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FRET-Based Direct and Continuous Monitoring of Human Fucosyltransferases Activity: An Efficient synthesis of Versatile GDP-L-Fucose Derivatives from Abundant D-Galactose**

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Abstract: We have developed a facile and versatile protocol for the continuous monitoring of human fucosyltransferases activity by using fluorescence energy resonance transfer (FRET), and have explored the feasibility of its use in an inhibitor screening assay. A convenient sugar nucleotide with a fluorogenic probe, 6-deoxy-6-*N*-(2-naphalene-2-yl-acetamide)-β-L-galactopyranos-1-yl-guanosine 5′-diphosphate disodium salt (**1**), was efficiently synthesized from naturally abundant D-galactopyranose via a key intermediate, 6-azide-1,2,3,4-tetra-*O*-benzoyl-6-deoxy-β-L-galactopyranose (**10**). It was demonstrated that the combined use of the glycosyl donor **1** and a dansylated acceptor substrate, sialyl-α2,3-LacNAc derivative (**2**) allowed us to carry out highly sensitive, direct, and continuous in vitro monitoring of the generation of sialyl Lewis X (SLe^x), which is catalyzed by human α-1,3-fucosyltransferase VI (FUT-VI). A kinetic analysis revealed that compound **1** was an excellent donor substrate ($K_M = 0.94$ μM and $V_{max} = 0.14$ μM min^{-1}) for detecting human FUT-VI activity. To the best of our knowledge, this synthetic fluorogenic probe is the most sensitive and selective donor substrate for FUT-VI among all of the known GDP-Fuc analogues, including the parent GDP-Fuc. When a dansylated asparagine-linked glycopeptide **20**, which is derived from egg yolk was employed as an alternate acceptor substrate, a FRET-based assay with compound **1** could be used to directly monitor the α1,6-fucosylation at the reducing terminal GlcNAc residue by human FUT-VIII ($K_M =$ 175 μM and $V_{max} = 0.06$ μM/min); this indicates that the present method might become a general protocol for the characterization of various mammalian fucosyltransferases in the presence of designated fluorogenic acceptor substrates. The present protocol revealed that compound **23**, which was obtained by a 1,3-dipolar cycloaddition between the disodium salt **16** and 1-ethynylnaphthalene exhibits highly potent inhibitory effects against the FUT-VI-mediated sialyl Lewis X synthesis ($IC_{50} = 5.4$ μM).

Keywords: carbohydrates · FRET (fluorescence resonant energy transfer) · fucose · fucosyltransferase · galactose

Introduction

Glycosylation, which is conducted by various glycosyltransferases, is one of the most important biological processes in the posttranslational modification of protein/lipid structures and functions. This modification greatly influences various molecular recognition processes, including bacterial/viral infections and cell adhesion in inflammation, as well as basic biological phenomena such as immune response, cellular differentiation, development, regulation, and many other intercellular communication and signal transductions.[1] It is evident that synthetic substrates and inhibitors of glycosyltransferases have become powerful tools, and they might lead to the development of novel therapeutic reagents, as well as specific molecular probes for characterizing enzymatic glycosylation processes.[2–11]

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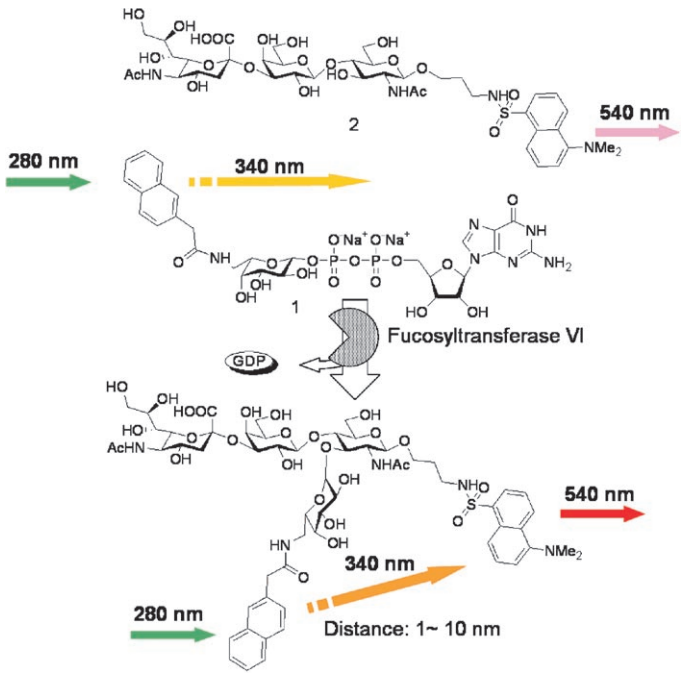
Fucosyltransferases (FucTs) are enzymes that catalyze the transfer of the L-fucose moiety from guanosine diphosphate β-L-fucose (GDP-Fuc) to various acceptors in an α1,2-, α1,3-, α1,4-, or α1,6-linkage.[12] It has been well documented that fucosylation conducted by mammalian FucTs is often the final glycosylation step in a range of biologically significant glycans such as sialyl Lewis X (sLe^x), sialyl Lewis A (sLe^a), and sialyl Lewis B (sLe^b) structures.[13] It was also suggested that the attachment of a L-fucose residue to the core chitobiose unit of asparagine-linked glycans reduces the antibody-dependent cellular cytotoxicity drastically.[14] Given that fucosylation is essential for basic biological events, especially fertilization, embryogenesis, lymphocyte trafficking, immune responses, and various diseases,[15] it is not surprising that extensive efforts have been made toward the synthesis of potential inhibitors of FucTs[2,9,16–29] and toward specific tagging methods to visualize fucosylated glycans in vivo.[30]

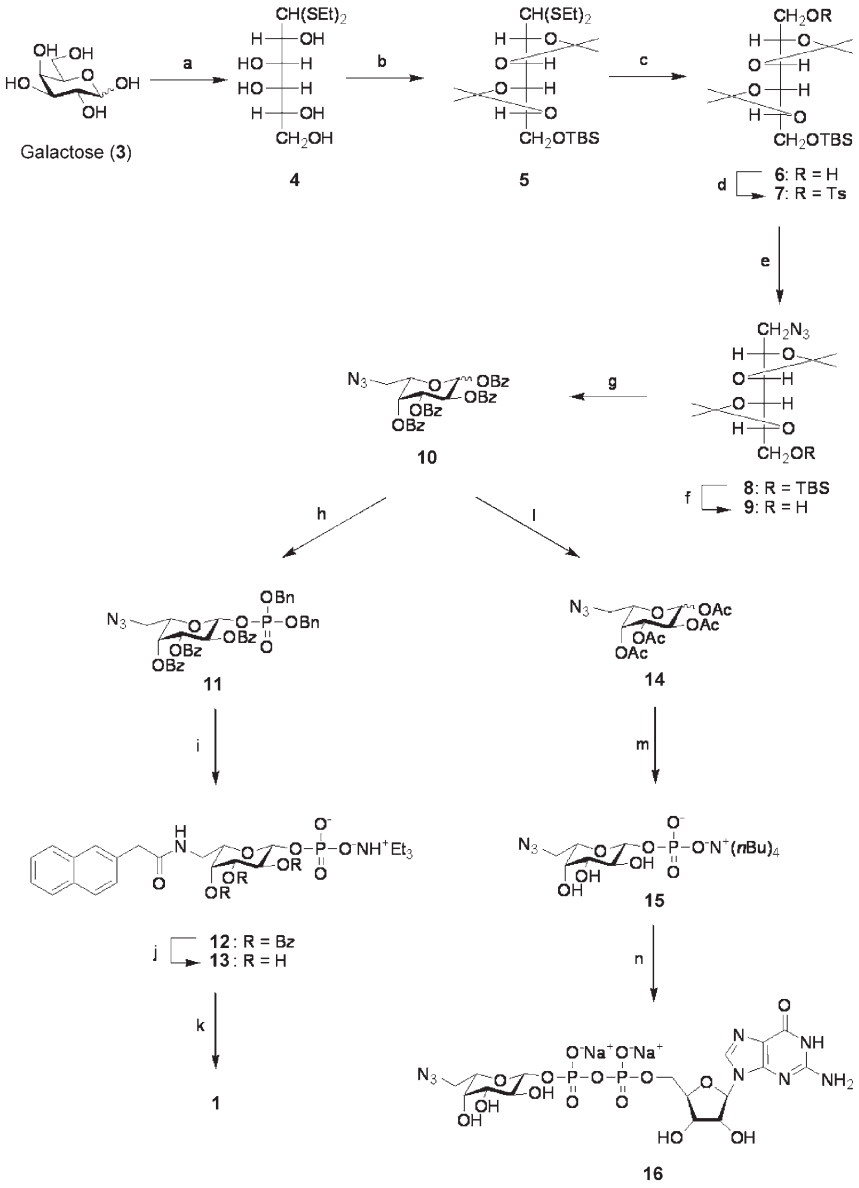
Our attention is now directed toward the need for a new method that allows for the direct, continuous, and quantitative monitoring of the FucTs reactions which yield fucosylated glycoconjugates of interest. The advent of facile and reliable screening methods should facilitate the process of searching for specific inhibitors against various FucTs. We hypothesized that an approach that uses fluorescent resonance energy transfer (FRET) might become a key to address this issue,[31,32] although modern mass spectrometry and surface plasmon resonance (SPR) approaches have been feasible in combination with the use of gold nanoparticles or high-performance microarrays to display glycosyl acceptor substrates or glycosyltransferases.[33–36] As illustrated in Scheme 1, we imagined that a FRET-based assay would allow a quite simple and specific sensing of the generation of the desired product (structures) as an enhanced fluorescence due to dansyl emission at 540 nm under an excitation of naphtylmethyl group at 290 nm, only when the FucT-mediated glycosylation occurs between the designated fluorescent donor **1** and acceptor **2** substrates. Its feasibility in continuous and quantitative kinetic analysis is evident because the fluorescence intensity at 540 nm directly represents the concentration of the sLe^x derivative generated by glycosylation. In the present study, we describe an efficient synthesis of versatile GDP-fucose analogues for direct, continuous, and quantitative monitoring of glycosylation catalyzed by human FucTs.



