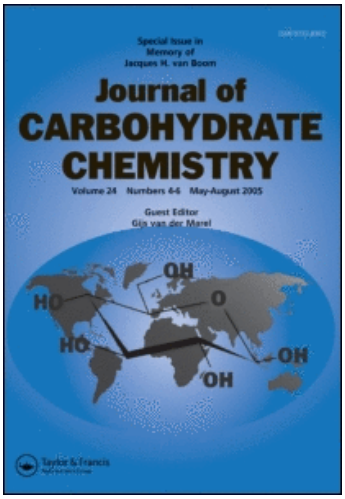

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Solid-Phase Enzymatic Synthesis of a Lewis a Trisaccharide Using an Acceptor Reversibly Bound to Sepharose

O. Blixt[a]; T. Norberg[a]
[a] Department of Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

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SOLID-PHASE ENZYMATIC SYNTHESIS OF A LEWIS A TRISACCHARIDE USING AN ACCEPTOR REVERSIBLY BOUND TO SEPHAROSE

O. Blixt and T. Norberg[1]

Department of Chemistry, Swedish University of Agricultural Sciences,
P.O. Box 7015, S-750 07 Uppsala, Sweden

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ABSTRACT

The disaccharide 2-aminoethyl *O*-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside was reacted with thiobutyrolactone to give a disaccharide with a thiol group on the aglycone. This disaccharide was reacted with activated Thiopropyl Sepharose, which gave a disaccharide bound to Sepharose via a disulphide bond. Enzymatic fucosylation, using GDP-fucose and partially purified human milk fucosyltransferase, gave a trisaccharide in good yield, which was cleaved from Sepharose by treatment with mercaptoethanol or dithiothreitol.

INTRODUCTION

Methods for the synthesis of oligosaccharides have developed considerably during the last twenty years, but a chemical oligosaccharide synthesis is still a relatively complicated task. Some recent attempts towards simplification have been made by applying solid-phase techniques,[2-4] which are routine in peptide and oligonucleotide synthesis. However, chemical glycosylation is neither regio- nor stereospecific, and consistent high coupling yields are therefore difficult to achieve. Furthermore, the elaborate protecting group patterns that have to be used for each monosaccharide component make their preparation tedious.

A radically different approach to oligosaccharide syntheses is to use enzymes (e.g., glycosyltransferases) as glycosylating catalysts. These give regio- and stereospecific glycosylations, without protecting groups on the monosaccharides. Here, progress has been dramatic,[5-7] mainly due to advances in enzyme production by genetic engineering. An example of what can be done with glycosyltransferases today is the recently reported[8] synthesis of a sialyl Lewis X derivative in kg amounts, using cloned glycosyltransferases for two crucial glycosylations.

Glycosyltransferase-catalyzed glycosylations in combination with solid-phase techniques should offer a particulary simple way to synthesize natural oligosaccharides on a laboratory scale. Some reports on such syntheses, using different transferases and solid-phase materials, have indeed appeared.[9-17] Further progress in this field is now critically dependent on the availability of enzymes, nucleotide sugars, and efficient, reversible solid-phase derivatization procedures. We here report such a reversible derivatization procedure based on a disulphide linkage, and its use in solid-phase fucosylation of a disaccharide immobilized on Sepharose.

RESULTS AND DISCUSSION

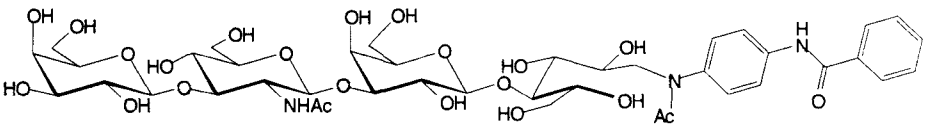
GDP-fucose preparation:

Nucleotide sugars have been prepared both chemically and enzymatically. In a particularly useful variation of enzymatic glycosylation,[18,19] the nucleotide sugar is generated in situ by a cyclic process during the enzymatic glycosylation. This latter procedure, however, requires additional enzymes, which, in the case of GDP-fucose, are not readily available. We therefore decided to prepare GDP-fucose chemically. Several chemical syntheses of GDP-fucose from fucose 1-phosphate and guanosine derivatives have been described,[20-27] low to moderate yields were reported throughout. We used a slight modification of Wong's procedure[24] involving silver carbonate-promoted reaction between tri-*O*-benzoyl-α-L-fucopyranosyl bromide (benzobromofucose) and dibenzylphosphate to give, after deprotection of the coupling product and purification by ion-exchange chromatography, fucose-1-phosphate bis(ammonium) salt in good yield (77 %). For the conversion from bis(ammonium) to bis(triethylammonium) salt, which is performed to get a more soluble derivative in the next step, we used coevaporation with triethylamine instead of an ion-exchange column, as reported by Wong.[24] In the final step, coupling of fucose-1-

phosphate with guanosine 5'-monophosphomorpholidate gave, after purification by ion-exchange chromatography (we found DEAE-Sephadex superior to Dowex-1-x8) and gel filtration, pure GDP-fucose bis(ammonium) salt in 22% yield. Although not optimal in terms of yield, this procedure was relatively simple, reproducible, and gave enough material for the current investigation. We consistently used the bis(ammonium) salt of GDP-fucose, it performed (not surprisingly) as well as the disodium or dilithium salt in the enzymatic fucosylations, but the tendency to decompose on concentration was less pronounced than with the alkali metal salts.

Enzyme preparation:

The use of partially purified human milk fucosyltransferase for enzymatic fucosylations has been reported before.[28-30] We used a slight modification of the reported isolation procedures, involving delipidation of human milk by centrifugation, ammonium sulfate precipitation, and ion-exchange chromatography. A new HPLC assay (UV detection) was developed for monitoring of the enzyme activity, where compound **1** was used as UV-absorbing substrate. This compound was obtained by treating lacto-*N*-tetraose (LNT, from human milk) with 4-aminobenzanilide[31] under reductive amination conditions,[32] followed by *N*-acetylation of the product with acetic anhydride.



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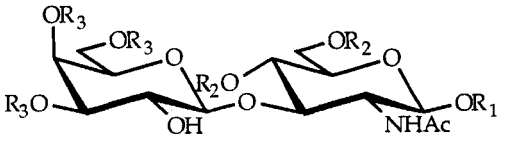
Disaccharide immobilization and fucosylation

Both 3-linked (type 1) and 4-linked (type 2) β-D-Gal*p*-D-GlcNAc*p* disaccharide derivatives are good substrates for human milk α-1-3/4-fucosyltransferase. We used a type 1 derivative, since a suitable precursor disaccharide **2** was available in quantity from earlier work.[33] Catalytic hydrogenation (Pd/C) of **2** gave the deprotected aminoethyl disaccharide **3** (92 %). Reaction of **3** with γ-thiobutyrolactone gave the derivative **4a** (94 %), which has a 7-atom spacer moiety terminating in a thiol group. Reaction of **4a** with activated Thiopropyl Sepharose resulted in immobilization, the extent of which was calculated from the ^{1}H NMR spectra (with added internal standard) of the



