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Exploring specificity of glycosyltransferases: synthesis of new sugar nucleotide related molecules as putative donor substrates

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Abstract—We investigated the specificity of glycosyltransferases toward donor substrates in two complementary directions. First we prepared simple *N*-acetyl-α-D-glucosamine 1-diphosphates: methyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, benzyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, 4-phenylbutyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, by the coupling of the corresponding activated alkyl phosphates with *N*-acetyl-α-D-glucosamine 1-phosphate. These diphosphates as well as 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate, tested as donors of *N*-acetylglucosamine in a reaction catalyzed by *Neisseria meningitidis N*-acetylglucosaminyltransferase (*LgtA*), proved to be devoid of activity. Evaluated as inhibitors, only 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate showed some inhibitory activity with an IC50 value of 7 mM.

In the second approach, we prepared sugar nucleotide mimics having the diphosphate bridge replaced by the oxycarbonyl-aminosulfonyl linker. The surrogate of GDP-Fuc was synthesized as a 9:1 α/β anomeric mixture, in 40% yield, starting from chlorosulfonyl isocyanate, perbenzylated L-fucopyranose, and a guanosine derivative, protected on the exocyclic amine and secondary hydroxyl functions of ribose. Then two deprotection steps, hydrogenolysis and enzymatic hydrolysis catalyzed by penicillin G amidase afforded the target molecule to be tested as fucose donor with recombinant human α-(1→3/4)-fucosyltransferase (FucT-III). Tested as a 4:1 α/β anomeric mixture, both in the absence and in the presence of cationic cofactors, this new guanosine fucose conjugate proved to be ineffective. Its inhibitory activity toward FucT-III evaluated through a competition fluorescence assay was very poor (IC50 value of 20 mM). The surrogate of UDP-GlcNAc that was already known as its protected acetylated derivative, tested as *N*-acetylglucosamine donor with *LgtA* in the presence of Mn^{2+} turned out not to be active either.

Keywords: Sugar nucleotides; Donor substrate; Surrogate; Fucosyltransferase; *N*-Acetylglucosaminyltransferase; Inhibitor

1. Introduction

Glycosyltransferases of Leloir-type that typically catalyze sugar units 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.[1,2] Through cloning strategy these enzymes, originally membrane-associated proteins, are now produced in the secreted form, in expression systems such as bacteria, yeasts, fungi, baculovirus-infected insect cells, making glycosyltransferases easily available to chemists. Adding an His tag to the primary sequence of recombinant enzymes is a further advantage that allows to immobilize enzymes on Ni^{2+}–agarose and use them in this form for synthetic purposes,[3] a procedure that is now widely applied in glycobiotechnology.[4] Nevertheless the major drawback to the enzymatic approach remains the requirement for sugar nucleotides, exhibiting high affinity constant for glycosyltransferases. A significant advance in this area would be the availability of donor substrates with lower affinity but simpler structures than sugar nucleotides.[5] Research work in this direction is rather limited. However a very recent report by Withers and co-workers is very worthy of note; these authors demonstrated that *p*-nitrophenyl α-sialoside could act as an alternative donor substrate for a

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bacterial α-(2→3/2→8)-sialyltransferase in the presence of catalytic amounts of CMP, thereby replacing CMP-NeuAc, the natural donor.[6] The validity of this elegant strategy remains to be proved for other glycosyltransferases, especially those from animal sources.

As for us we addressed the same topic according to two different and complementary approaches. Our previous work has shown a relative flexibility of glycosyltransferases toward the nucleotide part of the molecule, since we found that the ability of the sugar nucleotide to act as a donor substrate is retained with unnatural nucleobase.[7] In continuation of this work we further investigated the donor specificity of a bacterial glycosyltransferase, *Neisseria meningitidis* UDP-GlcNAc: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3′)-β-*N*-acetylglucosaminyl-transferase (EC 2.4.1.56, *LgtA*) cloned and expressed in *Escherichia coli*,[8,9] first by testing very simple 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphates. *LgtA* catalyzes transfer of *N*-acetylglucosamine from UDP-GlcNAc onto position-3 of the terminal galactose of lactose unit with an inversion of configuration at the anomeric carbon of glucosamine. By analogy with glycosidase reactions the acceptor hydroxyl group attacks the C-1 of glucosamine in an S_N2-like mechanism leading to the inversion of configuration (Chart 1). *LgtA* has an absolute requirement for Mn^{2+}, which co-ordinates the pyrophosphate group of the donor substrate. Mn^{2+} binding is fulfilled by the residues of the conserved D × D motif, which has been identified in many glycosyltransferases.[10]

Here we report the synthesis of diphosphates **1**, **2**, **3**, and **4** (Chart 2), related to UDP-GlcNAc, where the nucleotide part of the molecule was replaced by methyl, benzyl, hydrogen, or 4-phenylbutyl groups, which were tested as donors of *N*-acetylglucosamine in the reaction catalyzed by *LgtA*.

In the second approach we were interested in devising substrate analogues having the diphosphate bridge replaced by a more easily available linkage that, like diphosphate, could act as a leaving group too. As surrogate of the diphosphate, we selected the oxycarbonylaminosulfonyl linker, a non-charged isostere of pyrophosphate, for three reasons:

(i) Both carbonyl and sulfonyl groups have been used as phosphate substitutes in several examples.[11–13]
(ii) The carbamate group is cleaved by glycosidases with the release of CO_2;[14] thereby in view of some similarity between glycosidases and glycosyltransferases, we guessed there was a reasonable chance

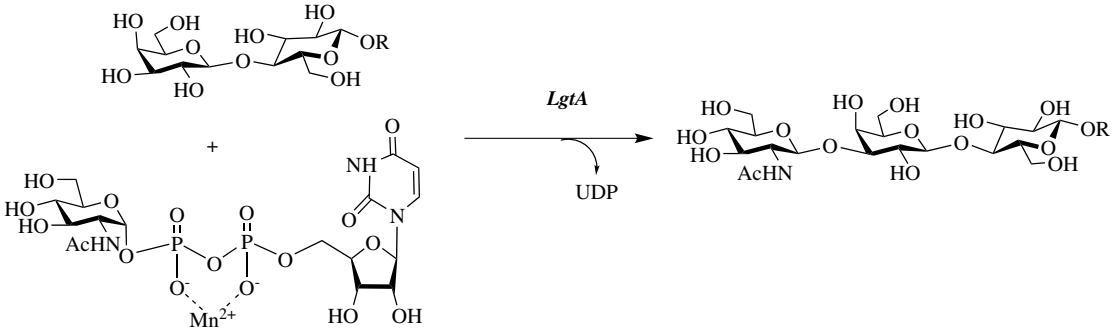


Chart 1. Reaction catalyzed by *Neisseria meningitidis* β-(1→3)-*N*-acetylglucosaminyltransferase (*LgtA*).

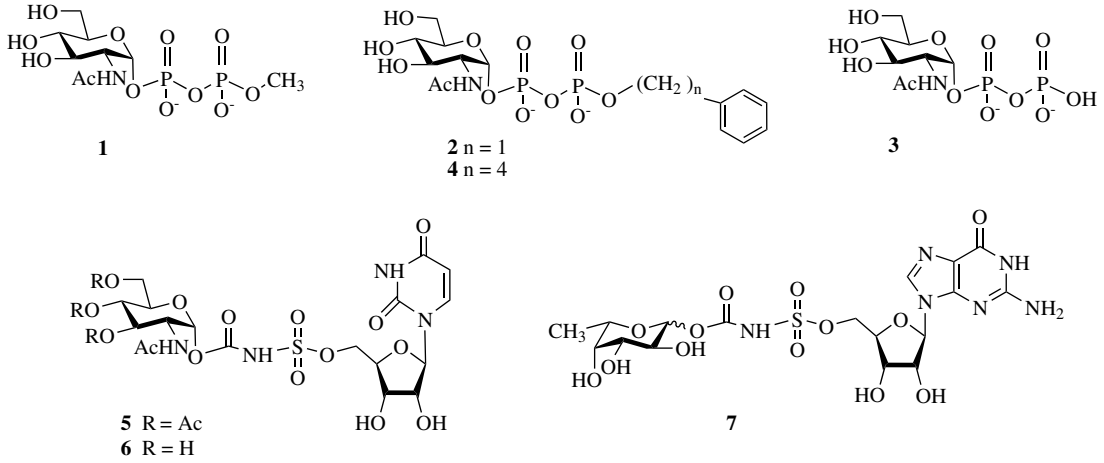


Chart 2. Structure of the sugar nucleotide related molecules that were synthesized and tested toward glycosyltransferases.

