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Synthesis of GDP-5-thiosugars and Their Use as Glycosyl Donor Substrates for Glycosyltransferases

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Two thiopyranoside analogues of GDP-sugars, GDP-5-thio-D-mannose (**14**) and GDP-5-thio-L-fucose (**15**), were synthesized. The syntheses included the phosphorylations of tetra-*O*-acetyl-5-thio-D-mannosyl bromide (**4**) and tri-*O*-benzoyl-L-fucosyl bromide (**6**) with silver dibenzyl phosphate, deprotection of the phosphate groups, and condensation of the deprotected phosphates with GMP-imidazolidate (**13**) in the presence of $MgCl_2$. These GDP-sugar analogues were found to be donor substrates for α(1,2)mannosyltransferase and α(1,3)fucosyltransferase, affording a 5-thiomannose-containing disaccharide (**18**) and a 5-thiofucose-containing trisaccharide (**21**), respectively. The conformation of the disaccharide analogue **18** was similar to that of its native counterpart by ROESY. These findings for GDP-5-thiosugars together with previous demonstrations of enzymatic transfer from UDP-5-thiosugars will allow the production of panels of oligosaccharide analogues with hydrolase-resistant properties.

Introduction

Glycosidase-resistant oligosaccharide analogues are expected to survive for a longer time in vivo than their native counterparts. As such, they would be able to confer metabolic stability to oligosaccharide-based drugs similar to drugs based on peptide mimetics, e.g., buserelin and octreotide.[1] Glycosidase-resistant oligosaccharides such as S-glycosides[2] and C-glycosides[3] can also be used for biochemical studies. For example, in the X-ray analysis of the complex between endoglucanase-I and a cellopentoside analogue in which all the interglycosidic bonds are sulfides and therefore hydrolase-resistant, a stable conformation was captured in the enzyme−substrate complex.[4] The availability of glycosidase-resistant oligosaccharide analogues would be valuable for the discovery of biologically active oligosaccharide ligands, even when the target receptor is a hydrolase or the assay system involves administration to cells or organisms rich in hydrolases. However, it may be difficult to construct a library containing S- or C-glycoside in all possible positions. In a simple case such as the construction of an S-linked glucose−glucose disaccharide library using a normal glucosyl donor, one needs to elaborate four intricate glucose analogues, each having a thiol group in place of a hydroxyl group from OH-2 to OH-6.

Alternatively, 5S-glycosides, the ring sulfur analogues of native glycosides, are also resistant to glycosidases.[5−7] Since 5S-glycosides can be synthesized by the usual glycosylation methods with various 5S-glycosyl donors, library construction appears as feasible as that of their corresponding native counterparts. However, chemical glycosylation with 5S-glycosyl donors gives mainly α-glycosides[7−13] even in the presence of an equatorial acetoxy group that would generally promote β-glycoside formation by anchimeric assistance. Therefore, it would be difficult to chemically synthesize 5S-analogues with the β-gluco or β-galacto configurations that are ubiquitous in natural oligosaccharides.

It has been shown that UDP-5-thiogalactose (UDP-5SGal)[5] and UDP-*N*-acetyl-5-thiogalactosamine (UDP-5SGalNAc)[14] are substrates for galactosyltransferase, giving 5SGalβ(1,4)GlcNAc and 5SGalNAcβ(1,4)GlcNAc

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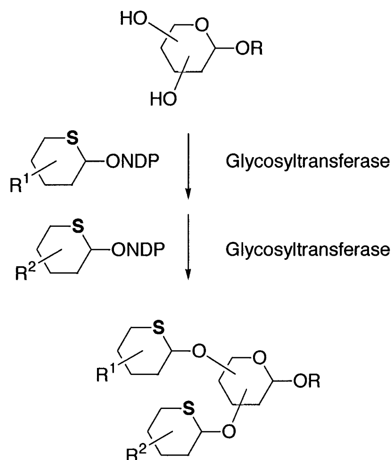
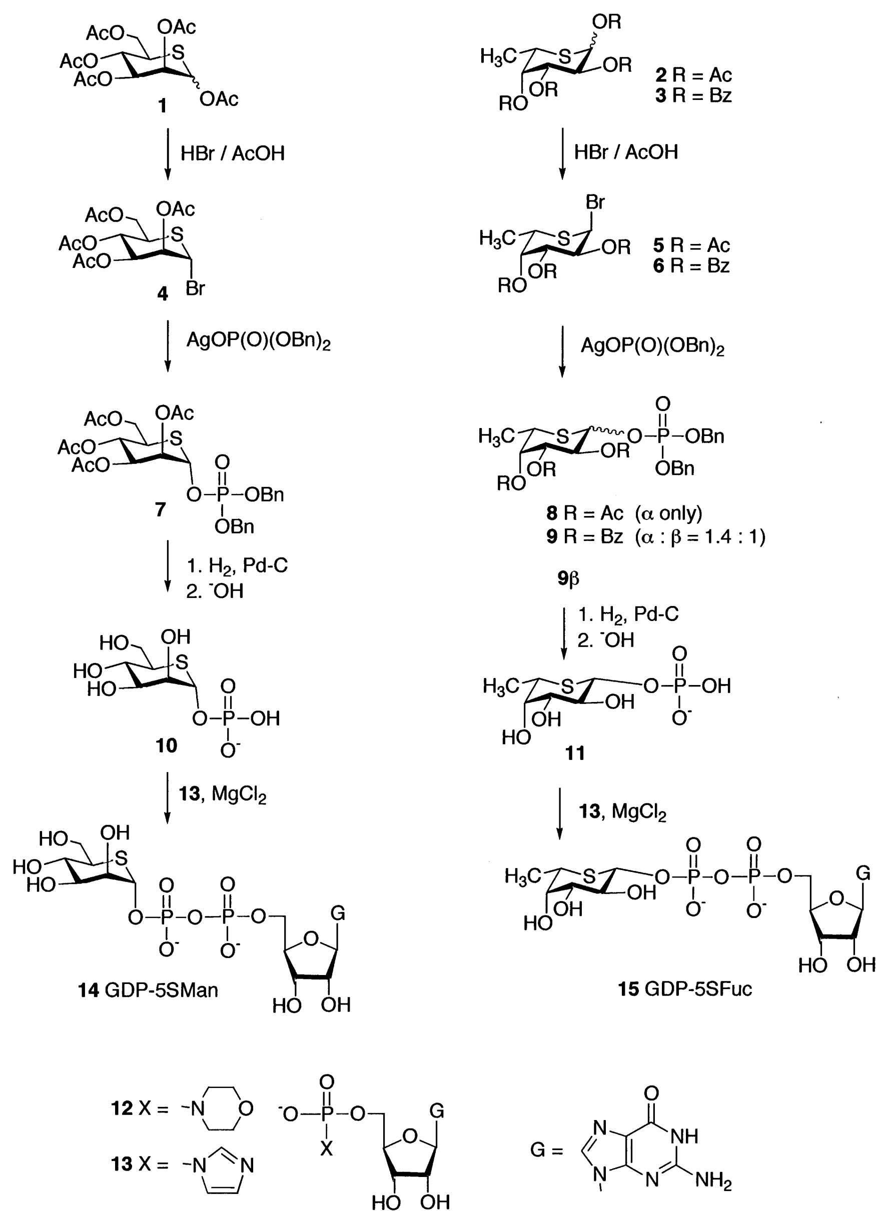