2) R_1 = -$(CH_2)_2$-N_3 R_2 = PhCH< R_3 = Bn

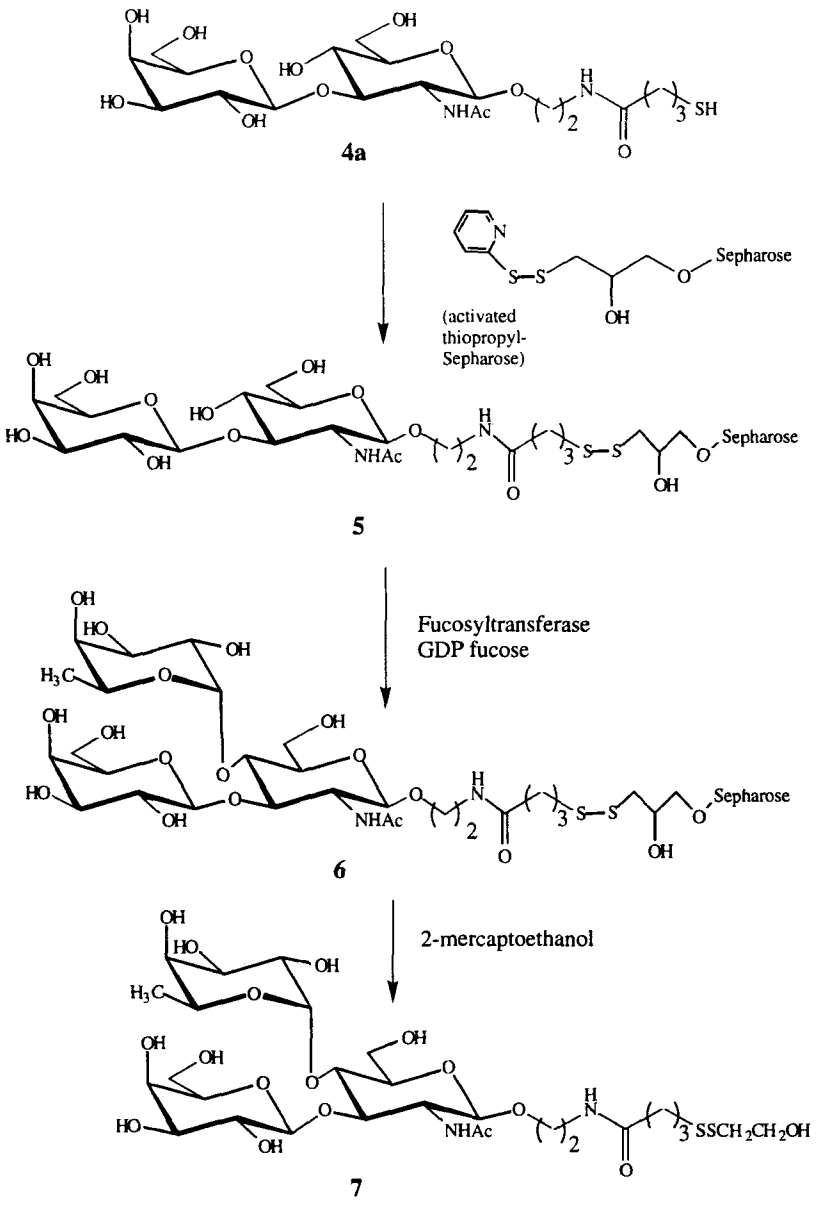
3) R_1 = -$(CH_2)_2$-NH_2 $R_2 = R_3$ = H

4a) R_1 = -$(CH_2)_2$-NHCO-$(CH_2)_3$-SH $R_2 = R_3$ = H

4b) R_1 = -$(CH_2)_2$-NHCO-$(CH_2)_3$-SS-$(CH_2)_2$-OH $R_2 = R_3$ = H

supernatant before and after the reaction. A value of 5-10 μmol of ligand/mL of wet gel was typical. To release the bound disaccharide, the gel was treated with mercaptoethanol at 60 °C. The expected amount (^{1}H NMR quantification) of disaccharide **4b,** containing a hydroxyethyl disulphide group in place of the original sulfhydryl group in **4a**, was obtained. Conversion of **4b** to **4a** could be effected by treatment with dithiothreitol (DTT) at pH 7. Alternatively, release from the gel could be effected by treatment directly with DTT at pH 7, giving the sulfhydryl disaccharide **4a** in similar high yield.

The Sepharose-bound disaccharide was reacted with partially purified human milk fucosyltransferase and GDP-fucose for 6 days, during which additional amounts of enzyme and nucleotide sugar were added every 48 hours. At the end of the period, 24 % of the totally added enzymatic activity remained. Release of the product with mercaptoethanol as above gave the trisaccharide **7** (68 % coupling yield). The total sugar recovery was 91 %, after Bio-gel P2 purification. If the Sepharose-bound disaccharide was treated, prior to the enzymatic reaction, with 25 mM dithiothreitol at pH 4.5 (at this pH, the Sepharose-disaccharide disulphide bond is not affected by DTT, but the remaining Sepharose thiopyridyl groups are converted to sulfhydryl groups) there was no noticable increase in reaction rate or coupling yield. This indicates, that enzyme inactivation by the remaining Sepharose thiopyridyl groups is not a serious problem in this case. The coupling yields reported here are by no means optimal, but in many cases they are acceptable for practical work. Preliminary experiments indicate, that the influence of factors like spacer length and solid-phase material on the coupling yields are considerable, this is currently under investigation.



