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Synthesis of unnatural sugar nucleotides and their evaluation as donor substrates in glycosyltransferase-catalyzed reactions

Amira Khaled, Tatiana Ivannikova and Claudine Augé*

Laboratoire de Chimie Organique Multifonctionnelle, UMR 8614, GDR 2590, Institut de Chimie Moléculaire et des Matériaux d'Orsay (ICMMO), Université Paris-Sud, Bât 420, F-91405 Orsay, France

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Abstract—New unnatural sugar nucleotides, UDP-Fuc and CDP-Fuc were synthesized from fucose-β-1-phosphate and nucleotide monophosphates activated as morpholidates. Furthermore, a nucleotide analogue was prepared by phosphorylation of 1-(β-D-ribofuranosyl)cyanuric acid, itself obtained as a protected derivative by condensation of the persilylated derivative of cyanuric acid with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in 74% yield. This phosphate activated according to the same procedure was condensed with fucose-β-1-phosphate, affording a new sugar nucleotide conjugate (NDP-Fuc) which was evaluated together with UDP-Fuc, CDP-Fuc and ADP-Fuc, as fucose donors in α-(1→4/3)-fucosyltransferase (FucT-III) catalyzed reaction. Fucose transfer could be observed with each of the donors and kinetic parameters were determined using a fluorescent acceptor substrate. Efficiency of the four analogues towards FucT-III was in the following order: UDP-Fuc = ADP-Fuc > NDP-Fuc > CDP-Fuc.

According to the same strategy ADP-GlcNAc was prepared from AMP-morpholidate and *N*-acetylglucosamine-α-1-phosphate; tested as a glucosaminyl donor towards *Neisseria meningitidis N*-acetylglucosaminyl transferase (*LgtA*), ADP-GlcNAc was recognized with 0.1% efficiency as compared with UDP-GlcNAc, the natural donor substrate.

Keywords: Sugar nucleotides; Donor substrate; Glycosyltransferases; Specificity

1. Introduction

Glycosyltransferases of Leloir-type that typically catalyze sugar unit transfer from a sugar nucleotide donor to an unprotected saccharide acceptor with complete regio- and stereoselectivity, have proved to be precious tools in preparative oligosaccharide synthesis.[1] Nowadays the number of glycosyltransferases cloned in research laboratories is increasing very rapidly and much improvement has been achieved in this area: these enzymes, originally membrane-associated proteins are now produced in soluble form, and mostly tagged with a six histidine tail, which turned out to be of benefit for synthetic purposes.[2] Supported synthesis with glycosyltransferases is also expected to develop.[3] However the major drawback to the enzymatic approach remains the requirement for stoichiometric amounts of sugar nucleotides. In situ regeneration of the sugar nucleotides is the most elegant way of using these cofactors, but it calls for additional enzymes and complicates the process.[4] For preparative purposes donor substrates with a simpler structure than sugar nucleotides, prepared at low cost, would be highly desirable. The pyrophosphate bridge plays a key role in enzyme mechanism: as a leaving group it interacts with Mn^{2+} when this cation is required for enzyme activity, which, according to the limited number of known glycosyltransferase crystal structures, is coordinated to two aspartate residues of the enzyme.[5] Mimics of the diphosphate bond that might act as alternate donor substrates would be of great interest, but searching for such analogues is somewhat hazardous. Therefore, we decided to first look at the possible changes, that glycosyltransferases are able to tolerate with respect to the nucleoside part of the sugar nucleotide. Thus, we report here the synthesis of

* Corresponding author. Tel.: +33 1 69 15 47 18; fax: +33 1 69 15 47 15; e-mail: clauauge@icmo.u-psud.fr

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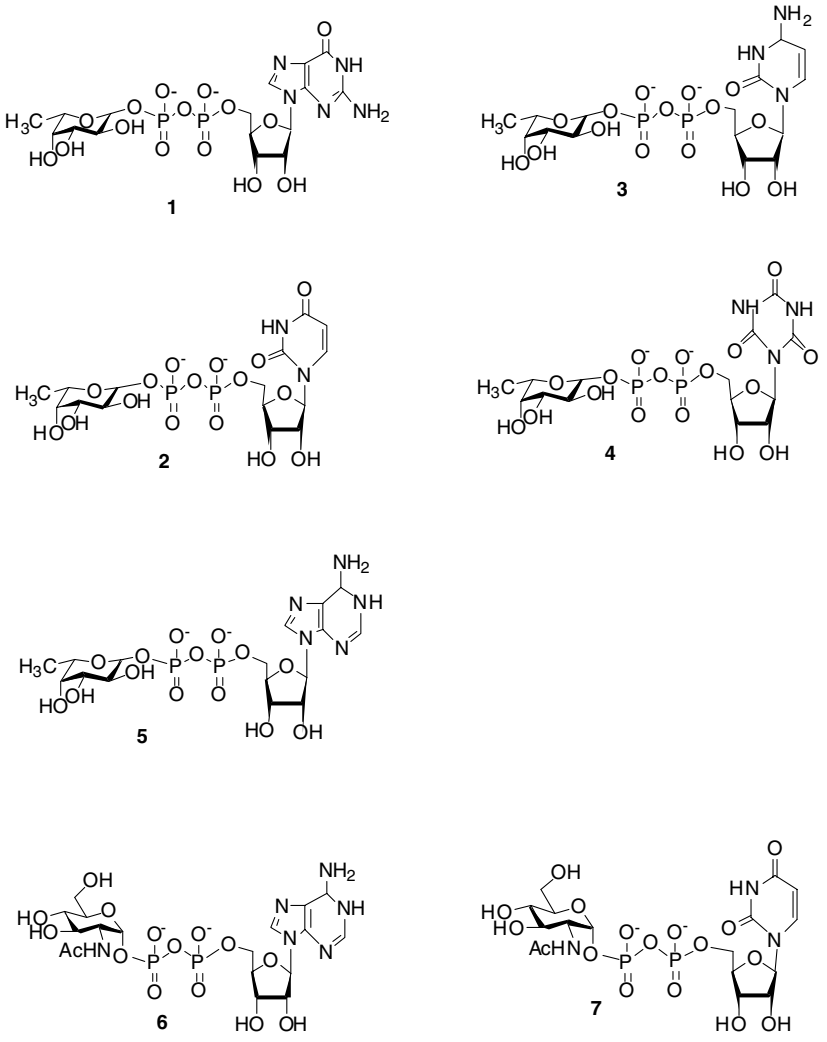


Chart 1. Unnatural sugar nucleotides UDP-Fuc (**2**), CDP-Fuc (**3**), NDP-Fuc (**4**), ADP-Fuc (**5**), ADP-GlcNAc (**6**) to be tested as donor substrates and natural donor substrates GDP-Fuc (**1**) and UDP-GlcNAc (**7**).

three new unnatural sugar nucleotide analogues of GDP-Fuc (**1**): UDP-Fuc (**2**), CDP-Fuc (**3**) and the so called NDP-Fuc (**4**) derived from 1-(β-D-ribofuranosyl)cyanuric acid (Chart 1). These analogues were evaluated along with the previously described ADP-Fuc (**5**),[6] as fucose donors in the reaction catalyzed by GDP-Fuc: β-D-Gal*p*-(1→3/4)-β-D-Glc*p*NAc-(1→4/3)-α-fucosyltransferase (EC 2.4.1.65, FucT-III). Furthermore, we describe the synthesis of another new sugar nucleotide ADP-GlcNAc (**6**), an analogue of UDP-GlcNAc (**7**), which was tested as an *N*-acetylglucosaminyl donor in the reaction catalyzed by *Neisseria meningitidis* UDP-GlcNAc: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3′)-β-*N*-acetylglucosaminyltransferase (EC 2.4.1.56, *LgtA*).

