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ERRATUM

Synthesis of oligosaccharides with oligoethylene glycol spacers and their conversion into glycoconjugates using *N,N,N′,N′*-tetramethyl(succinimido)uronium tetrafluoroborate as coupling reagent*

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Glycosides of glucose and lactose with di- and tetraethylene glycols, transformed into bifunctional (alcohol, ester) spacer molecules, have been synthesized. After deprotection, these spacer glycosides, containing a free carboxyl group, have been transformed efficiently into glycoconjugates using *N,N,N′,N′*-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU) for the formation of an active ester.

Keywords: oligoethylene spacer, oligosaccharide conjugation, glycoconjugate, protein coupling, *N,N,N′,N′*-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU)

Introduction

Syntheses of glycoconjugates are of great interest due to the many biological functions exhibited by these molecules [1, 2]. In the synthesis of neoglycoconjugates many different spacer molecules and coupling methods have been employed [3, 4]. Presently, spacer molecules derived from oligoethylene glycols have gained in interest, owing to their hydrophilic nature, and are frequently utilized [5–7]. Oligosaccharides with an ethylene glycol spacer have been synthesized and exploited by Verez-Bencomo *et al.* [5] and by Bertozzi and Bednarski [6], who used an aldehydo and an amino group, respectively, for the further coupling into glycoconjugates. We now describe the synthesis of ethylene glycol glycosides with a carboxylic acid as functional group.

The techniques normally employed for the formation of glycoconjugates from spacer glycosides with a carboxylic acid or ester as functional group are via the formation of an acyl azide [8] or carbodiimidated couplings. We now report that *N,N,N′,N″*-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU), shown to be a good reagent for the formation of carboxamides [9, 10], efficiently transforms carboxylic acid spacer oligosaccharide derivatives into active esters and glycoconjugates.

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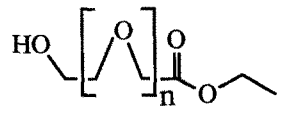
Results and discussion

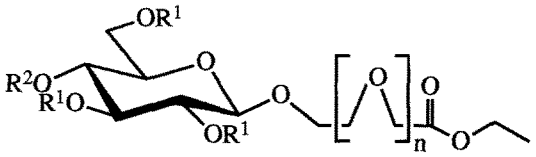
Treatment of di- or tetraethylene glycol with sodium hydride (2 equiv) followed by chloroacetic acid gave a mixture of mono- and dicarboxylic acids together with starting material. Esterification with ethanol and Dowex H^+ ion exchange resins followed by purification then gave the bifunctional, partially protected spacers **1** (17%) and **2** (15%). The syntheses could be performed easily on a 50 g scale to give 10–20 g of the spacer molecules. Similar substances have been synthesized earlier in comparable yields [11].

The spacer was introduced in various ways. Treatment of 1,2,3,4,6-penta-*O*-acetyl-β-D-glycopyranosyl and 1,2,2′,3,3′,4′,6,6′-octa-*O*-acetyl-β-D-lactopyranosyl with the spacer **1** or **2**, in the presence of boron trifluoride etherate [12], gave the β-glucosides **3**, **4**, **5** and **6** in 67, 58, 84 and 72% yields, respectively. The tetraethylene glycol spacer (**2**) was found to react much slower than the diethylene glycol spacer (**1**) under these conditions (48 h compared with 3 h), but the yields were about the same. Compound **2** was also coupled to perbenzoylated thioethyl glucopyranoside (using dimethyl (methylthio) sulfonium triflate (DMTST) as promoter [13]) and to perbenzoylated glycopyranosyl bromide (silver trifluoromethanesulfonate as promoter [14]) to give ethyl 14-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-3,6,9,12-tetraoxa-tetradecanoate (**7**) in 70 and 59% yield, respectively.