FIGURE 1. Construction of the 5-thiosugar-based oligosaccharide mimetics library with glycosyltransferases.

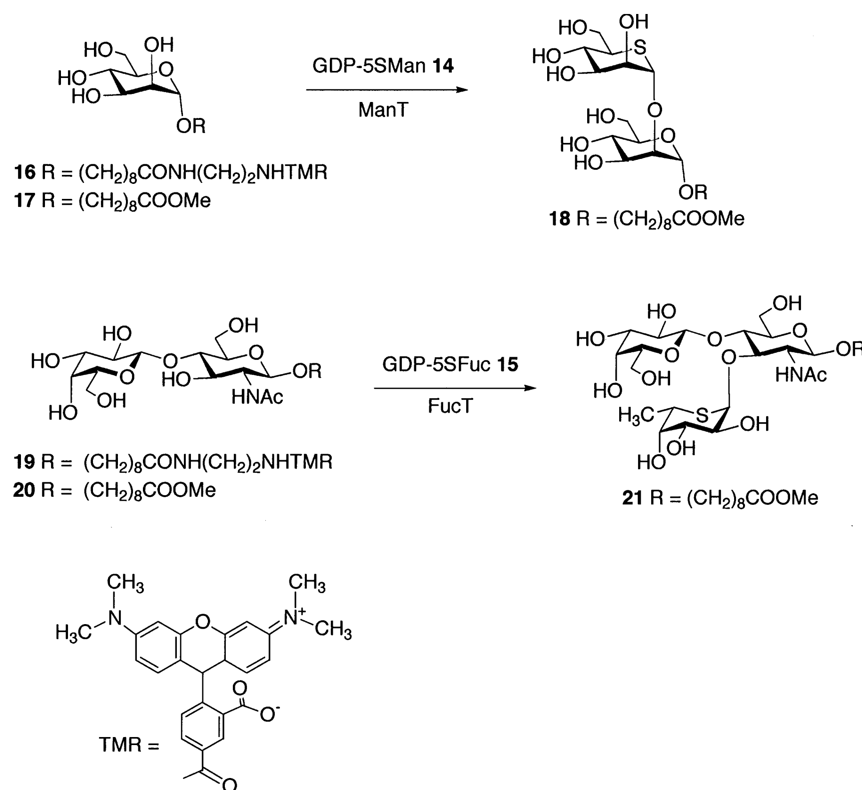
with complete regio- and stereoselectivity. This allows the custom syntheses of 5S-glycosides with native sequences and anomeric configurations. In addition to UDP-5S-sugars, if guanine-based 5S-sugar nucleotides were donor substrates for glycosyltransferases, the enzymatic syntheses of many important 5S-sugar-based oligosaccharide analogues would be possible (Figure 1).