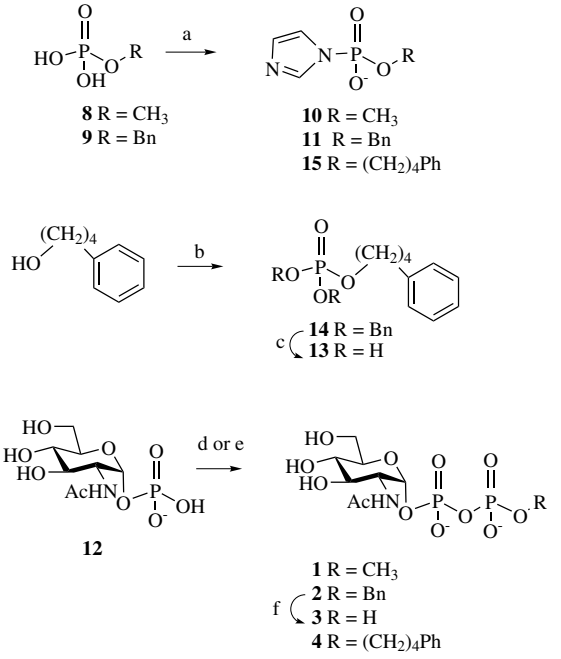
that the carbamate group might also be substrate for glycosyltransferases.

(iii) In chemical glycosylation, glycosyl carbamates[15] and glycosyl sulfonyl carbamates[16] can serve as glycosyl donors after activation with an electrophilic promoter.

The glucosaminyl derivative **5** belonging to this class of compounds had been previously synthesized and tested for its antiviral activity.[11] Thus, in this second part we report on the biochemical evaluation of the deprotected derivative **6** as a donor substrate toward *LgtA* and, in addition, the synthesis of the new GDP-Fuc mimic **7** (Chart 2), which was tested as a fucose donor toward recombinant human 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).[3] FucT-III catalyzes the transfer of fucose onto position-4 or position-3 of the *N*-acetylglucosamine unit of disaccharides β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc or β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc, respectively. Like *LgtA*, FucT-III is an inverting enzyme; β-linked GDP-Fuc is the donor substrate, giving rise to α-linked fuco-oligosaccharides (Chart 3), but as opposed to *LgtA* it does not show an absolute requirement for Mn^{2+} for activity, just exhibiting some activation by this cation, depending on the pH.[17] Therefore, it was of interest to test both sugar nucleotide mimics **6** and **7**.

2. Results and discussion

The synthesis of the diphosphates of *N*-acetylglucosamine relies on the coupling of 2-acetamido-2-deoxy-α-D-glucopyranose 1-phosphate and an activated form of the corresponding monoalkyl phosphates. Methyl and benzyl alcohols were phosphorylated according to an old procedure using inorganic phosphorous acid and mercuric chloride in the presence of triethylamine.[18] The monoalkyl phosphates **8** and **9** could be isolated and crystallized as their bis(cyclohexylammonium) salts. As an activated form of the phosphate, the imidazolidate, commonly used in the synthesis of nucleotides, was chosen.[19] Thus, the acidic forms of the monoalkyl phosphates **8** and **9** were treated with carbonyldiimidazole in the presence of triethylamine. The formation of the imidazolidate could be monitored by ^{31}P NMR spectroscopy[20]: the ^{31}P resonance signal is shifted from 0.14 (for compound **9**) to −7.3 (for compound **11**). The reaction of the mono-cyclohexylammonium salt of 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate **12** and activated methyl phosphate **10** was carried out in DMF (Scheme 1). Purification by anion exchange chromatography on DEAE-Sephadex afforded pure pyrophosphate **1** in 43% yield. Condensation of activated benzyl phosphate **11** with glucosamine phosphate **12** was performed in DMF in the presence of zinc chloride[21] reported as an activator for this coupling, but no increased yield was observed and diphosphate **2**



Scheme 1. Reagents and conditions: (a) CDI 2 equiv, NEt_3 1 equiv, DMF, 3 h, rt, then MeOH 1 equiv; (b) DIAD 1.5 equiv, PPh_3 1.5 equiv, dibenzyl phosphate 1.5 equiv, THF, 4 h, rt, 60%; (c) Pd 10%/C, EtOH–M $NaHCO_3$, H_2, overnight, 69%; (d) **10** 1 equiv or **15** 0.66 equiv, DMF, 3 d, rt, 43% for **1**, 28% for **4**; (e) **11** 1 equiv, $ZnCl_2$ 8 equiv, DMF, 2 d, rt, 38% for **2**; (f) Pd 10%/C, MeOH–0.1 M $NaHCO_3^-NHEt_3^+$, H_2, 3 h, 70%.

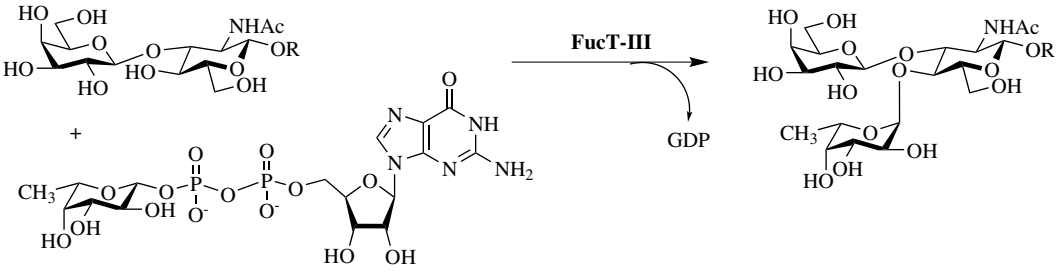


Chart 3. Reaction catalyzed by α-(1,3/4)-fucosyltransferase (FucT-III).

was finally isolated in 38% yield after two purification steps. The benzyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate **2** was hydrogenolyzed in the presence of palladium on charcoal giving the *N*-acetyl-α-D-glucosamine diphosphate **3** as its triethylammonium salt after purification on anionic exchange chromatography in 70% yield.

Mitsunobu reaction[22] was applied to the synthesis of 4-phenylbutylphosphate **13**: 4-phenyl-1-butanol was reacted with dibenzylphosphate in the presence of diisopropyl azodicarboxylate and triphenylphosphine to give after purification 4-phenylbutyl-dibenzylphosphate **14** in 60% yield. Catalytic hydrogenation of compound **14** in the presence of palladium on charcoal afforded compound **13** as its sodium salt in 69% yield. Phosphate **13** was converted to imidazolidate **15** as previously described, which in turn was reacted with the monotriethylammonium salt of 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate **12** used in excess. Two purification procedures, anionic exchange chromatography and gel permeation were necessary to isolate pure diphosphate **4** in 29% yield.

All synthesized *N*-acetyl-α-D-glucosamine diphosphates **1**, **2**, **3**, and **4** were tested as *N*-acetylglucosamine donor toward recombinant *N. meningitidis* UDP-GlcNAc: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3′)-β-*N*-acetylglucosaminyl-transferase *(LgtA)*. For the evaluation of these analogues, we applied a fluorescent assay utilizing β-D-Gal*p*-(1→4)-β-D-Glc*p*-*O*-$(CH_2)_6$-NH-dansyl disaccharide[23] as an acceptor substrate. This disaccharide was incubated with each of diphosphates **1**, **2**, **3** or **4** at a concentration of 60 mM. We speculated that, within the active site, simple juxtaposition of diphosphate **3** and uridine in the presence of Mn^{2+} might replace UDP-GlcNAc and function as a donor. Thus compound **3** was also tested in the presence of uridine, both compounds at the same concentrations (60 or 100 mM). But no conversion of substrate into product could be observed in any of these assays. Then, we studied the inhibitory activity of the four diphosphates toward *LgtA* through a competitive fluorescent assay. Only diphosphate **3** showed some inhibition. Inhibitory activity of *LgtA* by 2-acetamido-2-deoxy-α-D-glucopyranose diphosphate **3** is reported in Figure 1 exhibiting an IC50 value of 7 mM. It would seem clear that the diphosphate **3** is recognized even weakly by *LgtA*, as opposed to the other three diphosphates **1**, **2**, **4** that are not recognized.

In the second part of this work we focused on the synthesis of sugar nucleotide mimics in which the diphosphate bridge was replaced by the oxycarbonyl-aminosulfonyl linker: compounds **6** and **7**, analogues of UDP-GlcNAc and GDP-Fuc, respectively. The strategy of synthesis of these compounds was based on the coupling of a protected sugar derivative, free on its anomeric position, with chlorosulfonyl isocyanate, followed

