AB} 11.9 Hz, OC*H*$_2$Ph), 4.79, 4.53 (AB q, 2H, J_{AB} 11.4 Hz, OC*H*$_2$Ph), 4.65 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1), 4.50, 4.48 (AB q, 2H, J_{AB} 11.4 Hz, OC*H*$_2$Ph), 4.35 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′), 4.29, 4.21 (AB q, 2H, J_{AB} 11.9, OC*H*$_2$Ph), 3.97 (t, 1H, $J_{2,3}$ 8.4, $J_{3,4}$ 8.8, H-3), 3.87 (d, 1H, $J_{3',4'}$ 2.7 Hz, H-4′), 3.83 (m, 1H, H-2′), 3.71 (dd, 1H, $J_{3,4} = J_{4,5}$ 7.9 Hz, H-4), 3.66–3.24 (m, 6H, H-5, H-5′ H-6a, H-6b, H-6a′, H-06b′), 3.44–3.24 (m, 1H, H-3′), 3.40 (s, 3H, OCH$_3$), 1.78 (s, 3H, C*H*$_3$C(O)NH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.50, 138.79, 138.67, 137.89, 137.82, 128.62, 128.49, 128.29, 127.95, 127.75, 127.59, 127.48, 103.52, 101.18, 82.30, 78.62, 78.01, 74.77, 73.71, 73.64, 73.55, 72.78, 72.42, 71.60, 68.29, 56.71, 55.70, 51.44, 23.75. MALDI-TOF: *m*/*z* 806.3 [M + Na]$^+$. Anal. Calc. (%) for $C_{43}H_{50}N_4O_{10}$: C, 65.97; H, 6.44; N, 7.16; O, 20.44. Found: C, 66.04; H, 6.55; N, 7.26.

Methyl 2-acetamido-6-amino-3-*O*-benzyl-2,6-dideoxy-4-*O*-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside (10)

1,3-Propane dithiol (151 μL, 1.5 mmol) and triethylamine (209 μL, 1.5 mmol) were added to a stirred solution of **9** (120 mg, 0.15 mmol) in a mixture of pyridine and water (4 : 1, v/v, 20 mL). The mixture was stirred at room temperature for 6 h TLC (chloroform–methanol, 9 : 1, v/v) indicated completion of the reaction. The mixture was co-evaporated with toluene, concentrated *in vacuo* and then chromatographed over flash silica gel (gradient DCM–methanol, 1 : 0 to 9 : 1, v/v) to afford compound **10** (90.8 mg, 80%). $[\alpha]_D$= −14.9 (*c* 1.39, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40–7.10 (m, 20H, arom), 6.10 (br s, 1H, N*H*COCH$_3$), 5.02, 4.74 (AB q, 2H, J_{AB} 11.5 Hz, OC*H*$_2$Ph), 4.90, 4.64 (AB q, 2H, J_{AB} 11.3 Hz, OC*H*$_2$Ph), 4.78, 4.54 (AB q, 2H, J_{AB} 11.8 Hz, OC*H*$_2$Ph), 4.69 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.65 (d, 1H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.32, 4.22 (AB q, 2H, J_{AB} 11.8 Hz, OC*H*$_2$Ph), 4.10 (t, 1H, $J_{3,4}$ 9.3 Hz, H-3), 3.94 (t, 1H, $J_{4,5}$ 9.3 Hz, H-4), 3.92 (m, 1H, H-2), 3.85 (d, 1H, $J_{3',4'}$ 2.2 Hz, H-4′), 3.67 (m, 1H, H-5), 3.48 (m, 1H, H-2′), 3.58–3.40 (m, 5H, H-3′, H-2′, H-6a′, H-6b′), 3.44 (s, 3H, OCH$_3$), 3.33 (dd, 1H, $J_{6b,5}$ 4.4 Hz, $J_{6b,6a}$ 11.8 Hz, H-6b), 3.12 (dd, 1H, $J_{6a,5}$ 5.0 Hz, H-6a), 1.90 (s, 3H, C*H*$_3$C(O)NH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.68, 139.14, 138.88, 138.63, 137.88, 128.44, 128.19, 127.92, 127.78, 127.62, 127.48, 127.34, 104.85, 101.74, 82.33, 80.84, 78.68, 74.74, 73.93, 73.69, 73.56, 73.00, 72.56, 68.69, 57.18, 56.97, 41.92, 23.75. MALDI-TOF: *m*/*z* 779.5 [M + Na]$^+$. Anal. Calc. (%) for $C_{43}H_{52}N_2O_{10}$: C, 68.24; H, 6.92; N, 3.70; O, 21.14. Found: C, 68.35; H, 7.04; N, 3.81.