We report the synthesis of two GDP-5S-sugar nucleotides [GDP-5-thiomannose (GDP-5SMan) and GDP-5-thiofucose (GDP-5SFuc)] and their evaluation as substrates for mannosyltransferase (ManT) and fucosyltransferase (FucT), respectively. The demonstration that these sugar nucleotides are substrates for these glycosyltransferases implies that production of 5S-glycoside analogues of oligomannose,[15] H-type 2, and Le^{x}[11] is feasible. All of these have been chemically synthesized with considerable effort. Of particular interest are analogues containing 5SFuc, since the ring sulfur atom tends to interact preferably with receptors as exemplified by the strong

SCHEME 1



SCHEME 2



inhibitory activity of 5SFuc against fucosidases[16] and the 5SFuc-based H-type 2 mimetics against an anti-H-type 2 antibody.[11]

Results and Discussion

Synthetic schemes for GDP-5SMan and GDP-5SFuc are shown in Scheme 1. The key step in these schemes was the stereoselective formation of α- and *β*-glycosyl phosphates required for GDP-5SMan and GDP-5SFuc, respectively. The reported synthetic methods for α-mannosyl phosphate and *β*-fucosyl phosphate include the coupling reaction between the per-*O*-acetylated glycosyl bromide and a silver salt of dibenzyl phosphate.[17] The desired configuration predominated for each compound due to anchimeric assistance from the 2-acetate. We used the same methods for the syntheses of 5S-glycosyl phosphates. Thus, α-5-thiomannosyl phosphate **7** was stereoselectively obtained from per-*O*-acetyl-5-thiomannosyl bromide (**4**), as supported by optical rotation ($[\alpha]^{25}_{D}$ +57.3), which showed approximately the same value as that of the α-mannosyl phosphate ($[\alpha]^{25}_{D}$ +58).[18] In the case of 5SFuc, however, only the undesired α-phosphate **8α** was produced from per-*O*-acetyl-5-thiofucosyl bromide (**5**). This result exemplifies the unusual axial glycoside formation in 5S-glycosylation reactions[7−13] despite the presence of 2-acetate that would usually direct equatorial glycoside formation. The desired *β*-phosphate was obtained by the phosphorylation of per-*O*-benzoyl-5-thiofucosyl bromide (**6**), though the α-phosphate was still a major isomer (**9α**:**9*β*** = 1.4:1). The stereoselectivity of the phosphorylation might be under the control of competition between the kinetic anomeric effect favoring the α-anomer formation and the neighboring-group participation that assists the *β*-anomer formation. If so, the partial formation of the *β*-anomer with the benzoyl derivative **6** is due to an enhanced participating ability of the carbonyl oxygen of 2-benzoate by delocalization of *π*-electrons from benzene.

Hydrogenolysis with 10% Pd/C as catalyst and alkaline treatment of **7** and **9*β*** gave the deprotected phosphates **10** and **11**, respectively. When these compounds were subjected to condensation with GMP-morpholidate (**12**) in the usual manner,[19] poor results were obtained, i.e., long reaction times of more than a week and low yields (<10%), as was reported for the synthesis of GDP-Fuc.[20] Recently, Wittmann and Wong reported that the addition of 1*H*-tetrazole improved the GDP coupling reaction.[21] However, attempts to use 1*H*-tetrazole in the reaction of **10** with **12** also gave low yields. Kadokura and co-workers recently reported an efficient P−P coupling method.[22] They showed that addition of a divalent metal chloride

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promoted the formation of P^1-methyl-P^3-(5′-guanosyl)-triphosphate in the reaction of GDP with methyl phosphorimidazolidate, with a shorter reaction time.[22] We followed this method by the addition of 4 equiv of $MgCl_2$ to the reaction of the deprotected phosphates **10** and **11** with GMP-imidazolidate (**13**) in DMF, to give GDP-5SMan (**14**) and GDP-5SFuc (**15**) in acceptable to significant yields (22% and 54%, respectively) within 36 h.

The reactions of **14** and **15** with tetramethylrhodamine (TMR)-labeled glycosyl acceptors **16** and **19**[23] were carried out in the presence of yeast α(1,2)ManT[24] and milk α-(1,3)FucT,[25] respectively (Scheme 2). Analyses of these reactions by capillary electrophoresis and MALDI-TOF mass spectrometry revealed that the corresponding products were formed as a result of enzymatic 5S-saccharide transfer. The relative rates of transfer of the thiodonors compared to natural donor substrates were 0.1 % for **14** and 2.3% for **15**. The relative rate of transfer for **14** was considerably lower than that of **15**. Reactions of acceptors **17** and **20** with **14** and **15**, respectively, were carried out to assign the structures of the saccharide products. Product isolation on a reversed-phase preparative MPLC instrument afforded the 5-thiomannose-containing disaccharide **18** and the 5-thiofucose-containing trisaccharide **21**, whose structures were supported by their ^{1}H NMR spectra. The isolated yields of these reactions were 7.8% for **18** and 41% for **21**, respectively.