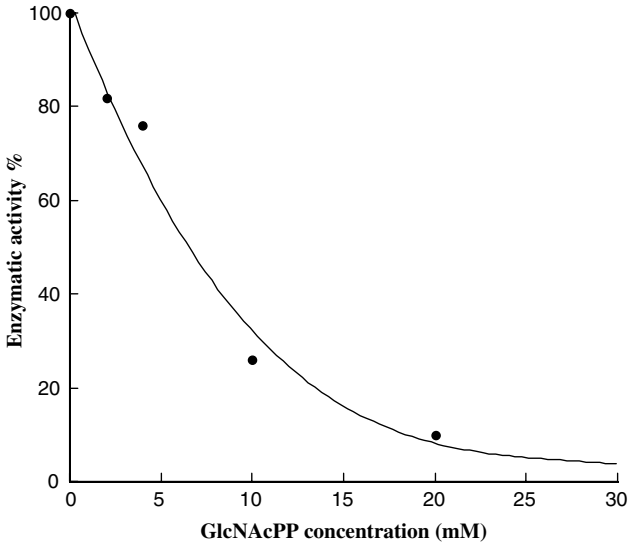
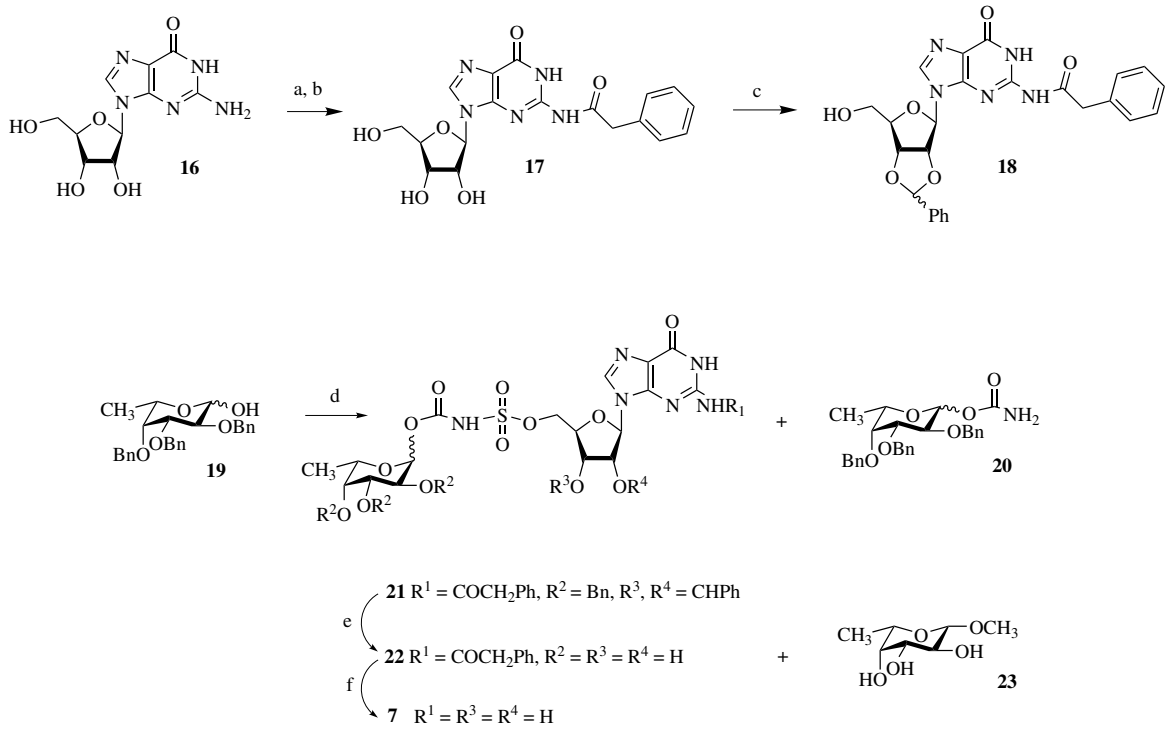


Figure 1. Inhibition of *LgtA* by 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate (GlcNAcPP) **3** as a function of the concentration.

by the in situ reaction of the unstable intermediate with the protected nucleotide derivative free on the primary hydroxyl function.

Whereas the synthesis of the protected *N*-acetylglucosamine derivative **5** had been previously reported,[11] for biochemical tests we had to prepare the deacetylated compound **6** that to our knowledge has not been described yet. Removal of the acetyl protecting groups of compound **5** by treatment with methanolic ammonia afforded compound **6** in a high yield (90%). The UDP-GlcNAc mimic **6** was tested as a *N*-acetylglucosamine donor toward *LgtA* at a concentration of 60 mM. No conversion of substrate into product could be observed and compound **6**, tested at concentrations up to 10 mM, through a competition fluorescence assay turned out not to show any inhibitory activity.

Nevertheless we decided to synthesize the new GDP-Fuc mimic **7** to be tested with FucT-III, an enzyme from animal source that could not involve the same enzymatic mechanism as *LgtA*, since in particular it does not show an absolute requirement for Mn^{2+}. We chose the benzyl ether as protecting groups on the fucose residue. Regarding the nucleoside part, as protection on the exocyclic amine of guanosine, we selected the phenylacetyl group, that can be cleaved off with penicillin G amidase under very mild conditions.[24] Thus, the formation of the phenylacetamide was performed by the treatment of guanosine **16** with phenylacetyl chloride after transient trimethylsilylation of hydroxyl functions,[25] affording the guanosine derivative **17** in 70% overall yield (Scheme 2). Then, the phenylacetamide derivative of guanosine was protected as its benzylidene acetal by the treatment of **17** with benzaldehyde and zinc chloride under standard conditions.[26] Acetal **18** was obtained as an



Scheme 2. Reagents and conditions: (a) TMSCl 7.5 equiv, pyridine, 8 h, rt, then $PhCH_2COCl$ 1.5 equiv, HOBt 1.6 equiv, CH_3CN, overnight, rt; (b) water, 25% NH_4OH, 0 °C, 30 min, 70% for two steps; (c) $ZnCl_2$ 3.4 equiv, PhCHO, 50 °C, 1 h, 88%; (d) (1) O=C=N–SO_2Cl 1 equiv, CH_2Cl_2, −35 °C, 2 h, then $t \rightarrow -10$ °C; (2) **18** 1 equiv, pyridine 2.5 equiv, CH_2Cl_2, rt, 20 h, **21**: 40%, α/β = 9:1, **20**: 17%; (e) Pd 10%/C, THF–MeOH–water, H_2 (40 psi), 4 d, 35%; (f) penicillin G amidase on Eupergit, 40 mM phosphate buffer pH 7.8, rt, overnight, 91%.

equimolar diastereomeric mixture in excellent yield. As the occurrence of both diastereomers appeared as a drawback, we tried another method to obtain one single benzylidene diastereomer,[27] but our effort was unsuccessful.

The perbenzylated fucopyranose **19** was first treated with chlorosulfonyl isocyanate at low temperature. An unstable intermediate was formed by the reaction of the anomeric hydroxyl group with isocyanate, the most reactive group.[28] Then when all starting fucose had reacted, the guanosine derivative **18** was added to the reaction mixture together with pyridine, to allow the in situ reaction of the chlorosulfonyl group with the 5′ hydroxyl function of **18**. The product was purified by chromatography on silica gel allowing to isolate first the carbamoyl fucose derivative **20** (17%) arising from the hydrolysis of the chlorosulfonyl intermediate, and the expected fucose guanosine conjugate still contaminated by byproducts. Then, a further purification by gel permeation on Sephadex LH-20 column afforded compound **21** as two fractions: the first eluted fraction was pure α anomer and the second one an anomeric mixture in 4:1 α/β ratio, estimated from ^{1}H NMR. Altogether compound **21** was obtained in 40% yield and 9:1 α/β overall ratio. None of both purification procedures was successful in isolating the pure β anomer. The occurrence of *R* and *S* benzylidene diastereomers for both anomers prevented the complete elucidation of ^{1}H NMR spectra. However, in the first fraction the α anomeric configuration was demonstrated by the presence of two doublets (δ 6.10, 6.06, J 3.5 Hz) assigned to the fucose α anomeric proton; concomitantly the H-8 guanosine proton appeared as two singlets (δ 7.99, 7.91), the benzylidene proton as two singlets (δ 6.09, 5.85), and the H-1 proton of the ribose as two doublets (δ 6.11, 6.02). Likewise the signal for the H-6 proton of the fucose residue was split into two doublets (δ 1.06, 1.03, J 6 Hz). By chance the β anomer eluted later than the α anomer from Sephadex LH-20, resulting in a β enriched mixture for the second fraction. The occurrence of the β anomer was ascertained on the basis of ^{1}H NMR data that revealed the presence of two extra doublets (δ 5.45, 5.43) with a typical large coupling constant (J 8.0 Hz) assigned to the fucose β anomeric proton, upfield shifted as compared to the α anomeric proton. In parallel the ^{1}H NMR spectrum showed three extra singlets, two for the H-8 proton of guanosine (δ 7.98, 7.95) and one for the benzylidene proton (δ 5.91). ^{13}C NMR data also exhibited two signals for the fucose anomeric carbon (δ 95.3, 91.0). Low ratios in the β anomer have already been reported in such reactions with 2-deoxy or 6-deoxyglucose derivatives[29] and seems to be reflecting the anomeric composition of the starting glycopyranoside hemiacetal in dichloromethane

solution at low temperature. Despite this unfavorable ratio, compound **21**, as a 4:1 α/β anomeric mixture, was immediately submitted to deprotection steps.