EXPERIMENTAL

General methods. Concentrations were performed under reduced pressure at < 40 °C bath temperature. NMR spectra were recorded at 30 °C using a Varian Unity 400 MHz instrument. The following reference signals were used: acetone δ 2.225 (^{1}H in D_2O), acetone δ 29.8 (^{13}C in D_2O). Only selected NMR data

are reported. Sep-Pak cartridges were from Waters Associates (Milford, Mass., USA). Thin-layer chromatography was performed on Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany). After elution with appropriate eluants, spots were visualized by UV light and/or by dipping in 5 % sulfuric acid, followed by charring. Water for all solutions was from a MilliQ water purification system (Millipore Corp., Bedford, Mass., USA), and was degassed by vacuum treatment before use. For HPLC, a Hitachi L-6200A pump and a L-4000 UV detector (set to 280 nm) was used with a 5 x 300 mm column packed with Lichrosorb straight-phase silica (particle diameter 5 μm, from Merck, Darmstadt, Germany). Elution parameters: flow rate; 3.0 mL/min, isocratic elution with 60:35:8 chloroform:methanol:0.15 M aqueous ammonia. The solid-phase enzymatic fucosylations were carried out in small columns (5-15 mL) with a fritted glass filter at the bottom end. The columns could be sealed at the top with a ground glass stopper, and at the bottom with a teflon stopcock. After charging with the appropriate reagents, the columns were sealed and slowly rotated in a 37 °C water bath for the specified time. Thiopyridyl-activated Thiopropyl Separose 6B was from Pharmacia Biotechnology AB (Uppsala, Sweden), Lacto-*N*-tetraose (LNT) was from IsoSep AB (Tullinge, Sweden), 1,2-*O*-isopropylidene-α-D-glucofuranose and guanosine-5'-monophosphomorpholidate were from Sigma Chemical Co., (St. Louis, Ill. USA). Buffer A had the following composition: 10 mM sodium cacodylate, 5 mM manganese(II)chloride, 0.05% sodium azide 2% glycerol, pH 6.8.

Guanosine 5'-(β-L-fucopyranosyl) diphosphate (GDP-fucose). Crude 2,3,4-tri-*O*-benzoyl-α-L-fucopyranosyl bromide[24] (9.3 g, 17.2 mmol) was mixed with dibenzylphosphate (14.4 g, 51.5 mmol), molecular sieves 3Å (12 g) and 1:1:1 dichloromethane-diethyl ether-acetonitrile (120 mL), then silver carbonate (9.5 g, 34.5 mmol) was added, and the mixture was stirred for 15 h and then further treated essentially as described,[24] to finally give an aqueous solution of crude β-L-fucopyranosyl 1-phosphate, which was applied to a column (2.5 x 50 cm) of Amberlite IRA-400 (HCO_3-form, particle size: 0.3-1.18 mm). The column was eluted with a gradient (0-0.6M, total elution volume: 2 L) of aqueous ammonium bicarbonate. A trace amount of fucose was eluted first, β-L-fucopyranosyl 1-phosphate was eluted between 0.2-0.3 M ammonium bicarbonate. Appropriate fractions were pooled, concentrated and lyophilized to constant weight (4.49 g). This material was dissolved in a mixture of triethylamine (6 mL), pyridine (60 mL) and water (20 mL), concentrated and lyophilized to constant weight to give the bis(triethylammonium) salt of β-L-fucopyranose 1-phosphate (5.82 g, 13.0 mmol, 77 % from benzobromofucose). This material (357 mg, 0.80 mmol, 1.16 eq) and guanosine-5'-monophosphate

morpholidate (0.5 g, 0.69 mmol, 1 eq) were combined in dry pyridine (8 mL). The mixture was stirred at room temperature for 4 days. Evaporation of the solvent gave a syrup which was dissolved in water (100 mL), this solution was slowly passed through a column of DEAE Sephadex (1.8x15 cm, HCO_3^--form). The column was washed with water (2 vol) and then eluted with a 0-0.3M ammonium bicarbonate gradient, obtained by connecting, to the column, a stirrer-equipped mixing vessel (containing 250 mL of water) which in turn was connected to a reservoir (containing 250 mL of 0.6M aqueous ammonium bicarbonate). The column was operated at room temperature with a flow rate of 1.0 mL/min, 5 mL fractions were collected. Fractions containing GDP-fucose (as indicated by TLC, 2-propanol-water-conc ammonia 5-2-3 by vol) were pooled, concentrated (<35 °C) and lyophilized. The residual white solid was dissolved in water (5 mL) and passed through a Sephadex G25 column (5 x 80 cm) using 5% 1-butanol in water as eluant. Fractions containing the product were collected, pooled and lyophilized. A white fluffy solid (90.1 mg, 22%) was obtained. The ^1H NMR parameters were identical to those reported for GDP-fucose.[22]

