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Carbohydrate Research 329 (2000) 309–316

CARBOHYDRATE RESEARCH

Synthesis of the Lewis b hexasaccharide and squarate acid–HSA conjugates thereof with various saccharide loadings

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Received 8 March 2000; accepted 20 June 2000

Abstract

The Lewis b hexasaccharide, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*, has been synthesised using a convergent synthesis. Starting from ethyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside, a thioglycoside tetrasaccharide donor block, was constructed through two orthogonal glycosylations with glycosyl bromide donors. First, the galactose moiety was introduced using silver triflate as a promoter and then the two fucose residues under halide-assisted conditions. Finally, this tetrasaccharide was linked to a spacer-equipped 3^{I},4^{I}-diol lactose acceptor in a DMTST-promoted coupling to give, after deprotection, the Lewis b hexasaccharide as its 2-aminoethyl spacer-equipped derivative. This was coupled to human serum albumin (HSA), using the squarate ester methodology, in various saccharide–protein ratios, to give neoglycoconjugates with different saccharide loadings in about 50% efficiency.

Keywords: Oligosaccharide synthesis; Glycoconjugates; *Helicobacter pylori*; Blood group antigens

1. Introduction

The Lewis b (Le^b) blood group antigen structure is known to have a number of important biological functions. Its effective binding to the *Griffonia simplicifolia* lectin [1,2] and interest in studying carbohydrate–protein interactions in general, prompted Lemieux and co-workers to synthesise the tetrasaccharide structure and numerous analogues thereof [2,3]. Rather recently it has been found that the Le^b structure is also a human receptor for the bacteria *Helicobacter pylori*, a binding event that causes gastritic and duodenal ulcers [4]. To investigate this binding, we synthesised the tetrasaccharide with the 8-methoxycarbonyloctyl spacer as described by Lemieux [5] and made BSA-conjugates of this. However, experiments showed that these conjugates did not bind to the bacteria and the free spacer tetrasaccharide was not an inhibitor to the binding [6]. Results that the free reducing tetrasaccharide did function as an inhibitor indicated that this might be due to a spacer effect, but the idea of an extended binding epitope could not be excluded. To allow investigation of multivalency effects [7], the aminoethanol glycoside of hexasaccharide Le^b structure was synthesised, and human serum albumin (HSA) conjugates thereof with different loadings were made using the squarate acid coupling chemistry [8,9].

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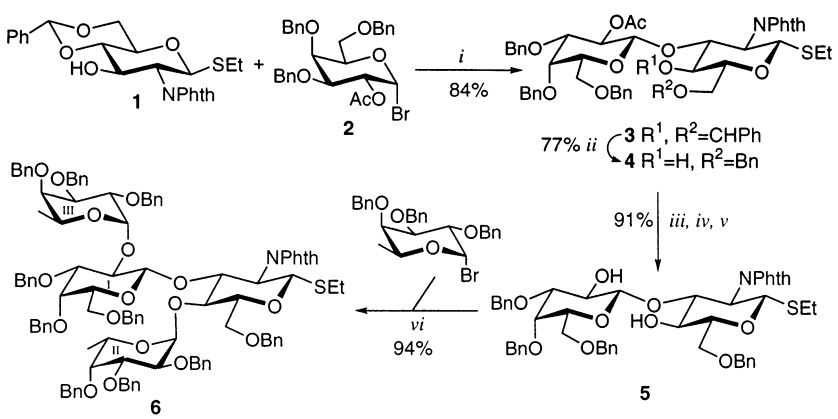
0008-6215/00/$ - see front matter
PII: S0008-6215(00)00189-0

2. Results and discussion

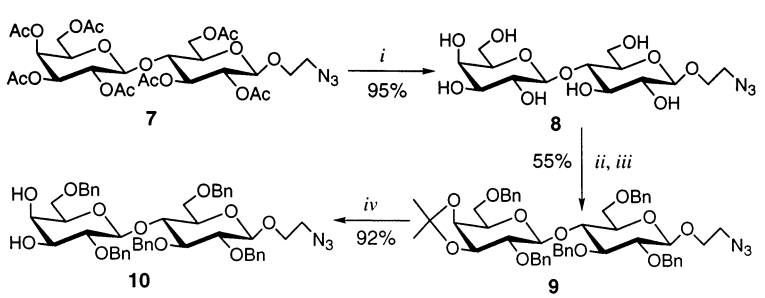
The Le[b] hexasaccharide has been synthesised earlier by Danishefsky et al. using glycals as glycosyl donors [10,11]. In our approach, bromide and thioglycoside donors were utilised. Since the tetrasaccharide structure with other spacer molecules might be of interest, we synthesised the hexasaccharide through a 4 + 2 block approach (Scheme 3), in which the formed tetrasaccharide thioglycoside donor block would be suitable also for the synthesis of various spacer tetrasaccharide derivatives. The required lactose acceptor block **7** could easily be prepared from lactose.