Scheme 1. Concept of FRET-based detection of sialyl Lewis X synthesized by FucTs. The increase of the dansyl emission at 540 nm under an excitation at 290 nm will be observed only when sialyl Lewis X tetrasaccharide is formed by glycosylation catalyzed by FucTs.

Results and Discussion

Synthesis of versatile GDP-L-fucose analogues from D-galactose: Because synthetic studies on GDP-L-fucose analogues had revealed a tolerance at the C6 position for FucTs-catalyzed glycosylations,[30,37,38] we selected compound **1** as an ideal fluorogenic donor (naphthylmethyl) substrate for use with acceptor substrate **2** (dansyl) as shown in Scheme 1. We established an efficient synthetic route to a novel fluorescence-labeled GDP-L-fucose derivative **1** via a key intermediate, 6-azide-1,2,3,4-tetra-*O*-benzoyl-6-deoxy-β-L-galactopyranose (**10**), by using D-galactose as a starting material. It seems likely that the use of D-galactose ($1.6 g^{-1}) would make the large-scale synthesis of various L-fucose derivatives possible through a series of established chemical manipulations; most C6-modified L-fucose derivatives have been prepared by starting from L-galactose, which is very expensive ($945 g^{-1}).[30,38] Conversion of the D-galacto into the L-galacto configuration was carried out by chemical modifications of compound **5**, which is derived from a known D-galactose diethyl dithioacetal **4**,[37] as shown in Scheme 2. Selective liberation of the thioacetal **5** yielded an alcohol **6**, which was tosylated and subjected to a subsequent substitution reaction to afford the fully protected azide **8** in high yield. After de-*O*-silylation, the primary hydroxyl group was oxidized by treatment with oxalyl chloride to afford a hemiacetal **9**, which was subjected to de-*O*-isopropylidenation. Subsequent *O*-benzoylation gave 6-azide-L-fucose as per-*O*-benzoate **10** in 73% overall yield from compound **9** (three steps). With key intermediate **10** in hand, we prepared compound **1** and another 6-azide analogue of GDP-L-fucose (**16**)[30] according to the published procedure.[39] As anticipated, it was demonstrated that our approach permits an efficient and large-scale synthesis of versatile 6-azide-L-fucose analogues; these have become key intermediates for a further derivatization study to develop potent inhibitors and novel molecular probes. We are currently investigating the feasibility of using compound **16** for the construction of potent inhibitor libraries.



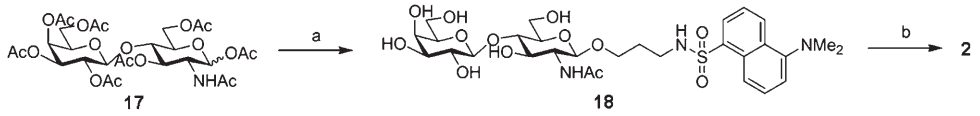
Scheme 2. Synthetic route of the GDP-L-fucose derivatives. a) ethanethiol, conc. HCl, 64%; b) i) *t*-butyldimethylsilyl chloride, pyridine, 0°C to RT; ii) 2,2-dimethoxypropane, pyridinium *p*-toluenesulfonic acid, CH_3CN, RT, 86%; c) i) *N*-bromosuccimide, 95% aq. acetone, 0°C; ii) $NaBH_4$, EtOH, RT, 79% (2 steps), d) *p*-toluenesulfonyl chloride, pyridine, RT, 97%; e) NaN_3, DMF, 60°C, 87%; f) $(tBu)_4NF$, THF, RT, 87%; g) i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78°C to 0°C; ii) 60% aq. AcOH, MeOH, 60°C; iii) benzoyl chloride, dimethylaminopyridine, pyridine, RT, 73% (3 steps); h) i) $TiBr_4$, EtOAc, CH_2Cl_2, RT; ii) $(BnO)_2PO_2H$, Ag_2CO_3, CH_2Cl_2, Et_2O, CH_3CN, RT, 70% (2 steps), i) i) H_2, Pd/C, MeOH, RT; ii) naphthyl acetic acid succinimidyl ester, diisopropyl ethyl amine, THF, RT, 64% (2 steps); j) Et_3N/MeOH, 60°C, 87%; k) GMP-morpholidate, 1*H*-tetrazole, pyridine, RT, 39%; l) i) NaOMe, MeOH, rt; ii) Ac_2O, pyridine, RT, quantitative yield; m) conditions described in reference [38] n) GMP-morpholidate, 1*H*-tetrazole, pyridine, RT, 3 days, 11%.

chemical and enzymatic procedure as indicated in Scheme 3.[32]

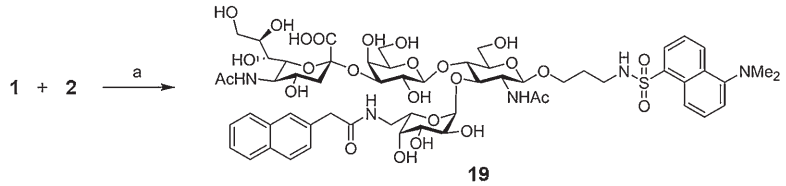
A coupling reaction between compounds **1** and **2** in the presence of human recombinant FucT VI was monitored under excitation at 290 nm, and the emission spectra were taken at appropriate intervals as shown in Figure 1. The results indicated that the fucosylation caused increases in the dansyl emission at 540 nm by FRET from the naphtylmethyl group in accordance with the progress of the reaction. In a similar manner, decreases in the naphtylmethyl emission at 340 nm, due to quenching by the dansyl group were also observed. In addition, the existence of the isofluorometric point at 480 nm clearly indicates that the attachment of the L-fucose residue to sialyl-LacNAc trisaccharide induces a drastic and convergent structural alteration. This result implies that the formation of a stable conformation of the sLe^x structure might be necessary for the specific interaction with partner carbohydrate-binding proteins such as P-selectin, E-selectin, and L-selectin. Figure 2 shows a typical FRET spectra, which was observed in the FucT VI-mediated reactions. The kinetic constants were determined to be $K_M = 0.94\ \mu M$ and $V_{max} = 0.14\ \mu M\,min^{-1}$, which indicates that compound **1** is a much better donor substrate than the parent GDP-L-fucose ($K_M = 5.5\ \mu M$).[9] The HPLC and MALDI analyses also suggested that the above assay condition generates only sLe^x derivative **19** from two fluorogenic substrates; the kinetic constants that were determined by HPLC methods were estimated to be $K_M = 3.5\ \mu M$ and $V_{max} = 0.46\ \mu M\,min^{-1}$ (Supporting Information).

Direct monitoring of human FucTs-mediated glycan synthesis by FRET: Fucosylation is often the final step in the biosynthetic pathways of many biologically significant glycan structures, such as sLe^x tetrasaccharides, which are critical selectin ligands that modulate cellular adhesion and lymphocyte recirculation.[13] To test the feasibility of **1** in the FRET-based direct monitoring of this unique glycan synthesis, a dansylated acceptor substrate **2** was readily synthesized by a

Next, our interest was directed to the feasibility of using compound **1** for the characterization of FucT VIII. For this purpose, we prepared the designated glycosyl acceptor substrate **20** according to the published procedure.[40] As expect-



Scheme 3. Preparation of a glycosyl acceptor substrate **2**. a) conditions described in reference [32]. b) CMP-Neu-5-Ac, rat recombinant α2,3-sialyltransferase, CIAP, pH 7.4, 37 °C, 2 days, 97 %.