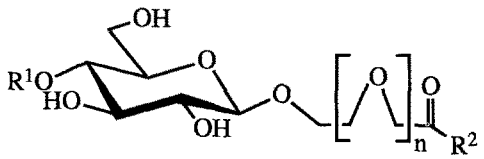
Deprotection of **3**–**6** with aqueous sodium hydroxide



1 n = 2
2 n = 4



3 $R^1 = R^2 = Ac$, n = 2
4 $R^1 = R^2 = Ac$, n = 4
5 $R^1 = Ac$, R^2 = 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl, n = 2
6 $R^1 = Ac$, R^2 = 2.3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl, n = 4
7 $R^1 = R^2 = Bz$, n = 4



8 $R^1 = H$, $R^2 = OH$, n = 2
9 $R^1 = H$, $R^2 = OH$, n = 4
10 R^1 = β-D-galactopyranosyl, $R^2 = OH$, n = 2
11 R^1 = β-D-galactopyranosyl, $R^2 = OH$, n = 4
12 $R^1 = H$, $R^2 = NH(CH_2)_4CH_3$, n = 4

followed by neutralization with Dowex H^+ resin, concentration and freeze-drying gave quantitatively the deacylated spacer glycosides **8**–**11**, with a carboxylic function accessible for activation and subsequent coupling to amines.

Bannwarth *et al.* have demonstrated the use of TSTU as an efficient reagent for the formation of carboxamides from carboxylic acids. The reagent is compatible with an aqueous solvent system, but activation in *N*,*N*-dimethylformamide (DMF) was more efficient [9, 10]. To dissolve hydrophilic, unprotected oligosaccharides the use of water as solvent is often a necessity and always an advantage. Therefore, different solvent systems with water:DMF:dioxane and water:dioxane were tried. All of these systems were found to function well. Dioxane:water, 4:1, was chosen in these experiments. Both the formation of the active ester and the conversion of this into the glycoconjugate could be monitored by TLC (EtOAc:MeOH:HOAc:H_2O, 12:3:3:1 by vol) and were found to be rapid and efficient. Slight modification of the published procedure [10] was necessary to achieve a high conversion into the intermediate active ester. Thus, lower yields were observed if an excess of base was used in the active ester formation. It was also important to use the protonated form of the acid, since incomplete neutralization after the deacylation resulted in low active ester formation. The use of 1 equiv of base and only a small excess of TSTU (1.6 equiv) gave almost quantitative conversion to the active ester in 60 min, according to TLC. An estimation of the speed and efficiency of the activation was made by adding pentylamine in borate buffer after 10 minutes of activation, which gave a 64% yield of the amide **12**.

The reaction of the activated ester with bovine serum albumin (BSA) gave a fast and very high incorporation of oligosaccharides. Since only a small excess of TSTU was needed, the formation of protein oligomers, a problem sometimes encountered in carbodiimide mediated couplings [15], was minimized. When the reaction was stopped after only 15–30 min by the addition of ethanolamine (1M, pH 8.5), the ratio of oligosaccharide units:BSA-molecule was found to be about 50:1, as determined by the phenol/sulfuric acid method [16]. Prolonged treatment (up to 18 h) gave ratios around 70:1, indicating that not only the lysine amino groups (57 in a BSA-molecule [17]) had been acylated, but also other amino acids [18]. That some of the incorporation was via ester linkages was shown by treatment of the latter BSA-conjugates with aqueous sodium hydroxide, which lowered the incorporation ratio to about 50:1. The formation of the BSA-conjugates was also shown by running an SDS-PAGE gel, which showed them to have a different mobility compared with BSA itself (Fig. 1). It also showed that no di- or trimerization of BSA had taken place.

The ease and efficiency of incorporation, the short reaction time, and the easy work-up make the use of TSTU in combination with oligoethylene spacer glycosides with a carboxylic acid as functional group a competitive alternative to existing methods for the preparation of oligosaccharide–protein conjugates.

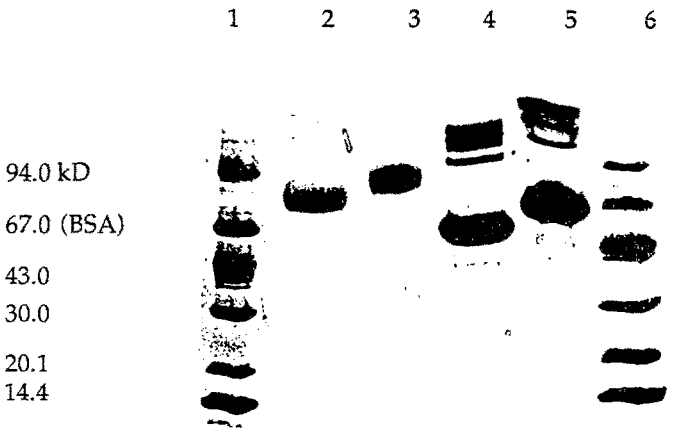


Figure 1. Coomassie stained SDS-PAGE gel showing the different mobility of the oligosaccharide conjugate of BSA(OLS-BSA) as compared to BSA itself: lane 1, LMW-kit; lane 2, OLS-BSA; lane 3, OLS-BSA (reduced); lane 4, BSA; lane 5, BSA (reduced); and lane 6, LMW-kit (reduced).

