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Synthesis of GDP-Fucose on a Soluble Support: A Donor Substrate for the Fucosyltransferases

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Abstract: The synthesis of GDP-fucose on a poly(ethylene glycol)-based soluble support is described. Our strategy relies on the attachment of the 6-deoxy-6-thio-L-galactose onto the polymer *via* a thioether linkage , and its conversion to GDP-fucose using established methods. We thus obtained GDP-fucose bound to the polymer that was tested as an acceptor for the recombinant fucosyltranferase FucT-III.

Keywords: carbohydrates; enzyme catalysis; fucosyltransferase; GDP-fucose; supported synthesis

Introduction

Glycoconjugates are known to play important roles in various biological events.[1] The breadth of glycoconjugate structures makes the synthesis of structurally well defined oligosaccharides an important challenge for synthetic chemists.

Glycosyltransferases have become widely used over the past decade as efficient tools for glycosylation since they are able to catalyze the transfer of sugar units with complete regio- and stereoselectivity.[2] They appear to be quite appropriate reagents for use in solid phase synthesis too.[3,4] However, enzymatic synthesis on solid support raises extra problems, such as the accessibility of the enzyme to the interior of the solid matrix[5] or the compatibility of the support with both aqueous and organic solvents. A potential solution would be the use of soluble supports which combine the advantages of both liquid phase (for the accessibility) and solid phase (for the purification) strategies.

Although poly(ethylene glycol) (PEG) is among the most studied soluble polymer supports for organic chemistry,[6] it suffers from a severe drawback: there are only two attachment points per polymer molecule. In order to increase its loading capacity, we coupled a pentaerythritol derivative at both ends of PEG 6000. The three-fold increase in attachment points gave a support with a loading capacity comparable to that of the Merrifield resin (1 mmol/g).[7]

In the classical approach to supported chemistry using glycosyltransferases, acceptor substrates are attached to the support at their anomeric positions and the oligosaccharides are built step by step (Figure 1). With this methodology, we have recently synthesized the Lewis^x trisaccharide and the lacto-*N*-tetraose tetrasaccharide using our soluble polymer based on poly(ethylene glycol).[8]

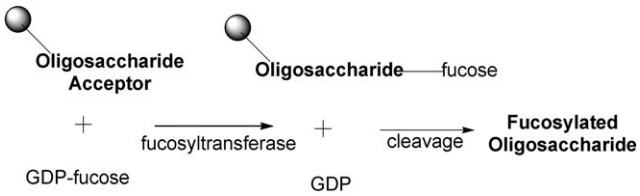


Figure 1. Strategy using a supported acceptor for the synthesis of fucosylated oligosaccharides.

We propose here another approach in which the sugar nucleotide donor is linked to the support instead of the acceptor. We have chosen GDP-fucose since fucose is generally the last sugar incorporated in the biosynthesis of an oligosaccharide. Moreover GDP-fucose is the sugar donor used by all known fucosyltransferases and may be transferred regio- and stereospecifically to different sugar acceptors. In this way our supported GDP-fucose can be an efficient tool to fish out a given oligosaccharide at the end of a synthesis using a correctly chosen fucosyltransferase.

Until now, only one example of this approach has been reported in the literature by Kajihara et al. who described the synthesis of CMP-sialic acids, on controlled pore glass and their enzymatic transfer in order to obtain immobilized sialyl glycoprotein [9]

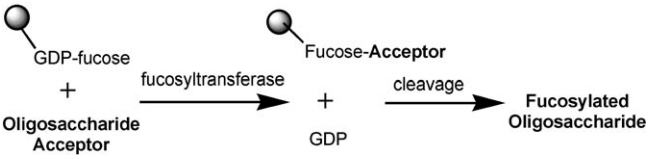


Figure 2. Strategy using the supported GDP-fucose for the synthesis of fucosylated oligosaccharides.

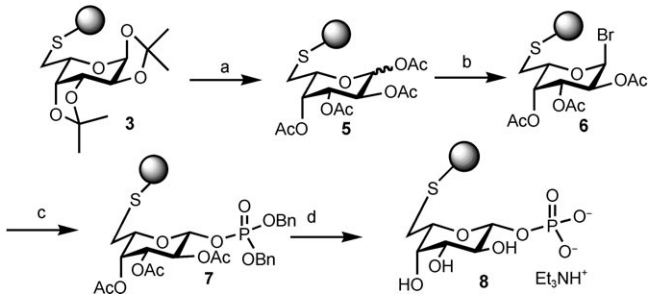
We report here the synthesis of GDP-fucose on a PEG-based soluble support that we have previously described, as well as the acceptor transfer onto the support using the fucosyltransferase FucT-III (Figure 2).