Partial Purification of Fucosyltransferase from Human milk. Human milk (2.4 L) was delipidated by centrifugation in a Beckman 6B-L centrifuge at 5000 x g for 60 min. The fat was finally removed by filtering through glass wool, then protein was precipitated from the supernatant at 65% ammonium sulfate (1016 g) overnight at room temperature. The solution was centrifugated at 27000 x g for 10 min, the pellet was dialyzed against water (3 x 10 L). After dialysis, any remaining undissolved protein was removed by centrifugation at 5000 x g for 30 min, the supernatant solution was loaded onto a column of CM-Sephadex C50 (5 x 20 cm) equilibrated with buffer A. The column was washed with buffer until no further protein was eluted (2-3 volumes). The column was then eluted with a gradient of sodium chloride (0-600 mM in buffer A, total elution volume 1 L) and the eluant was monitored by measuring absorbance at 280 nm.The fractions (510-660 mL) containing fucosyltransferase activity as determined by assay 1, were pooled and ultrafiltered down to 75 mL, then diluted with 75 mL buffer A and finally concentrated to 17 mL. The total activity of one preparation was estimated to 283 mU by assay 2.

4-Aminobenzanilide: 4-Nitroacetanilide (4.2 g, 23 mmol) was refluxed for 3 h with 50 % aqueous sulfuric acid (400 mL), the mixture was then cooled and neutralized with sodium hydroxide pellets and extracted with dichloromethane. The extract, containing 4-nitroaniline, was dried (magnesium sulfate), concentrated to 150 mL, mixed with benzoic anhydride (15.6 g, 69 mmol) and refluxed for 3 days. The mixture was then washed with aqueous sodium bicarbonate solution, dried (magnesium sulfate), and concentrated. The residue

was dissolved in 1:1 dichloromethane-ethyl acetate (100 mL), silica gel (8 g) was added, and the mixture was concentrated to dryness. The solid was applied to a dry silica gel column (5 x 25 cm), which was then eluted with toluene-ethyl acetate mixtures (0 %, 40 %, 60 %, 200 mL each). Fractions containing product (by TLC) were boiled with toluene (100 mL), the solid product (4-nitrobenzanilide[34]) was filtered off, dried, and hydrogenated at room temperature and atmospheric pressure using ethyl acetate (130 mL) as solvent and Pd/C (70 mg) as catalyst. Filtration and concentration gave product (0.87 g, 4.1 mmol), melting at 127- 128 °C (lit[31] mp 125-128 °C).

4-Aminobenzanilide conjugate of Lacto-*N*-tetraose (1): Lacto-*N*-tetrose (215 mg, 0.30 mmol) and 4-aminobenzanilide (420 mg, 1.98 mmol) were dissolved in ethanol-water (2:1, 15 mL). Sodium cyanoborohydride (420 mg) was added and the mixture was stirred until complete conversion into a faster-migrating, UV-absorbing material was observed (TLC, 3-3-3-1 ethyl acetate-methanol-acetic acid-water, 24 h). Acetic anhydride (2.0 mL) was added and the mixture was stirred for 24 h. The mixture was then diluted with water, washed twice with ethyl acetate, and the combined organic phases were back-extracted with water. The combined aqueous phases were concentrated to 10 mL and loaded onto a Sep-Pak C18 cartridge (10 g, conditioned with methanol and then water). Elution with water removed impurities, the desired material was then eluted with methanol. Appropriate fractions were concentrated, taken up in water (2 mL), and passed through a Bio-Gel P2 column (2.5 x 75 cm), packed and eluted with 5% 1-butanol in water. Appropriate fractions (TLC monitoring) were pooled and lyophilized to give **1** (112 mg, 40 %). NMR data: ^{1}H, δ 1.89 (s, 3H, C*H*$_3$CON), 2.02 (s, 3H, C*H*$_3$CONH), 4.440 and 4.445 (2 d, 2H, H-1 Gal), 4.73 (d, 1H, H-1 GlcNAc), 7.35-7.90 (m, 9H, aromatic protons); ^{13}C, d 21.6, 21.8 (*C*H$_3$CON and *C*H$_3$CONH), 50.9 (*C*H$_2$NH), 54.3 (*C*HNH), 102.1, 102.7, 103.0 (3 C-1), 168.9 (Ar*C*O), 174.1, 174.5 (CH$_3$*C*O). FAB-MS: *m/z* = 946.1 (M+H).

