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Enzymatic supported synthesis of lacto-*N*-neotetraose using dendrimeric polyethylene glycol

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Abstract—The lacto-*N*-neotetraose tetrasaccharide was synthesized on a new dendrimeric support, based on polyethylene glycol. Starting from 1-thio-β-D-lactose, the trisaccharide (2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-1-thio-β-D-glucopyranose was obtained using *Neisseria meningitidis* β-(1 → 3)-*N*-acetylglucosaminyltransferase according to a soluble synthesis approach, bound on the support and galactosylated using the milk β-(1 → 4)-galactosyl transferase to give after cleavage the tetrasaccharide lacto-*N*-neotetraose.

Keywords: Chemo-enzymatic synthesis; Dendrimeric support; Glycosyltransferase

1. Introduction

Chemo-enzymatic syntheses of oligosaccharides are now well documented.[1] This approach combines effective chemical methodologies in carbohydrate synthesis and use of glycosyltransferases for their high stereoselective and regioselective potential in glycosylation.[2] One of the major drawbacks of this approach was the poor availability of such enzymes but this problem has now been overcome by the use of recombinant DNA technology.

Many groups have reported solid phase enzymatic glycosylation and considerable efforts have been devoted to the improvement of the support.[3,4] Two important aspects must be considered, namely the loading capacity of the support and its compatibility with the glycosyltransferases.

In oligosaccharide syntheses, Krepinsky and Douglas[5] followed by others[6] proposed a very attractive alternative using polyethylene glycol as a soluble support. Our approach was to increase the loading capacity of such a polymer by coupling a pentaerythritol derivative at both extremities of PEG 6000 leading to a dendrimeric support.[7] The 3-fold increase attachment points, with respect to commercially available PEG, gave a support where loading was comparable to that of Merrifield resin (1 mmol/g). Proof of the efficiency of this new PEG derivative in soluble supported chemo-enzymatic oligosaccharide synthesis was illustrated by the preparation of the Lewis^x trisaccharide.[7]

According to this methodology, the synthesis of the 3 sulfo Lewis^x pentasaccharide, a good ligand for selectins,[8] could be considered. A way to obtain this compound would be to start from lactose and using the recombinant *Neisseria meningitidis* β-(1 → 3)-*N*-acetylglucosaminyltransferase (LgtA).[6a,9] Unfortunately, initial experiments have shown that this transferase was completely inactive on lactose bound to our dendrimeric polyethylene glycol, a result related to the previous observation by Yan et al.[10] with lactose bound on polyethylene glycol monomethylether (MPEG).

In this paper, we report a solution to overcome this problem. The trisaccharide **5** was obtained according to a soluble synthesis approach, then bound on the support and galactosylated using milk β-(1 → 4)-galactosyltransferase to give after cleavage the lacto-*N*-neotetraose **13**.

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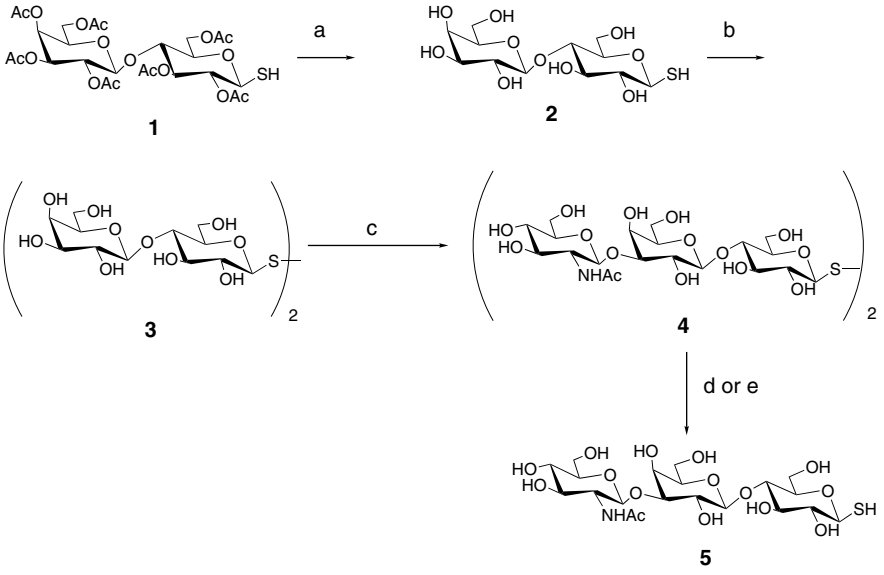
2. Results and discussion

