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Synthesis and Immunochemical characterization of *S*-linked Glycoconjugate Vaccines against *Candida albicans*

Xiangyang Wu, Tomasz Lipinski, Eugenia Paszkiewicz, and David R. Bundle*[a]

Abstract: Replacement of the glycosidic oxygen atom by a sulphur atom is a promising technique for creating glycoconjugates with increased resistance to hydrolysis by endogenous glycosidases. The synthesis and antigenic properties of two distinct (1→2)-β-mannan trisaccharides with inter residue-*S*-linked mannopyranose residues are described. Syntheses were based on an oxidation–reduction strategy to construct the *O*-linked β-mannopyranoside bonds and a S_N2 inversion to provide 1-thio-β-mannopyranoside residues. Subsequently the allyl trisaccharide glycosides were subjected to photo addition with cysteine amine and coupled to tetanus toxoid and bovine serum albumin with good efficiency via an adipic acid tether. Rabbit immunization studies revealed that the antibodies elicited by the two glycoconjugates were able to recognize the corresponding *O*-linked trisaccharide epitope conjugated to BSA and the native cell wall antigen of *Candida albicans.*

Keywords: antibodies · *Candida albicans* · glycoconjugates · oligosaccharides · vaccines

Introduction

Conjugate vaccines composed of microbial polysaccharides conjugated to immunogenic proteins have been shown to be an attractive and cost effective strategy to prevent deadly infectious diseases.[1–3] These successes have created renewed interest in the wider potential of glycoconjugate vaccines for both prophylactic and therapeutic applications. Completely synthetic carbohydrate based vaccines to combat *Haemophilus influenzae* type b have recently been reported and shown to be as effective as their semi-synthetic counterparts that are derived from bacterial fermentation, extraction of the polysaccharide and subsequent conjugation to carrier protein.[4] Strategies to create completely synthetic vaccines are therefore attracting attention.

A particularly attractive epitope for consideration as a synthetic vaccine is the *C. albicans*, (1→2)-β-mannan trisaccharide. *C. albicans* is the most common etiologic agent of candidiasis,[5] and commonly affects immunocompromised patients, as well as those undergoing long term antibiotic treatment.[6] Cutler and co-workers have shown the protective potential of monoclonal antibodies specific for the (1→2)-β-mannan trisaccharide antigen present in the *C. albicans* cell wall phosphomannan[7] in a disseminated candidiasis mouse model. A glycoconjugate vaccine prepared from this trisaccharide attached to tetanus toxoid was shown to be a highly effective immunogen in rabbits, although immunization of mice has so far failed to demonstrate a robust immune response.[8]

We have demonstrated that thiooligosaccharide conjugate vaccines could evoke antibodies specific for native antigens in mice.[9,10] For example synthetic ganglioside antigens containing a terminal *S*-linked sialic acid were able to generate antibodies that recognized the corresponding *O*-linked antigen. As part of a detailed study of the immunochemistry of the protective (1→2)-β-mannan antigen of *Candida albicans* we have investigated the synthesis of *C. albicans* trisaccharide vaccine candidates composed of oligosaccharides containing terminal and an internal interglycosidic *S*-linkages. Epitopes **I** and **II** appeared to be a good model to study the immunological properties of isosteric conjugates with increased resistance to catabolic processing by glycosidases. The substitution of the glycosidic oxygen atom by sulfur or carbon is a well known method to enhance the stability of the glycosidic linkage towards hydrolysis by either chemical or enzymatic means.[11,12]

[a] Dr. X. Wu, Dr. T. Lipinski, Dr. E. Paszkiewicz, Prof. Dr. D. R. Bundle
Alberta Ingenuity Centre for Carbohydrate Science
Department of Chemistry, University of Alberta
Edmonton, AB T6G 2G2 (Canada)
Fax: (+1) 780-492-7705
E-mail: dave.bundle@ualberta.ca

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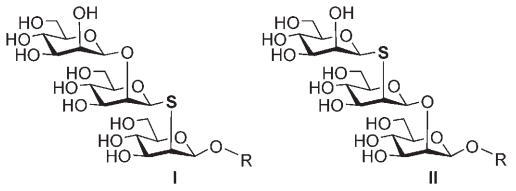
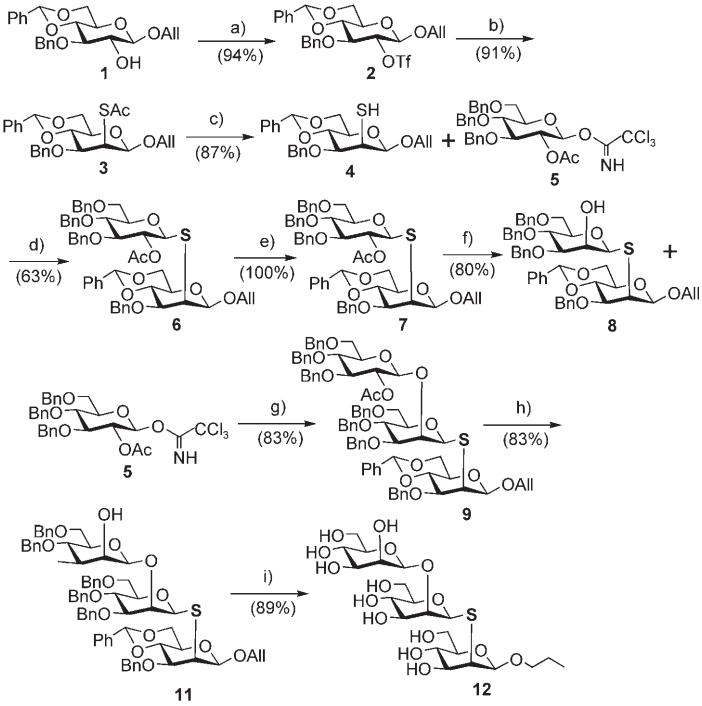


A significant number of publications report the synthesis of *S*-linked oligosaccharides.[11–13] The most convenient access to thioglycosides is based on S_N2 displacement of halogenoses because of the high nucleophilicity of thiolate groups.[14] Crich and co-workers have reported a direct stereoselective synthesis of β-thiomannosides based on the coupling of anomeric *S*-phenyl sulfoxide with sugar thiol.[15] Our group has successfully synthesized a *S*-linked tetrasaccharide with a novel enzyme-catalyzed technique employing a lactose thiol acceptor and UDP-gal donor.[16] Here, we introduce a related but simplified approach based on the glycosylation of a 2-deoxy-2-thiomannopyranoside acceptor by a glucopyranosyl trichloroacetimidate donor and an oxidation–reduction strategy to achieve the epimerization at C-2 to obtain *S*-linked β-mannose oligomers suitably functionalized for covalent attachment to immunogenic proteins.



Scheme 1. a) Tf_2O, CH_2Cl_2, pyridine; b) KSAc, DMF, 70 °C; c) NH_2NH_2·HOAc, THF; d) TMSOTf, CH_2Cl_2, −20 °C; e) NaOMe, MeOH; f) DMSO, Ac_2O; then L-selectride, THF; g) TMSOTf, CH_2Cl_2, −10 °C; h) NaOMe, MeOH; and DMSO, Ac_2O; then L-selectride, THF; i) 10 % Pd/C, H_2.

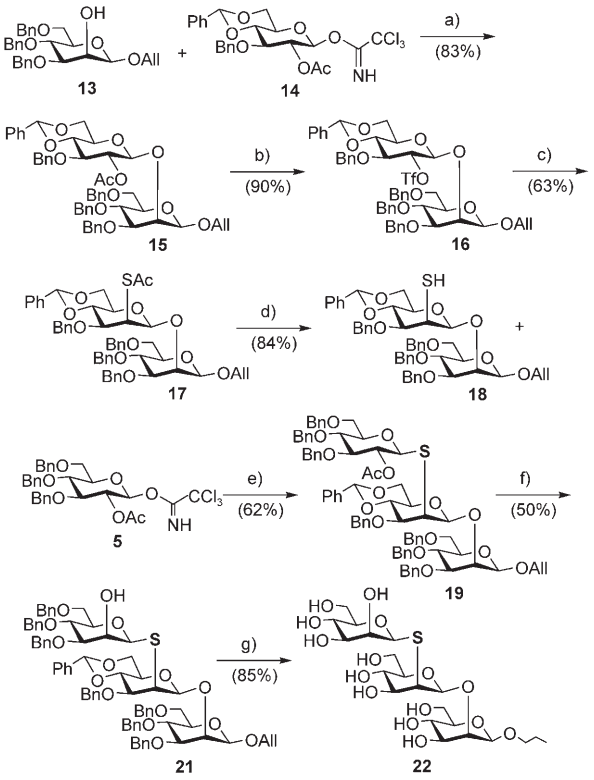
Results and Discussion

Synthesis of (1→2)-β-linked inter-thio trisaccharide (12): Building block **1** was synthesized according to a published procedure.[17,18] The reaction between alcohol **1** and trifluoromethanesulfonic anhydrate (Tf_2O) was performed in the presence of pyridine as base in CH_2Cl_2 at 0 °C to give triflate **2** in 94 % yield. Inversion of configuration with potassium thioacetate (KSAc)[17] furnished the desired protected thiol **3** in excellent yield (Scheme 1).