Material and methods

General methods

Concentrations were performed at <40 °C bath temperature. For optical rotations a Perkin-Elmer 241 polarimeter was used, and measurements were performed at room temperature. NMR spectra were recorded at 30 °C with a Jeol GSX-270 instrument. The following reference signals were used: Me_4Si δ 0.00 in C^2HCl_3 and dioxane δ 67.40 in ^2H_2O. Assignments of shifts for ethylene glycol carbons were based on published data [19]. Gel filtrations were performed on a column (2.6 cm × 90 cm) of Bio-Gel P-2 irrigated with water: (1% n-butanol) unless otherwise stated and a differential refractometer was used for monitoring the column effluents. Silica gel 60 F-254 (Merck, Darmstadt, Germany) was used for TLC and the spots were detected by charring with sulfuric acid or by staining with iodine vapour. Column chromatography was performed on Matrex™ Silica gel 60 (0.035–0.070 mm, Amicon Corp.). Pre-dried powdered molecular sieves (4 Å, Union Carbide) were used. Sodium hydride (55–60% in mineral oil) was prewashed in petroleum ether, b.p. 60–70 °C, prior to use. Dialysis tubing from Spectrum Medical Industries (Spectra/Por, m.w. cutoff 12.000–14.000) was used. SDS-PAGE 10–15 was run on a Pharmacia Phast System according to the manufacturer's instructions. A Pharmacia LMW-kit (low molecular weight) range 14.4–94 kDa together with BSA and mercaptoethanol-reduced BSA were used as reference, and the gel was stained by the standard Coomassie technique.

Elemental composition of compounds **8**–**12** was determined by use of the accurate mass technique [20, 21] and in the positive ionization FAB-mode [22] with a B-linear scan and detection in the centroid position mode. A Jeol SX102 mass-spectrometer with a xenon gun, nitrobenzyl alcohol as matrix and a mixture of poly(ethylene glycol) 400/poly(ethylene glycol) 600, 1:1, as standard, were employed [22]. A resolution of 3000 and an acceleration voltage of 10 kV were used. In all experiments the masses obtained deviated <0.005 mass unit. When more than one possible composition was suggested, the incorrect alternatives were excluded by NMR data.

Ethyl 8-hydroxy-3,6-dioxaoctanoate (**1**)

To a cooled (ice-bath) stirred suspension of sodium hydride (22.6 g, 520 mmol) in *N,N*-dimethylformamide (DMF), diethylene glycol (25 g, 235 mmol) in DMF (80 ml) was added over 1 h. When the evolution of hydrogen had ceased, chloroacetic acid (22.3 g, 235 mmol) in DMF (40 ml) was slowly added. When the addition was completed, the ice-bath was removed and the reaction mixture stirred at room temperature for 3 h. The mixture was concentrated to about 1/10 of its original volume and diluted with water (100 ml). Acidification with 12M hydrogen chloride (c 25 ml, pH c 1.5, paper) followed by concentration and co-concentration with ethanol (3 × 100 ml) gave a residue that was redissolved in ethanol (200 ml). The sodium chloride formed was removed by filtration, and Dowex H^+ resin (prewashed in ethanol, 35 g) was added. The reaction was stirred at 70 °C for 30 min and then filtered, neutralized (pH 7, paper) with triethylamine and concentrated to give a crude mixture (50 g). Partition between water (50 ml) containing sodium chloride (5 g) and chloroform (3 × 100 ml) gave a water phase containing unreacted diethylene glycol and salts and an organic phase with the diethyl- and monoethyl ester derivatives of diethylene glycol together with trace amounts of diethylene glycol. The organic phase was concentrated (28 g) and purified by silica gel chromatography (ethyl acetate:toluene:dichloromethane:methanol, 10:10:1:1.5 by vol) to give **1** (7.84 g, 17%) and the diester (12.3 g) as colourless liquids. ^{13}C-NMR data for **1** (C^2HCl_3): δ 14.2 (CH_3), 60.9 ($CH_3\underline{C}H_2O$), 61.7 ($\underline{C}H_2OH$), 68.7 (–$O\underline{C}H_2CO$), 70.4, 71.0, 72.6 (–$O\underline{C}H_2$), 170.5 (carbonyl C).