Removal of the benzyl and benzylidene protecting groups by hydrogenolysis proved to be particulary problematic. The sluggish deprotection of benzyl ethers had been previously observed with nucleosides and may be the result of inhibition due to the nucleobase that could adsorb onto the catalyst.[30] Therefore, we first studied hydrogenolysis of the guanosine derivative **18**: deprotection of **18** carried out under a hydrogen pressure of 40 psi in THF–methanol–water mixture, in the presence of 10% palladium on charcoal, required two days to reach complete reaction. These conditions were applied to compound **21** and the formation of the desired deprotected compound was monitored by HPLC (reverse phase column, elution with a gradient of acetonitrile–water). After 4 days, the reaction was stopped, but large decomposition of the fucose guanosine conjugate could not be avoided; finally compound **22** could be isolated on a C_{18} Sep-Pak cartridge by elution with a gradient of methanol–water in 35% yield. One major byproduct could be identified in the fraction eluted with water as methyl L-fucopyranoside **23**, predominantly β, arising from the nucleophilic attack of the predominant compound **21α** by methanol in the course of hydrogenolysis. The proportion of the β anomer in compound **22** was 20% as confirmed by 1D and 2D NMR experiments (COSY, HMQC, HMBC) and in good agreement with the α/β ratio of starting **21**. Enzymatic removal of the phenylacetyl group on the exocyclic amine of the guanosine by the treatment of compound **22** with penicillin G amidase (EC 3.5.1.11) immobilized on Eupergit, was straightforward. Purification on a C_{18} Sep-Pak cartridge eluted with 9:1 water–methanol afforded the target compound **7** (91% yield), the ^{13}C NMR, ^{1}H NMR, and mass spectral data of which were in complete agreement with the structure. The α/β mixture was not viewed as a problem to test compound **7** as a fucose donor because the α-(1→3/4)-fucosyltransferase (FucT-III) was expected to select the required β anomer.

We examined the ability of compound **7** to act as a fucose donor using β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-*O*-$(CH_2)_6$-NH-dansyl as a fluorescent acceptor substrate.[23] The biochemical tests were carried out at pH 6.0 using soluble or immobilized FucT-III, with or without the addition of a cationic cofactor: manganese chloride or ytterbium triflate. Indeed we previously observed that this lanthanide salt was able to replace manganese chloride for increasing FucT-III activity.[31] Compound **7** was not available in larger amount for assays at higher concentrations than 5 mM in the β anomer. However, this concentration is 100-fold higher than the K_m for GDP-Fuc. Unfortunately no conversion of substrate into product could be observed in any of the assays. Then, we studied the inhibitory activity of compound **7** toward FucT-III through a competition fluorescence assay. Compound **7** only exhibited a very poor inhibition with an IC50 of 20 mM. It is noteworthy that a related analogue of GDP-Fuc had been previously reported not to show any significant inhibition.[32]

In conclusion we prepared several new molecules related to the sugar nucleotides UDP-GlcNAc and GDP-Fuc. The series of *N*-acetyl-α-D-glucosamine diphosphates tested as *N*-acetylglucosamine donors in *LgtA*-catalyzed reaction turned out to be devoid of activity, even at high substrate concentrations (120-fold higher than the concentration commonly used with UDP-GlcNAc). Expected hydrophobic interactions between amino acid residues of the enzyme active site and either the benzyl group of compound **2** or the 4-phenylbutyl chain of compound **4** are not able to stabilize the molecule avoiding the anomeric carbon of 2-acetamido-2-deoxy-α-D-glucopyranose 1-diphosphate to be attacked by the 3-hydroxyl of the galactose. Furthermore each constituent of UDP-GlcNAc, that is uridine and *N*-acetyl-α-D-glucosamine diphosphate **3**, does not exhibit by itself an affinity high enough to allow the in situ formation of a functional intermediate able to play the role of UDP-GlcNAc. These experimental results confirm that the nucleotide part of the molecule is essential for donor recognition.

In the second part, we explored the ability of the oxycarbonylaminosulfonyl linker to replace the diphosphate bridge. We synthesized new sugar nucleotide conjugates that were supposed to be donor substrates for glycosyltransferases. But neither **6**, mimic of UDP-GlcNAc, nor **7**, mimic of GDP-Fuc, tested at a concentration 100-fold higher than the natural substrates, turned out to be functional. The non-inhibitory activity of compound **6** as well as the poor inhibitory activity of compound **7** prove that these mimics are unable to accommodate the enzyme active site. Molecular modeling experiments together with the X-ray crystallographic structure of the enzyme active sites would be essential to understand the binding hindrance of these analogues. In this regard the quite recent X-ray crystal structure of *Helicobacter pylori* α-(1→3/4)-fucosyltransferase could be helpful.[33] Furthermore, directed mutagenesis of the amino acid residues involved in the binding of the nucleotide part may provide an enzyme with a lower specificity toward the donor substrate.

3. Experimental

3.1. General methods

NMR spectra were recorded using Bruker AC-200, AC-250 or DRX-400 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. Optical rotations were measured with a Jasco digital micropolarimeter. Mass spectra were performed either on a Finigan MATT 95 apparatus using the ESI technique or on a Perspective Biosystems DE-STR-MALDI-TOF System. 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. Reversed phase HPLC (RP-HPLC) analyses were performed using a Waters 600 pump equipped with a reversed phase column (C_{18}, Nucleosil, 5 μm, 3.9 mm id × 300 mm; mobile phase, water–MeCN: 73:27; flow rate 1 mL/min), with a Luminescence Spectrometer LS50B (Perkin–Elmer) as the detection device. Fluorescence of substrates and products was read at 385 nm excitation/540 nm emission. For NMR spectroscopy complete signal assignments were based on COSY, HSQC, and HMBC correlations. Penicillin G amidase immobilized on Eupergit C was purchased from Fluka, ytterbium triflate from Aldrich.

3.2. Methyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, bis(triethylammonium)salt (1)

Methyl phosphate bis(cyclohexylammonium) salt (1 mmol) was dissolved in a small amount of water and passed through a column of Dowex-50, H^+ resin (20 cm × 1.8 cm). Methyl phosphoric acid (**8**) was eluted with 1:1 EtOH–water and the soln was freeze-dried. To the dry acid, carbonyldiimidazole (CDI) (0.324 g, 2 mmol), Et_3N (0.13 mL, 1 mmol), and DMF (5 mL) were added and the mixture was vigorously stirred at rt for 3 h. Then to neutralize the excess of CDI, MeOH (30 μL) was added and the reaction mixture was stirred additionally for 15 min. Solvents were evaporated affording crude methyl phosphorimidazolidate **10**; ^{31}P NMR (101 MHz, MeOD): δ −6.35. A soln of activated methylphosphate **10** (0.50 mmol) and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate mono-cyclohexylammonium salt[34] **12** (200 mg, 0.5 mmol) in dry DMF (6 mL) was stirred at rt under argon for 4 d. Then, the reaction mixture was concentrated to dryness and the residue dissolved in water was purified by anionic exchange chromatography on DEAE-Sephadex A-25 (HCO_3^- form) column (2 × 23 cm). Elution with a gradient of 0 to M triethylammonium hydrogenocarbonate buffer (pH 7.8) afforded **1** as a white solid (128 mg, 43%); $[\alpha]_D^{29}$ +49 (*c* 0.79, water); ^1H NMR (250 MHz, D_2O): δ 5.44 (dd, 1H, $J_{1,2}$ 3 Hz, $J_{1,P}$ 7 Hz, H-1), 3.99–3.71 (m, 5H, H-2, H-4, H-5, H-6, H-6′), 3.60 (d, 3H, $J_{H,P}$ 11.5 Hz, CH_3), 3.52 (t, 1H, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 3.10 (q, 12H, *J* 7 Hz, CH_2–CH_3), 2.03 (s, 3H, NHAc), and 1.21 (t, 18H, CH_2–CH_3); ^{13}C NMR (62.9 MHz, D_2O): δ 175.0 (CO), 94.7 (d, $J_{C,P}$ 6.3 Hz, C-1), 73.2, 71.1, 69.8, (C-2, C-3, C-4), 60.6 (C-6), 53.9 (d, $J_{C,P}$ 8.7 Hz, C-2), 53.5 (CH_3), 46.9 (CH_2–CH_3)$_3$, 22.3 (CH_3), and 8.6 (CH_2–CH_3)$_3$; ^{31}P NMR (101 MHz, D_2O): δ −9.70 and −13.23 (J_{PP} 22.9 Hz); ESIMS (negative mode): *m*/*z* 394.0 [M−2NHEt$_3$+H]$^-$. HRESIMS (negative mode) *m*/*z*: calcd for $C_9H_{18}O_{12}N_1P_2$, 394.0299; found, 394.0303.