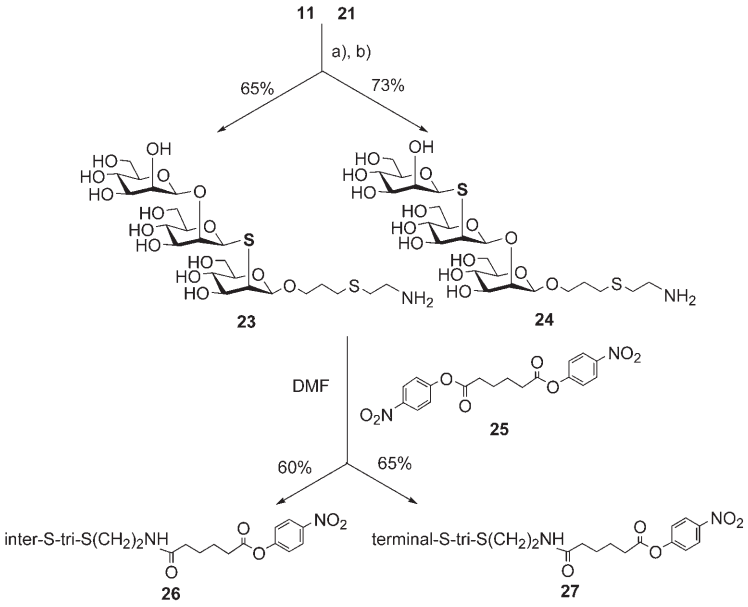
Interestingly, the *sec*-triflate **2** is sufficiently stable to be purified by chromatography without detectable decomposition. The treatment of **3** with hydrazine acetate in DMF afforded thiol **4** in 87 % yield. It should be mentioned that compound **4** was not oxidized to the corresponding disulfide even when exposed to air for a long time, possibly due to the steric hindrance caused by the 4,6-*O*-benzylidene protecting group. The glycosylation of acceptor **4** by imidate donor **5** was performed in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) (0.03 equiv) in CH_2Cl_2 at −20 °C to give disaccharide **6** in good yield. Transesterification gave the desired alcohol **7** in quantitative yield, and this was oxidized to the corresponding keto derivative by DMSO and Ac_2O (2:1). Subsequent stereoselective reduction with L-selectride at −78 °C in THF afforded disaccharide **8** in 80 % yield over two steps. Repetition of the glycosylation with donor **5** followed by transesterification, oxidation and reduction gave trisaccharide **11** in 69 % yield over four steps. Most importantly, excellent diastereoselectivity was observed in this strategy since in this reduction reaction only trace amounts of the β-*gluco* epimer could be detected by ^1H NMR. Replacement of the glycosidic oxygen atom by sulfur did not lead to either decreased yield or poor selectivity. Hydrogenolysis of trisaccharide **11** using 0.15 equivalent of palladium charcoal (10 % Pd) furnished the deprotected trisaccharide **12** in 89 % yield.

Synthesis of (1→2)-β-linked terminal-thio trisaccharide (22): Building blocks **13** and **14** were synthesized according to published procedures (Scheme 2).[17] Reaction of glycosyl donor **14** with the monosaccharide acceptor **13** in CH_2Cl_2 at −10 °C in the presence of TMSOTf as catalyst (0.02 equiv) afforded disaccharide **15** in 83 % yield. Transesterification of **15** in MeOH, followed by triflation with Tf_2O and pyridine in CH_2Cl_2 at 0 °C furnished **16** in high yield. The reaction of **16** with potassium thioacetate in DMF at 70 °C gave acetylated thiol **17**. Reaction of compound **17** with hydrazine acetate in DMF afforded the acceptor **18**, and subsequent glycosylation by glycosyl donor **5** in the presence of TMSOTf (0.02 equiv) furnished trisaccharide **19** in 62 % yield. Finally, deacetylation, oxidation and reduction afforded the desired trisaccharide **21** in 50 % yield over three steps. Hydrogenolysis of trisaccharide **21** using 0.15 equivalents of palladium/charcoal activated (10 % Pd) furnished the unprotected trisaccharide **22** in 85 % yield.

Synthesis of tether halfesters 26 and 27: For the conjugation of deprotected oligosaccharides to proteins, a terminal amine was chosen as a versatile handle from which glyco-



Scheme 2. a) TMSOTf, CH_2Cl_2, −10 °C; b) NaOMe, MeOH; then Tf_2O, CH_2Cl_2, pyridine; c) KSAc, DMF, 70 °C; d) NH_2NH_2·HOAc, THF; e) TMSOTf, CH_2Cl_2, −10 °C; f) NaOMe, MeOH; and DMSO, Ac_2O; then L-selectride, THF; g) 10 % Pd/C, H_2.



Scheme 3. a) 2-Aminoethanethiol hydrochloride, MeOH, CH_2Cl_2, *hν* 365 nm; b) Na/NH_3, *t*BuOH, THF.

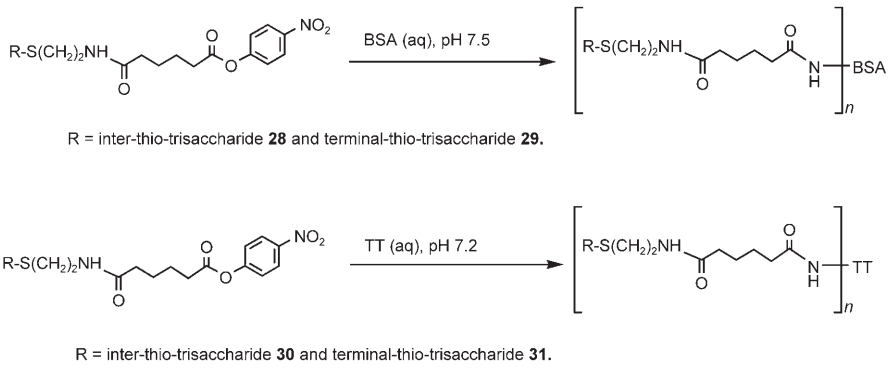
conjugates could be readily generated.[17] The protected oligosaccharides **11** and **21** were elaborated via photoaddition of 2-aminoethanethiol to the allyl glycosides to give the amine-functionalized glycosides,[17] then subsequently deprotection under Birch conditions to give the desired amino-functionalized glycosides **23** and **24** in good yields. For efficient attachment of oligosaccharides to proteins, a highly efficient conjugation strategy is required. Previously, coupling of such compounds to bovine serum albumin (BSA) protein was achieved through a squarate linker.[17] While conjugates prepared in this manner are perfectly acceptable for use in ELISA screening, its use in conjugate vaccine applications has been correlated with antibody response to the squarate residue itself.[17,19] Here, half esters of adipic acid phenyl ester were prepared according to our recently published procedure.[8,19,20] The oligosaccharide amines **23** and **24** were treated with five equivalents of the homobifunctional *p*-nitrophenyl ester **25** in dry DMF at room temperature for 5 h, affording the corresponding half esters **26** and **27** in good yields after purification on a reverse phase column, as shown in Scheme 3. The reaction is readily monitored by TLC or UV spectroscopy, and the half esters are stable during chromatography purifications on silica gel and reverse-phase under acidic conditions. Excess linker could be easily removed by washing with dichloromethane and the yields of this reaction were in the range of 60–65 %.

Formation of neoglycoproteins: With the required half esters at our disposal, coupling of **26** and **27** to BSA was performed by an 18 h incubation in buffer (pH 7.5) at ambient temperature. The BSA conjugates **28** and **29** were obtained as white powders after dialysis against deionized water followed by lyophilization (Scheme 4). In the same way, **26** and **27** were conjugated to tetanus toxoid (TT) in phosphate buffer (pH 7.2) overnight at ambient temperature. After dialysis against phosphate buffer saline (PBS) pH 7.2, the conjugates **30** and **31** were ready for vaccine formulation. Targeted and observed incorporations are tabulated below (Table 1). The degree of incorporation of the oligosaccharides on BSA and TT was established by MALDI-TOF MS using sinapinic acid as the matrix. Conjugation efficiencies of between 24 and 39 % were achieved, corresponding to the incorporation of 7–12 ligands on TT or BSA with a 30-fold molar excess of activated oligosaccharides similar to those published for the coupling of oligosaccharides to BSA.[8,19,20]

Table 1. BSA and tetanus toxoid mannopyranan conjugates.

Product	Saccharide [mg]	Molar ratio of protein/ester	Number of haptens	Incorporation efficiency [%]
28	2.0	1:30	11	36.7
29	0.5	1:20	7.8	39
30	1.2	1:30	7.2	24
31	0.6	1:30	8.7	29

Immunization studies in rabbits: To show the ability of synthetic *S*-analogues to mimic the corresponding *O*-linked native antigen we analyzed the binding of compounds **12**



Scheme 4. Synthesis of neoglycoproteins.

and **22** to monoclonal antibody C3.1 which is specific for the β-mannan oligosaccharide epitope of *C. albicans* phosphomannan. Figure 1 shows the results of the inhibition EIA.

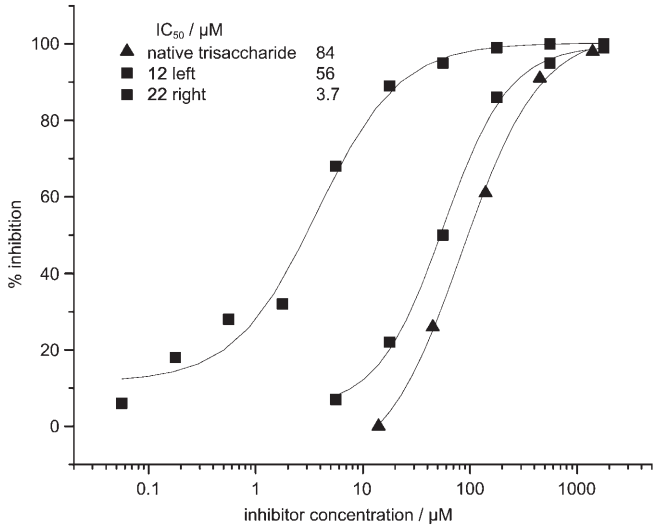


Figure 1. Inhibition assay (ELISA) for compounds **12** (■, left) and **22** (■, right) with β-mannan specific monoclonal Ab (C3.1). Plates were coated with trisaccharide–BSA conjugate and co-incubated with a constant concentration of monoclonal Ab and serially diluted **12** and **22**.