Results and Discussion

We have chosen to attach the L-galactose at the C-6 position *via* a thioether linkage since Hingdsgaul and coworkers have demonstrated that the fucosyltransferase FucT-III can accept bulky groups at the C-6 position of the fucose.[10] This traceless fixation will allow us to generate, after cleavage under desulfurization conditions, the desired L-fucose.

In the first step, the thiol sugar derivative **1**, easily obtained in a few efficient steps from L-galactose,[11] was attached to the support **2**. In a previous work, we have shown that the coupling of a thiol sugar derivative to a dendrimeric support **2** was very efficient.[6] Using this methodology, the sugar **1** was bound to the support **2** by nucleophilic displacement of the bromides in the presence of a slight excess of sodium methoxide. A small amount of sodium borohydride was also added to prevent the formation of a disulfide by-product resulting from the dimerization of the thiol generated by deacetylation of the compound **1**. In order to ensure complete consumption of **1**, 0.66 equivalent of this compound per Br was introduced in the reaction. In this way, a support with 4 molecules of L-galactose per molecule of PEG was obtained after simple precipitation from a CH_2Cl_2/*t*-BuOMe mixture (Scheme 1).

Starting from **3**, the synthesis of supported GDP-fucose was performed using established methods.[12] However, the use of the supported phase allowed easier product purification by precipitation or dialysis of the PEG derivatives (Scheme 2). It is important to note that most of the given yields are determined by weighing the products obtained, just like in solution phase chemistry.

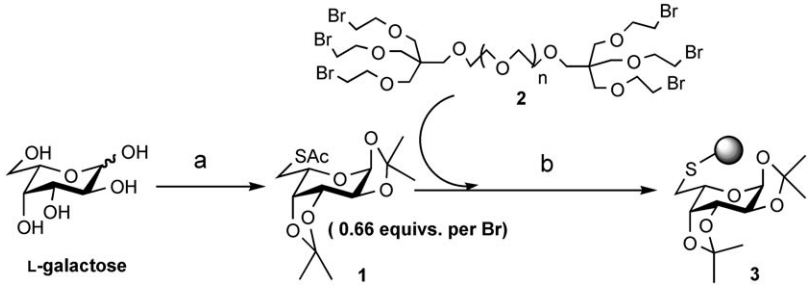


Scheme 2. *Reagents and conditions:* (a) (i) TFA 80%/H_2O, rt, 20 min, 97%; (ii) Ac_2O/pyridine, rt, 1 night, 100%; (b) TMSBr (40 equivs.), CH_2Cl_2, rt, 5 days, crude; (c) dibenzyl phosphate (15 equivs.), silver carbonate (10 equivs.), $CH_2Cl_2/Et_2O/CH_3CN$ (1/1/1), rt, 1 day, 98%; (d) (i) H_2, Pd/C (50%/weight), MeOH/NEt_3 (9/1), rt, 4 days; (ii) MeOH/NEt_3/H_2O (8/1/1), rt, 2 days, 74%.

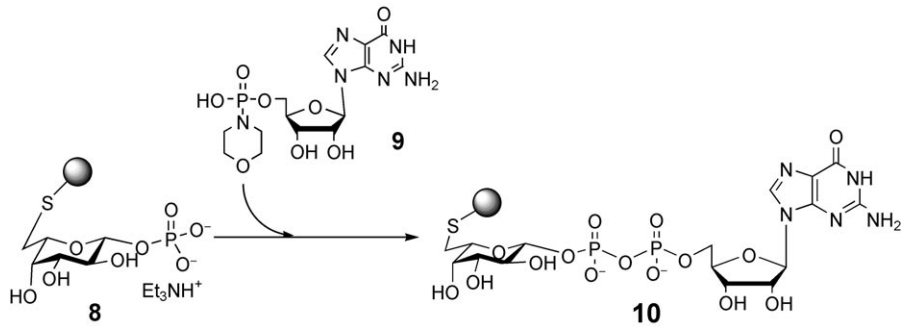
Acetal groups were first removed in aqueous acidic conditions and the product was acetylated to give compound **5**.