Assay 1: Ion-exchange column eluate (200 µL) was added to a vial containing **1** (0.1 µmol), GDP-fucose (0.3 µmol), and ATP (0.3 µmol) in buffer A (200 µL), the mixture was then incubated at 37 °C for 24 h. The incubation mixture was diluted with water (0.5 mL) and loaded onto a pre-conditioned Sep-Pak C-18 cartridge (100 mg), the cartridge was washed with water and then eluted with 0.5 mL methanol. The methanol eluate was filtered through a 0.22 µm cellulose filter before HPLC analysis.

Assay 2: Partially purified enzyme (100 µL) was added to a vial containing **1** (0.2 µmol), GDP-fucose (0.4 µmol), and ATP (0.4 µmol), the mixture was then incubated at 37 °C for 1 h. The mixture was purified on a Sep-Pak cartridge and filtered (as described under assay 1) before it was subjected to HPLC analysis.

HPLC analysis: The sample (10 μL) was injected under the specified conditions (see general methods), and the product/substrate ratio was determined by integration. In the case of assay 1, the ratio was directly used to estimate the relative activity of the ion-exchange column eluate fractions. In the case of assay 2, saturation conditions in both substrates were assumed, and the activity was calculated from the substrate and product peak areas. One unit (U) is defined as the amount of enzyme that fucosylates 1 μmol of **1** per minute.

2-(3'-Mercaptopropionylamido)ethyl β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (4a). Disaccharide **2** (1.25 g, 1.54 mmol[33]) was dissolved in ethanol (200 mL), acetic acid (15 mL) and Pd/C 10% (3.0 g) was added, whereafter the mixture was hydrogenated at atmospheric pressure. When ready (TLC: ethyl acetate-methanol-acetic acid-water, 3-3-3-1 by vol) the mixture was filtered, concentrated, and lyophilized to give **3** (acetate form, 0.69 g, 1.42 mmol, 92 %). This material was dissolved in a mixture of degassed aqueous sodium bicarbonate (0.5 M, 8 mL) and ethanol (7 mL), then γ-thiobutyrolactone (0.75 mL, 8.6 mmol) and DTT (0.55 g, 3.6 mmol) was added and the mixture was stirred at 50 °C under a nitrogen atmosphere overnight (TLC: ethyl acetate-methanol-acetic acid-water, 3-3-3-1 by vol.). The mixture was then acidified to pH 7 with 1 M HCl, concentrated, and the residue was dissolved in water (3 mL), filtered, and applied onto a BioGel P-2 column (2.5 x 75 cm, packed and eluted with 5% 1-butanol in water). The appropriate fractions were pooled and lyophilized to give **4a** (710 mg, 1.34 mmol, 94 %). NMR data: ^{1}H, δ 2.06 (s, 3H, C*H*$_3$CONH), 1.91 (quintet, 2H, CH$_2$-C*H*$_2$-CH$_2$-SH), 2.40 (t, 2H, C*H*$_2$-CH$_2$-CH$_2$-SH), 2.58 (t, 2H, CH$_2$-CH$_2$-C*H*$_2$-SH), 3.41 (t, 2H, OCH$_2$C*H*$_2$NH-), 3.76, 3.93 (2 m, 2H, OC*H*$_2$CH$_2$NH), 4.46 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1'), 4.59 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1); ^{13}C, δ 21.9, 22.6, 22.9, 33.9 (*C*H$_2$-*C*H$_2$-*C*H$_2$, *C*H$_3$CONH), 38.7 (OCH$_2$*C*H$_2$NH), 54.0 (C-2), 67.6, 68.0 (C-6, 6'), 67.6, 68.0, 68.3, 70.2, 72.0, 74.8, 74.9, 82.1 (O*C*H$_2$CH$_2$NH, C-3,4,5, C-2,3,4,5), 100.3, 103.0 (C-1, 1'), 174.0, 175.4 (CH$_3$*C*ONH, -CH$_2$*C*ONH). FAB-MS: *m/z* = 529.2 (M+H).