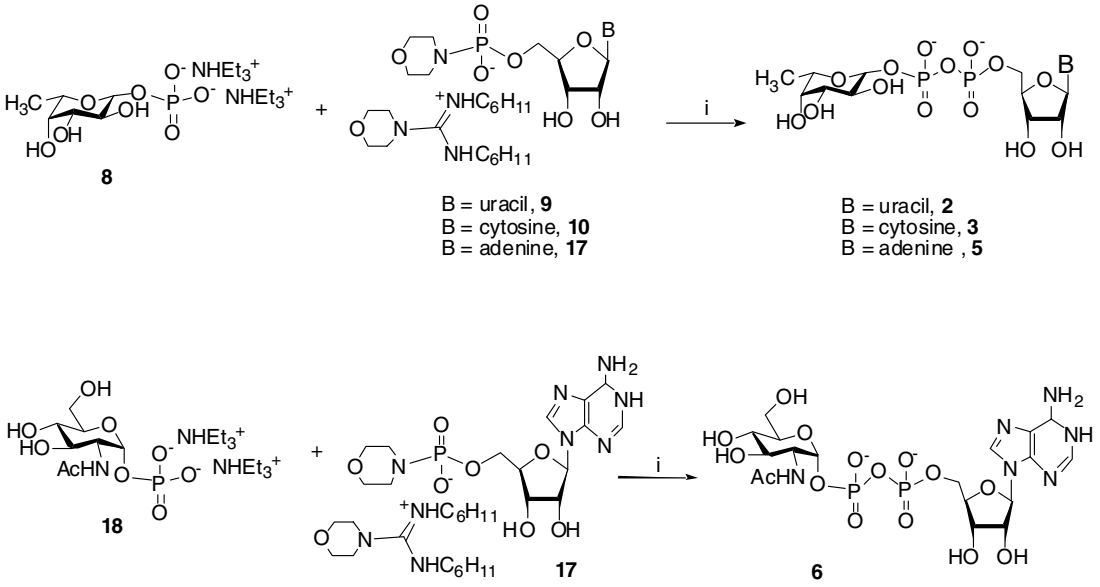
2. Results and discussion

Glycosyltransferases are known to exhibit strict donor specificity with a high affinity constant for the sugar nucleotide. To our knowledge there is only one report by Öhrlein et al. who have shown that two recombinant fucosyltransferases FucT-III and FucT-VI were able to accept unnatural donor substrates in which the guanine of the natural donor GDP-Fuc (**1**) has been replaced by other purine bases.[6,7] Following this work we decided to examine the base specificity in more details, and we prepared various fucose nucleotide conjugates, analogues to **1**, which have the purine base replaced either by the pyrimidine bases, like uracil (UDP-Fuc **2**) or cytosine (CDP-Fuc **3**), or cyanuric acid (NDP-Fuc **4**). Their syntheses rely on the coupling of fucose-β-1-phosphate and the activated form of the nucleotide monophosphate. We chose the morpholidate first introduced by Moffatt and co-workers[8] and commonly used in the synthesis of GDP-Fuc,[9–11] instead of the imidazolide alternatively described in the synthesis of ADP-Fuc.[12] These morpholidates, commercially available at a very high price, were synthesized in the laboratory.[13]

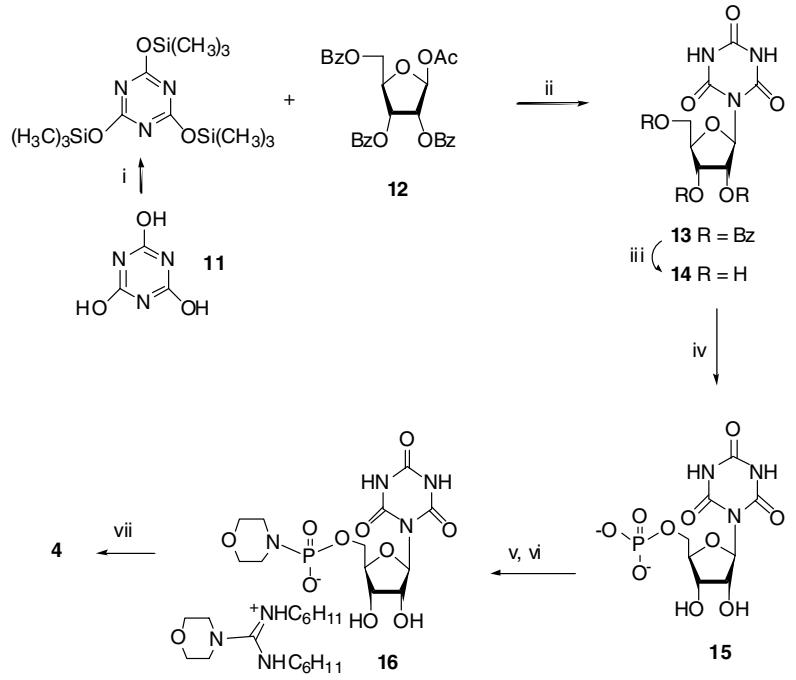
Reaction of the triethylammonium salt of β-L-fucosyl-1-phosphate[11] **8** with uridine 5′-monophosphoromorpholidate **9**[13] was carried out in pyridine. Work-up and purification with anionic exchange chromatography on DEAE-Sephadex afforded UDP-Fuc (**2**) in 33% yield (Scheme 1). In view of the modest yield, condensation of phosphate **8** with cytidine 5′-monophosphoromorpholidate **10** was performed in pyridine in the presence of 1*H*-tetrazole[14] reported as an activator for this coupling, but no increased yield was observed and CDP-Fuc (**3**) was obtained in the same yield.