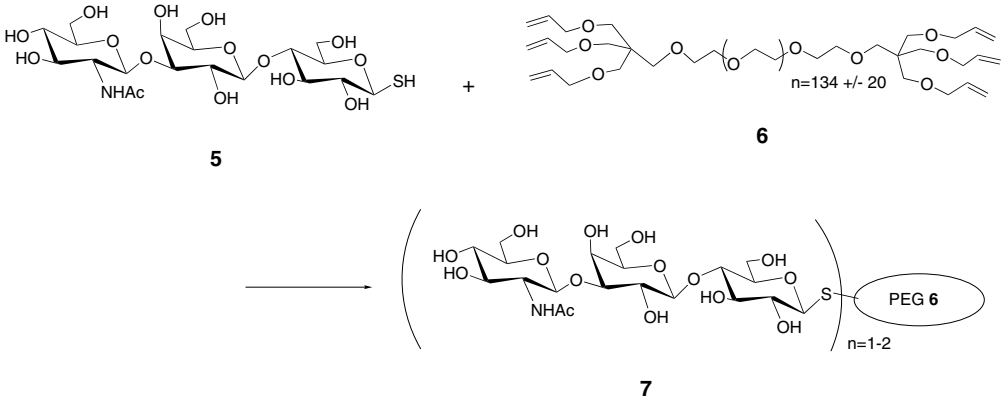
N. meningitidis β-(1 → 3)-*N*-acetylglucosaminyltransferase being inactive on lactose bound to PEG, we decided to perform the glycosylation prior to the coupling on the support and without isolation of the intermediate oligosaccharide.

In our previous work,[7] we have shown that coupling of a thiol sugar derivatives onto the dendrimeric support **6** was very efficient. We thus decided to enzymatically glycosylate the β-D-1-thiolactoside **2** and then couple the 1-thiotrisaccharide **5** to the dendrimeric PEG **6**. However, in the incubation reaction medium, thiolactose tends to lead to the dimer **3**. We choose to start from **3** easily obtained in 76% yield from **2** after oxidation using iodine in methanol and crystallization.[11] Compound **3** was then treated with UDP-GlcNAc (3 equiv) in presence of the β-(1 → 3)-*N*-acetylglucosaminyltransferase (Scheme 1). The complete transformation of **3** into the bis-trisaccharide **4** was shown on TLC analysis.

In order to conserve the advantage of the supported phase, namely easier product purification, we decided to continue our synthesis without isolation of compound **4**. Thus, after precipitation of proteins using acetone and deionization with ion-exchange resin, disulfide **4** was reduced with $NaBH_4$ or Bu_3P.[12] The reaction was followed using ^{13}C NMR analysis by monitoring the disappearance of the signal for C-1^I in **4** at 89.8 ppm and the appearance of the signal for C-1^I in **5** at 80.4 ppm. After completion of the reaction, the mixture was treated with the allyl functionalized PEG **6** under UV irradiation (Scheme 2). The reaction was dialyzed and the amount of trisaccharide covalently bound to the support **7** was quantified after cleavage with mercury(II) trifluoroacetate[13] by the phenol sulfuric acid method.[14] We found that only 50% of the theoretical trisaccharide



Scheme 1. Reagents and conditions: (a) MeONa–MeOH, one night, 95% yield; (b) I_2, EtOH, 76% yield; (c) GlcNAcTase, UDP-GlcNAc 3 equiv, 15 mM $MnCl_2$, 100 mM cacodylate buffer, pH 7.4, 3 days; (d) $NaBH_4$, 5 equiv; (e) Bu_3P, 4 equiv, MeOH 10%.