Methyl 2-acetamido-3-*O*-benzyl-6-chloroacetamido-2,6-dideoxy-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside (11)

Chloroacetic anhydride (363 mg, 2.12 mmol) was added to a solution of **10** (100 mg, 0.13 mmol) in a mixture of dichloromethane and methanol (1 : 1, mL). The mixture was stirred at room temperature for 16 h. TLC analysis (chloroform–methanol, 9 : 1, v/v) indicated completion of the reaction. The reaction mixture was diluted with dichloromethane (20 mL), washed successively with a saturated solution of $NaHCO_3$ (10 mL), water (2 × 10 mL) and brine (10 mL), followed by drying over $MgSO_4$. After evaporation of the solvent, the residue was purified by flash silica gel chromatography (hexane–acetone, gradient 3 : 1 to 1 : 1, v/v) followed by size exclusion column chromatography (LH20, MeOH–DCM, 1 : 1, v/v) to afford compound **11** (97.4 mg, 90%) as a white foam. $[\alpha]_D$ = −3.97 (*c* 1.05, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.40–7.2 (m, 20H, arom), 7.17 (m, 1H, NHCO), 5.56 (d, 1H, $J_{NH,2}$ 7.3 Hz, N*H*COCH$_3$), 4.89, 4.59 (AB q, 2H, J_{AB} 11.9 Hz, OC*H*$_2$Ph), 4.72, 4.64 (AB q, 2H, J_{AB} 11.9 Hz, OC*H*$_2$Ph), 4.69 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 4.63, 4.55 (AB q, 2H, J_{AB} 11.9 Hz, OC*H*$_2$Ph), 4.53 (d, 1H, $J_{1',2'}$ 7.0 Hz, H-1′), 4.34, 4.28 (AB q, 2H, J_{AB} 11.9 Hz, OC*H*$_2$Ph), 4.02 (s, 2H, CH$_2$CO), 4.03 (m, 1H, H-3), 3.97 (dd, 1H, $J_{2',3'}$ 8.8 Hz, H-2′), 3.91 (d, 1H, $J_{3',4'}$ 2.2 Hz, H-4′), 3.69 (t, 1H, $J_{4,5}$ 8.4 Hz, H-4), 3.52 (m, 1H, H-2), 3.47 (s, 3H, OC*H*$_3$), 3.41 (dd, 1H, $J_{3',4'}$ 11.8 Hz, H-3′), 3.88–3.58 (m, 4H, H-5, H-5′, H-6a′, H-6b′), 3.58 (m, 1H, H-6b), 3.36 (dd, 1H, $J_{6a,5}$ 4.4 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 1.84 (s, 3H, C*H*$_3$C(O)NH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.36, 166.39, 138.82, 138.74, 138.18, 137.90, 128.86, 128.63, 128.52, 128.42, 128.30, 128.03, 127.99, 127.92, 127.76, 127.67, 127.61, 127.56, 103.76, 101.27, 82.39, 79.37, 78.80, 74.79, 74.17, 74.05, 73.82, 73.72, 73.68, 73.03, 72.73, 72.04, 68.51, 57.01, 56.16, 42.94, 40.90, 23.81.

MALDI-TOF: *m*/*z* 855.33 [M + Na]$^+$. Anal. Calc. (%) for $C_{45}H_{53}N_2O_{11}$: C, 64.86; H, 6.41; N, 3.36; O, 21.12. Found: C, 64.97; H, 6.47; N, 3.47.