3.3. Benzyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, bis(triethylammonium)salt (2)

Benzyl phosphate bis(cyclohexylammonium) salt (1 mmol) was dissolved in a small amount of water and passed through a column of Dowex-50, H^+ resin (20 cm × 1.8 cm). Benzyl phosphoric acid (**9**) was eluted with 1:1 EtOH–water and the soln was freeze-dried. To the dry acid, carbonyldiimidazole (CDI) (0.324 g, 2 mmol), Et_3N (0.13 mL, 1 mmol), and DMF (5 mL) were added and the mixture was vigorously stirred at rt for 3 h. To neutralize the excess of CDI, MeOH (30 μL) was added and the reaction mixture was stirred additionally for 15 min. Solvents were evaporated affording crude activated benzyl phosphorimidazolidate **11**; ^{31}P NMR (101 MHz, MeOD): δ −7.3. To a soln of activated benzylphosphate **11** (0.25 mmol) and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate mono-cyclohexylammonium salt **12** (200 mg, 0.5 mmol) in dry DMF (6 mL) was added $ZnCl_2$ (272 mg, 2 mmol) and the mixture was stirred at rt under argon for 48 h. Then after evaporation to dryness the residue dissolved in 1:1 water–MeOH was purified by anion exchange chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. Elution with a gradient of 0 to M triethyl- ammonium hydrogenocarbonate buffer (pH 7.8) afforded **2** as its triethylammonium salt, but still contaminated by salts. After exchanging triethylammonium for sodium salt by passing through Dowex-50 (Na^+ form) resin, the product dissolved in water was applied onto a Biogel P-2 column (<45 μm, 2 × 63 cm); elution with 6 mM triethylammonium hydrogenocarbonate buffer (pH 7.8) afforded pure compound **2** as a white powder (64 mg, 38%); $[\alpha]_D^{29}$ +52 (*c* 0.66, water); ^1H NMR (250 MHz, D_2O): δ 7.50–7.35 (m, 5H, Ph), 5.47 (dd, 1H, $J_{1,2}$ 3 Hz, $J_{1,P}$ 7 Hz, H-1), 5.00 (d, 2H, $J_{H,P}$ 7 Hz, CH_2), 4.01–3.72 (m, 5H, H-2, H-4, H-5, H-6, H-6′), 3.52 (t, 1H, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 3.18 (q, 12H, CH_2–CH_3), 1.98 (s, 3H, NHAc), and 1.24 (t, 18H, CH_2–CH_3); ^{13}C NMR (62.9 MHz, D_2O): δ 174.9 (CO), 137.6 (d, $J_{C,P}$ 6 Hz, Ar-C), 128.9, 128.5, 128.1 (Ar-C), 94.7 (d, J_{C1-P} 5.7 Hz, C-1), 73.2, 71.2, 69.8 (C-3, C-4, C-5), 68.2 (d, $J_{C,P}$ 5.5 Hz, CH_2), 60.6 (C-6), 53.9 (d, $J_{C,P}$ 8.8 Hz, C-2), 46.9 (CH_2–CH_3), 22.3 (CH_3), and 8.5 (CH_2–CH_3); ^{31}P NMR (101 MHz, D_2O): δ −11.17 and −13.20 ($J_{P,P}$ 23.2 Hz); ESIMS (negative mode): *m*/*z* 470.0 [M−2NHEt$_3$+H]$^-$. HRESIMS (negative

mode) *m*/*z*: calcd for $C_{15}H_{22}O_{12}N_1P_2$, 470.0612; found, 470.0617.

3.4. 2-Acetamido-2-deoxy-α-D-glucopyranosyl-diphosphate, bis(triethylammonium)salt (3)

To a soln of compound **2** (43 mg, 64 μmol) in 1:1 MeOH–0.1 M triethylammonium hydrogenocarbonate buffer (pH 8) (2 mL), 10% Pd on charcoal (20 mg) was added and the mixture was stirred at rt under hydrogen pressure for 3 h. Catalyst was filtered, rinsed with MeOH and water; filtrate and washing were concentrated under diminished pressure and then freeze-dried. The residue was purified by anion exchange 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 a white solid (26 mg, 70%); $[\alpha]_D^{29}$ +58 (*c* 0.54, water); ^{1}H NMR (250 MHz, D_2O): δ 5.44 (br, 1H, H-1), 4.01–3.69 (m, 5H, H-2, H-4, H-5, H-6, H-6′), 3.47 (t, 1H, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 3.15 (q, 12H, C*H*$_2$–CH$_3$), 2.03 (s, 3H, NHAc), and 1.24 (t, 18H, CH$_2$–*C*H$_3$); ^{13}C NMR (62.9 MHz, D_2O): δ 175.1 (CO), 94.6 (C-1), 73.2, 71.2, 69.9 (C-3, C-4, C-5), 60.7 (C-6), 53.9 (C-2), 46.9 (*C*H$_2$–CH$_3$), 22.3 (CH$_3$), and 8.5 (CH$_2$–*C*H$_3$); ^{31}P NMR (101 MHz, D_2O): δ −9.20 and −12.80; ESIMS (negative mode): *m*/*z* 380.0 $[M-2NHEt_3+2H]^-$. HRESIMS (negative mode) *m*/*z*: calcd for $C_8H_{16}O_{12}N_1P_2$, 380.0142; found, 380.0160.

3.5. 4-Phenylbutyl-dibenzylphosphate (14)

Diisopropyl azodicarboxylate (1.2 mL, 6 mmol) was added dropwise to a soln of 4-phenyl-1-butanol (0.6 g, 4 mmol), dibenzylphosphate (1.67 g, 6 mmol), and triphenylphosphine (1.57 g, 6 mmol) in THF (4 mL). The mixture was stirred at rt for 4 h. Then solvent was evaporated, the residue was taken up in CH_2Cl_2, washed with satd aq $NaHCO_3$, the organic phase was evaporated to dryness and Et_2O was added to the residue. After 1 h at 0 °C the precipitate was filtered, solvent was evaporated, and the residue was purified by flash chromatography (5:1 petroleum ether–ethyl acetate) affording pure compound **14** as a colorless syrup (0.98 g, 60%); ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.45–7.05 (m, 15H, Ph), 5.03 (d, 2H, $J_{H,P}$ 7.5 Hz, CH$_2$), 5.02 (d, 2H, $J_{H,P}$ 7.5 Hz, CH$_2$), 4.00 (m, 2H, H-2, CH$_2$), 2.57 (m, 2H, CH$_2$), and 1.64 (m, 4H, 2CH$_2$); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 141.6, 135.8, 135.6, 128.3, 128.2, 128.1, 127.7, 125.6 (Ar-C), 69.4 (CH$_2$Ph), 68.9 (d, $J_{C,P}$ 6.9 Hz, CH$_2$Ph), 67.4 (d, $J_{C,P}$ 6.9 Hz, CH$_2$Ph), 34.9 (CH$_2$), 29.4 (d, $J_{C,P}$ 9.2 Hz, CH$_2$), and 26.9 (CH$_2$); ^{31}P NMR (101 MHz, $CDCl_3$): δ −0.26; ESIMS (positive mode): *m*/*z* 433.1 $[M+Na]^+$. HRESIMS (positive mode) *m*/*z*: calcd for $C_{24}H_{27}O_4Na_1P_1$, 433.1539; found, 433.1543.

3.6. 4-Phenylbutylphosphate disodium salt (13)

To a soln of compound **14** (0.41 g, 1 mmol) in 5:1 EtOH–M $NaHCO_3$ (12 mL), 10% Pd on charcoal (200 mg) was added and the mixture was stirred at rt under hydrogen atmosphere overnight. Catalyst was filtered, rinsed with 1:1 EtOH–water; filtrate and washing were evaporated, the residue was taken up in water, extracted with EtOAc, then the aqueous phase was concentrated and freeze-dried yielding pure 4-phenylbutylphosphate disodium salt **13** as a white solid (189 mg, 69%); ^{1}H NMR (360 MHz, MeOD): δ 7.30–7.10 (m, 5H, Ph), 3.83 (dt, 2H, $J_{H,P} = J_{H,H}$ 6.5 Hz, CH$_2$), 2.63 (t, 2H, *J* 7 Hz CH$_2$), and 1.67 (m, 4H, 2CH$_2$); ^{13}C NMR (62.9 MHz, MeOD): δ 143.8, 129.4, 129.2, 126.6 (Ar-C), 65.0 (CH$_2$Ph), 36.7 (CH$_2$), 32.0 (d, $J_{C,P}$ 7.8 Hz, CH$_2$), and 29.1 (CH$_2$); ^{31}P NMR (101 MHz, D_2O): δ 6.17; ESIMS (negative mode): *m*/*z* 229.1 $[M-2Na+H]^-$.

3.7. 4-Phenylbutyl-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-diphosphate, bis(triethylammonium) salt (4)

4-Phenylbutylphosphate **13** as its disodium salt (1 mmol) was dissolved in a small amount of water and passed through a column of Bio-Rad AG 50W-X8 resin (H^+ form). 4-Phenylbutylphosphoric acid was eluted with 1:1 EtOH–water and the soln was freeze-dried. To the dry acid, carbonyldiimidazole (CDI) (0.324 g, 2 mmol), Et_3N (0.13 mL, 1 mmol), and DMF (5 mL) were added and the mixture was vigorously stirred at rt for 3 h. To neutralize excess of CDI, MeOH (30 μL) was added and the reaction mixture was stirred additionally for 15 min. Solvents were evaporated affording crude imidazolide **15**; ^{31}P NMR (101 MHz, $(CD_3)_2SO$): δ −4.71. A soln of activated phosphate **15** (0.3 mmol) and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate mono-triethylammonium salt **12** (180 mg, 0.45 mmol) in dry DMF (5 mL) was stirred at rt under argon atmosphere for 3 d. Then after evaporation to dryness the residue dissolved in 1:1 water–MeOH was purified by anionic exchange 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 **4** as its triethylammonium salt, still contaminated by salts. After exchanging triethylammonium for sodium salt by passing through Dowex-50 (Na^+ form) resin, the product dissolved in water was applied onto a Biogel P-2 column (<45 μm, 2 × 63 cm); elution with 6 mM triethylammonium hydrogenocarbonate buffer (pH 7.8) afforded pure compound **4** as a white powder (63 mg, 29%); $[\alpha]_D^{29}$ +32 (*c* 0.55, water); ^{1}H NMR (250 MHz, D_2O): δ 7.40–7.20 (m, 5H, Ph), 5.43 (dd, 1H, $J_{1,2}$ 3 Hz, $J_{1,P}$ 7 Hz, H-1), 4.00–3.71 (m, 5H, H-2, H-4, H-5, H-6, H-6′), 3.56 (q, 2H, CH$_2$), 3.50 (t, 1H,