The tetrasaccharide thioglycoside **6** was synthesised using a strategy similar to that of Lemieux et al. in the synthesis of the corresponding methyl glycoside [2] (Scheme 1). Thus, ethyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**1**) [12] was coupled with 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl bromide (**2**) [13], [14], using silver trifluoromethanesulfonate (silver triflate) as a promoter, to give the thioglycoside disaccharide **3** in 84% yield. Reductive opening of the benzylidene acetal with $NaCNBH_3$–HCl in THF (→**4**, 77%) [15] followed by deacetylation gave the 4,2[I]-diol **5**. The normally straightforward Zémplen deacetylation was accompanied by extensive opening of the phthalimido ring. Reclosing the ring by treatment with trifluoroacetic anhydride followed by de-*O*-trifluoroacetylation ($Mg(OMe)_2$) was necessary to obtain **5** in an overall 91% yield. The subsequent coupling, between the thioglycoside acceptor **5** and the halide donor 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide [16], using halide-assisted coupling conditions [17], afforded the desired tetrasaccharide thioglycoside block **6** in 94% yield. Although efficiently prepared, the tetrasaccharide was found to be quite unstable. Even when kept in the freezer, substantial elimination of ethyl mercaptan was observed to produce the corresponding glucal derivative. Consequently, compound **6** had to be prepared just before use.

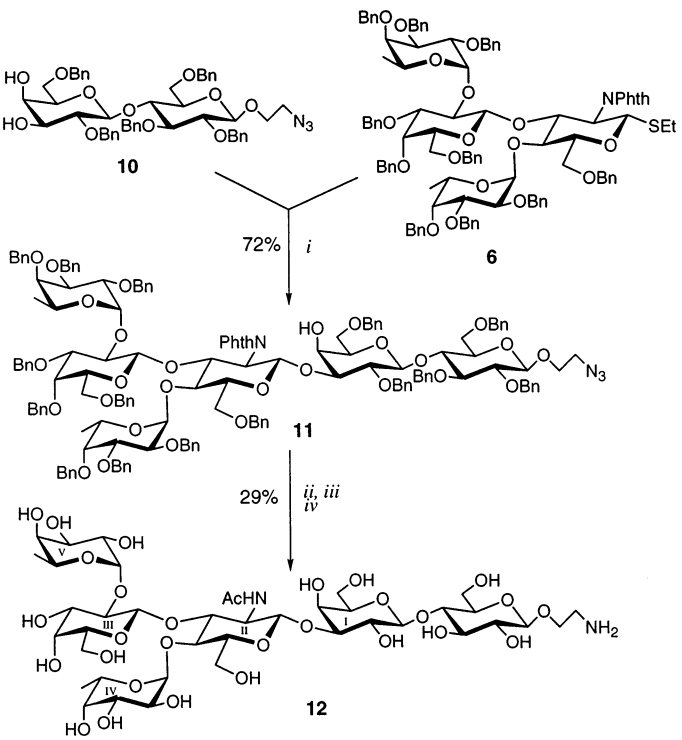
The lactoside acceptor **10** was synthesised according to Scheme 2. The 2-azidoethyl lacto-



Scheme 1. (i) AgOTf, 2,6-di-*tert*-butyl pyridine, CH_2Cl_2, toluene, −74°C; (ii) $NaCNBH_3$, HCl–Et_2O, THF; (iii) NaOMe, MeOH–CH_2Cl_2; (iv) TFAA, pyridine; (v) $Mg(OMe)_2$, MeOH–CH_2Cl_2; (vi) Et_4NBr, CH_2Cl_2.



Scheme 2. (i) NaOMe–MeOH; (ii) acetone, PTS; (iii) BnBr, NaH, DMF; (iv) 70% TFA, CH_2Cl_2.



Scheme 3. (i) DMTST, CH_2Cl_2, $-78°C \rightarrow$ r.t.; (ii) NH_2NH_2–H_2O, EtOH–dioxane, Δ; (iii) Ac_2O–MeOH–CH_2Cl_2; (iv) 10% Pd/C, H_2 (120 psi), EtOH–EtOAc, 60% HOAc.