Plates were coated with β-mannan trisaccharide–BSA conjugate and co-incubated with monoclonal antibody C3.1 and serially diluted **12** and **22** *S*-analogues. Both compounds were able to inhibit binding of antibodies to cell wall antigen. Trisaccharide **22** ($IC_{50}=3.7$ μM) proved to be the tighter binding inhibitor compared with **12** ($IC_{50}=56$ μM). While this result confirms the close conformational similarity of the sulphur containing oligosaccharides to native *O*-linked oligosaccharide it raises the question why **22** is so much more active than **12** or the native trisaccharide.

We previously reported a tetrasaccharide also bearing a terminal *S*-linked mannose residue (compound **33** of ref. [14]). It exhibited only slightly better inhibition than the corresponding *O*-linked tetrasaccharide (≈1.7-fold more active).[17,21,22] Initially we attributed this activity to the relatively small binding site of the monoclonal antibody C3.1 that we believed to be complementary to a trisaccharide and the rather rigid antigen conformation that occurs in β1,2-*manno* oligomers.[22] Larger oligosaccharide such as the tetra through hexsaccharide bind with significantly reduced affinity.[22] We attributed this to the helical conformation of these antigens which places the fourth residue close to the first mannose.[22] Since the native structure is relatively rigid there is likely a steric clash at the periphery of the binding site, which account for the lower activity of larger structures. The thio-linked tetrasaccharide is able to release this steric interaction because thio glycosidic bonds are far less constrained than the corresponding *O*-linked antigen.[17,22]

Recent binding site mapping studies on a disaccharide epitope (Nycholat and Bundle, unpublished results) have suggested that the preferred binding epitope is possibly the terminal reducing disaccharide rather than a terminal nonreducing disaccharide. In this context **22** can provide this binding element whereas trisaccharide **12** cannot. In addition because the terminal *S*-linked mannose residue has greater flexibility there is the potential for mutual fitting of this third residue to create higher affinity interactions than is the case for the native trisaccharide. On going modeling studies of the C3.1 binding site are intended to clarify this point.

Further studies established immunogenic properties of compounds **23** and **24** conjugated to TT. Groups of three rabbits were immunized with both glycoconjugates. The immunization results were evaluated by ELISA using plates coated with either *O*-linked trisaccharide–BSA conjugate or *C. albicans* cell wall extract. The results for the titrations of sera obtained after two immunizations with both conjugates are shown in Figure 2. The average titre of immune sera (IgG subclass) reached 1:300000 for **30** and 1:100000 for **31** when evaluated against native trisaccharide–BSA conjugate. The titre against cell wall antigen was similar for both compounds and reached about 1:12000. Similar results were observed for immunization with *O*-linked trisaccharide–TT conjugate (unpublished data). The lower titre against the cell wall antigen suggests that a portion of serum antibodies recognize an epitope consisting of oligosaccharide plus at least part of the tether. The data show the tether effect applies similarly to *S*- and native *O*-linked oligosaccharides.

Conclusion

Two analogues of thio-linked (1→2)-β-mannanpyranan trisaccharide were synthesized employing a oxidation-reduc-

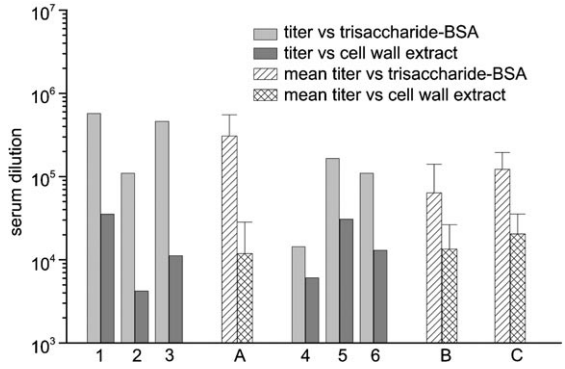


Figure 2. Titration of rabbit sera obtained by immunization with glycoconjugates **30**, **31** and *O*-linked trisaccharide–tetanus toxoid conjugate. Sera were titrated against *Candida* cell wall antigen and *O*-linked trisaccharide-BSA conjugate. Filled bars represents serum titre of individual rabbits, patterned bars show the geometric mean of titres for groups of animals, error bars represent the standard deviation. A) rabbits (1–3) immunized with **30**, B) rabbits (4–6) immunized with **31**, C) rabbits immunized with *O*-linked trisaccharide–tetanus toxoid conjugate (this group of eight animals was a part of another study, not yet published, for clarity individual titres are not shown).

tion methodology for the *O*-linked β-mannopranoside and S_N2 inversion for 1-thio-β-mannopyranoside. Conjugation of these oligosaccharides to BSA or tetanus toxoid using a linear homobifunctional linker was accomplished with high efficiency under mild conditions. Inhibition data show that the two *S*-linked trisaccharides are effective inhibitors of a monoclonal antibody generated to the native cell wall β-mannan, implying that these oligosaccharides can adopt conformations similar to the *O*-linked trisaccharide. Remarkably, antibody induced by the tetanus toxiod glycoconjugates **30** and **31** was cross-reactive with the native trisaccharide antigen and also exhibited affinity and titres for the cell wall of *C. albicans* similar to those induced by the corresponding *O*-linked conjugate.

Experimental Section

General methods: ^{1}H NMR spectra were recorded at either 400, 500, or 600 MHz, and are referenced to the residual protonated solvent peaks; δ_H 7.24 ppm for solutions in $CDCl_3$, and 0.1% external acetone (δ_H 2.225) for solutions in D_2O. Mass analysis was performed by positive-mode electrospray ionization on a hybrid sector-TOF mass spectrometer and for protein glycoconjugates by MALDI mass analysis, employing sinapinic acid as matrix. Analytical thin-layer chromatography (TLC) was performed on silica gel 60-F254 (Merck). TLC detection was achieved by charring with 5% sulfuric acid in ethanol. All commercial reagents were used as supplied. Column chromatography used silica gel (SiliCycle, Quebec City, Quebec, 230–400 mesh, 60 Å), and redistilled solvents. HPLC separations were performed on a Beckmann C18 semipreparative reversed-phase column with a combination of methanol and water containing 0.1% HOAC as eluents. Photoadditions were carried out using a spectroline model ENF-260C UV lamp and cylindrical quartz vessels.

Allyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-*O*-trifluoromethanesulfonyl-β-D-glucopyranoside (2): Alcohol **1** (485 mg, 1.28 mmol) was dissolved in dichloromethane (10 mL) and pyridine (570 μL) was added followed by *N*,*N*-dimethyl-4-aminopyridine (50 mg, 0.4 mmol). The resulting solution was cooled to 0°C and trifluoromethanesulfonic anhydride (384 μL, 2.27 mmol) was added dropwise, then the reaction mixture was warmed up to room temperature and stirred for 2.0 h. The solution was diluted with dichloromethane, washed with aqueous sodium bicarbonate, dried over sodium sulfate, and concentrated to a brown oil. The residue was purified by chromatography with ethyl acetate/hexane 1:4 to give product **2** (610 mg, 94%). $[\alpha]_D = -45.5°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.46–7.22 (m, 10H, Ar), 5.97–5.84 (m, 1H, $OCH_2CH{=}CH_2$), 5.57 (s, 1H, PhC*H*), 5.37–5.24 (m, 2H, $OCH_2CH{=}CH_2$), 4.92–4.71 (dd, 2H, CH_2Ph), 4.68–4.62 (m, 2H, 1-*H*, 2-*H*), 4.41–4.34 (m, 2H, 6-*H*, $OCH_2CH{=}CH_2$), 4.19–4.12 (m, 1H, $OCH_2CH{=}CH_2$), 3.91–3.73 (m, 3H, 3-*H*, 4-*H*, 6′-*H*), 3.51–3.43 ppm (m, 1H, 5-*H*); ESI HRMS: *m*/*z*: calcd for $C_{24}H_{25}O_8F_3SNa$: 553.11145; found: 553.11155.

Allyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thioacetyl-β-D-mannopyranoside (3): Compound **2** (106 mg, 0.2 mmol) and potassium thioacetate (456 mg, 4 mmol) were put together and DMF (5 mL) was added under argon. The resulting mixture was stirred at 70°C for 30 min. TLC check indicated that the starting material disappeared. The reaction mixture was diluted with toluene (50 mL), washed several times with water and brine, dried with sodium sulfate and concentrated to a brown oil. Purification by chromatography with ethyl acetate/hexane 1:4 gave **3** (83 mg, 91%). $[\alpha]_D = -53.3°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.48–7.24 (m, 10H, Ar), 5.86–5.83 (m, 1H, $OCH_2CH{=}CH_2$), 5.59 (s, 1H, PhC*H*), 5.32–5.20 (m, 2H, $OCH_2CH{=}CH_2$), 4.77 (m, 1H, 1-*H*), 4.75–4.62 (dd, 2H, CH_2Ph), 4.61 (m, 1H, 2-*H*), 4.36–4.33 (m, 1H, $OCH_2CH{=}CH_2$), 4.31 (m, 1H, 6-*H*), 4.15–4.11 (m, 1H, $OCH_2CH{=}CH_2$), 3.97 (dd, ^3J = 4.67, 9.71 Hz, 1H, 3-*H*), 3.83 (t, ^3J = 10.2 Hz, 1H, 6′-*H*), 3.69 (t, ^3J = 9.6 Hz, 1H, 4-*H*), 3.40 ppm (m, 1H, 5H); ESI HRMS: *m*/*z*: calcd for $C_{25}H_{28}O_6SNa$: 479.14988; found: 479.14986.

Allyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (4): Hydrazine acetate (35 mg, 0.34 mmol) was added to a solution of compound **3** (104 mg, 0.23 mmol) in THF (5 mL), and the mixture was stirred for 3 h. TLC check indicated that the reaction was complete. The mixture was diluted with ethyl acetate (30 mL), washed with water then brine and dried with sodium sulfate followed by concentration. The residue was purified by chromatography on silica gel using ethyl acetate/hexane 1:4 to give **4** (83 mg, 87%). $[\alpha]_D = -63.1°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.54–7.24, 5.96–5.77 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (s, 1H, PhC*H*), 5.36–5.22 (m, 2H, $OCH_2CH{=}CH_2$), 4.80–4.74 (m, 2H, CH_2Ph), 4.72 (s, 1H, 1-*H*), 4.38–4.41 (m, 1H, $OCH_2CH{=}CH_2$), 4.35–4.32 (m, 1H, 6-*H*), 4.26 (t, ^3J = 9.0 Hz, 1H, 4-*H*), 4.15–4.11 (m, 1H, $OCH_2CH{=}CH_2$), 3.92 (t, 1H, 6′-*H*), 3.87 (m, 2H, 3-*H*, 2-*H*), 3.41–3.37 ppm (m, 1H, 5-*H*); ESI HRMS: *m*/*z*: calcd for $C_{23}H_{26}O_5SNa$: 437.13932; found: 437.13909.

Allyl (2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1→2)-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (6): Glycosyl donor **5** (140 mg, 0.21 mmol), monosaccharide acceptor **4** (67 mg, 0.162 mmol) and activated 4 Å molecular sieves (20 mg) were dried together in a pear-shaped flask (10 mL) under vacuum for one hour then dichloromethane (3 mL)was added. The suspension was stirred for 10 min. at room temperature under argon, and then the temperature was reduced to −40°C and 0.1 M trimethylsilyl trifluoromethanesulfonate solution in dichloromethane (80 μL) was added dropwise. After 30 min., the reaction mixture was neutralized with triethylamine and concentrated in vacuum. The residue was purified by flash chromatography (*n*-hexane/ethyl acetate 6:1) to afford **6** (115 mg, 80%) as a white foam. $[\alpha]_D = -49.5°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.5–7.2 (m, 25H, Ar), 5.95–5.86 (m, 1H, $OCH_2CH{=}CH_2$), 5.56 (s, 1H, PhC*H*), 5.36–5.20 (m, 2H, $OCH_2CH{=}CH_2$), 5.09–5.05 (t, ^3J = 10 Hz, 1H, 2b-*H*), 4.92–4.78 (m, 4H, 1b-*H*, 3/2 CH_2Ph), 4.72–4.70 (d, ^2J = 12.0 Hz, 1H, $^1/_2CH_2Ph$), 4.65 (s, 1H, 1a-*H*), 4.58–4.47 (m, 4H, $2CH_2Ph$), 4.42–4.34 (m, 1H, $OCH_2CH{=}CH_2$), 4.28 (m, 1H, 6a-*H*), 4.10–4.02 (m, 1H, $OCH_2CH{=}CH_2$), 3.90 (dd, 1H, 2a-*H*), 3.80–3.87 (m, 2H, 3a-*H*, 6b-*H*), 3.78–3.62 (m, 5H, 6′a-*H*, 6′b-*H*, 3b-*H*, 4a-*H*, 4b-*H*), 3.54–3.48 (m, 1-H, 5b-*H*), 3.38–3.30 ppm (m, 1H, 5a-*H*); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.1–126.2, 116.9, 101.5, 100.8, 84.5, 84.1, 79.1, 78.1, 75.2, 75.22, 75.1, 73.4, 73.1, 69.7, 69.4, 68.6, 67.7 ppm; ESI HRMS: *m*/*z*: calcd for $C_{52}H_{56}O_{11}SNa$: 911.34356; found: 911.34321.

Allyl 3-*O*-benzyl-2-*O*-(3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (7): Sodium methoxide (0.7 mg, 0.013 mmol) was added to a solution of **6** (115 mg, 0.13 mmol) in methanol (5 mL) and the mixture was stirred at room temperature overnight. Then it was neutralized with IR 120 (H^+ form), and concentrated in vacuum. The residue was purified by flash chromatography (n-hexane/ethyl acetate 4:1) to afford **7** (110 mg, 100%) as a white foam. $[\alpha]_D = -38.5°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.5$–7.2 (m, 25H, Ar), 5.90–5.82 (m, 1H, $OCH_2CH{=}CH_2$), 5.60 (s, 1H, PhC*H*), 5.36–5.30 (m, 1H, $OCH_2CH{=}CH_2$), 5.20–5.16 (m, 1H, $OCH_2CH{=}CH_2$), 5.00 (d, $^2J = 11.4$ Hz, 1H, $^1/_2\,CH_2Ph$), 4.90–4.76 (m, 4H, $2\,CH_2Ph$), 4.64 (s, 1H, 1a-*H*), 4.58–4.54 (m, 2H, CH_2Ph), 4.49–4.46 (d, $^2J = 11.4$ Hz, 1H, $^1/_2\,CH_2Ph$), 4.40–4.34 (m, 2H, 1b-*H*, $OCH_2CH{=}CH_2$), 4.32–4.28 (m, 1H, 6a-*H*), 4.14–4.08 (m, 1H, $OCH_2CH{=}CH_2$), 3.90–3.82 (m, 3H, 3a-*H*, 4a-*H*, 6′a-*H*), 3.75 (m, 2H, 6b-*H*, 6′b-*H*), 3.68–3.58 (m, 4H, 2a-*H*, 2b-*H*, 3b-*H*, 4b-*H*), 3.50 (m, 1H, 5b-*H*), 3.44 (s, 1H, OH), 3.37–3.32 ppm (m, 1H, 5a-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 139.1–126.0, 117.8, 101.6, 99.9, 86.7, 85.7, 80.3, 79.7, 76.8, 75.7, 75.1, 75.0, 74.0, 73.7, 72.6, 69.9, 69.3, 68.6, 67.7 ppm; ESI HRMS: *m*/*z*: calcd for $C_{50}H_{54}O_{10}SNa$: 869.33299; found: 869.33291.

Allyl 3-*O*-benzyl-2-*O*-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (8): Disaccharide **7** (46 mg, 0.054 mmol) was dissolved in freshly distilled DMSO (4 mL) and acetic anhydride (2 mL) was added. The resulting solution was stirred for 18 h at room temperature then diluted with ethyl acetate, washed with water, aqueous sodium bicarbonate and a brine solution. Finally, the solution was concentrated at low pressure to give a yellow syrup. It was dissolved in THF (5 mL), cooled to −78 °C under argon, then L-selectride (1 M THF, 0.5 mL) was added dropwise and the reaction mixture was stirred for 15 min. The cooling bath was removed and the solution was allowed to warm up to room temperature. The reaction was quenched after 15 min with methanol (0.5 mL), and the mixture was diluted with dichloromethane. Washings with a solution of hydrogen peroxide (5%) and sodium hydroxide (1 M) followed by sodium thiosulfate (5%) and sodium chloride solutions gave a clear organic solution. It was dried over magnesium sulfate and concentrated to a colourless oil which was purified by flash chromatography (*n*-hexane/ethyl acetate 3:1) to afford **8** (37 mg, 80%) as a clear syrup. $[\alpha]_D = -45.6°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.46$–7.20 (m, 25H, Ar), 5.90–5.83 (m, 1H, $OCH_2CH{=}CH_2$), 5.56 (s, 1H, PhCH), 5.27–5.22 (m, 1H, $OCH_2CH{=}CH_2$), 5.20–5.16 (m, 1H, $OCH_2CH{=}CH_2$), 5.04 (s, 1H, 1b-*H*), 4.92 (m, 2H, CH_2Ph), 4.76 (d, $^2J = 12$ Hz, 1H, $^1/_2\,CH_2Ph$), 4.67 (s, 1H, 1a-*H*), 4.5–4.67 (m, 5H, $^5/_2\,CH_2Ph$), 4.44–4.40 (m, 1H, $OCH_2CH{=}CH_2$), 4.31 (dd, $^3J = 10.2$, 4.8 Hz, 1H, 4a-*H*), 4.25 (t, $^3J = 3.6$ Hz, 1H, 2b-*H*), 4.09–4.06 (m, 1H, $OCH_2CH{=}CH_2$), 3.94 (dd, $^3J = 4.8$, 1.8 Hz, 1H, 2a-*H*), 3.90–3.84 (m, 3H, 3a-*H*, 4b-*H*, 6a-*H*), 3.80–3.72 (m, 3H, 6b-*H*, 6′a-*H*, 6′b-*H*), 3.59 (dd, $^3J = 9.6$, 3.6 Hz, 1H, 3b-*H*), 3.51 (m, 1H, 5b-*H*), 3.37 ppm (m, 1H, 5a-*H*); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.2–126.1, 117.6, 101.6, 100.9, 83.6, 82.7, 79.7, 79.0, 75.4, 75.0, 74.4, 73.5, 71.3, 70.1, 69.9, 69.8, 68.9, 68.6, 67.9 ppm; ESI HRMS: *m*/*z*: calcd for $C_{50}H_{54}O_{10}SNa$: 869.33299; found: 869.33308.