$J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 3.16 (q, 12H, CH_2–CH_3), 2.65 (t, 2H, CH_2), 2.01 (s, 3H, NHAc), 1.65 (m, 4H, $2CH_2$), and 1.24 (t, 18H, CH_2–CH_3); ^{13}C NMR (62.9 MHz, D_2O): δ 174.8 (CO), 143.2, 128.7, 126.0 (Ar-C), 94.5 (C-1), 73.1, 71.1, 69.6 (C-3, C-4, C-5), 66.6 (CH_2), 60.5 (C-6), 53.8 (C-2), 46.8 (CH_2–CH_3), 34.7, 29.5, 27.1 (CH_2), 22.3 (CH_3), and 8.3 (CH_2–CH_3); ^{31}P NMR (101 MHz, D_2O): δ −10.78 and −13.23 ($J_{P,P}$ 21.4 Hz); ESIMS (negative mode): m/z 512.3 $[M-2NHEt_3+2H]^-$. HRESIMS (negative mode) m/z: calcd for $C_{18}H_{28}O_{12}N_1P_2$, 512.1081; found, 512.1092.

3.8. 5′-O-[[(2-Acetamido-2-deoxy-α-D-glucopyranosyl-oxy)carbonyl]sulfonyl]-uridine (6)

A suspension of compound **5**[11] (36 mg, 0.05 mmol) in saturated methanolic ammonia (4 mL) was kept at rt for 4 h. Then solvents were evaporated and the residue was purified on a column of Sephadex LH-20 in MeOH to afford compound **10** as a white solid (26 mg, 90%); $[\alpha]_D^{29}$ +49 (*c* 1, H_2O); ^{1}H NMR (400 MHz, MeOD): δ 7.93 (d, 1H, $J_{5,6}$ 8 Hz, H-6), 5.95 (d, 1H, $J_{1',2'}$ 4 Hz, H-1′), 5.90 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1″), 5.79 (d, 1H, H-5), 4.33 (d, 1H, $J_{5'a,5'b}$ 11.5 Hz, H-5′a), 4.27 (d, 1H, H-5′b), 4.20 (m, 3H, H-2′, H-3′, H-4′), 4.00 (dd, 1H, $J_{2,3}$ 10.5 Hz, H-2″), 3.76 (dd, 1H, $J_{3,4}$ 9.0 Hz, H-3″), 3.70 (m, 3H, H-6″a, H-6″b, H-5″), 3.40 (t, 1H, $J_{4,5}$ 9.0 Hz, H-4″), and 1.98 (s, 3H, NHAc); ^{13}C NMR (100.7 MHz, MeOD): δ 173.9 (CO), 166.2 (C-4), 156.9 (CO), 152.6 (C-2), 142.6 (C-6), 103.2 (C-5), 93.2 (C-1″), 90.0 (C-1′), 83.9 (C-4′), 75.5, 75.4, 72.6, 71.8, 71.6, (C-2′, C-3′, C-3″, C-4″, C-5″), 69.1 (C-5′), 62.4 (C-6″), 54.5 (C-2″), and 22.8 (CH_3); ESIMS (positive mode): m/z 593.1 $[M+Na]^+$. 615.0 $[M-H+2Na]^+$. HRESIMS (positive mode): $[M+Na]^+$ calcd for $C_{18}H_{26}N_4O_{15}NaS$, 593.1040; found, 593.1013. Anal. Calcd for $C_{18}H_{26}O_{15}N_4S$, H_2O: C, 36.74; H, 4.80. Found: C, 36.44; H, 4.92.

3.9. 2-*N*-(Phenylacetyl)-9-(β-D-ribofuranosyl)-guanine (17)

To a suspension of guanosine **16** (566 mg, 2 mmol) in anhyd pyridine (10 mL), was added trimethylsilyl chloride (1.9 mL, 15 mmol) and the mixture was stirred at rt for 8 h. Then a soln of phenylacetyl chloride (0.4 mL, 3 mmol) and 1-hydroxybenzotriazole (432 mg, 3.2 mmol) in CH_3CN (1 mL) and pyridine (1 mL) was added dropwise at 0 °C and the mixture was stirred at rt overnight. Then the reaction was cooled to 0 °C and stopped by the addition of water (2.5 mL). After 5 min, 25% aqueous ammonium hydroxide (6 mL) was added. The aminolysis of the silyl ethers was completed in 30 min. The solvents were evaporated and the residue was dissolved in water and extracted with ethyl acetate. The aqueous phases were concentrated until the product crystallized. The crystals were filtered, washed with water, and dried giving **17** (558 mg, 70%). $[\alpha]_D^{29}$ −10 (*c* 0.3, MeOH); mp 189 °C. ^{1}H NMR (250 MHz, $(CD_3)_2SO$): δ 11.9 (d, 1H, NH), 8.27 (s, 1H, H-8), 7.30 (m, 5H, Ar-H), 5.80 (d, 1H, $J_{1,2}$ 5.5 Hz, H-1′), 5.49 (d, 1H, *J* 6.0 Hz, OH), 5.18 (d, 1H, *J* 5.0 Hz, OH), 5.05 (t, 1H, *J* 6.0 Hz, OH), 4.41 (q, 1H, H-2′), 4.11 (q, 1H, H-3′), 3.89 (dt, 1H, H-4′), 3.79 (s, 2H, CH_2), 3.63 (m, 1H, $J_{5'a,5'b}$ 10.0, $J_{4',5'a}$ 2.5 Hz, H-5′a), and 3.53 (m, 1H, $J_{4',5'b}$ 3.0 Hz, H-5′b); ^{13}C NMR (100.7 MHz, MeOD–$CDCl_3$): δ 173.3 (CO), 155.2 (C-6), 148.2 (C-2), 147.1 (C-4), 137.7 (C-8), 132.8, 128.2, 127.5, 126.2 (Ar-C), 119.3 (C-5), 87.5 (C-1′), 84.6 (C-4′), 74.2 (C-2′), 69.5 (C-3′), 60.4 (C-5′), and 42.0 (CH_2); ESIMS (positive mode): m/z 402 $[M+H]^+$, 424 $[M+Na]^+$, 440 $[M+K]^+$; HRESIMS m/z: $[M+Na]^+$ calcd for $C_{18}H_{19}N_5O_6Na$, 424.1228; found, 424.1232.

3.10. 9-(2′,3′-*O*-Benzylidene-β-D-ribofuranosyl)-2-*N*-(phenylacetyl)-guanine (18)

A mixture of compound **17** (500 mg, 1.24 mmol), freshly distilled benzaldehyde (3.65 mL), and $ZnCl_2$ (576 mg, 4.23 mmol) was stirred until complete dissolution, and heated at 50 °C for 1 h. Excess of benzaldehyde was removed by co-evaporation with toluene; the crude product was precipitated with ether and then purified by flash chromatography (19:1 CH_2Cl_2–MeOH) to give the benzylidene acetal **18** as a white solid (mixture of two diastereomers, 528 mg, 88%). ^{1}H NMR (360 MHz, $CDCl_3$): δ 12.05 (NH), 9.84, 9.75 (NH), 7.70, 7.68 (2s, 1H, H-8), 7.53–7.28 (m, 10H, Ar-H), 6.13, 5.99 (2s, 1H, CH_aPh, CH_bPh), 5.90, 5.89 (2d, 1H, $J_{1',2'}$ 3.5 Hz, H-1′), 5.25, 5.26 (2dd, 1H, $J_{2',3'}$ 6.5 Hz, H-2′), 5.14, 5.09 (2dd, 1H, $J_{3',4'}$ 3.5 Hz, $J_{3',4'}$ 2.5 Hz, H-3′), 4.51, 4.36 (2m, 1H, H-4′), 3.96, 3.90 (2dd, 1H, $J_{5'a,5'b}$ 12.5 Hz, $J_{4,5'a}$ 2.5 Hz, H-5′a), 3.80, 3.77 (2dd, 1H, $J_{4,5'b}$ 2.5 Hz, H-5′b), and 3.79 (s, 2H, CH_2Ph); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 173.7 (CO), 155.5 (C-6), 147.7 (C-2), 139.0 (C-4), 135.7, 135.5 (C-8), 132.9, 129.3, 128.7, 128.3, 127.4, 126.6, 126.4 (Ar-C), 120.9, 120.8 (C-5), 107.3, 104.0 (CH_aPh, CH_bPh), 90.7, 89.9 (C-1′), 86.6, 85.3 (C-4′), 84.5, 83.8, 82.4, 80.6 (C-2′, C-3′), 62.0 (C-5′), and 42.0 (CH_2); ESIMS (positive mode): m/z 490 $[M+H]^+$, 512 $[M+Na]^+$, 528 $[M+K]^+$; HRESIMS m/z: $[M+H]^+$ calcd for $C_{25}H_{24}O_6N_5$, 490.1721; found, 490.1718. Anal. Calcd for $C_{25}H_{23}O_6N_5$: C, 61.20; H, 4.93. Found: C, 61.09; H, 4.93.