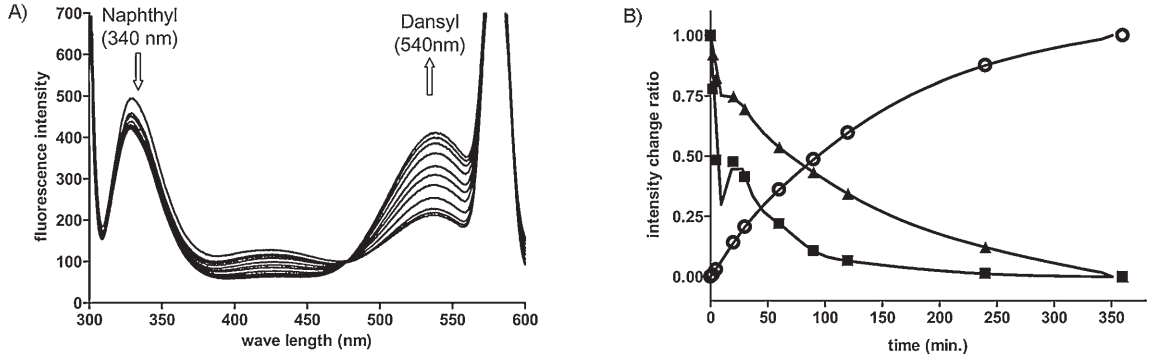


Figure 1. The FRET that was observed in FucT VI mediated sialyl Lewis X synthesis. Condition: a) **1** (20 μM), **2** (20 μM), FucT VI (3.5 mU), 20 mM HEPES buffer that contained 20 mM $MnCl_2$, pH 7.5, at 25 °C, total volume 2.5 mL; A) The time course of the relative fluorescence emission of **1** and **2** during sugar transferring with FucT VI. The slit width of excitation was 5 nm and emission was 20 nm, and emission spectra were scanned at 300 nm/min from 300 nm to 650 nm; B) Fluorescence change of naphthylmethyl (square), naphthalene excimer (triangle), and dansyl (circle).

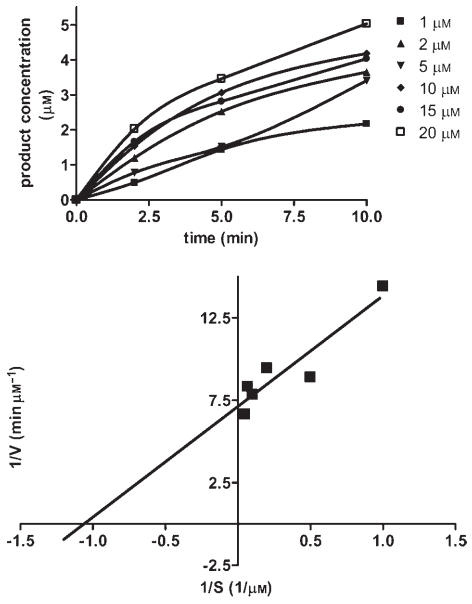


Figure 2. Determination of kinetic constants of **1** for FucT VI. Conditions: 1, 2, 5, 10, 15, and 20 μM of **1**, **2** (20 μM), and 3.5 mU FucT VI were incubated in 20 mM HEPES buffer that contained 20 mM $MnCl_2$, pH 7.5, at 25 °C for 10 min. A) Changes in the dansyl emission at 540 nm. B) Lineweaver–Burk plot of α1,3-fucosylation reactions measured as a function of the concentration of glycosyl donor substrate.

ed, the combined use of **1** and **20** allowed the FucT VIII-mediated synthesis of glycopeptide **21** to be monitored, as shown in Figure 3. The increase of the dansyl emission at 525 nm and the decrease of the naphtylmethyl emission at 340 nm were observed, and the spectra could be used for further kinetic studies. Interestingly, a typical isofluorometric point was not detected in the conversion of **20** into **21**; however one was observed in the case of the construction of sLe^x tetrasaccharide **19** from sialyl-LacNAc derivative **2** by fucosylation with FucT VI. Kinetic constants of **1** for human recombinant FucT VIII were estimated to be $K_M = 175$ μM and $V_{max} = 0.06$ μM min^{-1} (Figure 4). This means that modification at the C6 position of GDP-fucose with a sterically hindered aromatic group might slightly reduce the affinity of the parent GDP-fucose for this enzyme, because the K_M value of the GDP-fucose for recombinant FucT VIII was reported to be 19.3 μM by conventional HPLC analysis.[41] These results imply that the 3D structures of the donor-binding pockets of FucT VI and FucT VIII seem to be different. To achieve a desired FRET assay for FucT VIII-selective inhibitors, it is likely that an appropriate structural optimization might be required for enhancing the affinity and specificity of fluorogenic GDP-L-fucose donor substrates toward FucT VIII. However, it must be noted that the present strategy might not be feasible for discriminating

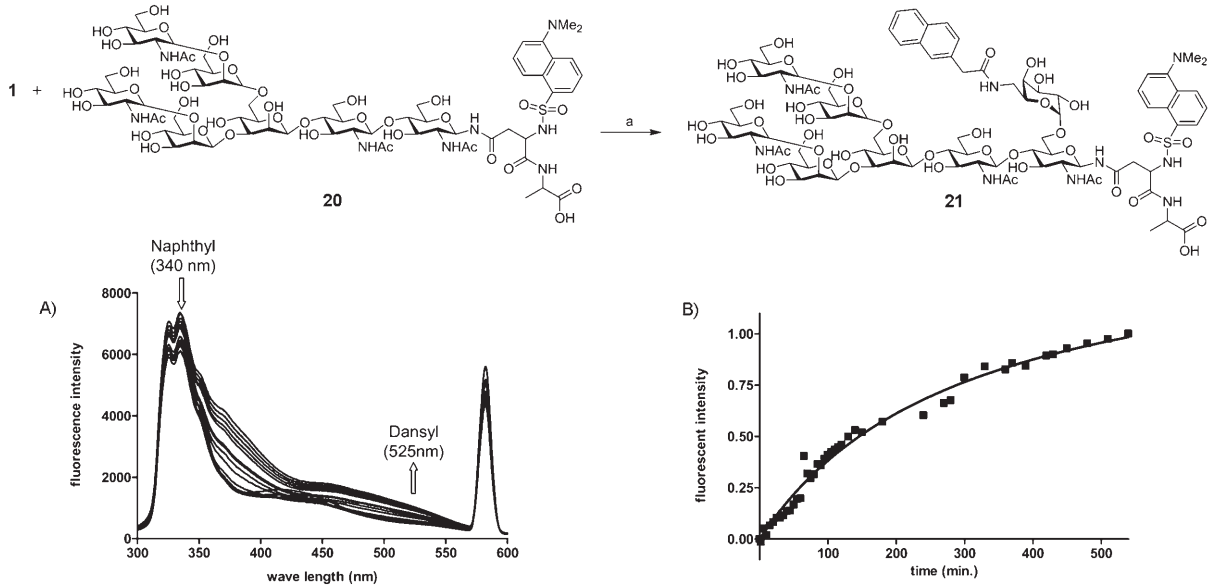


Figure 3. FRET analysis of the generation of glycopeptide **21** by FucT VIII in the presence of compound **1** and **20**. Conditions: a) **1** (50 μM), **20** (100 μM), 80 μU α1,6-FucT (FucT VIII), 50 mM sodium cacodyrate buffer, pH 7.5, total volume: 200 μL, 25 °C. A) The changes in the fluorescence emission spectra during the sugar-transfer reaction conducted by FucT VIII; B) Time course of the fluorescence intensity due to the dansyl emission at 525 nm.

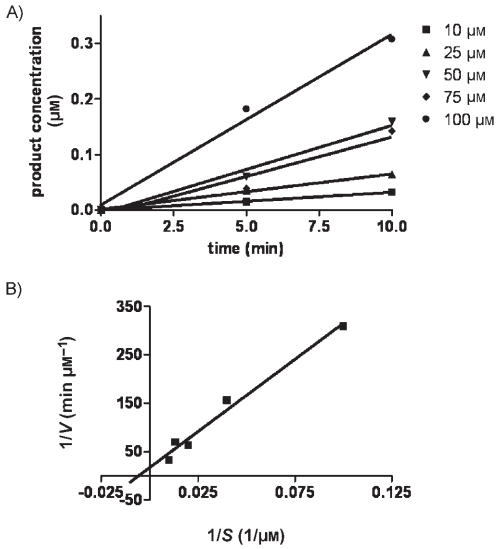
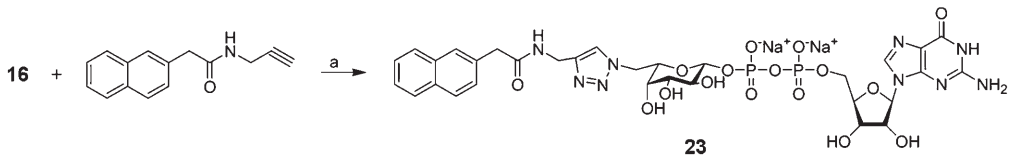


Figure 4. Determination of kinetic constants of **1** for FucT VIII. Conditions: 10, 25, 50, 75, 100 μM of **1**, **20** (100 μM), and 80 μU α1,6-FucT VIII were incubated in 50 mM sodium cacodylate buffer, pH 7.5, at 25 °C for 10 min. A) Changes in the dansyl emission at 525 nm. B) Lineweaver–Burk plot of α1,6-fucosylation reactions measured as a function of the concentration of glycosyl donor substrate.

the functions among several FucTs that exhibit very similar substrate specificity.