Thiopropyl Sepharose disaccharide Conjugate (5). Activated Thiopropyl Sepharose 6B gel (degree of functionalization: approximately 20 μmol/mL wet gel) was re-swollen in water for 15 min at rt, and the additives were washed away with water (200 mL/g powder). Compound **4a** (approximately 36 mg) and a solution of 1,2-*O*-isopropylidene-α-D-glucofuranose (200 μL of a 0.10 M deuterium oxide solution, 20.0 μmol) were dissolved in deuterium oxide (2 mL). An ^{1}H NMR spectrum was recorded, and the relative intensities of proton signals from the reference compound (doublet at 4.37 ppm) and **4a** (doublet at 4.46 ppm, H-1') indicated the presence of 66 μmol of **4a**. The solution was transferred with the aid of water (4 mL) to a small column (15 mL) containing

the gel (2 mL, wet) and the mixture was gently rotated at room temperature overnight. The gel (**5**) was filtered off, washed with water (10 mL) and the filtrate was lyophilized, redissolved in deuterium oxide, and analyzed by ^{1}H NMR. The relative intensities of the analyte and reference signals now indicated that 46 μmol of **4a** remained in the filtrate (assuming that the internal standard was not adsorbed to the gel). The total amount of **4a** adsorbed to the gel was therefore 66-46 = 20 μmol, and the degree of functionalization was 10 μmol/mL.

Release of disaccharide from the Sepharose Conjugate (5) with mercaptoethanol: An aliquot (0.5 mL) of the above conjugate **5** was treated with mercaptoethanol (0.1 mL) in water (1.0 mL) at 60 °C for 16 h. The gel was filtered off and washed with water (5 mL), and the filtrate was lyophilized. The residue, according to ^{1}H NMR analysis with added internal standard, contained the expected amount of disaccharide **4b**. Removal of mercaptoethanol-derived contaminants by Bio-Gel P2 gel filtration gave pure **4b**. The ^{1}H NMR spectrum of **4b** was similar to that of **4a**, but the original $CH_2CH_2CH_2$-SH triplet signal (2.58 ppm) had shifted downfield (2.80 ppm), and there were additional triplet signals (from the -S-CH_2CH_2-OH group) at 2.93 and 3.89 ppm.

Release of disaccharide from the Sepharose Conjugate (5) with dithiothreitol (DTT): An aliquot (0.5 mL) of the above conjugate **5** was treated with an aqueous solution of DTT (50 mM, 1.0 mL) at rt for 16 h. The gel was filtered off and washed with water (5 mL), and the filtrate was lyophilized. The residue, according to ^{1}H NMR analysis with added internal standard, contained the expected amount of disaccharide **4a**. Removal of DTT and DTT-derived contaminants by Bio-Gel P2 gel filtration gave pure **4a**, indistinguishable (NMR and TLC) from the original sample.

Conversion of 4b to 4a: A solution of **4b** (5 mg) was treated with an aqueous solution of DTT (50 mM, 5.0 mL) at rt for 4 h. The mixture was lyophilized and the solution was subjected to gel filtration on a Bio-Gel P2 column (1.8 x 80 cm). Appropriate fractions were pooled and lyophilized. Analysis of the residue (TLC and ^{1}H NMR) showed complete conversion into **4a**.

Enzymatic solid phase fucosylation. Thiopropyl Sepharose disaccharide conjugate **5** (1 mL, 5.5 μmol) was conditioned with buffer A, then partially purified fucosyltransferase (1.5 mL, 17 mU), and GDP-fucose (9 μmol in 0.5 mL of buffer A) were added. The mixture was gently shaken at 37 °C for 6 days, during which two more additions of the same amount enzyme and GDP-fucose were made. At the end, an activity test detected (assay 1) 24% of enzyme activity still remaining. The gel was filtered and washed with water (20 mL). A solution of 2-mercaptoethanol (0.2 mL) in water (2 mL) was added to the gel and the mixture

was gently rotated for 1 h at 60 °C. The gel was filtered and washed with degassed water (20 mL), the filtrate was concentrated and passed through a BioGel P-2 (1x 80 cm, elution with 5% 1-butanol in water). Fractions containing **4b** and **7** were pooled and lyophilized, the residue contained 5 μmol (91 % recovery) total oligosaccharide, by ^{1}H NMR with added internal standard, and the **4b**/**7** ratio corresponded to a 68% glycosylation yield.

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