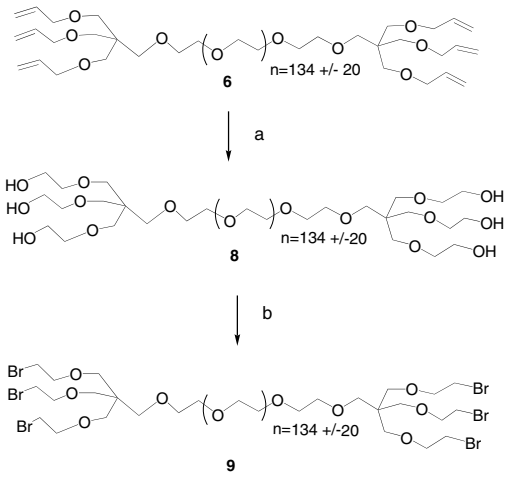


Scheme 2. Reagents and conditions: (**6**) 2.1 equiv, H_2O, UV, 36 h.

amount was fixed giving an average loading of 0.20 mmol of trisaccharide per gram of PEG, so we envisaged a more effective coupling strategy involving a nucleophilic substitution.

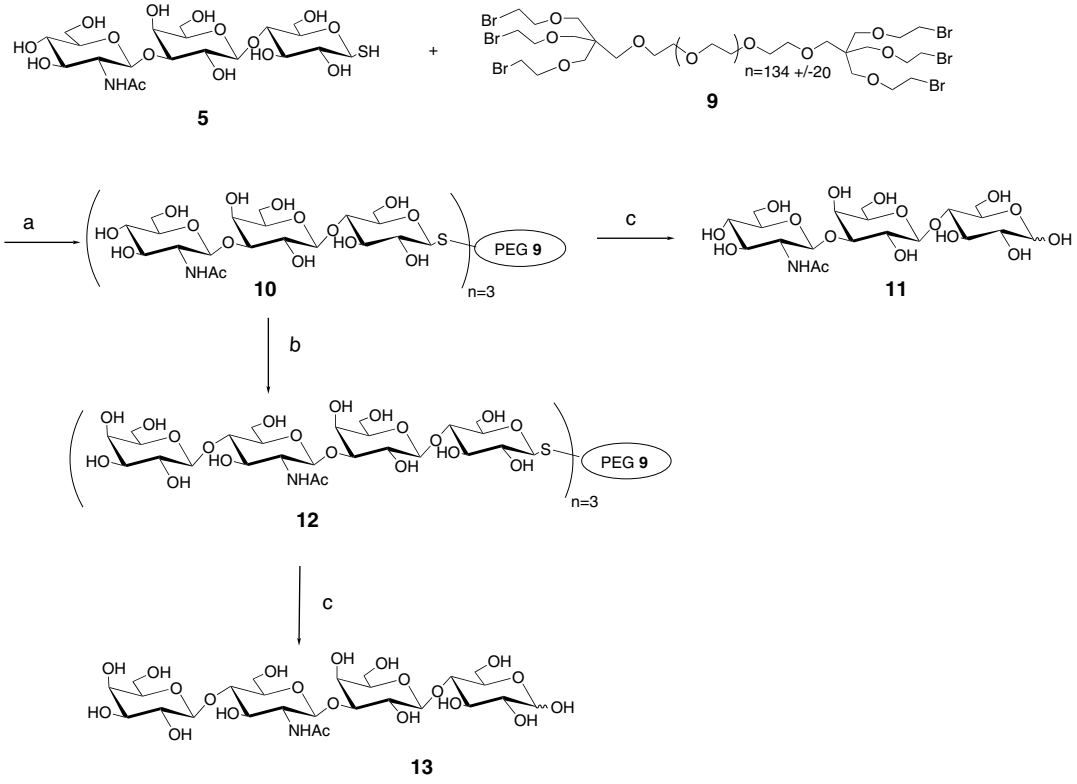
We designed a new support with bromide as leaving group, which was easily prepared in a high-yield three-steps sequence. First, **6** was ozonolyzed and the aldehyde functions directly reduced to afford PEG hexitol **8**. Bromination of this compound was attempted under various conditions including $SOBr_2$[15] or HBr in acetic acid, but the best result was obtained in 93% yield using triphenylphosphine and bromine (Scheme 3).[16] PEG derivatives **8** and **9** were characterized by NMR spectroscopy, permeation gel chromatography and MALDI-TOF analyses.