As a substitute for the nucleotide base to be tested with glycosyltransferases we next selected cyanuric acid for three reasons. Firstly it is a very cheap starting material, secondly it is easy to handle in coupling reactions because of its *C*-3 symmetry, and finally it is a component of dye reagents recognized as good ligands for enzymes exhibiting high affinity with nucleotide diphosphates, such as glycosyltransferases.[15] Cyanuric acid **11** was persilylated by refluxing with trimethylchlorosilane and hexamethyldisilazane, and the tris(trimethylsilyl)-derivative was treated with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose **12** in the presence of $SnCl_4$, according to Vorbrüggen conditions (Scheme 2).[16] We used an excess of cyanuric acid since stoichiometric amounts led to an equimolecular mixture of mono- and di-substituted cyanuric acid. In this way, the coupling compound could be isolated after chromatography on silica gel as the pure β-anomer **13** in 74% yield. Saponification with methanolic ammonia afforded the crystalline deprotected nucleoside analogue **14** (53%).[17] Compound **14** was then phosphorylated according to the phosphorylation procedure with phosphorus oxychloride in trialkyl phosphate.[18] Whereas triethyl phosphate was unsuitable due to low solubility of starting material in this solvent, phosphorylation of compound **14** performed in trimethyl phosphate, afforded the nucleotide analogue **15** as its ammonium salt in 48% yield after purification by chromatography on silica gel. To prepare the corresponding fucose phosphate conjugate, we again applied the procedure relying on nucleotide activation as the morpholidate. To this end compound **15**, as its acidic form, was heated under reflux with morpholine in the presence of dicyclohexylcarbodiimide in a mixture of water and *tert*-butyl alcohol.[13] After removing dicyclohexylurea, the phosphoromorpholidate could be isolated as a precipitate identified by ^{1}H NMR spectroscopy as its 4-morpholine *N*,*N*′,-dicyclohexylcarboxamidine salt **16**. The coupling reaction of compound **16** with fucose phosphate **8** was performed in the presence of 1*H*-tetrazole; contrary to the previous coupling reactions, an excess of sugar phosphate was used. The sugar nucleotide analogue (NDP-Fuc) **4** was isolated in poor yield (20%) in addition to 73% of the starting fucose **8**; the reaction was not optimized, but compound **4** was directly tested as a donor substrate with FucT-III. ADP-Fuc (**5**) known to serve as a donor substrate for FucT-III and FucT-VI was also synthesized in 54% yield by condensation of phosphate **8** and AMP-morpholidate **17**. This analogue was needed for comparison with the other unnatural substrates.

Finally, in order to extend this study to another glycosyltransferase, we prepared ADP-GlcNAc **6** as an analogue of UDP-GlcNAc **7**. The coupling reaction of *N*-acetylglucosamine α-1-phosphate[19] **18** and AMP-morpholidate **17** performed in the presence of 1*H*-tetrazole, provided after anionic chromatography, ADP-GlcNAc **6** (Scheme 1). This remained contaminated by traces of



Scheme 1. Reagents and conditions: (i) **9** 1.6 equiv, pyridine, 7 days, rt, **2**: 33%; **10** 1.6 equiv, 1*H*-tetrazole 3 equiv, pyridine, 6 days, rt, **3**: 32%; **17** 1.6 equiv, 1*H*-tetrazole 3 equiv, pyridine, 2 days, rt, **5**: 54%; **17** 1.1 equiv, 1*H*-tetrazole 3.3 equiv, pyridine, 3 days, rt, **6**: 58%.



Scheme 2. Reagents and conditions: (i) TMSi-Cl 0.003 equiv, HMDS 4.2 equiv, 6 h, reflux; (ii) **12** 0.59 equiv, $SnCl_4$ 0.38 equiv, CH_3CN, 1 h, rt, **13**: 74%; (iii) NH_3 in MeOH, 4 days, 0 °C, **14**: 53%; (iv) $POCl_3$ 3.3 equiv, $PO(OCH_3)_3$, 10 h, 0 °C, **15**: 48%; (v) Bio-Rad AG 50 H^+ form; (vi) $H_{11}C_6N{=}C{=}NC_6H_{11}$ 4 equiv, *tert*-butyl alcohol–water, morpholine 5 equiv, 4 h, reflux, **16**: 65%; (vii) **8** 1.7 equiv, 1*H*-tetrazole 2 equiv, pyridine, 2 days, rt, **6**: 20%.

1*H*-tetrazole and further purification with Bio-Gel P2 was required to obtain the pure compound **6** in 58% yield.

All synthesized sugar nucleotide analogues **2**, **3**, **4**, **5** were tested as fucose donors towards recombinant α-(1→3/4)-fucosyltransferase, which was produced in Baculovirus-infected insect cells.[2] This enzyme is able to catalyze transfer of a fucose residue to position-4 of the *N*-acetylglucosamine unit belonging to the disaccharide β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc or position-3 of the *N*-acetylglucosamine unit belonging to the disaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc. For evaluation of the analogues, we applied a fluorescent assay utilising as an acceptor substrate β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-*O*-$(CH_2)_6$-NH-dansyl.[20] This disaccharide was incubated with each of the analogues **2**, **3**, **4** or **5** at a concentration of 20 mM. The kinetics of the conversion are shown in Figure 1.

Surprisingly total conversion was observed after 3 h for UDP-Fuc (**2**) as well as for ADP-Fuc (**5**), after 12 h for NDP-Fuc (**4**), whereas for CDP-Fuc (**3**) conversion was only 70% after 18 h. It is worthy of mention that under these conditions, only 10 min were required for total conversion with the natural substrate, GDP-Fuc (**1**). Unexpectedly, FucT-III does not only tolerate the exchange of guanosine for adenine but may also accept a pyrimidine base. NDP-Fuc and CDP-Fuc were utilized with lower efficiency than UDP-Fuc, but nevertheless they could act as fucose donors. In order to get

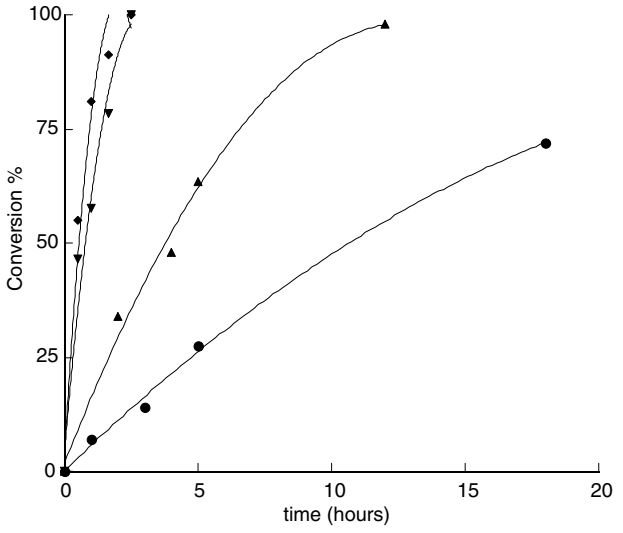


Figure 1. Kinetics of fucose transfer reaction catalyzed by FucT-III using analogues **2**, **3**, **4**, **5** as donor substrates; UDP-Fuc **2** (♦), CDP-Fuc **3** (●), NDP-Fuc **4** (▲), ADP-Fuc **5** (▼).