Table 1
Degree of incorporation of Le^b-hexaoligosaccharide–HSA conjugates

Hapten–protein ratio	Incorporation achieved [a]
2:1	1.5
10:1	6
20:1	11
30:1	14

[a] Confirmed by MALDI-TOF.

side **7** was deacetylated (Zemplén, 95%) and the product **8** was treated with acetone and *p*-TsOH to give the $3^I,4^I$-*O*-isopropylidene acetal derivative, which was directly benzylated to give **9** (55%). It was de-isopropylidenated by treatment with aqueous trifluoroacetic acid to give the $3^I,4^I$ diol acceptor **10** in 92% yield.

In the coupling between tetrasaccharide donor **6** and disaccharide acceptor **10** (Scheme 3), it was important to use a large excess of promoter to effectuate the glycosylation reaction, otherwise the elimination reaction of **6**, known to occur in similar couplings [12], became an important competing reaction resulting in a low yield of the desired product. However, with 20 molar equivalents of the promoter DMTST, a 72% yield of the hexasaccharide **11** could be obtained.

Deprotection of hexasaccharide **11** was rather complicated, mainly due to problems with the removal of the phthalimido group. When **11** was heated with hydrazine hydrate for 5 days, the corresponding free amine was obtained in 59% yield. Subsequent selective *N*-acetylation (acetic anhydride in methanol/CH_2Cl_2, 73%) and simultaneous catalytic debenzylation and reduction of the azido group gave the aminoethyl glycoside hexasaccharide **12** (67%) ready for conjugation to a protein.

The squaric acid diester chemistry seems to be one of the best choices for preparation of *neo*-glycoconjugates [8,9]. Recently, this approach has been successfully used for conjugation of various oligosaccharide haptens to proteins [18–20]. The effect of some parameters (reaction time, concentration, molar excess of hapten) upon the efficiency of conjugation was also described.

We used this approach, with some slight modifications, for the preparation of Le^b-tetra- and hexasaccharide conjugates with HSA. In order to avoid complications on the stage of derivatisation with diethyl squarate (caused by the ability of the amino group in a spacer to form salts) up to one equivalent of Et_3N was added to complete the reaction in a quantitative yield. To elute the oligosaccharide-squarate derivative from a C-18 Sep-Pak cartridge a step-wise gradient of aqueous methanol (usually 10–40%) was used. The effect of pH upon the efficiency of the coupling step was also investigated. We found that at pH 10 the coupling was more efficient than at pH 9.0, in contrast to Pozsgay et al., who obtained slightly higher incorporation at pH 9 [19]. In a model experiment with an appropriate globotrioside at a 20:1 hapten–protein ratio, the average incorporation was found to be 8 (MALDI-TOF data) at pH 9.0 and 10.5 at pH 10.0 [21]. In case of Le^b-tetrasaccharide (15:1 hapten–HSA), we have reached loading of 6 at pH 9.0 and 8 at pH 10.0. In our hands, in order to reach the targeted incorporation of hapten, a 2:1 hapten–protein ratio was needed (Table 1).

In conclusion, an efficient way to Le^b-hexasaccharide–HSA-conjugates based on synthetic oligosaccharide structures and with different carbohydrate loading is described. The synthetic strategy allows the formation of Le^b tetrasaccharide structures with various spacers. The conjugates are presently being evaluated for their binding to *Helicobacter pylori* bacteria.

3. Experimental

General methods.—Concentration of solutions was performed under reduced pressure at < 40 °C (bath). NMR spectra were recorded at 25 °C at 300 or 400 MHz (^{1}H) or 75 or 100 MHz (^{13}C) in $CDCl_3$ with Me_4Si as internal standard ($\delta = 0$ ppm) or in D_2O with acetone as an internal standard (^{13}C: $\delta = 31.0$ ppm; ^{1}H: $\delta = 2.225$ ppm). TLC was performed on Silica Gel F_{254} (E. Merck) with detection by UV light and/or by charring with 8% sulfuric acid. Silica Gel (0.040–0.063 mm, Amicon) was used for column chromatography.