Compound **5** was then coupled to this new support under aqueous basic conditions to give **10**. In order to ensure complete fixation of the elaborated oligosaccharide **5**, only 0.75 equiv of this compound per Br was used. Conjugated **10** was easily purified after coupling by dialysis (Scheme 4). The yield of the total sequence **3** to **10** and the loading ratio were determined after cleavage of the trisaccharide with mercury(II) trifluoroacetate. We found that the efficiency of the coupling was dependent on the reducing agent used to regenerate the thiol (79% with PBu_3 and 54% with $NaBH_4$) leading to a loading of 0.37 mmol/g using PBu_3 and 0.31 mmol/g using $NaBH_4$.



Scheme 3. Reagents and conditions: (a) (i) O_3, 30 min, CH_2Cl_2–MeOH; (ii) $NaBH_4$ (8.6 equiv); (b) Br_2, PPh_3, CH_3CN, 50 °C, 18 h, 92%.

The compatibility of this PEG-supported substrate **10** with enzymatic glycosylation was illustrated by galactosylation using milk bovine β-(1 → 4)-galactosyltransferase. Compound **10** was incubated with the commercial enzyme (E.C.2.4.1.22), manganese chloride, UDP-Glc and UDP-galactose 4-epimerase that epimerizes in situ UDP-Glc to UDP-Gal.[1a] We adopted the experimental conditions described by Brinkmann et al.[17]



Scheme 4. Reagents and conditions: (a) **9** (0.22 equiv), NaOH 0.1 M, 48 h, rt; (b) GaIT, UDPGE, UDP-Glc 1.5 equiv, 20 mM Hepes buffer pH 7.5, $MnCl_2$ 5 mM, 37 °C, 2 days; (c) $(CF_3CO_2)_2Hg$ (2 equiv), acetic acid 0.05 M, rt, 2 h.

who demonstrated that a dilute solution favoured the complete galactosylation on GlcNAc-DOX-MPEG. The glycosylation could be monitored by TLC after cleavage of an aliquot, using mercury(II) trifluoroacetate, giving the known lacto-*N*-neotetraose **13** tetrasaccharide. After 3 days, the reaction was complete, the enzymes were precipitated and the supernatant was dialyzed to give **12**. A small sample of **13**, prepared by cleavage of an aliquot of **12**, exhibited data similar to those already described.[18]

In conclusion, we propose an efficient solution to overcome the problem of the inactivation of LgtA by PEG support while keeping the advantages of purification procedures offered by soluble supported chemistry. This allows us to prepare the PEG-linked tetrasaccharide **12**, which may be used as a substrate in other chemo-enzymatic syntheses to obtain more elaborate selectin ligands.

3. Experimental

3.1. General methods

NMR spectra were recorded with Bruker AM-250 or AMX-360 spectrometers; the chemical shifts for ^{1}H and ^{13}C NMR specta are given relative to the signal of acetone (δ 2.22 and 30.5 ppm) in D_2O. Mass spectra were obtained with a Finnigan MATT 95 apparatus using ESI. Size exclusion chromatography was performed using a TSK-Gel G3000 PW_{XL} column or a SHODEX GF-7MHQ column and refractometer for the detection. Reactions were monitored by TLC on Silica Gel $60F_{254}$ with detection by charring with 10% H_2SO_4 in EtOH or 2% orcinol in 10% H_2SO_4. UDP-Glc was purchased from Sigma, bovine milk D-GlcNAc-β-(1 → 4)-galactosyltransferase and UDP-galactose 4-epimerase from Calbiochem. Recombinant *N. meningitidis* β-(1 → 3)-*N*-acetylglucosaminyltransferase was prepared as described by Blixt et al.[9] UDP-GlcNAc was prepared by chemical synthesis as described by Heidlas et al.[19]