Feasibility of the FRET protocol for inhibitor screening: The versatility of the present protocol in the inhibitor screening of FucTs is evident, because the inhibitory effect can be detected simply as the decrease of the fluorescence intensity at 540 nm, which is due to dansyl emission by FRET under an excitation of naphtylmethyl group at 290 nm. For the proof of this principle, we tested preliminarily the effect of compound **23** on the FucT VI-mediated sLe^x tetrasaccharide (**19**) synthesis from **1** and **2**. Here, compound **23**[42] is a GDP-L-fucose analogue that was obtained by means of Cu^I-catalyzed [3+2] cycloaddition of 6-azide-GDP-L-fucose **16** (the kinetic constants of **16** were estimated as follows: $K_M = 19.9\ \mu M$ and $V_{max} = 0.073\ \mu M\,min^{-1}$ for FucT VI, Supporting Information) with 1-ethynyl-naphthalene (Scheme 4). The IC_{50} value of **23** was determined by measuring the decrease of the fluorescence intensity at 540 nm that was caused through FRET as shown in Figure 5. When the inhibition assay was carried out on the basis of the present FRET strategy, FucT VI activity was determined with a fixed concentration of **1** (20 μM, **2** (20 μM), and FucT VI (1.4 mU) in the presence of various concentrations of **23** (5, 10, and 15 μM). The estimated inhibitory effect by **23** ($IC_{50} = 5.4\ \mu M$) was in good quite agreement with the result that was obtained by the HPLC-based inhibition assay ($IC_{50} = 3.6\ \mu M$). Judging from the difference in the chemical structures of the very similar GDP-L-fucose analogues **1** and **23**, it is evident that the triazole ring that connects the naphtylmethyl group and GDP-L-fucose moiety is an important motif for this compound to function as a potent inhibitor for FucT VI. Interestingly, the naphtylmethyl group that was combined simply by an amide bond did not exhibit any inhibitory effect; this suggests that the triazole ring might bind tightly with this binding pocket by forming multiple hydrogen bonds rather than by contributing to hydrophobic interactions. The relative inhibitory effects of related compounds such as 2-naphtylphosphate, GMP, and CMP (all at 20 μM) were preliminarily compared



Scheme 4. Synthesis of a potential inhibitor **23** by means of the Crick reaction[9] of 6-azide-GDP-L-fucose (**16**) with 1-ethynyl-naphthalene. a) 6-azide-GDP-L-fucose **16** (2 mM), 1-ethynyl-naphthalene (2 mM), $CuSO_4$ (0.2 mM), and sodium ascorbate (2 mM) in H_2O/methanol (75:25 *v*/*v*, the total volume was adjusted to 50 μL) were incubated at room temperature for 7 h. The completion of the coupling reaction was monitored and judged by mass spectrometry as described in the Supporting Information. (50 μL) was added to the mixture to adjust the concentration of product to be 1 mM, and this solution was used directly for the subsequent inhibition assay.

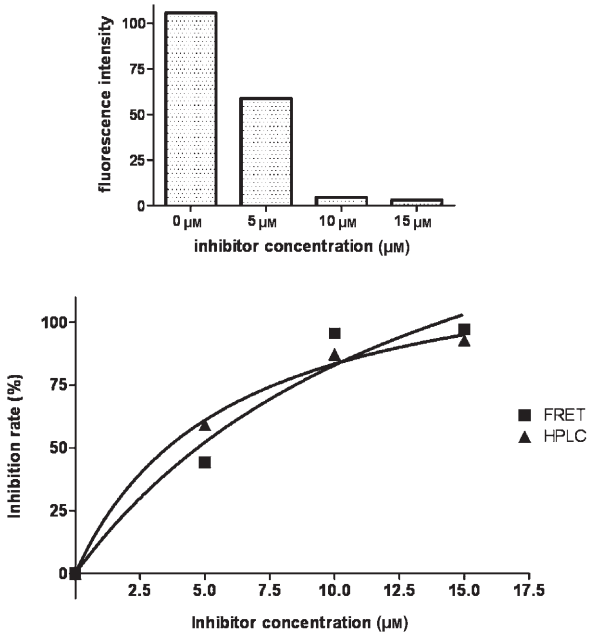


Figure 5. Inhibitory effect of compound **23** on the synthesis of sLe^x tetrasaccharide **19** by FucT VI in the presence of **1** and **2**. The IC_{50} value of **23** was determined by measuring the decrease of the fluorescence intensity at 540 nm (square) and by a conventional HPLC assay (triangle). FucT VI activity was determined with a fixed concentration of **1** (20 μM), **2** (20 μM) and FucT VI (1.4 mU) in the presence of various concentrations of **23** (5, 10, and 15 μM) in 20 mM HEPES buffer (pH 7.2) that contained 20 mM $MnCl_2$ (total volume 250 μL

by using **23** (20 μM) as a positive control, and the results are summarized in Table 1.

Table 1. The relative inhibitory effects determined by FRET assay. The effects of 2-naphtylphosphate, GMP, and CMP on the FucT VI-mediated sLe^x synthesis were evaluated by employing the following conditions: **1** (20 μM), **2** (20 μM), and FucT VI (0.7 mU) in 25 mM HEPES buffer (250 μL, pH 7.2) that contained 20 mM $MnCl_2$ at 25 °C for 1 h. The inhibition (%) was determined by comparing this result with the result that was obtained by using compound **23** as a positive control.

Compound[a]	Inhibition [%]	Compound[a]	Inhibition [%]
2-naphtylphosphate	26	GMP	56
CMP	35	**23**	100

[a] The concentration of each compound was 20 μM.

In conclusion, we have established an efficient synthetic route to highly useful GDP-L-fucose derivatives by starting from inexpensive D-galactose, through a key intermediate **10**. The use of these compounds in the FRET-based assay systems for the characterization of human recombinant FucT VI and FucT VIII has also been explored. This method allowed us to rapidly and easily evaluate the inhibitory effect of synthetic compounds on the FucT-mediated sLe^x synthesis, and we found that compound **23**, which is derived by click chemistry by using 6-azide GDP-fucose **16** is a potent inhibitor for FucT VI. It should also be noted that the present protocol might be suitable not only for investigating mammalian FucTs, but also for bacterial FucTs when a designated glycosyl acceptor substrate is optimized.

Experimental Section

Reagents and general methods: Recombinant human α1,3-fucosyltransferase VI (FucT VI, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of fucose from GDP-Fuc to *N*-acetyllactosamine per minute at 37 °C, pH 7.5) and recombinant rat α2,3-sialyltransferase (α2,3-siaT, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of sialic acid from CMP-Neu5 Ac to *N*-acetyllactosamine per minute at 37 °C, pH 7.4) were purchased from Calbiochem (San Diego, CA). Recombinant human α1,6-fucosyltransferase (FucT VIII, one unit of this enzyme is defined as the amount of enzyme that will transfer 1.0 μmol of fucose from GDP-Fuc to asparagine-linked *N*-acetyl-β-D-glucosamine of the chitobiose moiety per minute at 37 °C, pH 7.5) was purchased from Toyobo Co. Ltd.(Osaka, Japan). β-Galactosidase was purchased from Seikagaku Co. Ltd. (Tokyo, Japan). Solvents and other reagents for chemical synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Kasei (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification. ^1H and ^{13}C NMR was recorded with Bruker lambda 600 MHz (Bruker BioSpin, Rheinstten Germany). Chemical shifts are given in ppm and referenced to internal TMS (δ_H = 0.00 ppm in $CDCl_3$), $CHCl_3$ (δ_H = 7.26 ppm in $CDCl_3$), *tert*-BuOH (δ_H = 1.24 ppm in D_2O), or $CDCl_3$ (δ_C = 77.00 ppm). Assignments of the ^1H NMR spectra were made by first-order analysis, and were verified by H,H COSY, TOCSY, and HMQC experiments. Elemental analyses were performed with MT-6 CHN CORDER (Yanako, Japan). Thin-layer chromatography (TLC) was performed on Merck precoated plates (20 × 20 cm; layer thickness: 0.25 mm; silica gel $60F_{254}$); spots were visualized by spraying a solution of 90:5:5 (*v*/*v*/*v*) MeOH/*p*-anisaldehyde/concentrated sulfuric acid and by heating at 180 °C for about 30 s, and by UV light (256 or 365 nm), when applicable. Flash column chromatography was performed on silica gel 60 (spherical type, particle size 40–50 μm; Wako Pure Chemical, Osaka, Japan) with the solvent systems specified, and the ratio of solvent systems was given in (*v*/*v*). Organic extracts were