Allyl (2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-(1→2)-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (9): The procedure used was analogous to the preparation of **6**, using glycosyl donor **5** (63 mg, 0.1 mmol), disaccharide **8** (70 mg, 0.083 mmol), dichloromethane (3 mL), trimethylsilyl trifluoromethanesulfonate (33 μL) (0.05 M CH_2Cl_2), activated 4 Å molecular sieves (20 mg) and the temperature was −10 °C. Column chromatography in *n*-hexane/ethyl acetate 4:1 gave trisaccharide **9** (90 mg, 83%). $[\alpha]_D = -40.6°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 7.54–7.20 (m, 40H, Ar), 5.80 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (s, 1H, PhCH), 5.21–5.17 (m, 2H, 2c-*H*, $OCH_2CH{=}CH_2$), 5.20–5.16 (m, 1H, $OCH_2CH{=}CH_2$), 5.10–5.08 (m, 1H, $OCH_2CH{=}CH_2$), 4.98–4.77 (m, 8H, 1a-*H*, 1c-*H*, $3\,CH_2Ph$), 4.68 (s, 1H, 1b-*H*), 4.60–4.41 (m, 10H, 2a-*H*, $OCH_2CH{=}CH_2$, $4\,CH_2Ph$), 4.33 (m, 1H, 6b-*H*), 4.06–4.02 (m, 1H, $OCH_2CH{=}CH_2$), 3.93 (t, $^3J = 10.2$ Hz, 1H, 4b-*H*), 3.89–3.80 (m, 4H, 3b-*H*, 2b-*H*, 3c-*H*, 6c-*H*), 3.72–3.62 (m, 7H, 4a-*H*, 4c-*H*, 5c-*H*, 6a-*H*, 6′a-*H*, 6′c-*H*, 6′b-*H*), 3.50 (m, 1H, 5a-*H*), 3.47 (dd, $^3J = 3.0$, 9.0 Hz, 1H, 3a-*H*), 3.38 (m, 1H, 5b-*H*), 1.98 ppm (s, 3H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.6–117.7, 101.6, 101.2, 101.1, 84.4, 83.1, 81.3, 79.9, 78.7, 75.5, 75.3, 75.0, 74.8, 74.7, 74.5, 73.8, 73.5, 73.4, 73.3, 70.9, 70.3, 69.8, 69.7, 69.4, 68.5, 68.1 ppm; ESI HRMS: *m*/*z*: calcd for $C_{79}H_{84}O_{16}SNa$: 1343.53723; found: 1343.53749.

Allyl (3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-(1→2)-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thio-β-D-mannopyranoside (10): Compound **10** was prepared according to the procedure for preparation of **7** using trisaccharide **9** (90 mg, 0.068 mmol), sodium methoxide (0.4 mg), dichloromethane (2 mL), methanol (1 mL). Column chromatography in *n*-hexane/ethyl acetate 3:1 gave trisaccharide **10** (87 mg, 100%). $[\alpha]_D = -1.2°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.50$–7.38 (m, 40H, Ar), 5.86–5.80 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (s, 1H, PhCH), 5.23–5.19 (m, 1H, $OCH_2CH{=}CH_2$), 5.14–5.10 (m, 2H, $OCH_2CH{=}CH_2$), $^1/_2\,CH_2Ph$), 5.07 (s, 1H, 1a-*H*), 4.98–4.81 (m, 6H, $3\,CH_2Ph$), 4.75 (d, $^3J = 7.2$ Hz, 1H, 1c-*H*), 4.69 (d, $^3J =$ 1.2 Hz, 1H, 1b-*H*), 4.56–4.44 (m, 9H, $4\,CH_2Ph$, 2a-*H*), 4.40–4.39 (m, 2H, $^1/_2\,CH_2Ph$, $OCH_2CH{=}CH_2$), 4.31 (m, 1H, 6b-*H*), 4.07–4.04 (m, 1H, $OCH_2CH{=}CH_2$), 3.95 (dd, $^3J = 1.2$, 4.8 Hz, 1*H*, 2b-*H*), 3.91–3.86 (m, 3H, 3b-*H*, 4a-*H*, 6′b-*H*), 3.82 (t, $^3J = 7.8$, 9.0 Hz, 1H, 2c-*H*), 3.78–3.68 (m, 5H, 3c-*H*, 4b-*H*, 6a-*H*, 6′a-*H*, 6′c-*H*), 3.66–3.58 (m, 3H, 4c-*H*, 5c-*H*, 6c-*H*), 3.52 (dd, $^3J = 3.0$, 9.0 Hz, 1H, 3a-*H*), 3.48 (m, 1H, 5a-*H*), 3.38 (m, 1H, 5b-*H*), 3.22 ppm (br, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 139.3–117.8, 103.7, 101.6–101.1, 85.2, 83.7, 81.4, 79.9, 78.8, 76.9, 75.7, 75.5, 74.9, 74.88, 74.81, 74.2, 74.1, 73.5, 73.3, 70.2, 69.9, 69.7, 69.6, 68.5, 68.1 ppm; ESI HRMS: *m*/*z*: calcd for $C_{77}H_{82}O_{15}SNa$: 1301.52667; found: 1301.52639.

Allyl (3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-(1→2)-3-*O*-benzyl-4,6-O-benylidene-2-deoxy-2-thio-β-D-mannopyranoside (11): The procedure used was analogous to the preparation of **8** using trisaccharide **10** (60 mg, 0.047 mmol), DMSO (4 mL), acetic anhydride (2 mL), THF (3 mL), L-selectride (1 M, 0.5 mL). Column chromatography in *n*-hexane/ethyl acetate 5:2 gave trisaccharide **11** (50 mg, 83%). $[\alpha]_D = -68.4°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.50$–7.38 (m, 40H, Ar), 5.86–5.80 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (s, 1H, PhCH), 5.24–5.21 (m, 1H, $OCH_2CH{=}CH_2$), 5.16–5.14 (m, 1H, $OCH_2CH{=}CH_2$), 5.08 (s, 1H, 1b-*H*), 5.00–4.91 (m, 5H, 1c-*H*, $2\,CH_2Ph$), 4.83–4.81 (d, $^2J = 12.0$ Hz, 1H, $^1/_2\,CH_2Ph$), 4.71 (d, $^3J = 1.4$ Hz, 1H, 1a-*H*), 4.67 (d, $^3J = 3.4$ Hz, 1H, 2b-*H*), 4.62–4.41 (m, 10H, $OCH_2CH{=}CH_2$, $4\,CH_2Ph$, $^1/_2\,CH_2Ph$), 4.38–4.36 (d, $^2J = 12.0$ Hz, 1H, $^1/_2\,CH_2Ph$), 4.32 (m, 1H, 6b-*H*), 4.10 (m, 1H, $OCH_2CH{=}CH_2$), 4.05 (dd, $^3J = 4.9$, 1.4 Hz, 1H, 2a-*H*), 3.97 (t, $^3J = 9.5$ Hz, 1H, 4c-*H*), 3.91 (dd, $^3J =$ 4.9, 9.9 Hz, 1H, 3a-*H*), 3.86 (t, $^3J = 10.4$ Hz, 1H, 4b-*H*), 3.82–3.78 (m, 2H, 6a-*H*, 6c-*H*), 3.75–3.68 (m, 2H, 6′a-*H*, 6′c-*H*), 3.66–3.62 (m, 2H, 4a-*H*, 6′b-*H*), 3.58 (dd, $^3J = 2.9$, 9.1 Hz, 1H, 3c-*H*), 3.56–3.50 (m, 3H, 3b-*H*, 5a-*H*, 5c-*H*), 3.39 ppm (m, 1H, 5b-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.4–137.3, 133.3, 128.9–126.1, 117.7, 101.6, 101.1, 99.6, 83.5, 81.5, 81.4, 79.7, 78.7, 77.3, 77.0, 76.8, 75.3, 75.2, 75.1, 74.9, 74.3, 74.2, 73.3, 73.2, 71.8, 70.7, 70,3, 70.2, 69.5, 69.4, 68.5, 67.9, 67.8 ppm; ESI HRMS: *m*/*z*: calcd for $C_{77}H_{82}O_{15}SNa$: 1301.52667; found: 1301.52639.

Propyl (β-D-mannopyranosyl)-(1→2)-(1-thio-β-D-mannopyranosyl)-(1→2)-2-deoxy-2-thio-β-D-mannopyranoside (12): Compound **11** (27 mg, 0.02 mmol) and Pd/C-activated (10% Pd) (50 mg) were put in the flask. The whole system was vacuumed for 10 min. followed by the addition of hydrogen. Methanol (5 mL) was added and the reaction mixture was stirred overnight. TLC check indicated that the starting material disappeared. The reaction mixture was filtered through Celite to give the clear solution which was concentrated. The resulting residue was purified by HPLC on C18 silica to give product **12** (10 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.02$ (s, 1H, 1b-*H*), 4.88 (s, 1H, 1c-*H*), 4.87 (s, 1H, 1a-*H*), 4.33 (d, $^3J = 3.0$ Hz, 1H, 2b-*H*), 4.21 (d, $^3J = 4.8$ Hz, 1H, 2a-*H*), 3.95–3.57 (m, 14H, OCH_2, 3a-*H*, 3b-*H*, 3c-*H*, 4a-*H*, 4b-*H*, 6a-*H*, 6′a-*H*, 6b-*H*, 6′b-*H*, 6c-*H*, 6′c-*H*, 2c-*H*), 3.44–3.34 (m, 3H, 5a-*H*, 5b-*H*, 5c-*H*), 3.30 (t, 1H, 4c-*H*), 1.61 (m, 2H, $OCH_2CH_2CH_3$), 0.91 ppm (t, 3H, $OCH_2CH_2CH_3$); ESI HRMS: *m*/*z*: calcd for $C_{21}H_{38}O_{15}SNa$: 585.18236; found: 585.18243.