further insights into the specificity of the transferase, we determined the kinetic parameters of FucT-III for each donor analogue and compared their values with those for the natural substrate **1**. The Lineweaver–Burk plots for analogues **2**, **3**, **4** and **5** are reported in Figure 2. Kinetic parameters of FucT-III for GDP-Fuc(**1**) and its analogues **2**, **3**, **4** and **5** are gathered in Table 1. The affinity constant we measured for **1** (43 μM) is in good

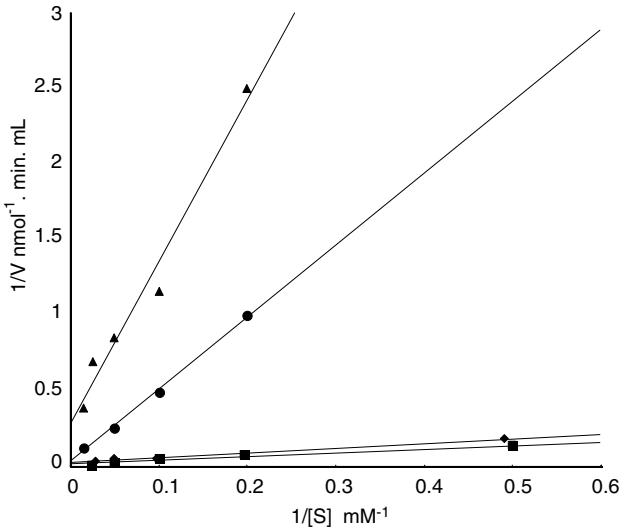


Figure 2. The Lineweaver–Burk plots for **2**, **3**, **4**, **5**; UDP-Fuc **2** (■), CDP-Fuc **3** (▲), NDP-Fuc **4** (●), ADP-Fuc **5** (♦).

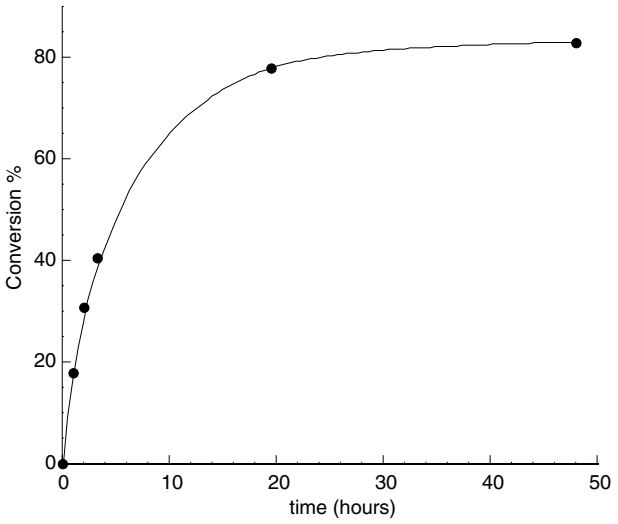


Figure 3. Kinetics of *N*-acetylglucosamine transfer reaction catalyzed by *LgtA* using analogue **6** as a donor substrate.

Table 1. Comparison of the kinetic parameters of FucT-III for GDP-Fuc (**1**) and analogues **2**, **3**, **4**, **5**

Donor substrates	V_{max} [nmol min^{-1} mL^{-1}]	K_m mM	V_{max}/K_m
GDP-Fuc **1**	148	0.043	3440
UDP-Fuc **2**	52	13	4
ADP-Fuc **5**	40	13	3
NDP-Fuc **4**	29	140	0.2
CDP-Fuc **3**	3	38	0.1

agreement with the literature value (33 μM).[21] The data corroborate the results observed in the kinetics experiments: UDP-Fuc **2** turns out to be as efficient a substrate as ADP-Fuc **5**. The K_m values are much higher for **2** and **3** than for **1** (13 mM vs 43 μM) and the V_{max}/K_m ratios are three orders of magnitude lower for these analogues than for **1**. NDP-Fuc **4** exhibits a lower affinity but a higher V_{max} than CDP-Fuc **3**, the poorest substrate out of the four analogues.

ADP-GlcNAc **6** was also tested as an analogue of UDP-GlcNAc **7** with *N. meningitidis* UDP-GlcNAc: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-*N*-acetylglucosaminyl-transferase (*lgtA*) cloned and expressed in *E. coli*.[22,23] This enzyme catalyzes transfer of *N*-acetylglucosamine onto position-3 of the terminal galactose of lactose unit. Thus β-D-Gal *p*-(1→4)-β-D-Glc*p* *O*-$(CH_2)_6$-NH-dansyl disaccharide[20] was incubated with analogue **6** at a concentration of 20 mM in the presence of the enzyme. Kinetics for the conversion are reported in Figure 3.

Again the donor specificity proved not to be as strict as was supposed, since ADP-GlcNAc turned out to serve as a donor substrate for *LgtA*. The kinetic parameters for ADP-GlcNAc (**6**) were measured and compared with those for the natural substrate, UDP-GlcNAc (**7**). The Lineweaver–Burk plot is reported in Figure 4. Data are similar to those previously observed

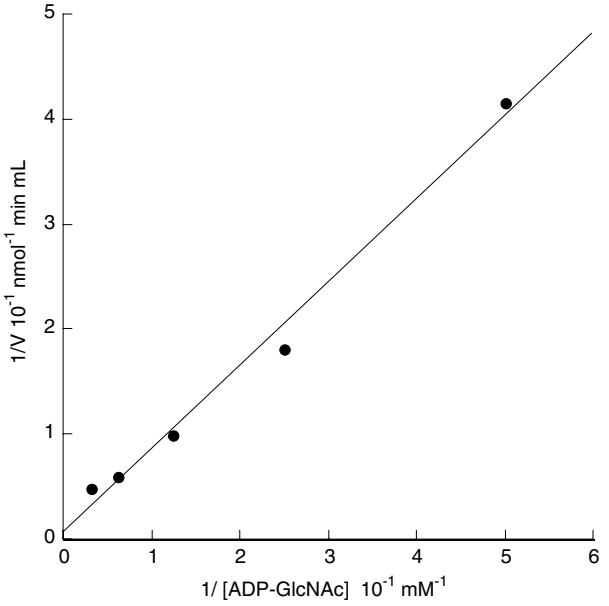


Figure 4. The Lineweaver–Burk plot for ADP-GlcNAc (**6**).

with fucose donor analogues and FucT-III: the V_{max}/K_m ratio is three orders of magnitude lower for analogue **6** than for **7**, with a K_m value multiplied by a factor of 135 for ADP-GlcNAc as compared to UDP-GlcNAc (Table 2). Nevertheless, working at high donor substrate concentrations allowed sugar transfer onto the acceptor substrate.