Methyl 2-acetamido-3-*O*-benzyl-2,6-dideoxy-2′,6-(2′-*O*-methylenelactam)-4-*O*-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-β-D-glucopyranoside (12)

Sodium hydride (60% in oil suspension, 40 mg, 1.1 mmol) was added to a solution of **11** (90 mg, 0.11 mmol) in dry *N*,*N*-dimethylformamide (20 mL). After stirring at room temperature 16 h, TLC (chloroform–methanol, 9 : 1, v/v) indicated completion of the reaction. The reaction mixture was quenched with methanol, diluted with ethyl acetate (50 mL) and successively washed with water (2 × 10 mL) and brine (10 mL), followed by drying over $MgSO_4$. After evaporation of the solvents, the residue was then purified by flash silica gel chromatography (hexane–acetone, gradient 3 : 1 to 1 : 1, v/v) to afford compound **12** (49 mg, 56%) as a white foam. $[\alpha]_D = -106$ (*c* 0.40, $CHCl_3$). ^{1}H NMR (DMSO, 300 MHz): δ 7.20–7.05 (m, 20H, arom), 7.94 (d, 1H, $J_{NH,2}$ 8.8 Hz, N*H*COCH$_3$), 7.74 (t, 1H, *J* 6.5 Hz, NHCO), 4.78 (d, 1H, $J_{1,2}$ 6.4 Hz, H-1), 4.76, 4.44 (AB q, 2H, J_{AB} 11.2 Hz, OC*H*$_2$Ph), 4.75, 4.51 (AB q, 2H, J_{AB} 11.7 Hz, OC*H*$_2$Ph), 4.73, 4.68 (AB q, 2H, J_{AB} 12.2 Hz OC*H*$_2$Ph), 4.72 (d, 1H, OC*H*–HCO), 4.57 (d, 1H, *J* 11.72 Hz, OCH–*H*CO), 4.27 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1′), 4.11, 3.98 (AB q, 2H, J_{AB} 12.2 Hz, OC*H*$_2$Ph), 3.88 (d, 1H, $J_{3',4'}$ <2 Hz, H-4′), 3.74 (m, 1H, H-3), 3.69 (dd, 1H, $J_{2,3}$ 9.3 Hz, H-2), 3.67 (m, 1H, H-6b′), 3.64 (m, 1H, H-6a′), 3.57 (m, 1H, H-5), 3.53 (m, 1H, H-4), 3.51 (m, 1H, H-3′), 3.34 (m, 1H, H-5′), 3.31 (m, 1H, H-6b), 3.30 (s, 3H, OCH$_3$), 3.29 (m, 1H, H-2′), 2.93 (dd, 1H, $J_{6a,5}$ 4.9 Hz, $J_{6a,6b}$ 8.3 Hz, H-6a), 1.89 (s, 3H, C*H*$_3$C(O)NH). ^{13}C NMR (DMSO, 125 MHz): δ 177.51, 171.98, 129.5–126.13 (arom.), 103.98, 101.79, 83.79, 82.71, 81.30, 74.58, 74.07, 73.89, 73.20, 72.90, 72.75, 72.32, 71.75, 68.31, 68.00, 56.35, 54.67, 40.30, 23.41. MALDI-TOF: *m*/*z* 820.2 [M + Na]$^+$. Anal. Calc. (%) for $C_{45}H_{52}N_2O_{11}$: C, 67.82; H, 6.58; N, 3.52; O, 22.08. Found: C, 67.93; H, 6.62; N, 3.59.

Methyl 2-acetamido-2,6-dideoxy-(2′-*O*-,6-*N*-methylenelactam)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (4)

10% Palladium on charcoal (32 mg) was added to a solution of **12** (30 mg, 0.04 mmol) in ethanol (3 mL). The mixture was vigorously stirred under an atmosphere of hydrogen for 16 h. TLC analysis (chloroform–methanol, 9 : 1, v/v) indicated completion of the reaction. After filtration using Celite and concentration of the filtrate under reduced pressure, the crude product was purified by column chromatography (Iatrobeads, chloroform–methanol–water, 74 : 24 : 2, v/v/v) to afford compound **4** (16.9 mg, 97%) as a white amorphous solid. $[\alpha]_D = -61.9$ (*c* 0.24, D_2O). ^{13}C NMR (D_2O, 125 MHz): δ 106.1 (C-1′), 103.8 (C-1), 90.1 (C-4), 87.1 (C-2′), 78.4 (C-5′), 76.1 (O*C*H$_2$CO), 75.7 (C-3), 74.6 (C-3′), 70.8 (C-4′), 68.5 (C-5), 63.2 (C-6′), 59.8 (OCH$_3$), 56.8 (C-2), 44.6 (C-6), 25.3 (CO*C*H$_3$). MALDI-TOF: *m*/*z* 459.3 [M + Na]$^+$. For ^{1}H NMR data see Table 3.

Methyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl-(2–6)-β-D-galactopyranosyl-(1–4)-2-acetamido-2,6-dideoxy-(2′-*O*-,6-*N*-methylenelactam)-4-*O*-β-D-glucopyranoside (5)

Incubations were carried out in 150 μL sodium cacodylate buffer (25 mM, pH 7.2) containing 0.5% Triton X100 and bovine serum albumin (1 mg ml^{-1}). Acceptor **4** (2.00 mg, 4.6 μmol), CMP-Neu5Ac (2.0 mg), alkaline phosphatase (3 U), and sialyltranferase (6.9 mU) were incubated at 37 °C for 36 h. Every 12 h, phosphatase (3 U), enzyme (6.9 mU) and CMP-Neu5Ac (2.0 mg) were added to the incubation mixture to compensate for any hydrolysis of the donor. The reaction was monitored by TLC on silica gel plates using $CHCl_3$–MeOH–H_2O (60 : 35 : 6,

Table 3 ^{1}H NMR chemical shifts (δ, ppm) and coupling constants (*J*/Hz) of compounds **1**(a), **2**(a) and **3**(b)

	1(a)		**2**(a)			**3**(b)		
	Gal	GlcNAc	Gal	GlcNAc	NeuAc	Gal	GlcNAc	Fuc
H-1 ($J_{1,2}$)	4.74 (8.3)	4.45 (8.4)	4.65 (7.8)	4.47 (8.3)	—	4.51 (8.3)	4.48 (7.0)	5.06 (5.1)
H-2 ($J_{2,3}$)	3.30 (9.2)	3.75	3.25 (9.3)	3.75 (9.8)	—	3.32	3.83	3.70 (11.2)
H-3a ($J_{3a,4}$, $J_{3a,3e}$)	3.78 (<0.2)	3.70	3.77–3.59 (<0.2)	3.77–3.59 (2.9)	2.61 (12.2)	3.71 (<0.2)	3.88	3.97–3.62
H-3e ($J_{3e,4}$)	—	—	—	—	1.69 (4.9)	—	—	3.95–3.60
H-4 ($J_{4,5}$)	3.96 (<1)	3.70	4.00	3.66	3.61 (12.2)	3.80	3.69	3.97–3.62
H-5 ($J_{5,6a}$)	3.78	4.05 (11.1)	3.93–3.79	3.63	3.88 (9.3)	3.82–3.57	4.08	4.78
H-6a ($J_{5,6b}$)	3.77 (5.4)	3.14 (5.4)	3.59	3.09	3.52	3.68	3.20	—
H-6b ($J_{6a,\ 6b}$)	3.77	4.05 (10.4)	3.82	4.01 (13.7)	—	3.81	3.93	—
H-7 ($J_{6,7}$) ($J_{7,8}$)	—	—	—	—	3.77–3.59	—	—	—
H-8^{iv} ($J_{8^{iv},9a}$, $J_{8^{iv},9b}$)	—	—	—	—	3.93–3.79	—	—	—
H-9 ($J_{9a,9b}$)	—	—	—	—	3.97, 3.74 (11.7)	—	—	—
CH_3-6 ($J_{5,Me\text{-}6}$)	—	—	—	—	—	—	—	1.18 (6.8)
CH_3O	—	3.50	—	3.47	—	—	3.51	—
CH_3CO	—	2.03	—	—	2.03, 1.99	—	2.03	—
OCH_2CO (H8R′, H-8S′) ($J_{8R',8S'}$)	4.56, 3.79 (16.5)	—	4.52, 3.79 (16.6)	—	—	4.47, 3.74 (17.6)	—	—

v/v/v) as the eluent. The acceptor **1** has an R_f of 0.32 and compound **2** had an R_f of 0.06. At the completion of the reaction, the sample was purified on ion-exchange chromatography using Dowex 1 × 8–200 (PO_4^{2-}, 100–200 mesh) followed by biogel P2 to afford compound **5** (3.20 mg, 99%). $[\alpha]_D = -2.54$ (*c* 2.8, D_2O). ^{13}C NMR (D_2O, 125 MHz): δ 181.34(*C*OCH$_3$), 181.25 (*C*OCH$_3$), 180.15 (CO_2H), 109.92 (C-1′), 107.66 (C-1), 94.18, 90.50, 80.00, 79.83(O*C*H$_2$CO), 79.25, 79.08, 77.93, 74.75, 74.21 (C-9″), 72.28, 69.22, 69.18, 68.87 (C-6′), 59.82, 57.92, 47.82 (C-6), 46.13 (C-3″), 28.32(CO*C*H$_3$), 28.28(CO*C*H$_3$), 23.03. MALDI-TOF: *m*/*z* = 727.3 [M^+ + Na]. For ^1H NMR data see Table 3.