*Ethyl (2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-*D*-galactopyranosyl)-(1→3)-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-*D*-glucopyranoside* (**3**).—A solution of ethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-β-D-galactopyranoside [22](4.194 g, 7.8 mmol) and bromine (459 μL, 9.0 mmol) in CH_2Cl_2 (30 mL) was stirred under argon for 40 min at rt, whereafter the mixture was concentrated and co-evaporated with CH_2Cl_2 twice. The residue, crude **2** (7.8 mmol) [13,14], **1** (2.0 g, 4.7 mmol) [12], and 2,6-di-*tert*-butylpyridine (1.89 mL, 8.4 mmol) was dissolved in CH_2Cl_2 (20 mL) and crushed 4 Å MS (3 g) was added. The stirred mixture was cooled to -74 °C under argon. After 1 h at this temperature, AgOTf (2.705 g, 10.5 mmol) dissolved in toluene (10 mL) was added. After another hour the reaction was left to attain rt over night. The reaction mixture was filtered through Celite, concd and purified by silica gel chromatography (6:1→2:1 light petroleum bp 45–60 °C–EtOAc) to produce **3** (3.58 g, 84%); $[\alpha]_D$ +3.3° (*c* 1.0, $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 14.7 (SCH_2CH_3), 20.3 (CH_3CO), 23.6 (SCH_2CH_3), 54.1 (C-2), 68.0, 68.5, 70.6, 71.4, 71.6, 72.0, 72.8, 73.3, 74.2, 75.9, 80.3, 80.9, 81.5 (C-1, 3- 6, 2^I-6^I, 3 CH_2Ph), 100.5, 101.0 (C-1^I, Ph*C*H), 122.4–138.2 (aromatic C), 166.5, 168.1 (Phth*C*O) and 168.5 (CH_3CO); ^{1}H, δ 5.13 (dd, $J = 8$ and 10 Hz, H-2^I). Anal. Calcd for $C_{52}H_{53}NO_{12}S$: C, 68.18; H, 5.83. Found: C, 68.28; H, 5.97.

*Ethyl 3,4,6-tri-*O*-benzyl-β-*D*-galactopyranosyl-(1→3)-6-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-*D*-glucopyranoside* (**5**).—$NaCNBH_3$ (2.0 g, 31.8 mmol) and crushed 3 Å MS (3.2 g) were added to a stirred solution of **3** (2.0 g, 2.2 mmol) in THF (50 mL) under Ar. After 5 min, HCl–Et_2O was added dropwise until the evolution of gas ceased. After an additional 20 min, the mixture was filtered through Celite, concentrated and purified on a silica gel column (5:1 toluene–EtOAc) to give ethyl (2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-6-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**4**, 1.55 g, 77%); $[\alpha]_D$ +16.4° (*c* 1.0, $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 14.9 (SCH_2CH_3), 20.0 (CH_3CO), 23.9 (SCH_2CH_3), 53.9 (C-2), 68.6, 69.8, 69.9, 70.9, 72.1, 72.2, 73.4, 73.6, 73.8, 74.4, 79.9, 80.4, 81.0, 82.7 (C-1, 3–6, 2^I–6^I, 4 CH_2Ph), 101.2 (C-1^I), 123.3–138.6 (aromatic C), 167.0, 168.4 (Phth*C*O) and 168.9 (CH_3CO). 1 M NaOMe (15 drops) was added to a solution of **4** (1.21 g, 1.3 mmol) in MeOH–CH_2Cl_2 (2:1, 30 mL), and the reaction was stirred for 72 h, during which time more 1 M NaOMe (15 drops) was added every 24 h. The mixture was neutralised with Dowex H^+ ion-exchange resin and filtered. The Dowex resin was washed thoroughly with CH_2Cl_2 to minimize losses. After concentration of the filtrate, the residue was dissolved in pyridine (30 mL) and TFAA (10 mL) was added at 0 °C. After 45 min the reaction mixture was diluted with CH_2Cl_2 and washed with water, 10% $CuSO_4$, 1 M HCl, $NaHCO_3$ and water, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in 2:1 MeOH–CH_2Cl_2 (60 mL) and the trifluoroacetyl groups were removed by the addition of 1 M $Mg(OMe)_2$ (20 drops). After 80 min, Dowex (H^+) ion-exchange resin was added, whereafter the mixture was filtered, concentrated and chromatographed (8:1→6:1 toluene–EtOAc) to yield **5** (1.05 g, 91%); $[\alpha]_D$ +5.1° (*c* 0.97, $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 15.0

(SCH_2CH_3), 24.1 (SCH_2CH_3), 53.9 (C-2), 68.2, 69.9, 70.1, 70.2, 72.0, 72.2, 73.3, 73.4, 73.6, 74.3, 79.3, 80.9, 81.6, 83.4 (C-1, 3–6, 2^I–6^I, 4 CH_2Ph), 103.9 (C-1^I), 122.9–138.2 (aromatic C), 167.6 and 168.2 (Phth*C*O). Anal. Calcd for $C_{50}H_{53}NO_{11}S$: C, 68.55; H, 6.10. Found: C, 68.27; H, 6.17.