Table 2. Kinetic parameters of *LgtA* for ADP-GlcNAc (**6**) as compared to those for UDP-GlNAc (**7**)

Donor substrates	V_{max} [nmol min^{-1} mL^{-1}]	K_m mM	V_{max}/K_m
UDP-GlcNAc **7**	640	0.64	1000
ADP-GlcNAc **6**	110	88	1.5

These kinetics studies with donor substrate analogues provide new and useful information on structure–activity relationships. Together with molecular modeling studies they may be helpful for the rational design of enzyme specificity.

3. Experimental

3.1. General methods

NMR spectra were recorded with a Bruker AC-200 or AC-250 spectrometers; the chemical shifts are given relative to the signal of tetramethylsilane in $CDCl_3$; for ^1H NMR and ^{13}C NMR spectra in D_2O, acetone (δ 2.22 and 30.5 ppm) was used as an internal reference. H_3PO_4 in D_2O was used as an external reference for ^{31}P NMR. Optical rotations were measured with a Jasco digital micropolarimeter. Mass spectra were performed either on a Finigan MATT 95 apparatus using the ESI technique. Reactions were monitored by TLC on Silica Gel $60F_{254}$ with detection by charring with 10% H_2SO_4 in EtOH or 2% orcinol in 10% H_2SO_4. HPLC measurements were performed using the Waters 600 pump equipped with a reverse-phase column (C_{18} Nucleosil, 5 μm, 4.6 mm id × 200 mm; mobile phase, water–MeCN: 65:35; flow rate 1 mL/min). Detection was done with a Luminescence Spectrometer LS50B from Perkin–Elmer. Fluorescence of substrate and product was read at 385 nm excitation/540 nm emission.

3.2. Uridine-5′-(β-L-fucopyranosyl)-diphosphate, bis(triethylammonium) salt (UDP-Fuc) (2)

To a soln of fucose-β-1-phosphate bis(triethylammonium) salt **8** (222 mg, 0.5 mmol) in dry pyridine (5 mL) was added UMP-morpholidate **9** (549 mg, 0.8 mmol) and the soln was stirred for 7 d at rt under argon. Then the mixture was concentrated in vacuo to dryness and the residue dissolved in water was purified by chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. Elution with a gradient of 0 to M triethylammonium hydrogenocarbonate buffer (pH 7.8) first afforded remaining **8** (88 mg, 40%) and then **2** as its triethylammonium salt (125 mg, 33%, 55% based on recovered fucose phosphate); $[\alpha]_D^{29}$ −5 (*c* 1, water); ^1H NMR (D_2O, 250 MHz): δ 7.90 (d, 1H, $J_{5,6}$ 8 Hz, H-6 uridine), 5.95 (d, 1H, $J_{1',2'}$ 4 Hz, H-1′), 5.93 (d, 1H, H-5 uridine), 4.88 (t, 1H, $J_{1,2} = J_{1,P}$ 8 Hz, H-1), 4.33 (m, 2H, H-2′, H-3′), 4.27–4.15 (m, 3H, H-4′, H-5′), 3.78 (q, 1H, $J_{5,6}$ 6.6 Hz, H-5), 3.70 (d, 1H, $J_{3,4}$ 3 Hz, H-4), 3.64 (dd, 1H, $J_{2,3}$ 9.5 Hz, H-3), 3.51 (dd, 1H, H-2), 3.16 (q, 12H, $2(CH_2–CH_3)_3$) and 1.22 (t, 21H, 2 $(CH_2–CH_3)_3$, H-6); ^{13}C NMR (D_2O, 50.3 MHz): δ 167.8, 153.3, 142.6, 103.6 (4C-uridine), 99.5 (d, $J_{C,P}$ 5 Hz, C-1), 89.2 (C-1′), 84.3 (d, $J_{C,P}$ 9 Hz, C-4′), 74.7, 73.6, 72.4, 72.2, 72.1, 70.8, (C-2′, C-3′, C-2, C-3, C-4, C-5), 65.9 (d, $J_{C,P}$ 4 Hz, C-5′), 47.5 ($CH_2–CH_3)_3$, 16.5 (C-6) and 9.2 $(CH_2–CH_3)_3$; ^{31}P NMR (D_2O, 101 MHz): δ −10.55 and −12.28 ($J_{P,P}$ 20.2 Hz); ESMS (negative mode): *m*/*z* 274 $[(M - 2\ NHEt_3)/2]^-$, 549 $[(M - 2NHEt_3 + 1]^-$. HRMS (negative mode): Calcd for $C_{15}H_{23}N_2O_{16}P_2$ $[(M - 2NHEt_3 + 1]^-$ 549.0517. Found: *m*/*z* 549.0527.

3.3. Cytidine-5′-(β-L-fucopyranosyl)-diphosphate, bis(triethylammonium) salt (CDP-Fuc) (3)

To a soln of β-L-fucose-1-phosphate bis(triethylammonium) salt **8** (100 mg, 0.225 mmol) and CMP-morpholidate **10** (246 mg, 0.36 mmol) in dry pyridine (5 mL) was added 1*H*-tetrazole (47 mg, 0.67 mmol) and the soln was stirred for 6 d at rt under argon. Then the mixture was concentrated under diminished pressure to dryness and the residue dissolved in water was purified by chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. Elution with a gradient of 0 to M triethylammonium hydrogenocarbonate buffer (pH 7.8) afforded **3** as its triethylammonium salt (56 mg, 32%); $[\alpha]_D^{30}$ +2 (*c* 1, water); ^1H NMR (D_2O, 250 MHz): δ 7.95 (d, 1H, $J_{5,6}$ 8 Hz, H-6 cytosine), 6.10 (d, 1H, H-5 cytosine), 5.95 (d, 1H, $J_{1',2'}$ 4 Hz, H-1′), 4.88 (t, 1H, $J_{1,2} = J_{1,P}$ 8 Hz, H-1), 4.40–4.15 (m, 5H, H-2′, H-3′, H-4′, H-5′), 3.75 (q, 1H, $J_{5,6}$ 6.6 Hz, H-5), 3.65 (d, 1H, $J_{3,4}$ 3 Hz, H-4), 3.60 (dd, 1H, $J_{2,3}$ 9.5 Hz, H-3), 3.50 (dd, 1H, H-2), 3.07 (q, 12H, $2(CH_2–CH_3)_3$ and 1.20 (t, 18H, $2(CH_2–CH_3)_3$, H-6); ^{13}C NMR (D_2O, 62.9 MHz): δ 166.9, 158.4, 156.4, 142.6 (4C-cytidine), 99.6 (d, $J_{C,P}$ 5.5 Hz, C-1), 90.1 (C-1′), 83.8 (d, $J_{C,P}$ 9.5 Hz, C-4′), 75.3, 73.6, 72.4, 72.2, 72.1, 70.3 (C-2′, C-3′, C-2, C-3, C-4, C-5), 65.6 (C-5′), 47.5 ($CH_2–CH_3)_3$, 16.5 (C-6) and 9.2 $(CH_2–CH_3)_3$; ^{31}P NMR (D_2O, 101 MHz): δ −10.51 and −12.28 ($J_{P,P}$ 21.5 Hz); ESMS (negative mode): *m*/*z* 273.5 $[(M - 2NHEt_3)/2]^-$; HRMS (negative mode): Calcd for $C_{15}H_{23}N_3O_{15}P_2$ $[(M - 2NHEt_3)/2]^-$ 273.5302. Found: *m*/*z* 273.5298.