3.2. Bis[β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl] disulfide (3)

A methanolic soln of MeONa (1 M, 10 mL) was added to a stirred soln of 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-1-thio-β-D-glucopyranose **1**[20] (4.6 g, 7 mmol) in dry MeOH (60 mL) and the reaction was allowed to stand one night at room temperature. After TLC indicated completion, the reaction mixture was neutralized with Dowex 50X8-200 (H^+ form) ion-exchange resin, filtered and concentrated to give **2** (2.38 g, 95%). ^{13}C NMR (D_2O, 62.9 MHz): δ103.4 (C-1^{II}), 80.4 (C-1^{I}), 79.5, 78.5, 76.0 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-5^{II}), 73.0 (C-3^{II}), 71.5 (C-2^{II}), 69.1 (C-4^{II}), 61.6 (C-6^{II}), 60.6 (C-6^{I}).

An ethanolic soln of I_2 (0.5 g in 20 mL of EtOH) was added dropwise[11] with stirring to an aq soln of **2** (1.5 g, 4.2 mm in 4.2 mL of water) until a persistant slight yellow colour. White needles began to appear at the end point. After standing for several hours at 0 °C, disulfide **3** (1.14 g, 76%) was filtered.

^{13}C NMR (D_2O, 62.9 MHz): δ103.4 (C-1^{II}), 89.8 (C-1^{I}), 79.6, 78.2, 76.1, 75.9 (C-2^{I}, C-3^{I}, C-4^{I}, C-5^{I}, C-5^{II}), 73.0 (C-3^{II}), 71.5 (C-2^{II}), 69.1 (C-4^{II}), 61.6 (C-6^{II}), 60.6 (C-6^{I}). LRMS (positive mode): m/z 737.2 [M+Na].

3.3. Enzymatic synthesis of bis[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl] disulfide (4)

β-(1 → 3)-*N*-Acetylglucosaminyltransferase (0.5 U) was added to a soln of acceptor **3** (108 mg, 0.15 mmol), UDP-GlcNAc (0.45 mmol) in cacodylate buffer 100 mM pH 7.4 containing 15 mM $MnCl_2$ (13 mL). The reaction was performed at 37 °C for 3 days and monitored by TLC (3:3:2 EtOAc–2-propanol–water). The mixture was then filtered through Amicon membrane and deionized using Dowex 50X8-200 H^+ and 1X8-200 HCO_3^- ion-exchange resins. The filtrate was used directly in the next step without any further purification.

^{13}C NMR (D_2O, 62.9 MHz): δ175.5 (C=O), 103.4 (C-1^{II}, C-1^{III}), 89.8 (C-1^{I}), 82.5 (C-3^{II}), 79.6 (C-4^{I}), 78.1 (C-2^{I} or C-3^{I}), 76.2 (C-5^{III}), 75.4 (C-5^{II}), 74.1 (C-3^{III}), 71.4 (C-2^{I} or C-3^{I}), 70.5 (C-2^{II}), 70.2 (C-4^{III}), 68.9 (C-4^{II}), 61.6 (C-6^{II}), 61 (C-6^{III}), 60.60 (C-6^{I}), 56.2 (C-2^{III}), 22.7 (OCH_3). LRMS (positive mode): m/z 1143.3 (M+Na).

3.4. Reductive cleavage of the disulfide 4 and fixation on the support 6

$NaBH_4$ (0.50 mmol, 19 mg) was added at 0 °C to a soln of the disulfide **4** (0.10 mmol) in 0.5 mL of water. After 2 h, when the starting material had disappeared as evidenced by TLC, (3/3/2 EtOAc–2-propanol–water), acetone was added to stop the reaction. The mixture was then neutralized with Dowex 50X8-200 H^+, filtered and coevaporated with MeOH.