*Ethyl (2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-[(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→4)]-6-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside* (**6**).—Ethyl 2,3,4-tri-*O*-benzyl-1-thio-α-L-fucopyranoside (1.191 g, 2.5 mmol) [12] in CH_2Cl_2 (7 mL) was treated with bromine (150 μL, 3.0 mmol) at 0 °C for 10 min and then concentrated and co-evaporated twice with CH_2Cl_2. The residue, crude 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide (2.5 mmol) [16], was dissolved in CH_2Cl_2 (2.5 mL) and added to a mixture of **5** (433 mg, 494 μmol), Et_4NBr (107 mg, 510 μmol) and crushed 4 Å MS (1.67 g) in CH_2Cl_2–DMF (2.5/1, 7 mL), which had been stirred under argon for 50 min. After 72 h MeOH (2 mL) was added. The mixture was then filtered through Celite, concentrated, co-evaporated with toluene and chromatographed (two columns: 6:1→2:1 light petroleum bp 45–60 °C–EtOAc and 15:1→12:1 toluene–EtOAc) to yield **6** (791 mg, 94%); NMR ($CDCl_3$): ^{13}C, δ 14.9 (SCH_2CH_3), 16.3 (C-6^{II}, 6^{III}), 24.3 (SCH_2CH_3), 55.8 (C-2), 66.4, 66.9, 67.3, 67.8, 70.6, 71.4, 71.8, 72.5, 72.6, 72.7, 72.8, 73.4, 73.5, 73.8, 74.5, 74.6, 74.8, 75.0, 75.2, 75.6, 77.7, 77.9, 79.4, 80.0, 80.3, 81.5, 83.4 (C-1, 3–6, 2^I–6^I, 2^{II}–5^{II}, 2^{III}–5^{III}, 10 CH_2Ph), 97.8, 98.3, 100.4 (C-1^I, 1^{II}, 1^{III}), 123.3–139.3 (aromatic C), 167.3 and 168.4 (Phth*C*O).

*2-Azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside* (**7**).—Peracetylated lactose (5.0 g, 7.4 mmol) was treated with HBr–HOAc (20 mL) and after 1 h, the reaction mixture was concentrated. The residue was dissolved in $CHCl_3$, washed with $NaHCO_3$ (aq sat) and water, dried ($MgSO_4$), filtered and concd. A soln of the residue in MeCN (10 mL) was added dropwise to a mixture of 2-azidoethanol (650 mg, 7.4 mmol), $HgCN_2$ (938 mg, 3.7 mmol), $HgBr_2$ (1.340 g, 3.7 mmol) and crushed 4 Å MS (1.2 g) in MeCN (10 mL), which had been stirred at ambient temperature under argon for 1 h. After 48 h the reaction mixture was diluted with $CHCl_3$, filtered through Celite and the filtrate was washed with water and NaI (aq 1 M), dried ($MgSO_4$), filtered and concd. The residue was chromatographed (6:1→2:1 toluene–EtOAc) to yield **7** (2.285 g, 44%); $[\alpha]_D$ −22° (*c* 1.1, $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 20.4, 20.6, 20.75 and 20.8 (CH_3CO), 50.4 ($OCH_2CH_2N_3$), 60.7, 61.7, 66.5, 68.5, 69.0, 70.6, 70.8, 71.4, 72.6, 72.7, 76.0 (C-2–6, 2^I–6^I, $OCH_2CH_2N_3$), 100.3, 100.9 (C-1, 1^I), 168.8, 169.4, 169.5, 169.7, 169.8, 170.0 (Me*C*O). Anal. Calcd for $C_{28}H_{39}N_3O_{18}$: C, 47.66; H, 5.57. Found: C, 47.76; H, 5.54.