3.4. 1-(β-D-Ribofuranosyl)cyanuric acid (14)

Cyanuric acid (636 mg, 4.93 mmol) and trimethylchlorosilane (0.1 mL, 0.013 mmol) were refluxed for 6 h with hexamethyldisilazane (4.4 mL, 20.9 mmol). Then solvents were evaporated to dryness (1.609 g, 95%). To the residue were added 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose[24] (1.458 g, 2.9 mmol), $SnCl_4$ (0.225 mL, 1.9 mmol) and dry MeCN (60 mL); the mixture was stirred at rt for 1 h. Then solvents were evaporated and the residue was extracted with hot toluene. The organic phase was concentrated and purified by flash column chromatography (4:1, toluene–EtOAc), affording **13** (1.24 g, 74%), which crystallized from MeOH–EtOAc–petroleum ether; mp 211 °C (lit.[16] 211–213 °C); ^1H NMR ($CDCl_3$, 250 MHz): δ 9.7 (s, 2H, NH), 8.06–

7.84 (3d, 6H, Bz), 7.60–7.20 (m, 9H, Bz), 6.45 (d, 1H, $J_{1',2'}$ 2 Hz, H-1′), 6.17 (dd, 1H, $J_{2',3'}$ 6.5 Hz, H-2), 6.08 (t, 1H, H-3) and 4.86–4.56 (m, 3H, H-4, H-5′); ^{13}C NMR ($CDCl_3$, 62.9 MHz): δ 166.3, 165.6, 165.2 (CO), 148.2 (CO), 129.4, 128.8, 128.6, 128.5, 128.2, 128.0 (C–Ar), 87.1 (C-1), 79.1 (C-4), 74.0 (C-2), 71.0 (C-3) and 63.8 (C-5); ESMS (positive mode): *m/z* 596.1 $[M + Na]^+$ $C_{29}H_{23}N_3O_{10}$.

Compound **13** (1.24 g, 2.16 mmol) was dissolved in MeOH (20 mL), saturated at 0 °C with ammonia and left at rt for 4 d. Then the methanolic ammonia was evaporated, the residue was taken up in water and extracted four times with ether. The aqueous phase was then evaporated to dryness and the residue crystallized from MeOH to give compound **14** (299 mg, 53%); mp 224–225 °C (lit.[16] 229–230 °C); $[\alpha]_D^{29}$ −24 (*c* 1, water); ^{1}H NMR (D_2O, 250 MHz): δ 6.10 (d, 1H, $J_{1',2'}$ 3.5 Hz, H-1′), 4.74 (dd, 1H, $J_{2',3'}$ 6 Hz, H-2′), 4.41 (t, 1H, H-3′), 3.99 (dt, 1H, $J_{4',5'a}$ 6 Hz, $J_{4',5'b}$ 3 Hz, H-4′), 3.90 (dd, 1H, $J_{5'a,5'b}$ 12 Hz, H-5′$_a$) and 3.76 (dd, 1H, H-5′$_b$); ^{13}C NMR (D_2O, 50.3 MHz) δ 155.1 (CO), 89.2 (C-1), 84.5 (C-4), 72.5 (C-2), 70.5 (C-3) and 62.6 (C-5).

3.5. 1-(β-D-Ribofuranosyl)cyanuric acid-5′-phosphate, ammonium salt (15)

To a soln of $POCl_3$ (0.172 mM, 2 mmol) in trimethyl phosphate (1 mL) cooled at 0 °C was added compound **14** (0.162 mg, 0.6 mmol) and water (0.004 mL, 0.2 mmol). The mixture was stirred at 0 °C for 10 h, then acidified to pH 1.5 by adding M NaOH and heated at 70 °C for 30 min. The solution was evaporated to dryness and the residue was purified by flash chromatography on silica gel (6:3:2 2-propanol–NH_4OH–water) to afford the phosphate **15** (129 mg, 48%); $[\alpha]_D^{27}$ −20 (*c* 0.5, water); ^{1}H NMR (D_2O, 200 MHz): δ 6.07 (d, 1H, $J_{1',2'}$ 3.2 Hz, H-1′), 4.70 (dd, 1H, $J_{2',3'}$ 6.5 Hz, H-2′), 4.40 (t, 1H, H-3′) and 4.05–3.80 (m, 3H, H-4′, H-5′); ^{13}C NMR (D_2O, 62.9 MHz): δ 155.1 (CO), 90.4 (C-1), 84.1 (d, $J_{C,P}$ 8 Hz, C-4), 73.7 (C-2), 71.6 (C-3) and 65.3 (d, $J_{C,P}$ 6 Hz, C-5); ^{31}P NMR (D_2O, 101 MHz): δ 4.34; HRMS (negative mode): Calcd for $C_8H_{11}N_3O_{10}P$ $[M–NH_4^+]^-$ 340.0182. Found: *m/z* 340.0185.