Analytical data: calculated for $C_8H_{16}O_5 + 0.3H_2O$, C 48.6, H 8.47; found, C 48.5, H 8.20.

Ethyl 14-hydroxy-3,6,9,12-tetraoxatetradecanoate (**2**)

Tetraethylene glycol (25 g, 129 mmol) was treated as described above for diethylene glycol to give **2** (5.6 g, 15%). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 60.7 ($CH_3\underline{C}H_2O$), 61.6 ($\underline{C}H_2OH$), 68.7 (–$O\underline{C}H_2CO$), 70.3–72.6 (7 × –$O\underline{C}H_2$), 170.5 (carbonyl C).

Analytical data: calculated for $C_{12}H_{24}O_7 + 0.5H_2O$, C 50.0, H 8.39; found, C 49.8, H 8.38.

General procedure for synthesis of the spacer glycosides **3**–**6** *from peracetylated glucose and lactose*

To a dry (4 Å molecular sieves), stirred solution of the peracetylated glycoside (100 mg) and the oligo-oxyethylene compound (1.2 equiv) in dichloromethane (5 ml), boron trifluoride etherate (10 equiv) was added and the reaction was left under an atmosphere of nitrogen and at room temperature for 3 h or 48 h; 3 h for compounds **3** and **5**, and 48 h for **4** and **6**. The reaction mixture was filtered through Celite and the flask and the Celite were washed with chloroform (20 ml). The combined filtrates were washed with saturated aqueous sodium hydrogen carbonate (5 ml) and water (5 ml). The combined aqueous phases were washed with chloroform (5 ml), whereafter the organic phases were combined, dried, filtered, concentrated and subjected to column chromatography. Elution with ethyl acetate:toluene:dichloro methane:methanol, 10:10:1:1.5 by vol, for compounds **4** and **6** and ethyl acetate:toluene:dichloromethane:methanol, 10:10:1:1 by vol, for **3** and **5** gave the target compounds **3** (67%), **4** (58%), **5** (84%) and **6** (72%).

Ethyl 8-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-3,6-dioxaoctanoate (**3**). $[\alpha]_D$-4° (*c* 0.81, chloroform). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 20.6–20.7 (4 × $\underline{C}H_3CO$), 60.8 ($CH_3\underline{C}H_2O$), 61.9 (C-6), 68.5–72.8 (C-2,3,4,5,5 × OCH_2), 100.8 (C-1, J_{CH} 160 Hz), 169.4–170.7 (5 × carbonyl C).

Analytical data: calculated for $C_{22}H_{34}O_{14}$, C 50.6, H 6.56; found, C 50.3, H 6.46.

Ethyl 14-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-3,6,9,12-tetraoxatetradecanoate (**4**). $[\alpha]_D$ −4° (*c* 0.58, chloroform). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 20.6–20.7 (4 × $\underline{C}H_3CO$), 60.8 ($CH_3\underline{C}H_2O$), 61.9 (C-6), 68.4–72.8 (C-2,3,4,5,9 × OCH_2), 100.8 (C-1, J_{CH}163 Hz), 169.4–170.6 (5 × carbonyl C).

Analytical data: calculated for $C_{26}H_{42}O_{16} + 0.5H_2O$, C 50.4, H 6.99; found, C 50.4, H 6.64.

Ethyl 8-O-(2, 2',3,3',4',6,6'-hepta-O-acetyl-β-D-lactopyranosyl)-3,6-dioxaoctanoate (**5**). $[\alpha]_D$ −3° (*c* 1.6, chloroform). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 20.5–20.8 (7 × $\underline{C}H_3CO$), 60.8, 60.9, 62.2 ($CH_3\underline{C}H_2O$, C-6,6'), 66.7–72.9 (C-2,2',3,3',4,4',5,5',5 × OCH_2), 100.6, 101.1 (C-1,1', J_{CH} 159, 161 Hz), 169.1–170.3 (8 × carbonyl C).

Analytical data: calculated for $C_{34}H_{50}O_{22}$, C 50.4, H 6.22, found, C 50.3, H 6.15.