Since PEG derivatives are sensitive to strongly acidic conditions, reagents such as hydrochloric or hydrobromic acid must be avoided. The next step involved the bromination of the anomeric position of **5** using TMSBr. The resulting bromide **6** was then coupled to dibenzyl phosphoric acid in the presence of silver carbonate giving **7**, in 98% yield for the two steps and where the β anomeric configuration was confirmed by ^1H NMR ($J_{1,2} = J_{1,P} = 7.8$ Hz).

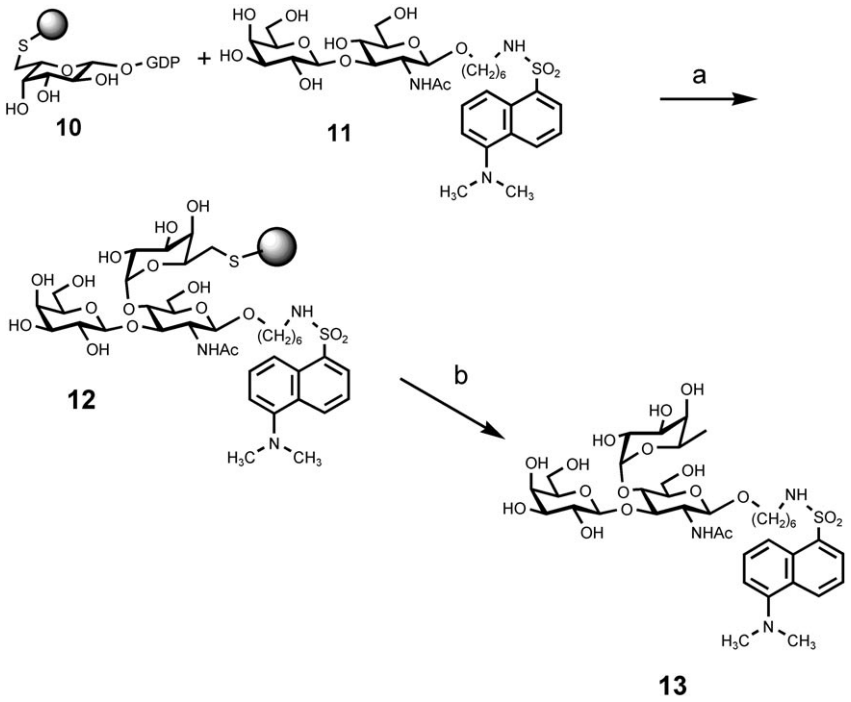
The benzyl groups were removed by hydrogenolysis in the presence of triethylamine and deacetylation was achieved in a mixture of methanol-triethylamine-water (8:1:1) giving compound **8** in 74% yield over the 2 steps. Finally, compound **8** was treated with GMP-morpholidate, using the conditions developed by Wittman and Wong,[13] to give the supported GDP-fucose **10** (Scheme 3).



Scheme 1. *Reagents and conditions:* (a) (i) $CuSO_4$ (2.25 equivs.), H_2SO_4 (cat.), acetone, 35 °C, 1 day; (ii) PPh_3 (3 equivs.), I_2 (2equivs.), imidazole (3.8 equivs.), toluene, 120 °C, 4 hours, 98%; (iii) potassium thioacetate (4 equivs.), acetone, reflux, 3 days, 99%; (b) **1** (4 equivs.), sodium methoxide (4.1 equivs.), sodium borohydride (1.8 equivs.), MeOH, rt, 3 days, 99%.



Scheme 3. *Reagents and conditions:* **9** (8 equivs.), 1*H*-tetrazole (12 equivs.), pyridine , rt, 3 days, 97%, 37% of supported GDP.



Scheme 4. *Reagents and conditions:* (a) **11** (2 equivs.), FucT-III (280 mU), MES buffer (100 mM, pH 6.4), 37 °C, 5.5 days, 47% of fluorescence transfer on **10**; (b) $NiCl_2$ (75 equivs.), $NaBH_4$ (225 equivs.), H_2 (1atm), MeOH, rt, 2 days, 18%.

UV quantification of GDP released from bound GDP-fucose after cleavage of a small amount in acidic conditions (HCl 10^{-2} M) indicated the synthesis of 1 molecule of GDP-fucose per molecule of PEG (0.12 mmol/g). The presence of GDP was also determined by TLC (PEI-cellulose) and compared to an authentic sample. No other nucleotides were observed.