The 2D ROESY spectrum for **18** (Figure 2) showed two strong interglycosidic ROE signals for H-1/H-5′ and H-1′/H-2, the latter being typical for the 1,2-glycosidic linkage. Peters reported similar NOE signals, including that of H-1/H-5′, for a natural Manα(1,2)Man disaccharide.[26] These results suggest a similarity for **18** and its natural counterpart with respect to their conformational populations on the basis of the flexibility of the glycosidic linkages. Peters carried out HSEA calculations on the conformation of the natural disaccharide with regard to the glycosidic dihedral angles (ϕ = H1′−C1′−O2−C2, ψ = C1′−O2−C2−H2) and estimated the global minimum A ($\phi = -47°$, $\psi = -20°$) and local minimum B ($\phi = 30°$, $\psi = 22°$) conformations, the energy difference being 2.4 kcal/mol.[26] Conformation A is consistent with the H-1/H-5′ cross-peak. According to an MM2 (92) calculation on the same disaccharide by Dowd and French, the global minimum C ($\phi = -25.5°$, $\psi = 60.1°$) is slightly (0.72 kcal/mol) more stable than local minimum D ($\phi = -42.4°$, $\psi = -21.3°$).[27] Conformation D is identical to conformation A, the global minimum from the HSEA calculation. Conformations A (=D) and C have glyconic dihedral angles (ϕ) in the range of the *exo*-anomeric effect and have an aglyconic dihedral angle (ψ) of −20° or 60°, only the former being consistent with the H-1/H-5′ cross-peak. Since the sulfur atom is electronegative enough to induce an *exo*-anomeric effect in 5-thioglycosides,[10] the glyconic dihedral angle of compound **18** would reside mainly between −30° and −60°. Then, the aglyconic dihedral must be around −20° to generate the H-1/H-5′ cross-peak. Overall, a large population of the dihedral angles for compound **18** should reside near those forming conformation A (Figure 3). The ^{1}H NMR spectrum of **21** shows full accordance with that of the same trisaccharide analogue synthesized by chemical methods.[11] As has been pointed out in a previous paper,[28] the chemical shift for H-5″ (5SFuc) resides at a rather lower field (δ ca. 3.9 ppm) than does that of 5-thiofucose (δ 3.64 ppm). A similar deshielding has been observed in the natural Le^x which is ascribed to the proximity of H-5″ to the galactose unit.[29] The proximity effect observed for both the natural and trisaccharide analogues suggests they have similar conformations.

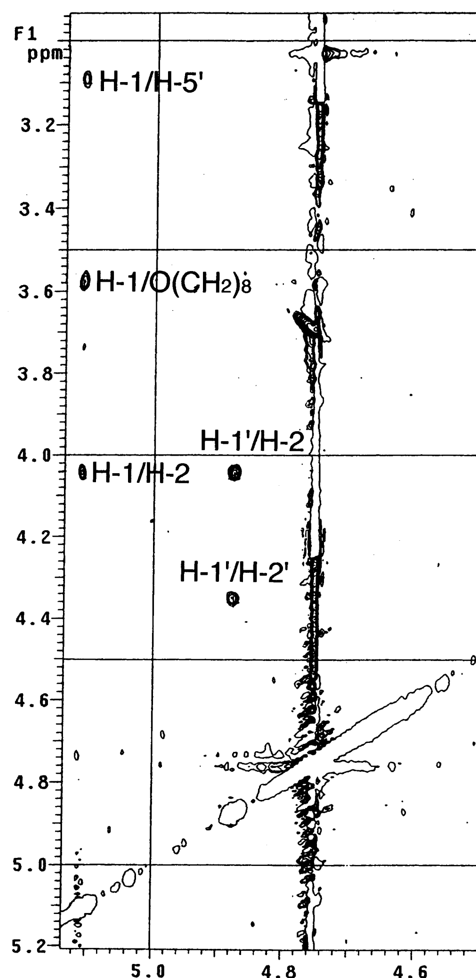

FIGURE 2. Relevant section of the TROESY spectrum of **18** in D_2O.

Accumulating data for 5S-glycosides, including this study, suggest they are conformationally similar to their natural counterparts.[10,13] In addition, 5S-glycosides are generally expected to be resistant to hydrolysis by glycosidases. These characteristics of 5S-glycosides lend themselves to the development of oligosaccharide-based drugs, analogous to the amide bond isosteres of drugs

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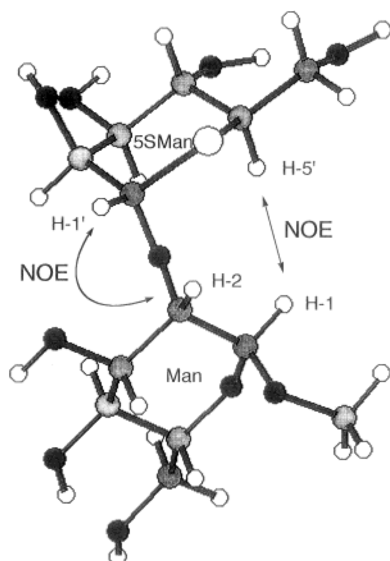

FIGURE 3. A presumed stable conformation of **18** with glycosidic torsion angles of $\phi = -47°$ and $\psi = -20°$.

based on peptide mimetics. Developments of such carbohydrate drugs are now viable, since we can enzymatically attach four different 5S-sugars, 5SGal, 5SGalNAc, 5SFuc, and 5SMan, to oligosaccharides. A further advantage expected for 5S-glycosides is their potentially stronger affinity for receptors compared to that of their natural counterparts. 5S-glycosides might find another application in hydrolase-resistant vaccines, since a part of the efforts to develop oligosacchride-based drugs has been focused on the cancer vaccines with oligosaccharide haptens.

Conclusions

We demonstrate that GDP-5SMan and GDP-5SFuc are donor substrates for α(1,2)ManT and α(1,3)FucT, affording a 5SMan-containing disaccharide and a 5SFuc-containing Le^x trisaccharide, respectively. The results of this study and previous studies with UDP-5SGal and UDP-5SGalNAc donors establish that 5-thiohexose nucleotides are substrates for glycosyltransferases. In principle, oligosaccharide analogues containing 5S-glycosides of all structures containing these monosaccharides can be enzymatically generated. 5S-glycosylation has the potential of becoming a routine modification in the development of oligosaccharide-mimetics-based drugs, similar to thioamide isosteres in peptide mimetics and phosphorothioate isosteres in antisense oligonucleotides.