*2-Azidoethyl (2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside* (**10**).—A solution of **7** (7.63 g, 10.8 mmol) in MeOH (100 mL) was made basic by the addition of 1 M NaOMe (4 mL). After 20 h the reaction was neutralised with Dowex (H^+) ion-exchange resin, filtered and concentrated to give crude 2-azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (**8**, 4.214 g, 95%); $[\alpha]_D$ +4.8° (*c* 1.0, water); NMR (D_2O): ^{13}C, δ 51.3 ($OCH_2CH_2N_3$), 60.8, 61.7, 69.2, 71.6, 73.2, 73.5, 75.0, 75.5, 76.0, 79.0, 85.5 (C-2–6, 2^I–6^I, $OCH_2CH_2N_3$), 102.8 and 103.6 (C-1, 1^I); 1 H, δ 4.36 (d, $J = 8$ Hz, 1 H) and 4.28 (d, $J = 8$ Hz, 1 H) (H-1 and H-1^I). A solution of **8** (4.2 g, 10.2 mmol) in acetone (200 mL) was acidified by the addition of *p*-TsOH. After 24 h the amount of solvent and *p*-TsOH was doubled and, after another 72 h, the solution was neutralised by the addition of Et_3N, and concd. The residue was dissolved in DMF (30 mL), benzyl bromide (7.3 mL, 61.5 mmol) was added and the mixture was added dropwise to a cooled (0 °C) slurry of 60% NaH (3.06 g, 76.5 mmol) in DMF (4 mL). After 1 h, the mixture was allowed to attain room temperature and, after another 3 h, the reaction was quenched by the successive addition of MeOH and ice–water. The mixture was extracted with toluene, the organic phase was washed with water, dried ($MgSO_4$), filtered, concd and the residue was chromatographed (20:1 toluene–EtOAc) to afford **9** (5.044 g, 55%); $[\alpha]_D$ +19.1° (*c* 1.0, $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 26.4 and 28.0

(Me_2CO_2), 51.0 ($OCH_2CH_2N_3$), 68.0, 68.2, 68.9, 71.9, 73.1, 73.3, 73.5, 74.97, 75.04, 75.3, 76.2, 79.3, 80.6, 81.7, 82.8 (C-2–6, 2^I–6^I, $OCH_2CH_2N_3$, 5 CH_2Ph), 101.7, 103.5 (C-1, 1^I), 109.6 (Me_2CO_2), 127.1–138.7 (aromatic C). 70% TFA (1 mL) was added to a solution of **9** (600 mg, 665 μmol) in CH_2Cl_2 (5 mL). After stirring at room temperature for 1 h more CH_2Cl_2 was added and the reaction mixture was washed with water, $NaHCO_3$ (aq sat), and water, dried ($MgSO_4$), filtered, concd and the residue was chromatographed (6:1 → 2:1 toluene-EtOAc) to give **10** (527 mg, 92%); $[\alpha]_D$ +16.8° (*c* 1.1 $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 50.8 ($OCH_2CH_2N_3$), 67.9, 68.1, 68.5, 68.6, 72.8, 73.0, 73.3, 73.4, 74.7, 74.8, 74.95, 75.0, 76.3, 79.9, 81.5, 82.5 (C-2–6, 2^I–6^I, $OCH_2CH_2N_3$, 5 CH_2Ph), 102.4, 103.4 (C-1, 1^I) and 127.0–38.8 (aromatic C). Anal. Calcd for $C_{49}H_{55}N_3O_{11}$: C, 68.28; H, 6.43. Found: C, 68.20; H, 6.56.

*2-Azidoethyl (2,3,4-tri-*O*-benzyl-α-*L*-fucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-*D*-galactopyranosyl)-(1→3)-[2,3,4-tri-*O*-benzyl-α-*L*-fucopyranosyl-(1→4)]-(6-*O*-benzyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-(1→3)-(2,6-di-*O*-benzyl-β-*D*-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-*D*-glucopyranoside* (**11**).—Tetrasaccharide **6** (791 mg, 463 μmol), diol **10** (559 mg, 649 μmol) and 4 Å MS (2 g) in CH_2Cl_2 (12 mL) were stirred under argon at −65 °C. After 40 min, a slurry of DMTST (2.388 g, 9.3 mmol) in CH_2Cl_2 (10 mL) was added via a syringe. The reaction was left to reach −25 °C, at which it was kept overnight, then stirred at room temperature for 1 h, when Et_3N (4 mL) was added, and the mixture was filtered through Celite, concd and chromatographed (4:1 → 2:1 light petroleum bp 45–60 °C–EtOAc) to give **11** (836 mg, 72%); $[\alpha]_D$ −30° (*c* 1.0 $CHCl_3$); NMR ($CDCl_3$): ^{13}C, δ 16.2, 16.4 (C-6^{IV}, 6^V), 50.7 ($OCH_2CH_2N_3$), 56.3 (C-2^{II}), 66.4, 66.9, 67.4, 67.6, 67.7, 67.8, 68.5, 70.6, 71.0, 71.3, 71.5, 71.8, 72.6, 72.6, 72.8, 72.9, 73.2, 73.5, 73.8, 74.5, 74.6, 74.7, 74.8, 75.1, 75.2, 75.3, 75.5, 77.6, 77.8, 78.0, 79.3, 80.2, 81.4, 82.6, 82.9, 83.4 (C-2–6, 2^I–6^I, 3^{II}–6^{II}, 2^{III}–6^{III}, 2^{IV}–5^{IV}, 2^V–5^V, $OCH_2CH_2N_3$, 15 CH_2Ph), 98.0, 98.2, 98.6, 100.4, 101.6, 103.3 (C-1, 1^I, 1^{II}, 1^{III}, 1^{IV}, 1^V), 122.9–139.3 (aromatic C), 167.0 and 168.4 (Phth*C*O). FABMS Calcd for $C_{151}H_{158}N_4O_{30}$ + Na: 2530.09. Found: 2530.17.