dried over anhydrous $MgSO_4$, and solutions were concentrated under diminished pressure below 50 °C. Column chromatography was performed on silica gel (Kanto Kagaku, Japan), Iatrobeads (6RS-8060, Jatron Laboratories, Mitsubishi Kagaku Iatron, Tokyo, Japan), Sephadex G-15 (GE Healthcare Bio-Sciences,Buckinghamshire England), Dowex 50W X8 (Muromachi Chemicals, Shizuoka, Japan), DEAE-Cellulose, or Wakogel C-18 (Wako Pure Chemical Industries Ltd., Osaka, Japan). Fluorescence measurement of emission spectra and excitation spectra was carried out by using a Hitachi F2500 fluorescence spectrometer. The excitation wavelength was 290 nm and the emission spectrum was scanned from 300 nm to 650 nm at 300 nm min^{-1}. The data were processed with FL-solution data manager software (Hitachi), Microsoft Excel, and GraphPad Prism. High-performance liquid chromatography (HPLC) was conducted on an Hitachi L-7100 HPLC system equipped with a Inertsil-ODS column (4.6 mm × 250 mm, GL Science Inc.) and Hitachi L-7485 fluorescence detector. Samples for MALDI-TOFMS were desalted and concentrated by using 10 μL C_{18} ZipTips™ (Millipore) according to the manufacturer's instructions. Typically, samples were dissolved in 1 μL of 10% trifluoroacetic acid in acetonitrile (*v*/*v*) and mixed with the same volume of a saturated solution of 2,5-dihydroxybenzoic acid in 33% acetonitrile that contained 0.1% trifluoroacetic acid. The above mixtures (1 μL) were applied to a stainless steel target MALDI plate and air-dried before analysis in the mass spectrometer. All measurements were performed by using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the Flexcontrol 1.2 software package (Bruker Daltonics GmbsH, Bremen, Germany). Ions were generated by a pulsed-UV laser beam (nitrogen laser, $\lambda = 337$ nm) and were accelerated to a kinetic energy of 23.5 kV. External calibration of MALDI mass spectra was carried out by using singly charged monoisotopic peaks of a mixture of human angiotensin II (*m*/*z*: 1046.542), bombesin (*m*/*z*: 1619.823), ACTH (*m*/*z*: 2465.199), and somatostatin 28 (*m*/*z*: 3147.472). The mixture of these peptides was measured on the central spot of a 3 × 3 square with external calibration. To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. The calibration of these mass spectra was performed automatically by using a customized macro command of the XMASS 5.1.2 NT software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the above-mentioned peptides.

Synthesis of novel GDP-fucose derivatives

6-*O*-*tert*-Butyldimethylsilyl-2,3:4,5-di-*O*-isopropylidene-D-galactose dimethyl dithioacetal (5): Compound **5** was synthesized from compound **4**[37] according to a previously reported procedure,[43] and the details are described in the Supporting Information.

6-*O*-*tert*-Butyldimethylsilyl-2,3:4,5-di-*O*-isopropylidene-D-galactitol (6): A solution of compound **5** (3.6 g, 7.49 mmol) in 95% aq. acetone (30 mL) was treated with *N*-bromosuccinimide (2.93 g, 16.5 mmol). The mixture was stirred at 0 °C for 5 min, and then $Na_2S_2O_3$ (5 g) and $NaHCO_3$ (5 g) were added. The reaction mixture was evaporated to remove acetone. The residue was dissolved in $CHCl_3$, and the solution was washed with brine, dried, and concentrated. The residual syrup was subjected to chromatography on silica gel (hexane/EtOAc 5:1). The fractions that contained the aldehyde were collected, concentrated, and the residue was dissolved in EtOH (20 mL). $NaBH_4$ (85 mg, 2.25 mmol) was added to this solution and the mixture was stirred at room temperature. After 40 min, the mixture was concentrated to a syrup and subjected to chromatography on silica gel (hexane/EtOAc 6:1) to afford the crude alcohol **6**. The syrupy **6** was dissolved in EtOH and the solution was stored in a refrigerator to yield pure compound **6** (2.22 g, 79%) as a white amorphous powder. $R_f = 0.53$ (hexane/EtOAc 2:1); ^{1}H NMR ($CDCl_3$): $\delta =$ 0.07–0.08 (m, 6H; $Si(CH_3)_2$), 0.87–0.94 (m, 9H; *t*Bu), 1.35, 1.36, 1.37, 1.38 (each s, 12H; *i*Pr), 3.59 (dd, $J_{5,6} = 12.0$ Hz, $J_{6\alpha,6\beta} = 5.6$ Hz, 1H; H6α), 3.72 (dd, $J_{1,2} = 11.3$ Hz, $J_{1\alpha,1\beta} = 4.6$ Hz, 1H; H1α), 3.79 (dd, $J_{5,6\beta} = 2.2$ Hz, 1H; H6β), 3.80 (dd, $J_{4,5} = 5.3$ Hz, $J_{3,4} = 2.4$ Hz, 1H; H4), 3.88 (t, $J_{1\beta,2} = 2.6$ Hz, 1H; H1β), 3.90 (t, $J_{2,3} = 1.3$ Hz, 1H; H3), 3.99–4.03 ppm (m, 2H; H2, H5); ^{13}C NMR ($CDCl_3$): $\delta = 0.10$, 0.19 ($Si(CH_3)_2$), 24.57 (*t*Bu), 31.73, 32.70, 32.73, 32.75 (*i*Pr), 68.73 (C6), 69.69 (C1), 88.19 (C4), 84.73 (C3), 88.19 (C5), 88.27 ppm (C2); HR-MS (FAB): *m*/*z* calcd for $C_{18}H_{36}O_6Si$: 377.2359; found: 377.2364 $[M+H]^+$.

6-*O*-*tert*-Butyldimethylsilyl-2,3:4,5-di-*O*-isopropylidene-1-*O*-*p*-toluenesulfonyl-D-galactitol (7): A cold solution of **6** (2.0 g, 5.31 mmol), 4-(dimethylamino)pyridine (1.30 g, 10.62 mmol) in pyridine (10 mL) was treated with *p*-toluenesulfonyl chloride (1.52 g, 7.97 mmol) in CH_2Cl_2 (2 mL) and the mixture was stirred at room temperature. After 15 h, the mixture was concentrated and the residue was dissolved in $CHCl_3$. The solution was washed with 1 N H_2SO_4, aq. sat. $NaHCO_3$, and brine. The organic layer was dried and concentrated. The residue was subjected to the purification by chromatography on silica gel (hexane/EtOAc 8:1), and the fractions that contained the product were collected and concentrated. The syrupy product was dissolved in EtOH and stored in a refrigerator to yield **7** (2.73 g, 97%) as white powdery precipitate. $R_f = 0.64$ (toluene/EtOAc 3:1); ^{1}H NMR ($CDCl_3$): $\delta = 0.09$ (m, 6H; $Si(CH_3)_2$), 3.89–3.96 (m, 9H; *t*Bu), 1.34, 1.35, 1.36. 1.39 (each s, 12H; *i*Pr), 2.48 (s, 3H; CH_3), 3.73 (dd, $J_{6\alpha,6\beta} = 11.3$ Hz, $J_{5,6\alpha} = 4.0$ Hz, 1H; H6α), 3.80 (t, $J_{2,3} = 7.7$ Hz, 1H; H2), 3.87 (d, 1H; H3), 3.89 (dd, $J_{5,6\beta} = 2.8$ Hz, 1H; H6β), 3.99 (m, 1H; H4), 4.13 (t, $J_{1\beta,2} = 5.1$ Hz, 1H; H1β), 4.15–4.17 (m, 1H; H5), 4.34 (dd, 1H; H1), 7.37, 7.84 ppm (each d, $J = 8.1$ Hz 4H; Ar); ^{13}C NMR ($CDCl_3$): $\delta =$ 0.59, 0.76 ($Si(CH_3)_2$), 24.44 (*t*Bu), 27.65 (CH_3), 31.96, 32.81, 33.03, 33.11 (*i*Pr), 68.94 (C6), 75.38 (C1), 83.40 (C3), 83.84 (C2), 84.20 (C5), 87.65 (C4), 134.11, 135.79 ppm (Ar); elemental analysis calcd (%) for $C_{25}H_{42}O_8SSi$: C 56.57, H 7.98, S 6.04; found: C 56.43, H 7.93, S 6.24; HR-MS (FAB): *m*/*z* calcd: 530.2370; found: 530.7467 $[M+H]^+$.