Allyl 2-*O*-(2-*O*-acetyl-3-*O*-benzyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (15): The procedure used here was analogous to the preparation of **6**. Glycosyl donor **14** (470 mg,

0.86 mmol), acceptor **13** (350 mg, 0.72 mmol) were used and dichloromethane (8 mL), trimethylsilyl trifluoromethanesulfonate (4.6 μL), activated 4 Å molecular sieves (100 mg) and the temperature was −10 °C. Column chromatography in *n*-hexane/ethyl acetate 4:1 gave trisaccharide **15** (520 mg, 83 %). $[\alpha]_D = -24.4°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.56–7.24 (m, 25 H, Ar), 5.98–5.92 (m, 1 H, $OCH_2CH{=}CH_2$), 5.62 (s, 1 H, PhCH), 5.38–5.34 (m, 1 H, $OCH_2CH{=}CH_2$), 5.25–5.20 (m, 2 H, 2b-H, $OCH_2CH{=}CH_2$), 5.05 (d, ^3J = 8.4 Hz, 1 H, 1b-*H*), 4.98–4.54 (m, 8 H, 4 CH_2Ph), 4.45–4.40 (m, 3 H, 1a-H, 6b-H, $OCH_2CH{=}CH_2$), 4.25 (d, ^3J = 3.0 Hz, 1 H, 2a-*H*), 4.48–4.45 (m, 1 H, $OCH_2CH{=}CH_2$), 3.94–3.82 (m, 4 H, 4b-*H*, 3b-*H*, 6′b-*H*, 6a-*H*), 3.72–3.64 (m, 2 H, 4a-*H*, 6a-*H*), 3.57–3.48 (m, 3 H, 3a-*H*, 5a-*H*, 5b-*H*), 2.12 ppm (s, 3 H, Ac); ESI HRMS: m/z: calcd for $C_{52}H_{56}O_{12}$Na: 895.36640; found: 895.36597.

Allyl 3,4,6-tri-*O*-benzyl-2-*O*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-*O*-trifluoromethanesulfonyl-β-D-glucopyranosyl)-β-D-mannopyranoside (16): Sodium methoxide (3.24 mg, 0.06 mmol) was added to a solution of **15** (520 mg, 0.60 mmol) in methanol (10 mL) and the resulting mixture was stirred overnight at room temperature. Then it was neutralized with IR 120 (H^+ form), and concentrated in vacuum. The crude product was dissolved in dichloromethane (10 mL) and pyridine (270 μL) was added followed by *N*,*N*-dimethyl-4-aminopyridine (24 mg, 0.19 mmol). The resulting solution was cooled to 0 °C and trifluoromethanesulfonic anhydride (180 μL, 1.08 mmol) was added dropwise, then the reaction mixture was warmed up to room temperature and stirred for 2.0 h. The solution was diluted with dichloromethane, washed with aqueous sodium bicarbonate, dried over sodium sulfate, and concentrated to a brown oil. The residue was purified by chromatography with ethyl acetate/hexane 1:4 to give **16** (520 mg, 90 %). $[\alpha]_D = -28.4°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.42–7.21 (m, 25 H, Ar), 5.96–5.90 (m, 1 H, $OCH_2CH{=}CH_2$), 5.54 (s, 1 H, PhCH), 5.29–5.20 (m, 3 H, $OCH_2CH{=}CH_2$, 1b-*H*), 4.95–4.90 (m, 2 H, CH_2Ph), 4.82–4.75 (m, 3 H, 2b-*H*, CH_2Ph), 4.66–4.50 (m, 4 H, 2 CH_2Ph), 4.47 (s, 1 H, 1a-H), 4.44–4.40 (m, 1 H, $OCH_2CH{=}CH_2$), 4.38 (m, 1 H, 6b-H), 4.33 (d, 1 H, 2a-*H*), 4.06–4.02 (m, 1 H, $OCH_2CH{=}CH_2$), 3.92 (t, ^3J = 9.0 Hz, 1 H, 3b-*H*), 3.86–3.80 (m, 2 H, 4b-*H*, 6a-*H*), 3.77 (t, 1 H, 6′b-*H*), 3.67–3.62 (m, 2 H, 4a-H, 6′a-H0, 3.58–3.46 ppm (m, 3 H, 3a-*H*, 5a-*H*, 5b-*H*).

Allyl 2-*O*-3,4,6-tri-*O*-benzyl-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-thioacetyl-β-D-mannopyranosyl)-β-D-mannopyranoside (17): The procedure used here was analogous to the preparation of **3**, using compound **16** (1.2 g, 1.25 mmol), potassium thioacetate (2.84 g, 24.9 mmol), and DMF (30 mL). Column chromatography in *n*-hexane/ethyl acetate 6:1 gave trisaccharide **17** (700 mg, 63 %). $[\alpha]_D = -77.0°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.52–7.20 (m, 25 H, Ar), 5.96–5.90 (m, 1 H, $OCH_2CH{=}CH_2$), 5.55 (s, 1 H, PhCH), 5.29 (s, 1 H, 1b-H), 5.28–5.18 (m, 2 H, $OCH_2CH{=}CH_2$), 4.90–4.74 (m, 4 H, 2b-*H*, $^3/_2$ CH_2Ph), 4.64–4.50 (m, 5 H, $^5/_2$ CH_2Ph), 4.46–4.42 (m, 1 H, $OCH_2CH{=}CH_2$), 4.41 (s, 1 H, 1a-*H*), 4.35 (d, ^3J = 3.0 Hz, 1 H, 2a-*H*), 4.29 (m, 1 H, 6a-*H*), 4.04–4.00 (m, 1 H, $OCH_2CH{=}CH_2$), 3.96 (dd, ^3J = 4.5, 9.5 Hz, 1 H, 3b-*H*), 3.82–3.68 (m, 5 H, 4a-*H*, 4b-*H*, 6′a-*H*, 6b-*H*, 6′b-*H*), 3.54 (dd, ^3J = 3.0, 9.0 Hz, 1 H, 3a-*H*), 3.46–3.40 (m, 2 H, 5a-*H*, 5b-*H*), 2.22 ppm (s, 3 H, SAc); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.6–126.1, 117.1, 101.5, 99.9, 99.6, 80.6, 80.2, 75.9, 75.5, 75.1, 74.6, 73.4, 72.1, 71.5, 70.4, 69.9, 69.6, 68.7, 67.6 ppm; ESI HRMS: m/z: calcd for $C_{52}H_{56}O_{11}$SNa: 911.34356; found: 911.34366.

Allyl 3,4,6-tri-*O*-benzyl-2-*O*-(3-*O*-benzyl-4,6-O-benzylidene-2-deoxy-2-thio-β-D-mannopyranosyl)-β-D-mannopyranoside (18): The procedure used was analogous to the preparation of **4** using compound **17** (0.7 g, 0.79 mmol), hydrazine acetate (165 mg, 1.58 mmol), and THF (20 mL). Column chromatography in *n*-hexane/ethyl acetate 5:1 gave trisaccharide **18** (560 mg, 84 %). $[\alpha]_D = -77.0°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.48–7.20 (m, 25 H, Ar), 5.94–5.86 (m, 1 H, $OCH_2CH{=}CH_2$), 5.62 (s, 1 H, PhCH), 5.26–5.19 (m, 2 H, $OCH_2CH{=}CH_2$), 5.18 (s, 1 H, 1b-*H*), 4.92–4.52 (m, 8 H, 4 CH_2Ph), 4.45–4.40 (m, 3 H, 1a-H, 2a-H, $OCH_2CH{=}CH_2$), 4.34 (t, 1 H, 4b-H), 4.29 (m, 1 H, 6b-H), 4.17 (m, 1 H, 2b-H), 4.05 (m, 1 H, $OCH_2CH{=}CH_2$), 3.90–3.80 (m, 4 H, 4a-H, 3b-H, 6′b-H, 6a-H), 3.74 (m, 1 H, 6a-*H*), 3.58 (dd, ^3J = 3.0, 9.0 Hz, 1 H, 3a-*H*), 3.48–3.40 (m, 2 H, 5a-*H*, 5b-*H*), 2.40 ppm (s, 1 H, SH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.4–126.1, 117.3, 101.6, 99.9, 99.6, 80.7, 78.1, 76.0, 75.7, 75.2, 74.2, 73.4, 72.0, 71.2, 70.5, 70.1, 69.5, 68.7, 67.8 ppm; ESI HRMS: m/z: calcd for $C_{50}H_{54}O_{10}$SNa 869.33299; found: 869.33323.

Allyl (2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1→2)-(3-*O*-benzyl-4,6-O-benzylidene-2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (19): The procedure used was analogous to the preparation of **6**. Glycosyl donor **5** (120 mg, 0.19 mmol) and disaccharide **18** (81 mg, 0.096 mmol) were used, dichloromethane (3 mL), trimethylsilyl trifluoromethanesulfonate (38 μL; 0.05 M CH_2Cl_2), activated 4 Å molecular sieves (20 mg) and the temperature was −10 °C. Column chromatography in *n*-hexane/ethyl acetate 5:1 gave trisaccharide **19** (78 mg, 62 %). $[\alpha]_D = -57.8°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.56–7.10 (m, 40 H, Ar), 5.92–5.86 (m, 1 H, $OCH_2CH{=}CH_2$), 5.56 (s, 1 H, PhCH), 5.29 (m, 1 H, 1c-*H*), 5.27–5.23 (m, 1 H, $OCH_2CH{=}CH_2$), 5.18–5.14 (m, 3 H, 1b-*H*, 2c-*H*, $OCH_2CH{=}CH_2$), 5.00–4.38 (m, 17 H, 8 CH_2Ph, $OCH_2CH{=}CH_2$), 4.20 (m, 1 H, 6b-*H*), 4.16 (m, 1 H, 2b-*H*), 4.07–4.03 (m, 1 H, $OCH_2CH{=}CH_2$), 3.91–3.85 (m, 3 H, 3c-*H*, 3b-*H*, 4a-*H*), 3.83–3.74 (m, 6 H, 4c-*H*, 6′b-*H*, 6a-*H*, 6′a-*H*, 6c-*H*, 6′c-*H*), 3.72–3.68 (m, 2 H, 4b-*H*, 5c-*H*), 3.60 (dd, ^3J = 3.0, 9.0 Hz, 1 H, 3a-*H*), 3.45 (m, 1 H, 5a-*H*), 3.38 (m, 1 H, 5b-H), 1.72 ppm (s, 3 H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$):δ = 138.5–126.2, 117.1, 102.3, 101.5, 100.2, 85.0, 84.4, 80.8, 78.9, 78.6, 78.5, 75.5, 75.4, 75.1, 75.1, 75.0, 73.4, 73.1, 73.0, 70.3, 69.8, 69.6, 69.5, 69.3, 68.6, 67.9 ppm; ESI HRMS: m/z: calcd for $C_{79}H_{84}O_{16}$SNa: 1343.53723; found: 1343.53728.