Experimental Section

General Procedures. Optical rotations were measured using a 0.5 dm cell. ^{1}H NMR spectra were obtained at 270 MHz with TMS as an internal standard in $CDCl_3$ and at 400 and 600 MHz with HOD (δ 4.80) as an internal standard in D_2O. ^{13}C NMR spectra were obtained at 67.8 MHz with TMS as an internal standard. ^{31}P NMR spectra were obtained at 109.25 MHz with 85% H_3PO_4 as an external standard. Column chromatography was performed with Kieselgel 60 or Wakogel C-300. TLC was carried out on plates precoated with Kieselgel 60 F254. α(1,2)Mannosyltransferase from *Saccharomyces cerevisiae* and calf intestine alkaline phosphatase were obtained commercially. α(1,3)Fucosyltransferase was isolated from human milk as has been described.[25]

2,3,4,6-Tetra-*O*-acetyl-5-thio-α-D-mannopyranosyl Dibenzyl Phosphate (7). To a solution of 1,2,3,4,6-penta-*O*-acetyl-5-thio-D-mannose (**1**)[30] (49 mg, 121 μmol) in CH_2Cl_2 (1.0 mL) was added dropwise 25% HBr−AcOH (0.5 mL) at 0 °C, and the mixture was stirred for 2 h at room temperature. The mixture was poured onto ice−water and extracted with $CHCl_3$. The extracts were washed with water and aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated to give 2,3,4,6-tetra-*O*-acetyl-5-thio-D-mannopyranosyl bromide (**4**). This compound was used in the next step without further purification. Silver dibenzyl phosphate (47 mg, 122 μmol) was added to a solution of the bromide in benzene (2.0 mL). The mixture was refluxed for 1 h in a round-bottom flask covered with aluminum foil to shield it from light and then was filtered through Celite, and the filtrate was concentrated. The residue was purified on a column of silica gel (3:2 hexanes−EtOAc) to give **7** (49 mg, 63%) as a syrup: $[\alpha]^{25}_D$ +57.3 (*c* 2.44, $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.39−7.34 (m, 10H), 5.44 (dd, 1H, J = 3.3, 4.3 Hz), 5.43 (t, 1H, J = 10.2 Hz), 5.41 (dd, 1H, J = 4.3, 6.9 Hz), 5.25 (dd, 1H), 5.27−5.22 (m, 4H), 4.25 (dd, 1H, J = 5.6, 12.2 Hz), 3.93 (dd, 1H, J = 3.3, 12.2 Hz), 3.40 (ddd, 1H, J = 3.3, 5.6, 12.2 Hz), 2.17, 2.04, 2.02, 2.00 (each s, 3H × 4); ^{13}C NMR ($CDCl_3$) δ 170.40, 169.56, 169.47, 128.68, 128.61, 128.12, 127.91, 78.08, 77.99, 70.39, 70.24, 69.83, 69.76, 69.69, 69.54, 68.41, 61.33, 39.91, 20.85, 20.54; ^{31}P NMR ($CDCl_3$) δ −2.23; HR-ESMS *m/z* calcd for $C_{28}H_{33}O_{12}PSNa$ $(M + Na)^+$ 647.1328, found 647.1330.

Guanosine 5′-Diphospho-5-thio-α-D-mannose Diammonium Salt (14). To a solution of **4** (76 mg, 122 μmol) in MeOH (2.0 mL) were added 10% Pd/C (100 mg) and tributylamine (30 mg, 162 μmol). The mixture was hydrogenated under hydrogen ballon with vigorous stirring for 24 h at room temperature. The mixture was filtered through Celite, and the filtrate was concentrated. The residue was allowed to stand for 24 h at room temperature in MeOH−H_2O−triethylamine (7:3:1, 3.3 mL). The solution was concentrated to give 5-thio-α-D-mannopyranosyl phosphate tributylammonium salt (**10**). In the meantime, the following operations were performed. To a solution of guanosine 5′-monophosphate (53 mg, 146 μmol) and tributylamine (38 mL, 160 μmol) in DMF (1.5 mL) was added *N,N*-carbonyldiimidazole (26 mg, 160 μmol), and the resulting mixture was stirred for 1 h at room temperature. The reaction was quenched with a small amount of MeOH and concentrated to give crude **13**. The glycosyl phosphate **10** and **13** thus obtained were coevaporated twice with pyridine and twice with DMF, respectively, and redissolved in DMF (2.0 mL). $MgCl_2$ (46 mg, 483 μmol) was added to the mixture, which gave rise to a clear homogeneous solution. It was stirred for 36 h at room temperature. After the mixture was concentrated, the residue was applied to a column of anion-exchange resin (Dowex 1-X-8, formate form) and fractionated by a gradient elution of aqueous NH_4HCO_3 (0−1 M). The fractions containing GDP-5-thiomannose were collected and concentrated. The residue was further purified with a column of Sephadex G-15. The appropriate fractions were collected and lyophilized to give **14** (18 mg, 22%) as a fluffy powder: $[\alpha]^{26}_D$ +39.5 (*c* 0.74, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 8.16 (s, 1H), 5.98 (d, 1H, J = 6.4 Hz), 5.33 (dd, 1H, J = 4.0, 9.2 Hz), 4.83 (dd, 1H, J = 5.2, 6.4 Hz), 4.57 (dd, 1H, J = 3.0, 5.2 Hz), 4.40 (m, 1H), 4.37 (dd, 1H, J = 2.3, 4.0 Hz), 4.28−4.25 (m, 2H), 3.94−3.78 (m, 4H), 3.32 (m, 1H); ^{31}P NMR (D_2O) δ −10.72 (J = 20.6 Hz), −12.63 (J = 20.6 Hz); HR-ESMS *m/z* calcd for $C_{16}H_{25}N_5O_{15}P_2SNa$ $(M + Na)^+$ 644.0441, found 644.0436.