1-Azide-1-deoxy-6-*O*-*tert*-butyldimethylsilyl-2,3:4,5-di-*O*-isopropylidene-D-galactitol (8): A mixture of tosylate **7** (2.73 g, 5.14 mmol) and sodium azide (1.34 g, 20.56 mmol) in dimethylfolmamide (20 mL) was stirred at 60 °C for 24 h. The mixture was concentrated and the residue was dissolved in EtOAc. The solution was washed with brine, dried, and evaporated. The residue was purified by chromatography on silica gel (hexane/EtOAc 45:1), and the fractions that contained the crude product were collected and concentrated. The residual syrupy material was dissolved in EtOH and the solution was stored in a refrigerator to yield a precipitate. The white amorphous powder was obtained by filtration to give the azide **8** (1.80 g, 87%). $R_f = 0.68$ (toluene/EtOAc 7:1); ^{1}H NMR ($CDCl_3$): $\delta =$ 0.08 (m, 6H; $Si(CH_3)_2$), 0.91 (m, 9H; *t*Bu), 1.34, 1.35, 1.36, 1.42 (each s, 12H; *i*Pr), 3.31 (dd, $J_{5,6\alpha} = 5.9$ Hz, $J_{6\alpha,6\beta} = 14.1$ Hz, 1H; H6α), 3.60 (dd, $J_{5,6\beta} = 2.8$ Hz, 1H; H6β), 3.72 (dd, $J = 9.5$, $J = 11.3$ Hz, 1H; H1α), 3.84–3.90 (m, 3H; H1β, H3, H4), 4.00 (m, 1H; H2), 4.13 ppm (m, 1H; H5); ^{13}C NMR ($CDCl_3$): $\delta = 1.21$, 1.34 ($Si(CH_3)_2$), 32.85 (*t*Bu), 33.65–33.83 (*i*Pr), 59.5 (C6), 70.69 (C1), 85.11 (C3), 86.28 (C4), 87.73 (C5), 89.52 ppm (C2); elemental analysis calcd (%) for $C_{18}H_{35}N_3O_5Si$: C 53.84, H 8.78, N 10.46; found: C 53.75, H 8.74, N, 10.39; HR-MS (FAB): *m*/*z* calcd: 402.2424; found: 402.2444 $[M+H]^+$.

1-Azide-1-deoxy-2,3:4,5-di-*O*-isopropylidene-D-galactitol (9): Compound **8** (1.80 g, 4.48 mmol) was dissolved in THF (10 mL) at 0 °C. Then, a 1 M THF solution of tetrabutyl ammonium fluoride (TBAF; 6.17 mL, 5.28 mmol) was added to this solution. The mixture was stirred at room temperature for 1 h. The mixture was concentrated and the residue was subjected to chromatography on silica gel (hexane/EtOAc 4:1). The fractions that contained product were collected and concentrated. The syrupy crude material was dissolved in EtOH and the solution was stored in refrigerator to give white powdery **9** (1.80 g, 87%). $R_f = 0.21$ (toluene/EtOAc 7:1); ^{1}H NMR ($CDCl_3$): $\delta = 1.34$, 1.36, 1.38, 1.42 (each s, 12H; *i*Pr), 3.32 (dd, $J_{6\alpha,6\beta} = 13.2$ Hz, $J_{5,6\alpha} = 5.1$ Hz, 1H; H6α), 3.60 (dd, $J_{1\alpha,1\beta} =$ 12.0 Hz, $J_{1\alpha,2} = 5.6$ Hz, 1H; H1α), 3.61 (dd, $J_{5,6\beta} = 2.8$ Hz, 1H; H6β), 3.77 (t, $J_{3,4} = 7.5$ Hz, 1H; H3), 3.79 (dd, $J_{1\beta,2} = 2.4$ Hz, 1H; H1β), 3.85 (t, $J_{4,5} =$ 8.0 Hz, 1H; H4), 4.03 (m, 1H; H2), 4.13 ppm (m, 1H; H5); ^{13}C NMR ($CDCl_3$): $\delta = 28.90$–29.80 (*i*Pr), 54.76 (C6), 64.04 (C1), 80.81 (C3), 81.50 (C4), 83.09 (C5), 84.82 ppm (C2); elemental analysis calcd (%) for $C_{12}H_{21}N_3O_5$: C 50.16, H 7.37, N 14.63; found: C 50.30, H 7.18, N 14.52; HR-MS (FAB): *m*/*z* calcd: 288.1559; found: 288.1556 $[M+H]^+$.

6-Azide-1,2,3,4-tetra-*O*-benzoyl-6-deoxy-β-L-galactopyranose (10): Compound **9** (1.20 g, 4.18 mmol) was added to a cooled solution (−78 °C) of oxalyl chloride (0.73 mL, 8.35 mmol) and DMSO (1.19 mL, 16.7 mmol) in CH_2Cl_2 (5 mL). After 15 min, Et_3N (2.91 mL, 20.88 mmol) was added and the mixture was stirred at 0 °C for 2 h. The mixture was then poured into

$CHCl_3$ and the solution was washed with brine, dried, and concentrated. The residue was purified roughly by silica gel column chromatography (hexane/EtOAc 8:1). The fractions that contained the aldehyde were collected and concentrated. The residue was dissolved in a mixture of methanol (5 mL) and 60% aq. acetic acid (10 mL), and stirred at 60°C. After 4 h, the solution was concentrated and co-evaporated with toluene to remove water, and the resulted syrup was dissolved in pyridine (30 mL). Benzoyl chloride (2.33 mL, 20.05 mmol) was added to the solution and the mixture was stirred at room temperature for 24 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$ and the organic layer was washed with 1 N H_2SO_4, aq. sat. $NaHCO_3$, and brine; dried; and concentrated. The residue was purified by chromatography on silica gel (hexane/EtOAc 5:1) to afford compound **10** (1.90 g, 73%). R_f=0.61 (toluene/EtOAc 7:1); ^{1}H NMR ($CDCl_3$): δ=3.42 (dd, $J_{5,6\alpha}$=5.6 Hz, $J_{6\alpha,6\beta}$=13.1 Hz, 1H; H6α), 3.61 (dd, $J_{5,6\beta}$=7.2 Hz, 1H; H6β), 4.59 (m, 1H; H5), 6.01–6.06 (m, 3H; H2, H3, H4), 6.94 (d, $J_{1,2}$=3.6 Hz, 1H; H1), 7.26–8.14 ppm (m, 20H; Ar); ^{13}C NMR ($CDCl_3$): δ=54.88 (C6), 71.90, 72.94, 73.34 (C2, C3, C4), 94.90 ppm (C1); elemental analysis calcd (%) for $C_{34}H_{27}N_3O_9$: C 65.70, H 4.38, N 6.76; found: C 65.66, H 4.43, N 6.89; HR-MS (FAB): *m*/*z* calcd for $[M+Na]^+$ $C_{34}H_{27}N_3O_9Na$: 644.1645; found: 644.1646.

6-Azide-2,3,4-tri-*O*-benzoyl-6-deoxy-β-L-galactopyranos-1-yl-dibenzyl phosphate (11): A solution of benzoate **10** (500 mg, 0.80 mmol) in CH_2Cl_2 (10 mL) and EtOAc (1 mL) was treated with $TiBr_4$ (1.18 g, 3.2 mmol) and the mixture was stirred at room temperature. After 24 h, sodium acetate (2.0 g) was added and the mixture was stirred for 10 min at room temperature. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The organic layer was washed with water, dried, and concentrated. The residual syrup was co-evaporated with toluene to remove water. The dried material was then dissolved in a mixture of CH_2Cl_2 (3 mL), acetonitrile (3 mL), and Et_2O (3 mL). Crushed molecular sieves (3 Å, 2 g) were added, and the mixture was stirred under nitrogen for 30 min. Dibenzyl phosphate (445.2 mg, 1.60 mmol) and silver carbonate (441.2 mg, 1.60 mmol) were added to this solution, and the mixture was stirred at room temperature under nitrogen. After 7 h, the molecular sieves were removed by filtration, and the filtrate was concentrated. The residue was subjected to the purification by chromatography on silica gel (0.5% *w*/*w* Et_3N in hexane/EtOAc 3:1). The fractions that contained the product were collected and concentrated. The syrupy material was lyophilized with benzene to afford a white amorphous powdery **11** (350 mg, 70%). R_f=0.25 (toluene/EtOAc 7:1); ^{1}H NMR ($CDCl_3$): δ=3.41 (m, 1H; H6α), 3.59 (m, 1H; H6β), 4.20 (m, 1H; H5), 4.85, 4.77 (each m, 2H; CH_2), 5.11–5.15 (m, 2H; CH_2), 5.58 (d, J=9.8 Hz, 1H; H3), 5.74 (t, $J_{1,P}$=$J_{1,2}$=7.2 Hz, 1H; H1), 5.89–5.94 ppm (m, 2H; H2, H4); ^{13}C NMR ($CDCl_3$): δ=53.10 (C6), 70.86 (C4), 72.07 (C2), 72.22 (CH_2), 72.26 (CH_2), 73.95 (C3), 76.38 (C5), 99.61 (C1), 129.08–136.38 ppm (Ar); HR-MS (FAB): *m*/*z* calcd for $C_{41}H_{36}N_3O_{11}P$: 778.2166; found: 778.2147 $[M+H]^+$.