3.11. 9-(2′,3′-*O*-Benzylidene-5′-*O*-[(2″,3″,4″-tri-*O*-benzyl-α,β-L-fucopyranosyl)oxycarbonylaminosulfonyl]-β-D-ribofuranosyl)-2-*N*-(phenylacetyl)-guanine (21), and 2,3,4-tri-*O*-benzyl-1-*O*-carbamoyl-α,β-L-fucopyranose (20)

Benzyl-protected fucose[35] **19** (412 mg, 0.95 mmol) was dissolved in dry CH_2Cl_2 (3 mL) and stirred with 4 Å

molecular sieves (1.3 g) for 20 min under argon. Chlorosulfonyl isocyanate (72 μl, 0.95 mmol) was then added to this soln cooled at −35 °C and the mixture was stirred at −35 °C for 2 h. Temperature was allowed to rise to −10 °C and the mixture was further stirred until all fucose derivative reacted to give a more polar intermediate. A soln of guanosine derivative **18** (465 mg, 0.95 mmol) in CH_2Cl_2 (4 mL) containing pyridine (0.2 mL, 2.5 mmol) was added and the mixture kept under argon was stirred for 20 h at room temperature. TLC (10:1 AcOEt–MeOH) monitoring of the reaction revealed a major spot (R_f 0.25). After filtration and evaporation of solvents, purification of the crude product by flash chromatography on a silica gel column (10:1 AcOEt–MeOH), afforded first the carbamoyl fucose derivative **20** and then the expected compound still contaminated by byproducts. This mixture was purified by gel permeation on Sephadex LH-20 (7:3 CH_2Cl_2–MeOH) affording two fractions of compound **21** as a white solid both as an *R*,*S* diastereomer mixture: the first one corresponding to the coupling product exclusively as α fucose (210 mg, 21.5%), the second one as 4:1 α/β anomeric mixture (180 mg, 18.5%). The overall yield was 40% with α/β ratio of 91:9; ^{1}H NMR of **21α** (250 MHz, MeOD–$CDCl_3$) (2 diastereomers 1:1 ratio) δ 7.99 (s, 0.5H, H-8a), 7.91 (s, 0.5H, H-8b), 7.50–7.00 (25H, 5Ar-H), 6.12 (d, 0.5H, $J_{1'',2''}$ 3.5 Hz, H-1″a), 6.09 (s, 0.5H, CH_bPh), 6.07 (d, 0.5H, $J_{1'',2''}$ 3.5 Hz, H-1″b), 6.06 (d, 0.5H, $J_{1',2'}$ 2.5 Hz, H-1′a), 6.03 (d, 0.5H, $J_{1'2'}$ 1.5 Hz, H-1′b), 5.85 (s, 0.5H, CH_aPh), 5.30 (dd, 0.5H, $J_{2',3'}$ 6.5 Hz, H-2′b), 5.19 (dd, 0.5H, H-2′a), 5.07 (dd, 0.5H, $J_{2',3'}$ 2.5 Hz, H-3′a), 4.99 (dd, 0.5H, H-3′b), 4.89 (d, 0.5H, *J* 11.5 Hz, CH_2Ph), 4.87 (d, 0.5H, *J* 11.5 Hz, CH_2Ph), 4.80–4.45 (m, 6H, H-4′, CH_2Ph), 4.30–3.50 (m, 8H, H-2″, H-3″, H-4″, H-5″, 2H-5′, CH_2), 1.06 (d, 1.5H, $J_{5'',6''}$ 6 Hz, H-6″a), and 1.03 (d, 1.5H, $J_{5'',6''}$ 6 Hz, H-6″b); ^{1}H NMR of **21β** in the 4:1 α/β mixture (250 MHz, MeOD–$CDCl_3$) δ 7.98 (s, 0.1H, H-8aβ), 7.95 (s, 0.1H, H-8bβ), 6.09 (s, 0.1H, CH_bPh), 5.91 (s, 0.1H, CH_aPh), 5.45 (d, 0.1H, $J_{1'',2''}$ 8 Hz, H-1″β), 5.43 (d, 0.1H, $J_{1'',2''}$ 8 Hz, H-1″β); ^{13}C NMR (62.9 MHz, MeOD–$CDCl_3$) (4:1 **21α/21β** ratio) δ 174.0 (CO), 158.5, 158.3 (CO), 155.5 (C-6), 147.3, 147.1 (C-2), 139.0 (C-4), 137.8, 137.6, 137.4 (Ar-C), 135.4, 135.1 (C-8), 132.9, 128.8, 127.5, 126.5, 125.9 (Ar-C), 120.6, 120.4 (C-5), 106.7 (*C*H_a-Ph) 103.5 (*C*H_b-Ph), 95.3 (C-1″β), 91.0 (C-1″α), 90.4 (C-1′), 83.7, 83.6, 82.9, 82.3, 81.6, 80.9 (C-2′, C-3′, C-4′), 78.7, 76.8, 75.9, 74.3, 74.0, 72.1, 71.9, 70.5 (C-2″, C-3″, C-4″, *C*H_2Ph), 67.8, 66.3 (C-5′, C-5″), 42.3, 42.2 (CH_2), and 15.6 (C-6″); ESIMS (positive mode): *m*/*z* 1029.5 $[M+H]^+$, 1051.5 $[M+Na]^+$, 1067.5 $[M+K]^+$. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{53}H_{52}O_{14}N_6S_1Na$, 1051.3154; found, 1051.3117.

Compound **20** first eluted from silica gel as a white solid (77 mg, 17%); ^{1}H NMR (250 MHz, $CDCl_3$–MeOD): δ 7.45–7.24 (m, 15H, Ar-H), 6.12 (d, 0.8H, $J_{1,2}$ 3.5 Hz, H-1α), 5.48 (d, 0.2H, $J_{1,2}$ 8 Hz, H-1β), 5.0–4.58 (m, 6H, 3CH_2Ph), 3.98 (q, 0.8H, H-5α) (dd, 0.8H, $J_{2,3}$ 10.0, $J_{3,4}$ 2.5 Hz, H-3α), 3.86–3.70 (m, 1H, H-5β, H-2α), 3.71 (d, 0.8H, H-4α), 3.67–3.56 (m, 0.6H, H-4β, H-3β, H-2β), 1.19 (d, 0.6H, $J_{5,6}$ 6.5 Hz, H-6β), and 1.12 (d, 2.4H, $J_{5,6}$ 6.5 Hz, H-6α); ^{13}C NMR (50.3 MHz, $CDCl_3$): δ 153.5 (CO), 138.7, 138.1, 137.5, 128.1, 127.6, 127.1 (Ar-C), 95.8 (C-1β), 92.8 (C-1α), 78.8, 75.1, 74.0, 73.8, 73.1, 72.7, 72.2, 71.6, 69.2 (C-2, C-3, C-4, C-5, *C*H_2Ph), and 16.4 (C-6); ESIMS (positive mode): *m*/*z* 500.0 $[M+Na]^+$; 977 $[2M+Na]^+$. HRESIMS *m*/*z*: $[M+Na]^+$ calcd for $C_{28}H_{31}O_6N_1Na$, 500.2044; found, 500.2052.

3.12. 9-(5′-*O*-[(α,β-L-Fucopyranosyl)oxycarbonyl-aminosulfonyl]-β-D-ribofuranosyl)-2-*N*-(phenylacetyl)-guanine (22)

To a soln of compound **21** (27 mg, 26 μmol, 4:1 α/β) in 5:3:2 THF–MeOH–water (2.5 mL), 10% Pd on charcoal (27 mg) was added and the mixture was stirred under hydrogen pressure (40 psi) for 4 d. Catalyst was filtered, rinsed with MeOH and water; filtrate and washing were concentrated under diminished pressure and then freeze-dried. The residue taken up in water was purified on Sep-Pak C_{18}. Elution with water afforded methyl L-fucopyranoside **23**, predominantly β (2 mg, 45%); then elution with 4:1 and 7:3 water–MeOH afforded pure compound **22** as a white powder (6 mg, 35%).^{1}H NMR (400 MHz, D_2O,): δ 8.20 (s, 1H, H-8), 7.35 (m, 5H, Ar-H), 6.12 (d, 1H, $J_{1',2'}$ 5.5 Hz, H-1′), 5.98 (d, 0.8H, $J_{1\alpha'',2''}$ 3.0 Hz, H-1″α), 5.30 (d, 0.2H, $J_{1\beta'',2''}$ 8.0 Hz, H-1″β), 4.78 (t, 1H, superimposed with HOD, H-2′), 4.57 (dd, 1H, $J_{2',3'}$ 2.5 Hz, H-3′), 4.40 (m, 3H, H-4′, H-5′a, H-5′b), 4.02 (q, 1H, $J_{5'',6''}$ 6.0 Hz, H-5″α), 3.88 (s, 2H, CH_2), 3.84 (m, 1.6H, H-2″α, H-3″α), 3.79 (m, 0.2H, H-5β), 3.74 (d, 0.8H, $J_{3'',4''}$ 2.5 Hz, H-4″α), 3.72–3.52 (m, 0.6H, H-2″β, H-3″β, H-4″β), 1.15 (d, 0.6H, H-6″β), and 1.07 (d, 2.4H, H-6″α); ^{13}C NMR (100.7 MHz, D_2O): δ 175.0 (CO), 158.7, (CO, C-6), 151.8 (C-2), 149.5 (C-4), 139.6 (C-8), 133.5, 129.5, 128.9, 127.5 (Ar-C) 120.3 (C-5), 95.6 (C-1″β), 93.2 (C-1″α), 87.9 (C-1′), 82.4 (C-4′), 73.4 (C-2′), 72.6, 71.5 (C-4″), 70.2 (C-3′), 69.4 (C-3″), 68.4, 68.3 (C-5′, C-5″), 67.0 (C-2″), 42.8 (CH_2), and 15.3 (C-6″); ESIMS (positive mode): *m*/*z* 715 $[M-H+2Na]^+$; MALDI-TOFMS *m*/*z*: $(M-H+2Na)^+$ calcd for $C_{25}H_{29}N_6O_{14}Na_2S$, 715.1258; found, 715.1284.