2,3,4-Tri-*O*-acetyl-5-thio-α-L-fucopyranosyl Dibenzyl Phosphate (8). To a solution of 1,2,3,4-tetra-*O*-acetyl-5-thio-L-fucose (**2**)[31] (70 mg, 201 μmol) in CH_2Cl_2 (2.0 mL) was added

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dropwise 25% HBr−AcOH (1.0 mL) at 0 °C, and the mixture was stirred for 2 h at room temperature. The mixture was poured onto ice−water and extracted with $CHCl_3$. The extracts were washed with water and aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated to give 2,3,4-tri-*O*-acetyl-5-thio-L-fucopyranosyl bromide (**5**). This compound was used in the next step without further purification. To a solution of the bromide in benzene (1.0 mL) was added silver dibenzyl phosphate (93 mg, 241 μmol). The mixture was refluxed for 1 h in a round-bottom flask covered with aluminum foil to shield it from light and then was filtered through Celite, and the filtrate was concentrated. The residue was purified on a column of silica gel (3:2 hexanes−EtOAc) to give **8** (67 mg, 59%) as a syrup: $[\alpha]^{26}_{D}$ −160.5 (*c* 0.91, $CHCl_3$); ^1H NMR (270 MHz, $CDCl_3$) δ 7.36−7.26 (m, 10H), 5.68 (br d, 1H, J = 8.6 Hz), 5.46 (br s, 1H), 5.37−5.29 (m, 2H), 5.16−5.03 (m, 4H), 3.44 (br q, 1H, J = 6.9 Hz), 2.17, 1.99, 1.88 (each s, 3H × 3), 1.08 (d, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 170.55, 170.04, 169.74, 135.40, 128.60, 128.57, 128.54, 127.95, 127.83, 78.03, 77.92, 72.19, 70.01, 69.92, 69.58, 69.49, 69.40, 68.43, 35.20, 20.63, 20.56, 20.50, 15.55; ^{31}P NMR ($CDCl_3$) δ −1.31; HR-ESMS *m*/*z* calcd for $C_{26}H_{32}O_{10}PS$ (M + H)$^+$ 567.1454, found 567.1453.

2,3,4-Tri-O-benzoyl-5-thio-*β*-L-fucopyranosyl Dibenzyl Phosphate (9*β*). To a solution of **2** (100 mg, 287 μmol) in MeOH (3 mL) was added NaOMe (catalytic). After being stirred overnight, the solution was neutralized with Dowex 50W-X8 (H^+), and the resin was filtered off. The filtrate was concentrated in vacuo, and the residue was dissolved in pyridine (3 mL). Benzoyl chloride (0.2 mL, 1.72 mmol) was added dropwise at 0 °C, and the mixture was stirred overnight at room temperature. The mixture was diluted with $CHCl_3$, washed with 1 M HCl and water, dried over $MgSO_4$, and concentrated. The residue was purified on a column of silica gel (10:1 hexanes−EtOAc) to give 1,2,3,4-tetra-*O*-benzoyl-5-thio-L-fucopyranose (**3**) concomitant with a small amount of reagent. This mixture was used in the next step without further purification. To a solution of this crude residue in CH_2Cl_2 (5 mL) was added dropwise 25% HBr−AcOH (1.5 mL) at 0 °C, and the mixture was stirred for 2 h at room temperature. The mixture was poured onto ice−water and extracted with $CHCl_3$. The extracts were washed with water and aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated to give 2,3,4-tri-*O*-benzoyl-5-thio-L-fucopyranosyl bromide (**6**). This compound was used in the next step without further purification. To a solution of the bromide in benzene (11 mL) was added silver dibenzyl phosphate (150 mg, 390 μmol). The mixture was refluxed for 1 h in a round-bottom flask wrapped with aluminum foil to shield the light and filtered through Celite, and the filtrate was concentrated. The residue was purified on a column of silica gel (3:1 hexanes−EtOAc) to give **9α** (112 mg, 52%) and **9*β*** (78 mg, 36%) each as a syrup. Data for **9α**: $[\alpha]^{26}_{D}$ −254.3 (*c* 1.26, $CHCl_3$); ^1H NMR (270 MHz, $CDCl_3$) δ 8.14−7.18 (m, 25H), 5.98 (dd, 1H, J = 2.0, 7.9 Hz), 5.93−5.91 (m, 3H), 5.09−5.00 (m, 4H), 3.69 (br q, 1H, J = 6.9 Hz), 1.20 (d, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 165.88, 165.73, 165.50, 135.49, 135.29, 133.53, 133.28, 133.15, 129.96, 129.70, 129.63, 129.27, 129.04, 128.95, 128.66, 128.52, 128.32, 128.25, 127.82, 127.75, 78.53, 78.42, 73.17, 71.39, 71.30, 69.54, 69.36, 35.99, 15.80; ^{31}P NMR ($CDCl_3$) δ −1.45; HR-ESMS *m*/*z* calcd for $C_{41}H_{37}O_{10}PSNa$ (M + Na)$^+$ 775.1743, found 775.1744. Data for **9*β***: $[\alpha]^{25}_{D}$ −147.7 (*c* 1.38, $CHCl_3$); ^1H NMR (270 MHz, $CDCl_3$) δ 8.30−6.94 (m, 25H), 6.12 (dd, 1H, J = 9.2, 9.6 Hz), 5.92 (dd, 1H, J = 1.7, 2.6 Hz), 5.86 (dd, 1H, J = 8.6, 9.2 Hz), 5.43 (dd, 1H, J = 2.6, 9.6 Hz), 5.09, 4.79 (each dd, 1H · 2, J = 7.3, 11.6 Hz), 5.02, 4.68 (each dd, 1H × 2, J = 7.3, 11.6 Hz), 3.59 (dq, 1H, J = 1.7, 6.9 Hz), 1.35 (d, 3H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 165.77, 165.46, 165.34, 135.35, 135.24, 133.50, 133.24, 129.99, 129.74, 129.69, 129.22, 128.95, 128.70, 128.60, 128.48, 128.34, 128.30, 128.23, 127.85, 127.46, 76.43, 72.76, 72.13, 72.02, 71.90, 69.76, 69.67, 69.50, 69.44, 38.20, 16.03; ^{31}P NMR ($CDCl_3$) δ −1.64; HR-ESMS *m*/*z* calcd for $C_{41}H_{37}O_{10}PSNa$ (M + Na)$^+$ 775.1743, found 775.1784. Anal. Calcd for $C_{41}H_{37}O_{10}PS$: C, 65.42; H, 4.95. Found: C, 65.48; H, 5.31.