The supported GDP-fucose was then tested as a substrate of a recombinant fucosyltransferase FucT-III.[14] The transfer assay was performed in a solution of MES buffer (50 mM, pH 6.5) containing the supported donor **10**, the dansyl Gal-β(1–3)GlcNAc **11** as an acceptor and $MnCl_2$ (20 mM). After 5 days at 37 °C, the reaction was stopped, the enzyme was removed by precipitation and the PEG derivative purified by gel filtration chromatography. Quantification of the enzymatic transfer was performed by the measurement of the fluorescence intensity of the fucosylated dansylated sugar bound onto the polymer (44 μm/g of PEG, 47%). No transfer was observed without enzyme.

Unfortunetly, Raney nickel was inefficient for the cleavage of the supported trisaccharide, we turned to the "Nickel Boride" reagent[15] which allowed the release of the dansylated trisaccharide **13** from the support. This compound was obtained with only 18% isolated yield based on the quantity of trisaccharide found bound onto the polymer. The final trisaccharide **13** was found to be identical to an authentic sample previously described in our laboratory by ^{1}H NMR, MS and HPLC analyses.[16]

In summary, we have performed the first supported synthesis of GDP-fucose to a soluble support in 8 chemical steps with 37% yield. The efficiency of this supported synthesis is similar to the one obtained with its solu-

tion phase cognate (unpublished results). The use of the supported phase allowed easier product purification by precipitation or dialysis.

The most important point of this work was that the fucosyltransferase FucT-III was able to recognize the supported GDP-fucose as a substrate. Work is in progress to extend the reaction to other fucosyltransferase substrates and to increase the yield of the cleavage.

Experimental Section

General Remarks

All moisture-sensitive reactions were performed under argon using oven-dried glassware. Anhydrous solvents were dried over standard drying reagents and freshly distilled prior to use. Reactions were monitored by TLC on silica gel 60 F_{254} or PEI-cellulose F. Detection was performed using UV light, and/or 5% sulphuric acid in ethanol or 2% orcinol in ethanol or 10% phosphomolybdic acid in ethanol, followed by heating. Flash column chromatography was performed on silica gel 6–35 μm. ^{1}H and ^{13}C NMR spectra were recorded at room temperature unless otherwise specified. Chemical shifts are reported in ppm (δ) *vs.* Me_4Si when recorded in $CDCl_3$, *vs.* acetone when recorded in D_2O or *vs.* DMSO signal when recorded in DMSO-d_6. Optical rotations were measured on an Electronic Digital Jasco DIP-370 polarimeter. Fluorescence detection was done with a Luminescence Spectrometer LS50B from Perkin-Elmer. Fluorescence of substrate and product was read at 385 nm excitation/540 nm emission. FucT-III was obtained from a French network (G3) devoted to the production and studies of recombinant glycosyltransferases. The cDNA coding for the human FucT-III gene was expressed in baculovirus-infected insect cells. Mass spectra were recorded on a Finnigan MAT 95 S spectrometer using electrospray ionization. Elemental analyses were performed at the Service Central de Microanalyses du CNRS (Gif sur Yvette, France).

Yield and Recuperation of PEG-Supported Compounds

The yields of the PEG-supported compounds were determined by weighing, with the assumption that the Mw of the PEG fragment was 7795 for compound **2** as determined by MALDI-TOF MS.[17]

After reaction, the PEG-supported compounds were purified by dialysis using a regenerated cellulose membrane (Spectra/Por 6 MWCO 2000) or by precipitation. PEG-supported compounds were precipitated after dissolution with CH_2Cl_2 followed by the addition of a 10-fold excess of *tert*-butyl methyl ether at 0 °C with vigorous stirring or by crystallisation from absolute ethanol. This precipitate was filtered, dried under vacuum and used in the following step.