An aliquote of **11** was benzoylated to give ^1H NMR: δ 5,67 (d, J = 3.7 Hz, 1 H, H-4^I).

2-Aminoethyl (α-L-fucopyranosyl)-(1→2)-(β-D-galactopyranosyl)-(1→3)-[α-L-fucopyranosyl-(1→4)]-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (**12**).—Hexasaccharide **11** (203 mg, 81 μmol) in 3:1 dioxane–EtOH 95% (12 mL) was treated with hydrazine hydrate (1 mL) and the mixture heated at 90 °C for 5 days, whereafter concentration, co-evaporation with toluene and chromatography (2:1 toluene–EtOAc) gave 2-azidoethyl(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-*D*-galactopyranosyl)-(1→3)-[(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→4)]-(6-*O*-benzyl-2-deoxy-2-amino-β-*D*-glucopyranosyl)-(1→3)-(2,6-di-*O*-benzyl-β-*D*-galactopyranosyl) -(1→4)-2,3,6-tri-*O*-benzyl-β-*D*-glucopyranoside (114 mg, 59%); NMR ($CDCl_3$): ^{13}C, δ 16.3, 16.5 (C-6^{IV}, 6^V), 50.9 ($OCH_2CH_2N_3$), 59.0 (C-2^{II}), 66.6, 66.8, 67.4, 67.9, 68.2, 68.6, 71.0, 71.5, 72.4, 72.6, 72.8, 73.0, 73.3, 73.4, 73.6, 74.5, 74.6, 74.7, 74.8, 74.9, 75.3, 75.4, 75.8, 75.9, 77.1, 77.8, 78.1, 78.9, 79.5, 79.6, 80.2, 81.5, 82.1, 82.6, 83.8 (C-2–6, 2^I–6^I, 3^{II}–6^{II}, 2^{III}–6^{III}, 2^{IV}–5^{IV}, 2^V–5^V, $OCH_2CH_2N_3$, 15 CH_2Ph), 97.8 and 98.1 (C-1^{IV}, 1^V), 101.5, 102.1, 103.4, 103.8 (C-1, 1^I, 1^{II}, 1^{III}), 126.0–139.0 (aromatic C). Ac_2O (800 μL) was added to the above free amine (114 mg, 50 μmol) in 4:1 MeOH–CH_2Cl_2 (10 mL). After 3.5 h the reaction mixture was concentrated and co-evaporated with toluene. Purification by chromatography (3:1 → 2:1 toluene–EtOAc) yielded 2-azidoethyl (2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-(1→3)-[(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-(1→4)]-(6-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→3)-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (85 mg, 73%); NMR ($CDCl_3$): ^{13}C, δ 16.3, 16.4 (C-6^{IV}, 6^V), 23.3 ($NHCOCH_3$), 50.9 ($OCH_2CH_2N_3$), 56.6 (C-2^{II}), 66.7, 67.4, 67.9, 68.1, 68.2, 68.5, 71.3, 71.5, 72.0, 72.6, 72.9, 73.1, 73.3, 73.4, 73.6, 74.2, 74.5, 74.6, 74.8, 74.9, 75.1, 75.3, 75.4, 76.1, 77.5, 77.9, 79.3, 79.6, 80.2, 81.5, 81.6, 82.5, 83.7 (C-2–6, 2^I–6^I, 3^{II}–