A soln of **6** (426 mg, 0.066 mmol, 2 equiv) in water (1.5 mL) was added and the mixture was allowed to react under UV illumination using a germicide lamp for 36 h at room temperature. At the end of the reaction, the mixture was dialyzed and lyophilized to give **7** (487 mg, 100%). Hydrolysis of an aliquot (11 mg) with mercuric(II) trifluoroacetate (4 mg, 10 μmol) in 100 μL 0.05 M HOAc, 2 h at room temperature and phenol sulfuric detection[13] gave 0.10 mmol of fixed trisaccharide (50%).

3.5. Hexa-ol-polyethylene glycol 6000 (8)

A soln of hexa-allyl-polyethylene glycol 6000[7] (**6**) (940 mg, 0.145 mmol) in dry 1:1 MeOH–CH_2Cl_2 (v/v) (28 mL) was cooled to (−78 °C). Then O_3 (15 min) was bubbled through the stirred soln. Excess of O_3 was removed with a stream of O_2. Then, $NaBH_4$ (24 mg, 0.6 mmol) was added. After stirring overnight at room temperature, the mixture was neutralized by addition of Dowex 50W-X8 (H^+). Afterwards, the resin was filtered off and the filtrate coevaporated with MeOH and concentrated. A white solid **8** (936 mg) was obtained (99% yield). ^{1}H NMR (Me_2SO-d_6, 360 MHz): δ 3.2–3.6 (m, –CH_2–CH_2–O–), 4.55 (s, 6H, OH). ^{13}C (90 MHz, Me_2SO-d_6): δ 72.76 (O–CH$_2$–CH$_2$OH), 60.08 (O–CH$_2$–CH$_2$OH). MALDI-TOF MS: found for the central peak: 7350 (commercial PEG 6000, found for the central peak: 6952). SEC: 7.5 mL.

3.6. Hexa-bromo-polyethylene glycol 6000 (9)

To a soln of triphenylphosphine (2 g, 7.7 mmol) in MeCN (4.5 mL), Br_2 (0.4 mL, 7.7 mmol) was added at 0 °C. The mixture was stirred 15 min at room temperature and if necessary, more triphenylphosphine was added until a discoloured soln was obtained. Compound **8** (5 g, 769 mmol) in MeCN (20 mL) was added to the mixture and the reaction was stirred overnight at 50 °C. Then, the soln was neutralized with satd $NaHCO_3$, filtered and evaporated. The residue was precipitated with 10:1 CH_2Cl_2–*t*-BuOMe (v/v) to yield **9** (4.9 g, 97%) as a white solid.

^{1}H NMR (Me_2SO-d_6 360 MHz): δ 3.2–3.6 (m, –CH_2–CH_2–O–), 3.7 (t, 6H, CH_2Br). ^{13}C (90 MHz, Me_2SO-d_6): 32.33 (O–CH$_2$–CH$_2$Br). MALDI-TOF MS: found for the central peak: 7795 (commercial PEG 6000, found for the central peak: 6952). SEC: 9 mL.

3.7. Reductive cleavage of the disulfide 4 and fixation on the support 9

3.7.1. Method A (using PBu_3). Bu_3P (0.3 mmol, 75 µl) was added to a soln of disulfide **4** (0.076 mmol) in aqueous MeOH 10% (5 mL). When the starting material had disappeared on TLC (3:3:2 EtOAc–2-propanol–water), **9** (260 mg, 0.038 mmol, 1.5 equiv) and aq NaOH (0.5 M, 1 mL) were added. After 2 days, the reaction was dialyzed and lyophilized to give **10** (320 mg, 100%). Hydrolysis with mercuric(II) trifluoroacetate and phenol sulfuric detection gave 0.12 mmol of fixed trisaccharide (79%) on support.

3.7.2. Method B (using $NaBH_4$). $NaBH_4$ (0.75 mmol, 28.5 mg) was added at 0 °C to a soln of the disulfide **4** (0.15 mmol). When the starting material had disappeared, Dowex H^+ was added to stop the reaction. After filtration, the mixture was coevaporated with MeOH.