6-*S*-Acetyl-6-deoxy-1,2,3,4-di-*O*-isopropylidene-6-thio-α-L-galactopyranose (1)

A solution of 6-deoxy-1,2,3,4-di-*O*-isopropylidene-6-iodo-α-L-galactopyranose (6.29 g, 17 mmol) and potassium thioacetate (7.76 g, 68 mmol) in acetone (250 mL) was refluxed for 3 days. After cooling, the excess of potassium thioacetate was removed by filtration and the layer was washed first with a saturated solution of $KHCO_3$ and then 3 times with water. The organic phase was dried on $MgSO_4$ and concentrated. Flash chromatography of the residue (toluene/AcOEt/Et_3N, 95:5:0.1) gave **1** as an oil; yield: 5.34 g (16.7 mmol, 99%); $[\alpha]_D^{34}$: +15 (*c* 1.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 250 MHz): δ = 1.32 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 2.34 (s, 3H, CO-CH_3), 3.04 (A of an ABX system, 1H, $J_{5,6}$ = 8.7 Hz, $J_{6,6'}$ = 13.8 Hz, *H-6*), 3.16 (B of ABX, 1H, $J_{5,6'}$ = 5.0 Hz, *H-6′*), 3.85 (ddd, 1H, $J_{4,5}$ = 1.8 Hz, *H-5*), 4.24–4.32 (m, 2H, *H-2, H-4*), 4.61 (dd, 1H, $J_{2,3}$ = 2.5 Hz, $J_{3,4}$ = 7.9 Hz, *H-3*), 5.52 (d, 1H, $J_{1,2}$ = 5 Hz, *H-1α*); ^{13}C NMR ($CDCl_3$, 63 MHz): δ = 24.33 (*C*H_3), 24.90 (*C*H_3), 25.86 (2*C*H_3), 29.59 (C_6), 30.46 (*C*H_3 acetate), 66.71 (C_5), 70.41 (C_3), 70.84 (C_2), 71.94 (C_4), 96.44 ($C_1\alpha$), 108.72 (O-*C*-O), 109.38 (O-*C*-O), 195.79 (*C*=O); anal. calcd. for $C_{14}H_{22}O_6S$: C 52.81, H 6.96, O 30.15, S 10.07; found: C 52.91, H 6.93, O 29.96, S 9.91.

Compound 3

The PEG derivative **2** (3 g, 385 μmol) and the compound **1** (555 mg, 1.74 mmol) were diluted in MeOH (20 mL). At 0 °C $NaBH_4$ (5 mg, cat) was added, followed a few minutes later by Na (41 mg, 1.79 mmol). After stirring for 3 days at room temperature, the reaction mixture was treated by the addition of resin Dowex 1X8 (OH^- form), filtered and acidified by the addition of resin Dowex 50X8 (H^+ form). The mixture was filtered and concentrated. The residue was precipitated from a 1/10 mixture of CH_2Cl_2/*t*-BuOMe to give **3** as a white amorphous solid; yield: (3.3 g, 383 μmol, 99%); ^{1}H NMR (DMSO-d_6, 400 MHz, 60 °C): δ = 1.30 (s, 12H, 4CH_3), 1.31 (s, 12H, 4CH_3), 1.37 (s, 12H, 4CH_3), 1.47 (s, 12H, 4CH_3), 2.63–2.75 (m, 16H, 8H_6, 4CH_2-S), 3.44–3.60 (m, PEG), 3.72 (t, 4H, 2CH_2-Br), 3.80 (td, 4H, $J_{4,5}$ = 1.8 Hz, $J_{5,6}$ = 6 Hz, 4*H-5*), 4.25–4.33 (m, 8H, 4*H-2,* 4*H-4*), 4.61 (dd, 4H, $J_{2,3}$ = 2.5 Hz, $J_{3,4}$ = 7.8 Hz, 4*H-3*), 5.45 (d, 4H, $J_{1,2}$ = 5 Hz, 4*H-1α*); ^{13}C NMR (DMSO-d_6, 100 MHz, 60 °C): δ = 24.07 (4*C*H_3), 24.50 (4*C*H_3), 25.60 (8*C*H_3), 31.02 (4*C-6*), 31.48 (4*C*H_2-S), 67.32 (4*C-5*), 69–70 (PEG), 70.15 (4*C-3*), 70.88 (4*C-2* + 4*C-4*), 95.66 (4*C-1α*).