Guanosine 5′-Diphospho-5-thio-*β*-L-fucose Diammonium Salt (15). To a solution of **9*β*** (138 mg, 183 μmol) in MeOH (15 mL) were added 10% Pd/C (50 mg) and triethylamine (28 μL, 200 μmol). The mixture was hydrogenated under hydrogen ballon with vigorous stirring for 12 h at room temperature. The mixture was filtered through Celite, and the filtrate was concentrated. The residue was allowed to stand for 2 days in aqueous NH_3 (28%)−pyridine (4:1, 15 mL) at room temperature. The solution was concentrated, and the residue was applied to a reversed-phase (C-18) column and eluted with water. The elute was lyophilized to give 5-thio-β-fucopyranosyl phosphate diammonium salt (53 mg, 98%) as a fluffy powder. A solution of this ammonium salt (18 mg, 61 μmol) in a small volume of water was passed through a column of cation-exchange resin (Dowex 50W-X-8, pyridinium form) with water, and the elute was concentrated. To a solution of the residue in pyridine (1.0 mL) was added tributylamine (16 μL, 67 μmol). The mixture was concentrated and coevaporated twice with pyridine. A solution of this residue in a small volume of water was lyophilized to give 5-thio-L-fucopyranosyl phosphate tributylammonium salt (**11**). In the meantime, the following operations were performed. To a solution of guanosine 5′-monophosphate (44 mg, 121 μmol) and tributylamine (32 mL, 134 μmol) in DMF (1 mL) was added *N*,*N*′-carbonyldiimidazole (24 mg, 148 μmol), and the resulting mixture was stirred for 2 h at room temperature. The reaction was quenched with a small amount of MeOH and concentrated to give crude **13**. This imidazolide **13** was coevaporated twice with pyridine and twice with DMF and dissolved in DMF (1.0 mL) together with the lyophilized phosphate **11**. To the mixture was added $MgCl_2$ (23 mg, 244 μmol), and the mixture was stirred for 36 h at room temperature. The mixture was purified in the same manner as described for the preparation of **14** to give **15** (21 mg, 54%) as a fluffy powder: $[\alpha]^{26}_{D}$ −29.9 (*c* 0.64, H_2O); ^1H NMR (400 MHz, D_2O) δ 8.17 (s, 1H), 5.98 (d, 1H, J = 6.3 Hz), 5.15 (t, 1H, J = 8.9 Hz), 4.86 (dd, 1H, J = 5.3, 6.3 Hz), 4.60 (dd, 1H, J = 3.3, 5.3 Hz), 4.40 (t, 1H, J = 3.3 Hz), 4.28 (m, 2H), 3.95 (br s, 1H), 3.84 (dd, 1H, J = 8.9, 9.6 Hz), 3.52 (dd, 1H, J = 3.1, 9.6 Hz), 3.26 (br q, 1H, J = 7.0 Hz), 1.21 (d, 3H, J = 7.0 Hz); ^{31}P NMR (D_2O) δ −10.42 (J = 20.6 Hz), −11.81 (J = 20.6 Hz); HR-ESMS *m*/*z* calcd for $C_{16}H_{26}N_5O_{14}P_2S$ (M + H)$^+$ 606.0672, found 606.0680.