Then, **9** (459 mg, 0.066 mmol) in aq NaOH (0.1 M 4 mL) was added and after, 2 days, the reaction was dialyzed and lyophilized to give **10** (510 mg, 95%). Compound **10** (100 mg) was cleaved using mercuric(II) trifluoroacetate (0.06 mmol, 25 mg) in HOAc (0.05 M, 5 mL) and after 2 h at room temperature, the mixture was purified on Biogel P2 to give 0.031 mmol of trisaccharide **11** (17 mg, 54% yield), This result agreed with colorimetric determination and spectral data were similar to those already published.[17]

^{13}C NMR of **5** (D_2O) δ175.5 (C=O), 103.4 (C-1^{II}, C-1^{III}), 82.5 (C-3^{II}), 80.4 (C-1^{I}), 79.6 (C-4^{I}), 78.5, 76.2, 76 (C-2^{I}, C-3^{I}, C-5^{I}, C-5^{III}), 75.4 (C-5^{II}), 74.1 (C-3^{III}), 70.5 (C-2^{II}), 70.2 (C-4^{III}), 68.9 (C-4^{II}), 61.6 (C-6^{II}), 61 (C-6^{III}), 60.60 (C-6^{I}), 56.2 (C-2^{III}), 22.7 (OCH_3).

^{1}H NMR of **10** (360 MHz, D_2O): δ 5.45 (d, 0.2 H, $J_{1\alpha,2}$ 3H_2, H-1^{I} α), 4.65 (d, 1H, $J_{1,2}$ 8 Hz, H-1^{III}), 4.53 (d, 0.8H, $J_{1\beta,2}$ 9 Hz, H-1$^{I}\beta$), 4.40 (d, 1H, $J_{1,2}$ 8 Hz, H-1^{II}), 4.11 (d, $J_{3,4}$ 2 Hz, H-4^{II}), 3.10–2.70 (m, 2H, CH$_2$–S), 2.00 (3H, NHCOCH$_3$).

3.8. Galactosylation

Compound **10** (200 mg, 0.025 mmol, 0.062 mmol of trisaccharide), UDP-Glc (0.093 mmol, 60 mg), bovine milk D-GlcNAc β-(1 → 4)-galactosyltransferase (0.25 U) and UDP-glucose epimerase (0.35 U) were incubated at 37 °C in 20 mM Hepes buffer pH 7.5 (15 mL) containing 5 mM $MnCl_2$. The reaction was monitored after cleavage of an aliquot using mercuric(II) trifluoroacetate by TLC on silica gel (3:3:2 EtOAc–2-propanol–water). After 48 h, the reaction was stopped by addition of acetone to precipitate the proteins. After filtration, the surnageant was treated with Dowex 50X8 H^+ to remove trace of $MnCl_2$, dialyzed and lyophilized to give 220 mg of galactosylated compound **12**.

^{1}H NMR of **12** (360 MHz, D_2O): δ 5.45 (d, 0.2 H, $J_{1^{\alpha},2}$ 3 Hz, H-1$^{I}\alpha$), 4.65 (d, 1H, $J_{1,2}$ 8 Hz, H-1^{III}), 4.57 (d, 0.8H, $J_{1\beta,2}$ 9 Hz, H-1$^{I}\beta$), 4.45 (d, 1H, $J_{1,2}$ 8 Hz, H-1^{IV}), 4.40 (d, 1H, $J_{1,2}$ 7.2 Hz, H-1^{II}), 4.13 (d, $J_{3,4}$ 2 Hz, H-4^{II}), 3.10–2.70 (m, 2H, CH_2–S), 2.00 (3H, NHCOCH_3).

Compound **12** (100 mg) was cleaved using mercuric(II) trifluoroacetate (0.06 mmol, 25 mg) in aq HOAc (0.05 M, 5 mL), 2 h at room temperature, then the mixture was purified on Biogel P2 to give 0.025 mmol of tetrasaccharide **13** (18 mg). This result agreed with colorimetric determination and spectral data were similar to those already published.[17]

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