Compound 4

Compound **3** (1 g, 116 μmol) was dissolved in 80% TFA in water (20 mL) at 0 °C. After stirring for 20 minutes at room temperature, the mixture was coevaporated 3 times with water (20 mL) and then 3 times with toluene (20 mL). The residue was precipitated from a 1/10 mixture of CH_2Cl_2/*t*-BuOMe to give **4** as a white amorphous solid; yield: 930 mg (112 μmol, 96%). ^{1}H NMR (D_2O, 400 MHz): δ = 2.76–2.88 (m, 16H, 8H_6, 4CH_2-S), 3.44–3.60 (m, PEG), 4.10 (t large, 2H, 2*H-5α*), 4.54 (d, 4H, $J_{1,2}$ = 8 Hz, 2*H-1β*), 5.2 (d, 4H, $J_{1,2}$ = 3 Hz, 2*H-1α*); ^{13}C NMR (D_2O, 100 MHz): δ = 30.75, 30.96, 31.45, 31.61 (2*C-6* + 2CH_2-S), 69–70 (PEG), 92.02 (*C-1α*), 96.17 (*C-1β*).

Compound 5

Compound **4** (453 mg, 54.8 μmol) was diluted in a 1/1 mixture of pyridine/anhydride acetic (10 mL). After stirring overnight the mixture was evaporated and coevaporated 3 times with toluene. The residue was precipitated from a 1/10 mixture of CH_2Cl_2/*t*-BuOMe to give **5** as a white amorphous solid; yield: 494 mg (55 μmol, 100%); ^{1}H NMR (DMSO-d_6, 400 MHz, 60 °C): δ = 1.90–2.15 (8 s, 48H, 16CH_3 acetate), 2.63–2.75 (m, 16H, 8*H-6*, 4CH_2-S), 3.44–3.60 (m, PEG), 3.81 (t, 4H, 2CH_2-Br), 4.27 (t, 4H, 2*H-5β* and 2*H-5α*), 5.07 (m, 4H, 2*H-3β* and 2*H-3α*), 5.31 (m, 4H, 2*H-2β* and 2*H-2α*), 5.37 (dd, 2H, 2*H-4α*), 5.48 (dd, 2H, 2*H-4β*), 5.83 (d, 2H, $J_{1,2}$ = 8 Hz, 2*H-1β*), 6.21 (d, 2H, $J_{1,2}$ = 4 Hz, 2*H-1α*); ^{13}C NMR (DMSO-d_6, 100 MHz, 60 °C): δ = 20.84 (*C*H$_3$ acetate), 31.16–31.65 (2*C-6* + 2*C*H$_2$-S), 69–70 (PEG), 89.25 (*C-1α*), 91.79 (*C-1β*), 169.13–170.39 (*C*=O acetate).

Compound 6

Compound **5** (450 mg, 50 μmol) was dissolved in CH_2Cl_2 (0.7 mL). At 0 °C was added TMSBr (296 μL, 2.24 mmol). After stirring for 5 days at room temperature the mixture was evaporated and coevaporated 3 times with toluene to give 6 as a yellow oil. This compound was used without further purification. ^{1}H NMR (DMSO-d_6, 360 MHz, 60 °C): δ = 1.90–2.15 (3 s, 36H, 12CH_3 acetate), 2.63–2.75 (m, 16H, 8*H-6*, 4CH_2-S), 3.40–3.60(m, PEG), 3.75 (t, 4H, 2CH_2-Br), 4.05 (t, 4H, *J* = 5 Hz, 4H_5), 4.95 (dd, 4H, $J_{3,4}$ = 3.6 Hz, 4*H-3*), 5.22 (dd, 4H, $J_{2,3}$ = 10.3 Hz, 4*H-2*), 5.42 (m, 4H, 4*H-4*), 6.90 (d, 4H, $J_{1,2}$ = 3.5 Hz, 4*H-1α*).

Compound 7

Dibenzyl phosphate (451 mg, 1.62 mmol) was added to a solution of compound **6** (875 mg, 97 μmol) in a 1:1:1 mixture (16.1 mL) of CH_2Cl_2/Et_2O/CH_3CN. After cooling to 0 °C silver carbonate (298 mg, 1.08 mmol) was added and the mixture was stirred for 1.5 days in the dark. The solution was filtered through a pad of celite and concentrated. The residue was precipitated from absolute EtOH to give **7** as a white amorphous solid; 938 mg (95 μmol, 98%); ^{1}H NMR (DMSO-d_6, 360 MHz, 60 °C): δ = 1.90–2.15 (3 s, 36H, 12CH_3 acetate), 2.63–2.75 (m, 16H, 8*H-6*, 4CH_2-S), 3.44–3.60 (m, PEG), 3.76 (t, 4H, 2CH_2-Br), 4.2 (t, 4H, *J* = 5 Hz, 4H_5), 4.90–5.10 (m, 20H, 4H_3 and 16*H* benz.), 5.25–5.35 (m, 8H, 4H_2 and 4H_4), 5.55 (t, 4H, $J_{1,2}$ = 7.8 Hz, $J_{1,P}$ = 7.8 Hz, 4*H-1*), 7.3–7.4 (*H*aromat.); ^{31}P NMR (DMSO-d_6, 100 MHz): δ = 2.33.