3.6. 1-(β-D-Ribofuranosyl)cyanuric acid-5′-(β-L-fucopyranosyl)-diphosphate, bis(triethylammonium) salt (4)

A soln of the ammonium salt **15** (82 mg, 0.23 mmol) was passed through a column of Bio-Rad AG 50W-X8 resin (H^+ form); the soln was freeeze-dried and the residue was dissolved in a mixture of water (2.3 mL), *tert*-butyl alcohol (2.3 mL) and distilled morpholine (0.078 mL, 1.14 mmol). To this soln heated under reflux was added dropwise dicyclohexylcarbodiimide (190 mg, 0.92 mmol) in *tert*-butyl alcohol (3.5 mL) and the reaction mixture was maintained under reflux for 3–4 h until complete reaction. Conversion of **15** into the nucleoside morpholidate **16** was evidenced by TLC on silica gel (6:3:2 2-propanol–NH_4OH–water). The mixture was then cooled to rt, the crystalline dicyclohexylurea was filtered and washed with water. The filtrate was concentrated and then extracted with ether to remove excess dicyclohexylcarbodiimide. The aqueous soln was evaporated to dryness. The residue was taken up in MeOH (1 mL); addition of dry ether (8 mL) to this soln gave a precipitate as a gum, which upon trituration with fresh ether gave the salt of 4-morpholine *N*,*N*′-dicyclohexylcarboxamidine **16** as a white powder (106 mg, 65%); ^{1}H NMR (D_2O, 200 MHz): δ 6.13 (d, 1H, $J_{1',2'}$ 3 Hz, H-1′), 4.73 (dd, 1H, $J_{2',3'}$ 6.3 Hz, H-2′), 4.50 (t, 1H, $J_{3',4'}$ 6.3 Hz, H-3′) and 4.10–3.95 (m, 3H, H-4′, H-5′), 3.83–3.06 (m, 18H, 8CH_2 morpholidate, 2CH–N), 2.10–1.25 (m, 20H, cyclohexyl).

To a soln of fucose-β-1-phosphate bis(triethylammonium) salt **8** (120 mg, 0.26 mmol) and morpholidate **16** (105 mg, 0.15 mmol) in dry pyridine (1 mL) was added 1*H*-tetrazole (19 mg, 0.28 mmol). Reaction was monitored by TLC on silica gel (6:3:2 2-propanol–NH_4OH–water). The soln was stirred for 2 d at rt under argon. Then the mixture was concentrated to dryness and the residue dissolved in water was purified by chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. Elution with a gradient of 0 to M triethylammonium hydrogenocarbonate buffer (pH 7.8) first afforded remaining **8** (84 mg, 73%) and then **4** as its triethylammonium salt (23 mg, 20%); $[\alpha]_D^{29}$ −11 (*c* 1.18, water); ^{1}H NMR (D_2O, 200 MHz): δ 6.10 (d, 1H, $J_{1',2'}$ 3.5 Hz, H-1′), 4.92 (t, 1H, $J_{1,2} = J_{1,P}$ 8 Hz, H-1), 4.74 (dd, 1H, $J_{2',3'}$ 6.5 Hz, H-2′), 4.48 (t, 1H, H-3′), 4.24 (t, 1H, $J_{3',4'}$ 6.5 Hz, H-4′), 4.30 (m, 2H, H-5′), 3.84 (q, 1H, $J_{5,6}$ 6.6 Hz, H-5), 3.75 (d, 1H, $J_{3,4}$ 3 Hz, H-4), 3.69 (dd, 1H, $J_{2,3}$ 10 Hz, H-3), 3.57 (dd, 1H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10 Hz, H-2), 3.21 (q, 12H, 2(CH_2–CH_3)$_3$) and 1.28 (t, 21H, *J* 7 Hz, 2(CH_2–CH_3)$_3$, H-6); ^{13}C NMR (D_2O, 50.3 MHz): δ 151.2 (CO), 98.7 (d, $J_{C,P}$ 5.5 Hz, C-1), 88.6 (C-1′), 82.1 (d, $J_{C,P}$ 9 Hz, C-4′), 72.8, 71.7, 71.5, 71.3, 72.1, 69.7 (C-2′, C-3′, C-2, C-3, C-4, C-5), 65.8 (d, $J_{C,P}$ 6 Hz, C-5′), 46.8 (CH_2–CH_3)$_3$, 15.8 (C-6) and 8.5 (CH_2–CH_3)$_3$; ^{31}P NMR (D_2O, 101 MHz): δ −10.42 and −12.37 ($J_{P,P}$ 20.3 Hz); HRMS (negative mode): Calcd for $C_{14}H_{21}N_3O_{17}P_2$ $[(M - 2NHEt_3)/2]^-$ 282.5173. Found: *m/z* 282.5175.

3.7. Adenosine-5′-(β-L-fucopyranosyl)-diphosphate, bis(triethylammonium) salt (ADP-Fuc) (5)

To a soln of β-L-fucose-1-phosphate bis(triethylammonium) salt **8** (222 mg, 0.5 mmol) and AMP-morpholidate **17** (565 mg, 0.8 mmol) in dry pyridine (7.5 mL) was added 1*H*-tetrazole (105 mg, 1.5 mmol). The reaction was monitored by TLC on silica gel (6:3:2 2-propanol–NH_4OH–water). The soln was stirred for 2 d at rt under

argon, then concentrated to dryness and the residue dissolved in water was purified by chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. Elution with a gradient of 0 to M triethylammonium hydrogenocarbonate buffer (pH 7.8) afforded **5** as its triethylammonium salt (209 mg, 54%); $[\alpha]_D^{30}$ −17 (*c* 1, water); ^{1}H NMR (D_2O, 250 MHz): δ 8.50 (s, 1H adenosine), 8.25 (s, 1H adenosine), 6.12 (d, 1H, $J_{1',2'}$ 5.5 Hz, H-1′), 4.88 (t, 1H, $J_{1,2} = J_{1,P}$ 8 Hz, H-1), 4.74 (t, 1H, $J_{2',3'}$ 5.5 Hz H-2′), 4.53 (dd, $J_{3',4'}$ 4 Hz, 1H, H-3′), 4.38 (m, 1H, H-4′), 4.2 (m, 2H, 2H-5′), 3.72 (q, 1H, $J_{5,6}$ 6.6 Hz, H-5), 3.69–3.58 (m, 2H, H-3, H-4), 3.55 (dd, 1H, $J_{2,3}$ 9.5 Hz, H-2), 3.16 (q, 12H, *J* 7 Hz, 2(CH_2–CH_3)$_3$), 1.24 (t, 18H, *J* 7 Hz, 2(CH_2–CH_3)$_3$) and 1.18 (d, 3H, $J_{5,6}$ 6.6 Hz, H-6); ^{13}C NMR (D_2O, 62.5 MHz): δ 158.6, 153.6, 149.7, 140.6, 119.3 (5C-adenosine), 99.5 (d, $J_{C,P}$ 6 Hz, C-1), 87.9 (C-1′), 84.8 (d, $J_{C,P}$ 10 Hz, C-4′), 75.3, 73.5, 72.4, 72.1, 71.4, (C-2′, C-3′, C-2, C-3, C-4, C-5), 66.3 (C-5′), 47.6 (CH_2–CH_3)$_3$, 16.4 (C-6) and 9.2 (CH_2–CH_3)$_3$; ^{31}P NMR (D_2O, 101 MHz): δ −10.85 and −12.71 ($J_{P,P}$ 20.4 Hz); ESMS (negative mode): *m*/*z* 285.5 [(M − 2NHEt$_3$)/2]$^-$ $C_{16}H_{23}N_5O_{14}P_2$.