Methyl α-D-fucopyranosyl-(1–3)-[β-D-galactopyranosyl-(1–4)]-2-acetamido-2,6-dideoxy-(2′-*O*-6-*N*-methylenelactam)-D-glucopyranoside (6)

Incubations were carried out in 150 μL sodium cacodylate buffer (25 mM, pH 6.5) containing $MnCl_2$ (8 mM), ATP (1.6 mM) and NaN_3 (1.6 mM). Acceptor **4** (1.10 mg, 2.5 μmol), GDP-Fucose (1.0 mg), alkaline phosphatase (4 U) and fucosyltransferase V (7.5 mU) are incubated at 37 °C for 4 days. Every 24 h, GDP-Fucose (1.0 mg), alkaline phosphatases (4 U) and FucT V (7.5 mU) were added to the incubation mixture to compensate for any hydrolysis of the donor. The reaction was monitored by TLC on silica gel plates using $CHCl_3$–MeOH–H_2O (60 : 35 : 6, v/v) as the eluent. The acceptor **4** has an R_f of 0.51 and compound **3** has an R_f of 0.33. After the completion of the reaction, the sample was purified on ion-exchange chromatography on a Dowex 1 × 8–200 (Cl^-, 100–200 mesh) followed by biogel P2 to afford compound **6** (1.44 mg, 99%). $[\alpha]_D = -26.86$ (*c* 0.1, D_2O). ^{13}C NMR (D_2O, 125 MHz): δ 180.85 (*C*OCH$_3$), 106.14, 105.04, (C-1, C-1′), 101.72 (C-1″), 91.82, 86.84, 86.53, 84.04, 78.25, 77.89, 77.46, 75.42, 74.60, 71.69, 71.86, 70.83, 70.22, 69.22, 68.40, 68.10, 63.66, 59.82, 57.95. MALDI-TOF: *m*/*z* = 605.23 [M^+ + Na]. For ^1H NMR data see Table 3.

Guanosine-5′-diphosphate-β-L-fucose, disodium salt (14)

GDP-Fucose was synthesized following a reported procedure.[31] Bis(cyclohexylammonium) β-L-fucopyranosyl phosphate (354 mg, 0.80 mmol) was dissolved in H_2O (10 mL), applied to a Bio-Rad AGW-X2 cation-exchange column (Et_3N^+) and eluted with H_2O (100 mL). The solution was evaporated, coevaporated with MeOH (2 × 10 mL), and dried under vacuum to give triethylammonium) β-L-fucopyranosyl phosphate **13** (290 mg). A mixture of **14** (290 mg, 0.80 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium guanosine 5′-monophosphomorpholidate (1 g, 1.27 mmol) was co-evaporated with dry pyridine (2 × 2 mL). 1*H*-Tetrazole (165 mg, 2.4 mmol) and dry pyridine (1.5 mL) were added and the solution was stirred at room temperature. After a while, product started to precipitate. The reaction was monitored by TLC (*i*-PrOH–NH_4OAc (1 M)–H_2O (3 : 2 : 1, v/v/v)). The residue was passed through Dowex resin 50 Na^+ with water, followed by purification using C-18 reverse phase chromatography in H_2O to afford compound **13** (211 mg, 45%) as a white amorphous solid. ^1H NMR data was in agreement with those reported.[31]

Molecular modeling

Nomenclature. Torsion angles at the glycosidic linkage have been defined with reference to the hydrogen atom: $\Phi_H = H_1'$–C_1'–O_1'–C_4 and $\Psi_H = C_1'$–O_1'–C_4–H_4.

Conformational analysis of 4. All energy calculations were performed using the TRIPOS force field[32] complemented by carbohydrate energy parameters.[33] The possible conformations of compound **4** were determined using the SEARCH procedure of SYBYL. The ring closure bond was chosen to be ν2. Tolerance for ring closure was 0.3 Å for bond length and 20° for bond angles. The systematic search was performed with steps of 5° on all torsion angles except for the amine group (angle μ2) for which only values of 0° and 180° were tested. The van der Waals cut off (steric penetration allowed) was fixed to 0.6. The hydroxylic hydrogen atoms were not taken into account during this rigid search. The energy was evaluated for each conformation but the electrostatic contribution was not included. Conformations were accepted in an energy window of 50 kcal mol^{-1}. The resulting conformations were then analyzed by a home-made clustering program to determine conformational families.