Compound 8

Compound **7** (925 mg, 94 μmol) was dissolved in a 9/1 mixture of MeOH/Et_3N (7.5 mL). Pd/C 10% (460 mg, 50% weight) was added and the mixture was hydrogenated (30 psi) during 4 days. The reaction mixture was decanted for 24 hours, filtered through a pad of celite and concentrated. The residue was diluted in a 3:3:1 mixture (7 mL) of MeOH/H_2O/Et_3N. After stirring for 2 days at room temperature, the mixture was evaporated and coevaporated with toluene. The residue was lyophilised to give **8** as an amorphous white solid; yield: 550 mg (68 μmol, 72%); ^{1}H NMR (D_2O, 250 MHz, 50 °C): δ = 2.63–2.75 (m, 16H, 8*H-6*, 4CH_2-S), 3.44–3.60 (m, PEG), 3.81 (t, 4H, 2CH_2-Br), 4.85 (t, 4H, $J_{1,2}$ = 7.8 Hz, $J_{1,P}$ = 7.8 Hz, 4*H-1*); ^{31}P NMR (D_2O, 100 MHz): δ = 2.11.

Compound 10

Compound **8** (92 mg, 11.3 μmol) and GMP-morpholidate **9** (74.5 mg, 102.9 μmol) were coevaporated 3 times with anhydrous pyridine and dried under vacuum for few hours. Anhydrous pyridine (1 mL) and tetrazole (11 mg, 153.6 μmol) were added. After stirring for 3 days at room temperature, the mixture was evaporated and coevaporated 3 times with water. The residue was purified on gel filtration column and then lyophilized to give **10** as an amorphous white solid; yield: 100 mg (10.5 μmol, 93%). The quantity of supported GDP-fucose was dosed by UV absorption at 254 nm (ε = 12200) and GDP quantity determined to be 37% with regard to **3**.

Supported Trisaccharide Type 1 (12)

Compound **10** (143 mg, 15 μmol) was shaken at 37 °C in a solution composed of **11** (27 mg, 37.7 μmol), $MnCl_2$ (12 mg, 20 mM), FucT-III (120 mU) and MES buffer (3 mL, 100 mM, pH 6.4). After 1 day, alkaline phosphatase (1 U), FucT-III (60 mU) and $MnCl_2$ (150 μL of a 200 mM solution) were added. After 2 days FucT-III (100 mU) and $MnCl_2$ (250 μL of a 200 mM solution) were added. After 2.5 days the enzyme was precipitated using MeOH and the mixture was centrifuged at 13000 rpm for 45 minutes. The supernatant was concentrated. The residue was purified on gel filtration column and then lyophilized to give **12** as an amorphous white solid; yield: 159 mg. The quantity of supported trisaccharide was dosed by emission of fluorescence at 540 nm and determined to be 47% (yield given with respect to GDP determination).

Dansylated Trisaccharide Type 1 (13)

Compound **12** (82 mg, 3.6 μmol of supported trisaccharide) was dissolved in MeOH (1 mL). At 0 °C was added first $NiCl_2$ (116 mg, 488 μmol) and then $NaBH_4$ (55 mg, 1.45 mmol). After 30 minutes, the reaction mixture was allowed to warm to room temperature and H_2 (1 atm) was applied. After stirring for 2 days at room temperature, the mixture was filtered through a pad of celite and concentrated. The residue was purified on a C18 reverse-phase silica gel column (using a gradient of water to MeOH) and the fractions containing the product were concentrated and lyophilised. The yield was obtained by emission of fluorescence at 540 nm and determined to be 18% (0.66 μmol of **13**). Its characteristics (^{1}H NMR, mass spectra) were identical to those of the product already published.[13]

Acknowledgements

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