Methyl β-L-fucopyranoside **23**; ^{1}H NMR (250 MHz, D_2O): δ 4.30 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1), 3.81 (q, 1H, $J_{5,6}$ 6 Hz, H-5), 3.74 (d, 1H, $J_{4,3}$ 3.0 Hz, H-4), 3.64 (dd, 1H, $J_{2,3}$ 10 Hz, H-3), 3.55 (s, 3H, OCH_3), 3.47 (dd, 1H, H-2), and 1.27 (d, 3H, H-6); ^{13}C NMR (90.5 MHz, D_2O): δ 103.7 (C-1), 72.9 (C-3), 71.3, 70.9, 70.4 (C-2, C-4, C-5), 51.1 (CH_3), and 15.3 (C-6); ESIMS (positive mode): *m*/*z* 201.1 $[M+Na]^+$.

3.13. 9-(5′-*O*-[(α,β-L-Fucopyranosyl)oxycarbonyl-aminosulfonyl]-β-D-ribofuranosyl)-guanine (7)

To a soln of compound **22** (6 mg, 9 μmol) in 40 mM phosphate buffer pH 7.8 (2.2 mL) Eupergit-immobilized penicillin amidase (75 mg) was added and the mixture was gently shaken at 25 °C overnight. Enzyme was filtered, rinsed with water, and the aqueous soln was freeze-dried. The residue taken up in water was purified on Sep-Pak C_{18}. Elution with 9:1 water–MeOH afforded pure compound **7** as a white foam (4.5 mg, 91%). ^{1}H NMR (400 MHz, D_2O): δ 8.05 (s, 1H, H-8), 5.95 (d, 1H, $J_{1',2'}$ 6.0 Hz, H-1′), 5.86 (d, 0.8H, $J_{1a'',2''}$ 3.5 Hz, H-1″α), 5.28 (d, 0.2H, $J_{1b'',2''}$ 8.0 Hz, H-1″β), 4.75 (t, 1H, superimposed with HOD, H-2′), 4.48 (dd, 1H, $J_{2',3'}$ 3.0 Hz, H-3′), 4.38 (m, 3H, H-4′, 2H-5′), 3.99 (q, 0.8H, $J_{5'',6''}$ 6.0 Hz, H-5″α), 3.88 (dd, 0.8H, $J_{2'',3''}$ 10.0, $J_{3'',4''}$ 3.0 Hz, H-3″α), 3.83 (dd, 0.8H, H-2″α), 3.80 (q, 0.2H, H-5″β), 3.74 (d, 0.8H, $J_{3'',4''}$ 2.5 Hz, H-4″α), 3.69 (d, 0.2H, $J_{3'',4''}$ 3.0 Hz, H-4″β), 3.64 (dd, 0.2H, H-3″β), 3.60 (dd, 0.2H, H-2″β), 1.14 (d, 0.6H, H-6″β), and 1.09 (d, 2.4H, H-6″α); ^{13}C NMR (100.7 MHz, D_2O): δ 160.4 (CO), 158.6 (C-6), 155.0 (C-2), 151.4 (C-4), 137.2 (C-8), 125.1 (C-5), 95.3 (C-1″β), 93.2 (C-1″α), 86.8 (C-1′), 82.4 (C-4′), 73.4 (C-2′), 71.6, 71.5 (C-4″), 70.3 (C-3′), 69.3 (C-3″), 68.4, 68.2 (C-5′, C-5″), 66.9 (C-2″), and 15.3 (C-6″); ESIMS (positive mode): *m*/*z* 553.3 $[M+H]^+$, 575.3 $[M+Na]^+$, 597.2 $[M-H+2Na]^+$; MALDI-TOFMS *m*/*z*: $(M+Na)^+$ calcd for $C_{17}H_{24}N_6O_{13}NaS$, 575.1014; found, 575.1010.

3.14. Assay of compounds 1, 2, 3, 4, 6 as *N*-acetylglucosamine donors toward *LgtA*

Assay conditions: 400 μM β-D-Gal*p*-(1→4)-β-D-Glc*p*-*O*-$(CH_2)_6$-NH-dansyl, 60 mM compound **1** or **2** or **3** or **4** or **6** and 15 mM MnCl were incubated at 37 °C for 15 h in a total volume of 200 μL with *LgtA* (40 μL, 2.5 mU) in 50 mM sodium cacodylate buffer pH 7.2. Two supplementary assays were run with compound **3** at concentrations of 60 and 100 mM in the presence of uridine used at the same concentrations. Reaction mixtures were analyzed by TLC (3:1:1 AcOH–AcOEt–water). A blank test was run in parallel, for which compounds to be tested were replaced by 500 μM UDP-GlcNAc. In these conditions 50% substrate was converted into the product in 5 min.

3.15. Inhibitory activity of compounds 1, 2, 3, 4, 6 toward *LgtA*

Enzyme assay conditions: 25 mM cacodylate buffer pH 7.2 containing 15 mM $MnCl_2$, 800 μM β-D-Gal*p*-(1→4)-β-D-Glc*p*-*O*-$(CH_2)_6$-NH-dansyl, 225 μM UDP-GlcNAc, 0.5 mU *LgtA* and compound **1** or **2** or **3** or **4** or **6** (at concentrations of 1, 2, 5, 10, and 20 mM) in 50 μL total volume. Assays were incubated at 37 °C for 11 min. Incubation was stopped by immersion in a boiling water bath for 2 min; the soln was diluted with water (100 μL) and filtered. The samples were analyzed by RP-HPLC using fluorescence detection. The percentage of conversion was quantified from the fluorescence intensity of the peaks corresponding, respectively, to acceptor substrate and product. Assays were duplicated and a control reaction without the inhibitor was run simultaneously. In each assay, the amount of the trisaccharide formed was less than 25% of the total amount of UDP-GlcNAc.

3.16. Assay of compound 7 as a fucosyl donor toward FucT-III

Assay conditions with soluble enzyme: 400 μM β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-*O*-$(CH_2)_6$-NH-dansyl, 4 mM compound **7** (4:1 α/β ratio) were incubated at 37 °C for 30 h in a total volume of 50 μL with FucT-III (20 μL, 1 mU) either in 25 mM sodium cacodylate buffer pH 6.0, or in 25 mM sodium cacodylate buffer pH 6.0 containing 10 mM $MnCl_2$, or 25 mM sodium cacodylate buffer pH 6.0 containing 10 mM $Yb(OTf)_3$. Assay conditions with immobilized enzyme[3]: 200 μM β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-*O*-$(CH_2)_6$-NH-dansyl, 35 mM compound **7** (6:1 α/β ratio) were stirred at 30 °C for 30 h in a total volume of 50 μL with FucT-III immobilized on Ni^{2+}–agarose (1 mU) in 25 mM sodium cacodylate buffer pH 6.0 containing 20 mM $MnCl_2$ or 20 mM $Yb(OTf)_3$. Reaction mixtures were analyzed by TLC (3:1:1 AcOH–AcOEt–water). A blank test was run in parallel, for which compound **7** was replaced by 100 μM GDP-Fuc. In these conditions 50% substrate was converted into the product in 10 min.

3.17. Inhibitory activity of compound 7 toward FucT-III

Enzyme assay conditions: 10 mM $MnCl_2$, 25 mM cacodylate buffer pH 6.0, 200 μM β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-*O*-$(CH_2)_6$-NH-dansyl, 50 μM GDP-Fuc, 0.05 mU soluble FucT-III and compound **7** (4:1 α/β ratio, 1, 2, 4, 7.5, 15, and 30 mM) in 50 μL total volume. Assays were incubated at 37 °C for 9 min. Incubation was stopped by immersion in a boiling water bath for 2 min; after filtration, samples were analyzed by RP-HPLC and the percentage of conversion of each assay was quantified as above. Assays were conducted in duplicate and a control reaction without the inhibitor was run simultaneously. In each assay, the amount of the trisaccharide formed was less than 25% of the total amount of GDP-Fuc.

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