3.8. Adenosine-5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, bis(triethylammonium) salt (ADP-GlcNAc) (6)

To a solution of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate bis(triethylammonium) salt **18** (87 mg, 0.173 mmol) in dry pyridine (5 mL) was added AMP-morpholidate **17** (135 mg, 0.191 mmol) and 1*H*-tetrazole (40 mg, 0.573 mmol) and the soln was stirred for 3 d at rt under argon. The reaction mixture was then concentrated to dryness and the residue dissolved in water was purified by anionic exchange chromatography on DEAE-Sephadex A-25 (HCO_3^- form) column eluted with a gradient of triethylammonium hydrogenocarbonate. Fractions containing compound **6** were still contaminated with traces of 1*H*-tetrazole; they were freeze-dried, and the residue taken up in water was purified on Bio-Gel P2 to afford pure **6** (84 mg, 58%); $[\alpha]_D^{29}$ 21 (*c* 0.45, water); ^{1}H NMR (D_2O, 250 MHz): δ 8.45 (s, 1H adenosine), 8.19 (s, 1H adenosine), 6.08 (d, 1H, $J_{1',2'}$ 5.5 Hz, H-1′), 5.45 (dd, 1H, $J_{1,2}$ 3.5, $J_{P,1}$ 7.5 Hz, H-1), 4.70 (dd, $J_{2,3}$ 5 Hz, 1H, H-2′), 4.46 (dd, $J_{3,4}$ 4 Hz, 1H, H-3′), 4.32 (m, 1H, H-4′), 4.15 (m, 2H, 2 H-5′), 3.96–3.65 (m, 5H, H-2, H-4, H-5, 2H-6), 3.45 (t $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 3.14 (q, *J* 7 Hz, 12H, 2(CH_2–CH_3)$_3$), 1.98 (s, 3H, NHAc) and 1.22 (t, *J* 7 Hz, 18H, 2 (CH_2–CH_3)$_3$); ^{13}C NMR (D_2O, 50.3 MHz): δ 174.9 (CO), 154.9, 152.0, 149.1, 146.9, 140.3 (5C-adenosine) 94.8 (d, $J_{C,P}$ 4.5 Hz, C-1), 87.3 (C-1′), 84.1 (d, $J_{C,P}$ 9 Hz, C-4′), 74.6, 73.4, 71.2, 70.7, 69.9 (C-2′, C-3′, C-3, C-4, C-5), 65.5 (C-5′), 60.7 (C-6), 53.9 (d, $J_{C,P}$ 9 Hz, C-2) 46.8 (C_2–CH_3)$_3$ 22.4 (CH_3) and 8.5 (CH_2–CH_3)$_3$ ^{31}P NMR (D_2O, 101 MHz): δ −10.77 and −12.40; HRMS (negative mode): Calcd for $C_{18}H_{26}N_6O_{15}P_2$ [(M − 2NHEt$_3$)/2]$^-$ 314.0465. Found: *m*/*z* 314.04656.

3.9. GDP-Fuc: β-D-Galp-(1→3/4)-β-D-GlcpNAc-(1→4/3)-α-fucosyltransferase kinetics studies using donor analogues

FucT-III was a 20-fold concentrated culture supernatant of recombinant enzyme produced in baculovirus-infected insect cells.[2] Each GDP-Fuc analogue (**2** or **3** or **4** or **5**, 2 μmol) and β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-*O*-$(CH_2)_6$-NH-dansyl (40 nmol) were incubated at 37 °C with FucT-III (20 μL, 0.02 U) in 50 mM sodium cacodylate buffer pH 6 containing 10 mM $MnCl_2$ in a total volume of 100 μL. Aliquots of the reaction mixture were withdrawn at different incubation times and analyzed by HPLC to determine the percentage of conversion.

3.10. Determination of kinetic parameters of FucT-III for GDP-Fuc, UDP-Fuc, CDP-Fuc, NDP-Fuc, ADP-Fuc

Enzyme assay conditions: 50 mM sodium cacodylate buffer pH 6.0, 15 mM $MnCl_2$, 0.4 mM Gal-β-(1→3)-GlcNAc-β-*O*-$(CH_2)_6$-NH-dansyl disaccharide, sugar-nucleotide analogue concentrations varying from 2 to 30 mM; FucT-III, 5 μL (0.4 mU); for GDP-Fuc, concentrations were 20, 40, 80, 160 μM. Total volume incubation was 50 μL. Assays were incubated at 37 °C for 15–60 min. Incubation was stopped by immersion in a boiling water bath for 1 min; after centrifugation and filtration, samples were analyzed by HPLC.

3.11. Preparation of *N. meningitidis* UDP-GlcNAc: β-D-Galp-(1→4)-β-D-Glcp-(1→3′)-β-*N*-acetyl-glucosaminyl-transferase (*LgtA*)

LgtA was produced in *E. coli* transfected with the plasmid carrying the *LgtA* gene.[22,23] The crude enzyme was prepared according to the procedure of Blixt et al.[25] with some modifications. At the end of the fermentation, the cells were harvested and frozen. After thawing, the cells were suspended in 0.05 M sodium cacodylate buffer pH 7.2 containing protease inhibitor cocktail (from Roche Diagnostics) and lysed with (106 microns size) glass beads using the mixer mill MM 200 from Retsch at the maximum frequency for 2 × 10 min; the lysate was then centrifuged at 20,000*g*, at 4 °C for 10 min and the supernatant was ready to be used for enzymatic assays.

3.12. *LgtA* kinetics studies using ADP-GlcNAc as a donor analogue

ADP-GlcNAc (**6**, 2 μmol) and β-D-Gal*p*-(1→4)-β-D-Glc*p*-*O*-$(CH_2)_6$-NH-dansyl (80 nmol) were incubated at 37 °C with *LgtA* (30 μL, 0.01 U) in 50 mM sodium caco-

dylate buffer pH 7.4 containing 15 mM $MnCl_2$ in a total volume of 100 μL. Aliquots of the reaction mixture were withdrawn at different incubation times and analyzed by HPLC to determine the percentage of conversion.

3.13. Determination of the kinetic parameters of *LgtA* for ADP-GlcNAc and UDP-GlNAc

Enzyme assay conditions: 50 mM sodium cacodylate buffer pH 7.4, 15 mM $MnCl_2$, 1 mM Gal-β-(1→4)-Glc-β-*O*-$(CH_2)_6$-NH-dansyl disaccharide, ADP-GlcNAc concentration: 32, 16, 8, 4, 2 mM; *LgtA*, 9 μL (3 mU); for UDP-GlcNAc, concentrations were 0.05, 0.1, 0.2, 0.5, 0.9 mM. Total volume incubation was 30 μL. Assays were incubated at 37 °C for 20–100 min. Incubation was stopped by immersion in a boiling water bath for 1 min; after centrifugation and filtration, samples were analyzed by HPLC.

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