α(1,2)ManT Capillary Electrophoresis Assay. 14 (10 nmol) was incubated with α-Mn-TMR (**16**; 5 nmol) in the presence of α(1,2)ManT (745 μU) in a buffer (100 mM MOPS, 20 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/mL BSA, pH 7.7, total volume 5 μL) at 37 °C for 2 h. As a reference, a reaction mixture consisting of GDP-D-mannose (10 nmol), **16** (5 nmol), and α(1,2)ManT (5.7 μU) in the same buffer was incubated at 37 °C for 2 h. An aliquot (0.5 μL) of each reaction mixture was diluted with water (50 μL) and then loaded onto a C-18 Sep-Pak cartridge preequilibrated with MeOH (10 mL) and water (10 mL). The cartridge was washed with water (10 mL), 30% MeOH (8 mL), and 50% MeOH (4 mL). The substrates and products bearing TMR were eluted with MeOH (4 mL) and concentrated under vacuum. The analysis of the relative rate by capillary electrophoresis with laser-induced fluorescence detection was performed as described before.[32]

Enzymatic Reaction with Donor 14. The mannosyl acceptor **17** (1.00 mg, 2.85 μmol) was incubated with **14** (2.4. mg, 3.71 μmol) in the presence of α(1,2)ManT (280 mU, 175 μL) and alkaline phosphatase (1 U/μL, 1 μL) in buffer (100 mM MOPS, 5 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/mL BSA, 175 μL) at room temperature. Reaction was continued for 44 days. During the reaction, 1.2 mg of **14** at the 12th and 33rd days, 1.72 μL of 1 M $MnCl_2$ at the 33rd day, and 172 μL of the buffer at the 22nd day were added to the reaction mixture.

(32) Sujino, K.; Uchiyama, T.; Hindsgaul, O.; Seto, N. O. L.; Wakarchuk, W. W.; Palcic, M. M. *J. Am. Chem. Soc.* **2000**, *122*, 1261.

The enzyme retained 75% and 25% of its original activity after 12 and 33 days, respectively. The yield of this reaction was estimated from TLC char to be 20% based on acceptor **17**. The reaction mixture was loaded onto a C18 Sep-Pak cartridge preequilibrated with MeOH (10 mL) and water (10 mL). The cartridge was washed with water (50 mL), and then the product was eluted with MeOH (40 mL). The MeOH eluate was concentrated, and the resulting residue was loaded onto a column of Iatrobeads (4.0 g) which was eluted with 10:1 CH_2Cl_2–MeOH (25 mL), 4:1 CH_2Cl_2–MeOH (24 mL), and 80:20:1 CH_2Cl_2–MeOH–H_2O (101 mL). The fractions containing the product were collected and concentrated. The residue was again loaded onto a preequilibrated C-18 Sep-Pak cartridge, and the cartridge was washed with water (30 mL) and then MeOH (10 mL). The MeOH eluate was concentrated, and the product was dissolved in water (10 mL). This solution was passed through a filter (0.22 μm), and the filtrate was lyophilized to give **18** as a fluffy white powder (0.117 mg, 7.8%): ^{1}H NMR δ 5.11 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 4.87 (d, 1H, $J_{1',2'}$ = 3.7 Hz, H-1′), 4.35 (dd, 1H, $J_{2',3'}$ = 2.6 Hz, H-2′), 4.04 (dd, 1H $J_{2,3}$ = 3.3 Hz, H-2), 3.94 (dd, 1H, $J_{6'a,5'}$ = 3.1, $J_{6'a,6'b}$ = 11.9 Hz, H-6′a), 3.93 (dd, 1H, $J_{3,4}$ = 9.8 Hz, H-3), 3.89 (dd, 1H, $J_{6a,5}$ = 2.3, $J_{6a,6b}$ = 11.6 Hz, H-6a), 3.82 (dd, 1H, $J_{6'b,5'}$ = 6.9 Hz, H-6′b), 3.80 (t, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 9.8 Hz, H-4′), 3.79–3.76 (m, 1H, H-3′), 3.78 (dd, 1H, H-6b), 3.75–3.72 (m, 1H, –CH_2O–), 3.70 (t, 1H, $J_{4,3}$ = $J_{4,5}$ = 9.8 Hz, H-4), 3.69 (s, 3H, OMe), 3.65–3.62 (m, 1H, H-5), 3.60–3.56 (m, 1H, –CH_2O–), 3.09 (m, 1H, H-5′), 1.67–1.53 (m, 2H, –CH_2(O)–), 1.39–1.28 (m, 12H, –$(CH)_2$–); HR-ESMS *m*/*z* calcd for $C_{22}H_{40}O_{12}NaS$ (M + Na)$^+$ 551.2138, found 551.2138.

Enzymatic Reaction with Donor 15. The *N*-acetyllactosaminyl acceptor **20** (0.5 mg, 0.9 μmol) was incubated with **15** (0.6 mg, 0.9 μmol) in the presence of milk α(1,3)FucT (33.75 mU, in 450 μL of 25 mM sodium cacodylate buffer, pH 6.5, containing 5 mM $MnCl_2$ and 25% glycerol) and 50 μL of concentrated buffer (200 mM Hepes, pH 7.0, containing 200 mM $MnCl_2$ and 2% BSA) at 37 °C. Additional **12** (0.6 mg, 0.9 μmol) was added 8, 24, and 48 h after the initiation of the reaction. The reaction was terminated after 7 days. The yield of this reaction was estimated from TLC char to be 75% based on acceptor **20**. The reaction mixture was purified in the same manner as described for the reaction of **14** with α(1,2)ManT to give the trisaccharide **21** containing 25% starting compound **20** as a fluffy powder (0.5 mg, 41%). The ^{1}H NMR spectrum of **21** shows a full accordance with that of the same trisaccharide analogue synthesized by chemical methods.[10] MALDI-TOF MS *m*/*z* 716.3 (M + H)$^+$, 738.3 (M + Na)$^+$.

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