6^{II}, $2^{III}-6^{III}$, $2^{IV}-5^{IV}$, $2^{V}-5^{V}$, $OCH_2CH_2N_3$, 15 CH_2Ph), 97.7, 97.9 (C-1^{IV}, 1^{V}) 101.2, 101.8, 102.0, 103.4 (C-1, 1^{I}, 1^{II}, 1^{III}), 126.1–139.0 (aromatic C) and 169.6 ($NHCOCH_3$). This compound (85 mg, 35 μmol) was dissolved in 10:15:2 EtOH (95%)–EtOAc–HOAc (60% aq) (17 mL) and 10% Pd–C (150 mg) was added. Hydrogenolysis was carried out under H_2 at 120 psi for 2 days. The suspension was filtered through silanated Celite, which was washed with EtOAc, EtOH and water. The combined filtrate was concd and co-evaporated with EtOH–toluene and toluene. The residue was purified on a Biogel P2 column eluted with water (1% *n*-BuOH)) to give **12** (24 mg, 67%) after lyophilization; $[\alpha]_D$ −49° (*c* 1.0 water); NMR (D_2O): ^{13}C (acetone, δ 31.0), δ 16.1, 16.2 (C-6^{IV}, 6^{V}), 23.0 ($NHCOCH_3$), 40.2 ($OCH_2CH_2N_3$), 56.5 (C-2^{II}), 60.2, 60.6, 61.7, 62.3, 66.5, 66.9, 67.7, 68.5, 69.0, 69.3, 69.4, 69.8, 70.1, 70.8, 72.5, 72.7, 73.4, 74.3, 74.9, 75.1, 75.4, 75.5, 75.5, 75.5, 75.8, 77.2, 78.8, 82.2 (C-2–6, $2^{I}-6^{I}$, $3^{II}-6^{II}$, $2^{III}-6^{III}$, $2^{IV}-5^{IV}$, $2^{V}-5^{V}$, $OCH_2CH_2N_3$), 98.4, 100.2, 101.2, 102.6, 103.6, 103.8 (C-1, 1^{I}, 1^{II}, 1^{III}, 1^{IV}, 1^{V}), and 174.6 (*C*O); ^1H (25 °C, acetone, δ 2.225), δ 1.26 (t × 2, 6 H, H-6^{IV}, 6^{V}), 2.06 (s, 3 H, $NHCOCH_3$), 3.27 (t, 2 H), 3.37 (m, 1 H), 3.52–3.99 (m, 32 H), 4.13 (m, 3 H), 4.34 (q, 1 H), 4.42 (d, 1 H, $J_{1,2} = 7.8$ Hz), 4.54 (d, 1 H, $J_{1,2} = 8.3$ Hz), 4.60 (d, 1 H, $J_{1,2} = 8.8$ Hz), 4.66 (d, 1 H, $J_{1,2} = 7.8$ Hz) (H-1, 1^{I}, 1^{II}, 1^{III}), 4.87 (q, 1 H), 5.02 (d, 1 H, $J_{1,2} = 3.9$ Hz), 5.15 (d, 1 H, $J_{1,2} = 3.9$ Hz) (H-1^{IV}, 1^{V}). HRMS Calcd for $C_{40}H_{71}O_{29}N_2$ $[M+H]^+$: 1043.4143. Found 1043.4159.

General procedure for derivatization of spacer glycoside with diethyl squarate.—Diethyl squarate (0.95 equiv) as a solution in MeOH (10/50 μL of MeOH) was added with stirring to a solution of the spacer glycoside (1–7 mg) in MeOH (in some cases in aq MeOH, 1 mL). After 12 h, TLC on silica gel (4:3:3:2 $CHCl_3$–MeOH–AcOH–water) showed complete conversion to a faster moving squarate derivative. In some cases addition of Et_3N (up to 1 equiv) was required to complete the conversion. The mixture was concd, dissolved in Me_2SO (0.4 mL), and diluted with water (4 mL). The soln was passed through a Sep-Pak C18 Plus (Waters) cartridge which was subsequently washed with water (20 mL). The product was eluted with a step-wise gradient of MeOH in water (10%, 1 mL of eluent each step) collecting fractions of 1 mL. The product was usually eluted with 10–40% aq MeOH. The pooled fractions were concd and dried to yield 90–100% of the oligosaccharide-squarate derivative.

General procedure for coupling to HSA.—The required amount of HSA (Sigma) was dissolved in a Labassco buffer (1 mL, pH 10.0). Then, with stirring, the oligosaccharide-squarate derivative was added as a soln in the same buffer (1 mL). The mixture was stirred for 16 h at rt and then dialyzed against 15 changes of deionized water in an Amicon ultrafiltration cell (10 mL) with PM-10 membrane, filtered through Millex-GS 0.22 μm filter (Millipore), and lyophilized to give the glycoprotein.

Acknowledgements

Financial support from EU (Contract Number BIO 4 CT 960158), from the Swedish Foundation for Strategic research (the Glycoconjugates in Biological Systems programme) and from the Swedish Natural Science Research Council are gratefully acknowledged. We thank Professor Per-Erik Jansson at the Clinical Research Center, University College of South Stockholm, Sweden, for running the MALDI-TOF spectra.

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