Geometry optimization. Compound **4** was fully optimized with the TRIPOS force-field. Partial charges were assigned according to the PIM energy parameters for carbohydrates[34] and the dielectric constant was set to a value of 4.0. Energy minimisation was performed with inclusion of one distance constraint (H1 ··· H3 set to 3.0 Å).

Heteronuclear $^3J_{C-H}$ coupling constants across the glycosidic linkages were calculated using the Karplus-type equation proposed by Tvaroska.[25]

$$^3J_{C-H} = 5.7\cos^2\Phi - 0.6\cos\Phi + 0.5$$

NMR Spectroscopy

Data were collected on Varian 500, 600 and 800 MHz spectrometers. The methyl amide disaccharide (5 mg) was lyophilized from D_2O and re-dissolved in 0.45 ml D_2O. The data for signal assignment was acquired on this sample at 25 °C with standard Varian gradient COSY and HSQC pulse sequences. Data was processed with nmrPipe software.[35] For the measurement of heteronuclear coupling constants and the measurement of NOEs, 0.12 ml of pyridine-d_5 was added to the sample, and the temperature was raised to 50 °C to change the chemical shifts of the overlapped H3 and H4 signals and remove second-order effects. Proton–proton coupling constants were extracted from the one-dimensional proton spectra in D_2O and in 21% pyridine–D_2O. For the observation of NOEs to the amide proton, the sample was recovered, and dissolved in 10% DMSO-d_6–pyridine-d_5. A two-dimensional ROESY experiment was used to obtain qualitative NOE data.

A 10 mg sample of LacNac was dissolved in D_2O and pyridine-d_5 added to give a final solution of 30% pyridine–D_2O. Chemical shifts of this sample at 40 °C were correlated to the data obtained in pure D_2O at 25 °C, using standard HSQC and TOCSY.

For LacNAc, a variation of the one-dimensional HSQMBC[24] was used in analogy to work done on lactose.[37] In the experiment, the initial proton pulse of the one-dimensional HSQMBC sequence was replaced by a selective one-dimensional TOCSY sequence, and the H1 of the glucosamine residue was selectively irradiated. Thus, only protons of the glucosamine spin system continue through the remainder of the pulse sequence. The result is a multiplet which arises from coupling between glucosamine H4 and galactosyl C1′, thus avoiding any contribution to the multiplet from $^3J_{C1-H3}$ or $^3J_{C1-H3'}$.

Heteronuclear coupling constants. For compound **4**, the three-bond proton–carbon coupling constants ($^3J_{CH}$) were obtained from a two-dimensional HSQMBC experiment, according to Williamson *et al.*[24] The data set was 1325 × 64 complex points, covering a ^1H range of 2.3 ppm and ^{13}C range of 78.3 ppm at 800 MHz. Two-dimensional data sets were zero-filled in t2 to 16384 points, and linear predicted and zero-filled in t1 to 512 points, giving 0.11 Hz/point resolution in t2 and 30 Hz/points in t1. Coupling constants were measured by fitting a one-dimensional reference spectrum to a one-dimensional trace through the center of the cross-peak of interest, which contains

the anti-phase heteronuclear coupling.[36] The reference spectra were constructed by adding a one-dimensional trace through the center of the appropriate auto-peak (*e.g.* H1′–C1′) with a copy of that trace, inverted 180° in phase and offset by a variable number of points. The coupling constant was estimated from the number of points offset for the reference spectrum that best matched the cross-peak pattern, based on minimization of their difference.

NOE experimental procedures. For each compound, selective one-dimensional NOESY experiments[27] were used to obtain buildup curves for the anomeric and other resolved protons. Linearity of the NOE buildup curves was observed at mixing times beyond 300 ms. The isolated spin-pair approximation was used to estimate the distances, by using the intra-ring H1′–H2′ NOE as an internal reference (3.09 Å).[34] Qualitative NOEs were also observed in the DMSO–pyridine solution with a two-dimensional ROESY experiment.

Acknowledgements

This research was supported by the Office of the Vice President of Research of the University of Georgia.

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