Allyl (3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl)-(1→2)-(3-*O*-benzyl-4,6-O-benzylidene-2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (20): The procedure used was analogous to the preparation of **7** using trisaccharide **19** (75 mg, 0.057 mmol), sodium methoxide (0.3 mg), dichloromethane (2 mL) and methanol (1 mL). Column chromatography in *n*-hexane/ethyl acetate 4:1 gave trisaccharide **20** (72 mg, 100 %). $[\alpha]_D = -57.8°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.52–7.20 (m, 40 H, Ar), 5.92–5.86 (m, 1 H, $OCH_2CH{=}CH_2$), 5.59 (s, 1 H, PhCH), 5.31 (s, 1 H, 1b-*H*), 5.26–5.18 (m, 2 H, $OCH_2CH{=}CH_2$), 5.05 (d, ^2J = 10.8 Hz, 1 H, $^1/_2$ CH_2Ph), 5.0–4.84 (m, 4 H, 2 CH_2Ph), 4.75 (d, ^3J = 10.2 Hz, 1 H, 1c-*H*), 4.68–4.52 (m, 8 H, 2a-*H*, $^7/_2$ CH_2Ph), 4.5–4.38 (m, 5 H, 1a-*H*, CH_2Ph, $OCH_2CH{=}CH_2$), 4.35 (m, 1 H, 6b-*H*), 4.07–4.0 (m, 3 H, 2b-H, 4a-H, $OCH_2CH{=}CH_2$), 3.92–3.76 (m, 5 H, 3b-*H*, 4b-*H*, 6′b-*H*, 6a-*H*, 6′a-*H*), 3.73–3.66 (m, 3 H, 3c-*H*, 4c-*H*, 6c-*H*), 3.60–3.54 (m, 3 H, 3a-*H*, 2c-*H*, 5c-*H*), 3.45–3.38 ppm (m, 3 H, 5a-*H*, 5b-*H*, 6′c-*H*); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.9–126.2, 117.5, 101.6, 100.4, 100.3, 89.6, 87.2, 80.5, 79.4, 78.9, 76.9, 75.7, 75.5, 75.4, 75.2, 75.1, 75.0, 74.5, 73.6, 73.4, 70.9, 70.6, 70.3, 69.9, 69.8, 69.5, 68.7, 67.9 ppm; ESI HRMS: m/z: calcd for $C_{77}H_{82}O_{15}$SNa: 1301.52667; found: 1301.52659.

Allyl (3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-(1→2)-(3-*O*-benzyl-4,6-O-benzylidene-2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-3,4,6-tri-*O*-benzyl-β-D-mannopyranoside (21): The synthesis of **21** was accomplished using the procedure for preparation of **8**. Trisaccharide **20** (60 mg, 0.047 mmol), dimethyl sulfoxide (4 mL), acetic anhydride (2 mL), THF (3 mL) and L-selectride (1 M, 0.5 mL) were used. Column chromatography in *n*-hexane/ethyl acetate 5:2 gave trisaccharide **21** (30 mg, 50 %). $[\alpha]_D = -33.8°$ (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.50–7.05 (m, 40 H, Ar), 5.89–5.82 (m, 1 H, $OCH_2CH{=}CH_2$), 5.57 (s, 1 H, CHPh), 5.36 (s, 1 H, 1c-*H*), 5.32 (s, 1 H, 1b-*H*), 5.24–5.14 (m, 2 H, $OCH_2CH{=}CH_2$), 4.93–4.8 (m, 4 H, 2 CH_2Ph), 4.56–4.46 (m, 8 H, 2a-*H*, 7/2 CH_2Ph), 4.43 (s, 1 H, 1a-*H*), 4.41–4.3 (m, 4 H, $OCH_2CH{=}CH_2$, $^3/_2$$CH_2$Ph), 4.23 (d, 1 H, $^1/_2$$CH_2$Ph), 4.14–4.13 (m, 2 H, 2b-*H*, 2c-*H*), 4.00 (m, 1 H, $OCH_2CH{=}CH_2$), 3.92–3.86 (m, 3 H, 3b-*H*, 4a-*H*, 4c-*H*), 3.80 (t, ^3J = 11.4 Hz, 1 H, 4b-*H*), 3.74–3.59 (m, 8 H, 6a-*H*, 6′a-*H*, 6b-*H*, 6′b-*H*, 6c-*H*, 6′c-*H*, 5a-*H*, 3a-*H*), 3.58 (dd, ^3J = 3.0, 10.8 Hz, 1 H, 3c-*H*), 3.43–3.37 ppm (m, 2 H, 5b-*H*, 5c-*H*); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 138.5–126.1, 117.4, 101.6, 100.6, 83.7, 83.5, 80.6, 79.5, 79.1, 77.3, 77.1, 76.8, 75.6, 75.4, 75.3, 75.2, 75.1, 74.5, 73.5, 73.4, 71.6, 70.9, 70.5, 70.2, 69.7, 69.2, 68.7, 68.6, 68.1 ppm; ESI HRMS: m/z: calcd for $C_{77}H_{82}O_{15}$SNa: 1301.52667; found: 1301.52664.

Propyl (1-thio-β-D-mannopyranosyl)-(1→2)-(2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-β-D-mannopyranoside (22): Compound **22** was obtained according to the procedure described for preparation of **12** using trisaccharide **21** (30 mg, 0.023 mmol), activated 10 % Pd/C (50 mg) and MeOH (5 mL). Purification by HPLC on C18 silica provided product **22**

(11 mg, 85%). ^{1}H NMR (600 MHz, D_2O): $\delta = 5.13$ (s, 1H, 1c-*H*), 5.05 (s, 1H, 1b-*H*), 4.76 (s, 1H, 1a-*H*), 4.27 (d, $^3J = 3.6$ Hz, 1H, 2a-*H*), 4.10 (d, $^3J = 3.0$ Hz, 1H, 2c-*H*), 3.96–3.58 (m, 13H, OCH_2, 2b-*H*, 3b-*H*, 4b-*H*, 3c-*H*, 3a-*H*, 6a-*H*, 6′a-*H*, 6b-*H*, 6′b-*H*, 6c-*H*, 6′c-*H*), 3.45 (t, 1H, 4a-H), 3.42–3.34 (m, 3H, 5a-*H*, 5b-*H*, 5c-*H*), 3.26 (t, 1H, 4c-*H*), 1.62 (m, 2H, $OCH_2CH_2CH_2CH_3$), 0.92 ppm (t, 3H, $OCH_2CH_2CH_2CH_3$); ESI HRMS: *m*/*z*: calcd for $C_{21}H_{38}O_{15}SNa$: 585.18236; found: 585.18243.

3-(2-Aminoethylthio)-propyl (β-D-mannopyranosyl)-(1→2)-(1-thio-β-D-mannopyranosyl)-(1→2)-2-deoxy-2-thio-β-D-mannopyranoside (23): Photoaddition of 2-aminoethanethiol (164 mg, 1.45 mmol) to compound **11** (37 mg, 0.029 mmol) in dichloromethane (1 mL) and methanol (5 mL) with subsequent debenzylation under Birch condition as reported by literature[16] afforded free amine **23** (12 mg, 65%). ^{1}H NMR (600 MHz, D_2O): $\delta = 5.0$ (s, 1H, 1b-*H*), 4.90 (s, 1H, 1c-*H*), 4.86 (s, 1H, 1a-H), 4.33 (d, $^3J = 3.0$ Hz, 1H, 2b-*H*), 4.21 (d, $^3J = 3.6$ Hz, 1H, 2a-*H*), 4.00 (m, 1H, OCHa), 3.95–3.6 (m, 13H, OCHb, 2c-*H*, 3a-*H*, 3b-*H*, 3c-*H*, 4a-*H*, 4b-*H*, 6a-*H*, 6b-*H*, 6c-*H*, 6′a-*H*, 6′b-*H*, 6′c-*H*), 3.46–3.32 (m, 3H, 5a-*H*, 5b-*H*, 5c-*H*), 3.30 (t, 1H, 4c-*H*), 3.28–3.22 (m, 1H, $SCH_2CH_2NH_2$), 2.58 (t, 1H, $SCH_2CH_2NH_2$), 2.55–2.52 (m, 4H, $CH_2CH_2SCH_2CH_2NH_2$), 1.92 ppm (m, 2H, $OCH_2CH_2CH_2S$); ESI HRMS: *m*/*z*: calcd for $C_{23}H_{44}NO_{15}S_2Na$: 638.21469; found: 638.21474.