6-Deoxy-2,3,4-tri-*O*-benzoyl-6-*N*-(2-naphthalen-2-yl-acetamide)-β-L-galactopyranos-1-yl-phosphate triethylammonium salt (12): A mixture of compound **11** (145 mg, 0.19 mmol) and 10% palladium/charcoal (300 mg) in methanol (5 mL) was stirred at room temperature under a hydrogen atmosphere. After 1 h, the palladium/charcoal was removed by filtration, and the filtrate was concentrated. Naphthyl acetic acid succinimidyl ester (80.7 mg, 0.29 mmol) and diisopropyl ethylamine (33.0 μL, 0.19 mmol) were added to a solution of this material in THF (5 mL), and the mixture was stirred at room temperature for 24 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The organic layer was washed with water, dried, and concentrated. The residual syrup was purified by chromatography on silica gel (0.5% *w*/*w* Et_3N in $CHCl_3$/MeOH 4:1) to afford a white amorphous powdery **12** (92.2 mg, 64%). R_f=0.85 ($CHCl_3$/MeOH/water 5:4:1); ^{1}H NMR (MeOD): δ=3.34 (dd, $J_{5,6\alpha}$=8.2 Hz, $J_{6\alpha,6\beta}$=13.9 Hz, 1H; H6α), 3.69 (dd, $J_{5,6\beta}$=4.6 Hz, 1H; H6β), 3.69 (s, 2H; CH_2), 4.27 (m, 1H; H5), 5.56 (t, $J_{1,2}$=$J_{1,P}$=7.9 Hz, 1H; H1), 5.66 (dd, $J_{2,3}$=10.3 Hz, $J_{3,4}$=3.4 Hz, 1H; H3), 5.75 (dd, $J_{1,2}$=7.9, $J_{2,3}$=10.3 Hz, 1H; H2), 5.84 (d, J=3.4 Hz, 1H; H4), 7.24–8.03 (m, 22H; Ar); ^{13}C NMR (MeOD): δ=22.52 (C6), 72.50 (C4), 73.83 (C2), 75.36 (C3), 76.31 (C5), 98.91 (C1), 130.20–132.59 (ar); HR-MS (FAB): *m*/*z* calcd for $C_{39}H_{34}NO_{12}P$: 738.1746; found: 738.1747 $[M-H]^-$.

6-Deoxy-6-*N*-(2-naphthalen-2-yl-acetamide)-β-L-galactopyranos-1-yl-phosphate triethylammonium salt (13): A mixture of tri-*O*-benzoate **12** (86.5 mg, 0.12 mmol) and Et_3N (1 mL) in MeOH (5 mL) was stirred at 60°C for 4 h. The mixture then was concentrated and the residual syrup was purified by chromatography on Iatrobeads (0.5% *w*/*w* Et_3N in $CHCl_3$/MeOH 2:1). The fractions of the product were collected and concentrated and the residual syrup was lyophilized with water to afford a white amorphous **13** (49.4 mg, 87%). R_f=0.52 (chloroform/MeOH/water 5:4:1); ^{1}H NMR (MeOD): δ=3.36 (dd, $J_{5,6\alpha}$=8.3 Hz, $J_{6\alpha,6\beta}$=14.1 Hz, 1H; H6α), 3.55 (dd, $J_{5,6\beta}$=4.6 Hz, 1H; H6β), 3.60 (dd, $J_{2,3}$=10.0 Hz, $J_{1,2}$=7.6 Hz, 1H; H2), 3.66 (dd, $J_{3,4}$=3.4 Hz, 1H; H3), 3.70 (m, 1H; H5), 3.78 (s, 2H; CH_2), 3.80 (d, $J_{4,5}$=3.2 Hz, 1H; H4), 4.90 (t, $J_{1,P}$=7.7 Hz, 1H; H4), 7.55–7.57 (m, 3H; Ar), 7.90–7.92 (m, 4H; Ar); ^{13}C NMR (MeOD): δ=43.10 (C6), 45.14 (CH_2), 71.94 (C4), 74.81 (C2), 75.25 (C3), 76.18 (C5), 100.57 (C1), 129.00–131.25 (ar); HR-MS (FAB): *m*/*z* calcd for $C_{18}H_{22}NO_9P$: 426.0959: found: 426.3349 $[M-H]^-$.

6-Deoxy-6-*N*-(2-naphthalen-2-yl-acetamide)-β-L-galactospyranos-1-yl-guanosine 5′-diphosphate disodium salt (1): Compound **13** (40 mg, 0.08 mmol) was co-evaporated with pyridine three times, and the residual syrup was dissolved in dry pyridine (4 mL). GDP-morphoridate (114.2 mg, 0.16 mmol) and 1*H*-tetrazole (22.4 mg, 0.32 mmol) was added to this solution and the mixture was stirred at room temperature under nitrogen for 72 h. Then, the mixture was concentrated and the residue was subjected to purification by chromatography on DEAE-Cellulose (0.1 M aq. ammonium bicarbonate). The fractions that contained the target material were collected and directly subjected to treatment with Dowex 50W×8 (Na^+ form). The solution was then concentrated and the residue was purified by chromatography on Wako-gel C18 (water only). The obtained material was finally purified by chromatography on a Sephadex G15 column (water only) to yield white amorphous **1** (25.2 mg, 39%). ^{1}H NMR (D_2O): δ=3.33 (dd, J= $J_{5,6\alpha}$=14.1, $J_{6\alpha,6\beta}$=9.8 Hz, 1H; H6″α), 3.63 (m, 2H; H2″, H6″β), 3.71 (m, 3H; H3″, CH_2), 3.76 (dd, $J_{4'',5''}$=2.3 Hz, 1H; H5″), 3.89 (d, $J_{3'',4''}$=3.0, 1H; H4″), 4.30 (s, 2H; H5′), 4.35 (s, 1H; H4′), 4.49 (m, 2H; H2′, H3′), 5.00 (t, $J_{1'',2''}$=$J_{1'',P}$=7.9 Hz, 1H; H4″), 5.72 (d, $J_{1',2'}$=4.4 Hz, 1H; H1′), 7.27–7.96 (m, 7H; naphthyl), 8.59 ppm (s, 1H; H8); ^{13}C NMR (D_2O): δ=43.14 (C6″), 44.91 (CH_2), 67.78 (C5′), 71.98 (C4″), 75.01 (C3″), 76.49 (C5″), 86.18 (C4′), 89.84 (C1′), 101.26 (C1″), 127.64, 128.58, 128.97, 129.76, 130.18, 130.29. 130.78, 131.261 ppm (naphthyl); HR-MS (FAB): *m*/*z* calcd for $C_{28}H_{35}N_6O_{16}P_2$: 773.1585: found: 773.5556 $[M+H]^+$.

6-Azide-6-deoxy-β-L-galactopyranos-1-yl-guanosine 5′-diphosphate disodium salt (16):[30] Compound **10** (820 mg, 1.32 mmol) and NaOMe (0.4 equiv, 17.28 mg) were dissolved in EtOH (5 mL) and the solution was stirred for 1 h. Then the mixture was neutralized with Dowex 50W×8 (H^+ form) and filtered. The filtrate was concentrated and the residue was dissolved in pyridine (3 mL). Acetic anhydride (2 mL) was added to this solution and the mixture was stirred at room temperature for 4 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The organic layer was washed with 1 N HCl, sat. $NaHCO_3$, and brine; dried; and concentrated. The residue was subjected to chromatography on silica gel (hexane/EtOAc 3:1) to provide a known 6-azide-6-deoxy-1,2,3,4-tetra-*O*-acetyl-α-L-galactopyranose (**14**)[38] (493 mg, quantitative yield). ^{1}H NMR ($CDCl_3$): δ=2.01, 2.03, 2.17, 2.18 (each s, 3H; Ac), 3.22 (dd, $J_{5,6\alpha}$=7.5, $J_{6\alpha,6\beta}$=5.4, 1H; H6α) 3.45 (dd, $J_{5,6\beta}$=7.5 1H; H6β), 4.23 (t, $J_{5,6\alpha}$=$J_{5,6\alpha}$=5.7 Hz, 1H; H5), 5.34 (m, 2H; H2, H3), 5.48 (s, 1H; H4), 6.40 ppm (s, 1H; H1). This material **14** (373 mg, 1.0 mmol) was converted directly into 6-azide-6-deoxy-β-L-galactopyranosyl phosphate *n*-tetrabutylammonium salt (**15**)[38] (72 mg, overall yield 18%). ^{1}H NMR (D_2O): δ=1.31, 3.17 (Et_3N) 3.49 (dd, $J_{5,6\alpha}$=12.9, $J_{6\alpha,\beta}$=7.2 Hz, 1H; H6a), 3.53 (dd, $J_{2,3}$=9.7, $J_{3,4}$=3.4 Hz, 1H; H3), 3.54–3.57 (m, 2H; H5, H6β), 3.66 (t, $J_{1,2}$=$J_{2,3}$=5.3 Hz, 1H; H2), 3.77 (d, $J_{3,4}$=4.0 Hz, 1H; H4), 4.89 ppm (t, $J_{1,2}$=$J_{1,P}$=7.6 Hz, 1H; H1) by phosphorylation and the subsequent de-*O*-acetylation according to the reported procedure.[38]