3-(2-Aminoethylthio)-propyl (1-thio-β-D-mannopyranosyl)-(1→2)-(2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-β-D-mannopyranoside (24): Compound **21** (80 mg, 0.063 mmol) and 2-aminoethanethiol (500 mg) in methanol/dichloromethane 5:1 (6 mL) were irradiated with UV light (385 nm) and the product was subjected to subsequent debenzylation reaction under Birch condition as reported by literature[16] to give free amine **24** (58 mg, 73%). ^{1}H NMR (600 MHz, D_2O): $\delta = 5.13$ (s, 1H, 1c-*H*), 5.07 (s, 1H, 1b-*H*), 4.76 (s, 1H, 1a-*H*), 4.29 (d, $^3J = 3.0$ Hz, 1H, 2b-*H*), 4.11 (d, $^3J = 3.0$ Hz, 1H, 2c-*H*), 4.02 (m, 1H, OC*H*a), 3.93 (dd, 1H, 1H, 3a-*H*), 3.92–3.86 (m, 3H, 6a-*H*, 6b-*H*, 6c-*H*), 3.83 (d, $^3J = 3.0$ Hz, 1H, 2a-*H*), 3.76–3.56 (m, 7H, OC*H*b, 3b-*H*, 3c-*H*, 6′b-*H*, 6′c-*H*, 6′a-*H*, 4b-*H*), 3.45 (t, 1H, 4a-*H*), 3.42–3.34 (m, 3H, 5a-*H*, 5b-*H*, 5c-*H*), 3.28–3.22 (m, 2H, 4c-*H*, $SCH_2CH_2NH_2$), 2.58 (t, 1H, $SCH_2CH_2NH_2$), 2.55–2.52 (m, 4H, $CH_2CH_2SCH_2CH_2NH_2$), 1.92 ppm (m, 2H, $OCH_2CH_2CH_2S$); ESI HRMS: *m*/*z*: calcd for $C_{23}H_{44}NO_{15}S_2Na$: 638.21469; found: 638.21463.

7-Aza-8,13-dioxo-13-(4-nitrophenoxy)-4-thiatridecanyl (β-D-mannopyranosyl)-(1→2)-(1-thio-β-D-mannopyranosyl)-(1→2)-2-deoxy-2-thio-β-D-mannopyranoside (26): To a solution of free amine **23** (5 mg, 7.85 μmol) in dry DMF (1 mL), diester **25** (30 mg, 78.5 μmol) was added under argon and the mixture was stirred for 5.0 h then TLC indicated the reaction was almost complete. Finally, the reaction mixture was co-evaporated with toluene to remove DMF and the residue was dissolved in CH_2Cl_2 (10 mL), washed with H_2O (10 mL) containing 0.3% acetic acid. The water solution was then passed through a C18-Sep-Pac cartridge and eluted with methanol containing 0.3% acetic acid, to remove any compound that would be irreversibly absorbed to the reverse phase silica column. The solution was concentrated at low pressure to afford crude product as a solid. Final purification on reverse phase silica (C18) was accomplished with a water methanol mixture containing 0.3% acetic acid gradient to yield pure half ester **26** (4.1 mg, 60%). ^{1}H NMR (600 MHz, CD_3OD): $\delta = 8.30$–8.28 (m, 2H, C_6H_2), 7.38–7.36 (m, 2H, C_6H_2), 4.91 (s, 1H, 1b-*H*), 4.75 (1H, 1c-*H*), 4.73 (s, 1H, 1a-*H*), 4.20 (d, $^3J = 3.6$ Hz, 1H, 2b-*H*), 4.06 (d, $^3J = 3.6$ Hz, 1H, 2c-*H*), 4.00 (m, 1H, OC*H*a), 3.89–3.83 (m, 3H, 6a-H, 6b-H, 6c-H), 3.76 (dd, $^3J = 4.2$, 9.0 Hz, 1H, 3a-*H*), 3.70–3.62 (m, 4H, OC*H*b, 4b-*H*, 4c-*H*, 6′a-*H*), 3.60 (d, $^3J = 4.2$ Hz, 1H, 2a-*H*), 3.55–3.51 (m, 2H, 6′b-*H*, 6′c-*H*), 3.47–3.43 (m, 2H, 3b-*H*, 3c-*H*), 3.37 (t, 2H, CH_2COO), 3.30–3.19 (m, 4H, 4a-*H*, 5a-*H*, 5b-*H*, 5c-*H*), 2.67–2.62 (m, 6H, $NHCOCH_2$, CH_2CH_2NH, $COCH_2CH_2$), 2.26 (t, 2H, SCH_2CH_2NH), 1.97–1.87 (m, 2H, $OCH_2CH_2CH_2S$), 1.78–1.72 ppm (m, 4H, $CH_2CH_2CH_2CH_2$); ESI HRMS: *m*/*z*: calcd for $C_{35}H_{54}N_2O_{20}S_2Na$: 909.94; found: 909.28.

7-Aza-8,13-dioxo-13-(4-nitrophenoxy)-4-thiatridecanyl (1-thio-β-D-mannopyranosyl)-(1→2)-(2-deoxy-2-thio-β-D-mannopyranosyl)-(1→2)-β-D-mannopyranoside (27): The procedure used was analogous to the preparation of **26** using free amine **24** (2.7 mg, 4.2 μmol), diester **25** (16 mg, 42 μmol) and DMF (0.5 mL). Purification by HPLC on C18 silica gave product **27** (2.4 mg, 65%). ^{1}H NMR (600 MHz, CD_3OD): $\delta = 8.30$–8.28 (m, 2H, C_6H_2), 7.38–7.36 (m, 2H, C_6H_2), 5.04 (s, 1H, 1c-*H*), 4.93 (1H, 1b-*H*), 4.58 (s, 1H, 1a-*H*), 4.13 (d, $^3J = 3.6$ Hz, 1H, 2a-*H*), 4.03–4.01 (m, 2H, 2c-*H*, OCH_2), 3.90–3.16 (m, 19H, 2b-H, 3a-H, 3b-H, 3c-H, 4a-H, 4b-H, 4c-H, 5a-H, 5b-H, 5c-H, 6a-H, 6′a-H, 6b-H, 6′b-H, 6c-H, 6′c-H, OCH_2, CH_2CO), 2.67–2.62 (m, 6H, $NHCOCH_2$, CH_2CH_2NH, $COCH_2CH_2$), 2.26 (t, 2H, SCH_2CH_2NH), 1.97–1.87 (m, 2H, $OCH_2CH_2CH_2S$), 1.78–1.72 ppm (m, 4H, $CH_2CH_2CH_2CH_2$); ESI HRMS: *m*/*z*: calcd for $C_{35}H_{54}N_2O_{20}S_2Na$: 909.94; found: 909.28.

Glycoconjugates: The general procedure for generating protein-carbohydrate conjugates employing the hapten/protein molar ratios of Table 1 was as follows: BSA (10 mg) was dissolved in phosphate buffer pH 7.5 (2 mL), and the half ester dissolved in DMF (100 μL) was slowly injected into the reaction medium, and the reaction mixture was left for one day at room temperature. The reaction mixture was then diluted with deionized water and dialysed against deionized water (2 L, changed 5×). The tetanus toxoid (TT) (0.5 mL at 10 mgs/mL in PBS pH 7.2) was conjugated as described for BSA and after 24 h were dialyzed against PBS. The solution of BSA conjugate was lyophilized to provide the product as a white solid, while the TT conjugates were not freeze dried. MALDI-MS (positive mode, matrix sinapinic acid, H_2O): inter-thio-trisaccharide–BSA conjugate **28** (74677); terminal-thio-trisaccharide–BSA conjugate **29** (72247); inter-thio-trisaccharide–TT conjugate **30** (158430); terminal-thio-trisaccharide–TT conjugate **31** (159542).

Preparation of antigens: Glycoconjugates were absorbed on alum by mixing a PBS solution of TT conjugate (0.723 mL conjugate **30**, 0.663 mL conjugate **31**) with a PBS suspension of alum (3.337 and 3.277 mL, respectively) and the mixture was rotated for at least 2 h or overnight at 4°C. Each vaccine formulation contained 300 μg of conjugate per 1 mL dose.

Immunization of rabbits: Groups of three New Zeeland white rabbits (weighing approximately 3 kg) were given injections of 1 mL of alum deposited glycoconjugate formulation (0.2 mL into quadriceps/posterior thigh, lumbar muscles (both sides) and 3 subcutaneous sites, 0.2 mL each). A booster injection was given on day 21 and blood samples were drawn on days 0 and 31.

ELISA experiments: BSA–carbohydrate–protein conjugates (10 $\mu g\,mL^{-1}$ in PBS) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4°C. The plate was washed 5× with PBST (PBS containing 0.05% (v/v) Tween 20). Sera were diluted with PBST containing 0.1% BSA. The solutions were distributed on the coated microtiter plate and incubated at room temperature for 2 h. The plate was washed with PBST (5×) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:2000 dilution in 0.1% BSA/PBST; 100 μL per well) was added. The mixture was then incubated for 1 hour. The plate was washed 5× with PBST before addition of a 1:1 mixture 3,3′,5,5′-tetramethylbenzidine (0.4 $g\,L^{-}$) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL per well). After 2 min, the reaction was stopped by addition of 1 M phosphoric acid (100 μL per well). Absorbance was read at 450 nm. Titres are recorded as the dilution giving an absorbance 0.2 above background.

Inhibition EIA: *O*-linked trisaccharide–BSA conjugate (5 $\mu g\,mL^{-1}$ in PBS) was used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4°C. The plate was washed 5 times with PBST (PBS containing 0.05% (v/v) Tween 20). The IgG monoclonal antibody (C3.1) was premixed with various concentrations of inhibitors and added to wells (100 μL per well) in triplicates, and incubated at room temperature for 2 h. The plate was washed with PBST (5×) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:2000 dilution in 0.1% BSA/PBST; 100 μL per well) was added. The mixture was then incubated for 1 h. The plate was washed 5 times with PBST before addition of a 1:1 mixture 3,3′,5,5′-tetramethylbenzidine (0.4 $g\,L^{-1}$) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL per well). After 2 min, the reaction was stopped by addition of 1 M phosphoric acid (100 μL per well). Absorbance was read at 450 nm and percent inhibition was calculated relative to wells containing sera without inhibitor.

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