Compound **15** (50 mg, 0.098 mmol) was co-evaporated with pyridine three times to remove water and the residual syrup was dissolved in dry pyridine (1 mL). GMP-morpholidate (142 mg, 2.0 equiv) and 1*H*-tetrazole (27.4 mg, 4.0 equiv) were added to this solution and the mixture was stirred at room temperature under nitrogen for 48 h. The mixture was

concentrated and the residue was dissolved in water. The aqueous solution was washed with Et_2O and concentrated. The residue was subjected to purification by a series of chromatography on Wako-gel C18 (water only) and DEAE-Cellulose (0.06 mM ammonium bicarbonate aq.), Sephadex G25 (water only), and Dowex 50W×8 (Na^+ form, water only). The factions that contained the product were collected and lyophilized to yield a white amorphous **16** (9.6 mg, 11%). ^{1}H NMR (D_2O): δ=8.52 (s, 1H; H8), 5.99 (d, $J_{1,2}$=5.1 Hz, 1H; H1′), 4.96 (t, $J_{1'',2''}=J_{1'',P''}$=7.8 Hz, 1H; H1″), 3.88 (d, $J_{3,4}$=3.2 Hz, 1H; H4″), 3.79 (m, 1H; H5″), 3.67 (dd, $J_{2'',3''}$= 9.6, $J_{3'',4''}$=3.6 Hz, 1H; H3″), 3.61–3.59 (m, 2H; H2″, H6α″), 3.46 ppm (dd, $J_{5,6}$=13.0 Hz, $J_{6\alpha,6\beta}$=5.5 Hz, 1H; H6β″); ^{13}C NMR (D_2O): δ=98.52 (C1″), 86.84 (C1′), 73.66 (C5″), 72.16 (C2″), 70.48 (C3″), 68.57 (C4″), 22.61 ppm (C6″); HR-MS (FAB): *m*/*z* calcd for $C_{16}H_{22}N_8O_{15}P_2Na$: 651.0583; found 651.0587 $[M+Na]^+$. These physical data were found to be in good agreement with the data that were reported recently by Wong et al. (see reference [30]).

Preparation of dansylated sialyl-LacNAc (2): Dansyl-*N*-acethyllactosamine (**18**), which was prepared from per-*O*-acetate **17** by the previously reported procedure[32] was subjected to the sialylation by using recombinant rat α2,3-siaT as follows: α2,3-siaT (50 mU) was added to a solution of **18** (11.0 mg, 0.16 mmol) in 50 mM sodium cacodyrate buffer (300 μL, that contained 5 mM $MnCl_2$, 1 mM NaN_3, 0.05% *v*/*v* CF-54, 300 μg BSA, pH 7.4) and CMP-Neu5 Ac (14.51 mg, 1.35 equiv) . The reaction mixture was incubated at 37°C for 2 d. After ultracentrifugation, the mixture was concentrated and the residue was subjected to purification on DEAE-cellulose column chromatography with 30 mM aq. ammonium bicarbonate. The fractions that contained the product were collected and concentrated, and the residue was treated with Dowex 50W×8 (Na^+ form). The solution was concentrated and subjected to the chromatography on Sephadex G25 (water only). The fractions that contained the product were collected and lyophilized to afford white amorphous **2** (15.7 mg, 97%). ^{1}H NMR (D_2O) δ=1.57–1.62 (m, 2H; CH_2), 1.72 (t, $J_{3''ax,3''eq}=J_{3''ax,4}$= 11.6 Hz, 1H; H3″ax), 1.95, 2.03 (each s, 6H; Ac), 2.84–2.85 (m, 7H; NMe×2, H3″eq), 2.90–2.93 (m, 2H; CH_2), 3.39–3.43 (m, 1H; CH_2), 3.49 (d, J=9.0 Hz, 1H; H8″), 3.55–3.78 (m, 13H; H3, H5, H6α, H6β, H2′, H5′, H6α′, H6β′, H4″, H7″, H9α″, H9β″, CH_2), 3.83–3.91 (m, 5H; H2, H4, H4′, H5″, H6″), 4.04 (dd, $J_{2,3}$=9.7 Hz, $J_{3,4}$=3.0 Hz, 1H; H3′), 4.28 (d, $J_{1,2}$=8.4 Hz, 1H; H1), 4.43 (d, $J_{1'2}$=7.8 Hz, 1H; H1), 7.28 (d, J=7.6 Hz, 1H; Ar), 7.57–7.61 (m, 2H; Ar), 8.18 (dd, J=1.2, J=7.3 Hz, 1H; Ar), 8.35 (d, J=8.7 Hz, 1H; Ar), 8.56 ppm (d, J=8.5 Hz, 1H; Ar); ^{13}C NMR (D_2O): δ=62.75 (C5), 63.74 (C5′), 65.34 (C4), 69.87 (C6″), 70.21 (C4′), 71.07 (C2), 72.10 (C2′), 74.49 (C3), 77.38 (C6), 78.24 (C3′), 103.72 (C1), 105.32 (C1′), 118.30, 122.10, 126.966, 131.54, 132.47, 134.23 ppm (ar); HR-MS (FAB): *m*/*z* calcd for $C_{40}H_{60}N_4O_{21}SNa$: 987.3369; found: 987.3383 $[M+Na]^+$.

Preparation of dansylated *N*-glycopeptide 20: Glycopeptide acceptor **20** was prepared by a modification of a known asparagine-linked glycopeptide that carries a biantennary asialoglycan that was isolated from egg yolk.[40] The crude asialoglycopeptide was dansylated[44] and subsequently treated with 25 mU β-galactosidase in 50 mM sodium acetate buffer (pH 5.0) at 37°C for 14 h. The reaction mixture was directly subjected to purification by reverse phase C_{18} HPLC to afford pure compound **20**. HR-MS (FAB): *m*/*z* calcd for $C_{69}H_{106}O_{41}N_8S$: 1733.6098; found: 1733.6233 $[M-H]^-$.

FRET-based continuous assay of glycosylation reactions by FucTs: The coupling reaction of donor substrate **1** and an acceptor **2** by FucT VI was carried out in a quartz cuvette (5 mL) at 25°C in 20 mM HEPES buffer (2.0 mL, pH 7.5) that contained 20 mM $MnCl_2$ (total volume: 2.5 mL). An emission spectrum under an excitation at 290 nm was taken immediately after the enzyme addition, and this was regarded as the 0 min spectrum, and the time course of the relative fluorescence emission of **1** (20 μM) and **2** (20 μM) during sugar transfer with FucT VI (3.5 mU) was investigated. The slit width of excitation was 5 nm and emission was 20 nm, and emission spectra were scanned from 300 nm to 650 nm at 300 nm min^{-1}. As for the determination of the kinetic constants of **1** for FucT VI, the reactions were carried out in 20 mM HEPES buffer (2.0 mL, pH 7.5) that contained 20 mM $MnCl_2$, in the presence of **1** (1–20 μM), **2** (20 μM), and FucT VI (3.5 mU).

In the case of FucT VIII, the general condition was as follows: glycosyl donor **1** (10–100 μM), an acceptor **20** (100 μM), and FucT VIII (80 μU) were incubated in 50 mM sodium cacodyrate buffer (200 μL, pH 7.5) by using a 700 μL cuvette.

Inhibition assay by FRET method: The FRET-based inhibition assay was performed by employing a fixed condition as follows: **1** (20 μM), **2** (20 μM), and FucT VI (1.4 mU) in 20 mM HEPES buffer (250 μL, pH 7.5) that contained 20 mM $MnCl_2$ at 25°C for 2 h. To measure the IC_{50} of compound **23**, the inhibitory effect by compound **23** (5, 10, and 15 μM) was evaluated by measuring the change (decrease) of the dansyl emission at 540 nm in comparison with the standard emission spectrum in the absence of the inhibitor. The glycosylation reactions were stopped by the addition of 250 μL of acetonitrile. The reactions were monitored by FRET and HPLC (Supporting Information). Relative inhibitory effects by 2-naphtylphosphate, GMP, and CMP on FucT VI activity were determined by employing the following condition: **1** (20 μM), **2** (20 μM), each compound (20 μM), and FucT VI (0.7 mU) in 25 mM HEPES buffer (250 μL, pH 7.2) that contained 20 mM $MnCl_2$ at 25°C for 1 h.

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