Ethyl 14-O-(2,2',3,3',4',6,6'-hepta-O-acetyl-β-D-lactopyranosyl)-3,6,9,12-tetraoxatetradecanoate (**6**). $[\alpha]_D$ −6° (*c* 1.4, chloroform). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 20.5–20.8 (7 × $\underline{C}H_3CO$), 60.8, 60.8, 62.0 ($CH_3\underline{C}H_2O$, C-6,6'), 66.7–72.8 (C-2,2',3,3',4,4',5,5'), 9 × OCH_2), 100.6, 101.0 (C-1,1', J_{CH} 159, 163 Hz), 169.0–170.4 (8 × carbonyl C).

Analytical data: calculated for $C_{38}H_{58}O_{24}$, C 50.8, H 6.50; found, C 50.6, H 6.44.

Ethyl 14-O-(2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl)-3,6,9,12-tetraoxatetradecanoate (**7**) (from 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide). Silver trifluoromethanesulfonate (57 mg, 0.20 mmol) suspended in toluene (1 ml) was added to a dry (molecular sieves), cooled (−30 °C) and stirred solution of 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl bromide (125 mg, 0.19 mmol) and **2** (62 mg, 0.22 mmol) in dichloromethane (5 ml). After 30 min, additional silver trifluoromethanesulfonate (57 mg, 0.20 mmol) suspended in toluene (1 ml) was added, due to incomplete comsumption of the starting glucoside (TLC), and the reaction was maintained at −30 °C for 2.5 h. Triethylamine (3 drops) were added and the solvent was evaporated under a stream of air. The residue was subjected to silica gel chromatography (ethyl acetate:toluene:dichloromethane: methanol 10:10:1:0.4 by vol) to give **7** (95 mg, 59%), $[\alpha]_D$ −19° (*c* 1.2, chloroform). ^{13}C-NMR data (C^2HCl_3): δ 14.2 (CH_3), 60.8 ($CH_3\underline{C}H_2O$), 63.2 (C-6), 68.7–72.9 (C-2,3,4,5,9 × OCH_2), 101.4 (C-1, J_{CH} 161 Hz), 128.3–133.4 (aromatic C), 165.0–170.4 (5 × carbonyl C).

Analytical data: calculated for $C_{46}H_{50}O_{16}$, C 64.3, H 5.87; found, C 64.2, H 5.93.

Ethyl 14-O-(2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl)-3,6,9,12-tetraoxatetradecanoate (**7**) (from ethyl 2,3,4,6-tetra-*O*-benzoyl-1-thio-β-D-glycopyranoside. Dimethyl-(methylthio) trifluoromethanesulfonate (DMTST) (160 mg, 0.62 mmol) was added to a stirred solution of ethyl 2,3,4,6 tetra-*O*-benzoyl-1-thio-β-Lglucopyranoside (135 mg, 0.21 mmol) and **2** (69 mg, 0.25 mmol) in dry dichloromethane (5 ml). The reaction was kept at room temperature for 1 h and then purified as described above to give **7** (127 mg, 70%).

General procedure for the synthesis of the deacylated spacer glycosides **8–11**

The acetylated glycoside (100 mg, 0.16–0.19 mmol) was dissolved in ethanol (5 ml) and deacetylation was accomplished by addition of 0.25M sodium hydroxide (4 ml) and stirring at room temperature for 1 h. Neutralization with Dowex H^+ resins, filtration and concentration afforded the desired products **8–11** in quantitative yields.

8-O-(β-D-Glucopyranosyl)-3,6-dioxaoctanoic acid (**8**). $[\alpha]_D$ −10° (*c* 1.0, water). ^{13}C-NMR data (^2H_2O, $p^2H = 2$): δ 61.6 (C-6), 68.4–76.7 (C-2,3,4,5,5 × OCH_2), 103.1 (C-1), 175.1 (carbonyl C).

Analytical data: calculated for $C_{12}H_{22}O_{10}$, C 44.2, H 6.80, O 49.0; mass deviation found, M + Na 1.2 mmu.

14-O-(β-D-Glucopyranosyl)-3,6,9,12-tetraoxatetradecanoic acid (**9**). $[\alpha]_D$ −12° (*c* 1.1, methanol). ^{13}C-NMR data (^2H_2O, $p^2H = 2$): δ 61.6 (C-6), 68.6–76.8 (C-2,3,4,5,9 × OCH_2), 103.1 (C-1), 175.2 (carbonyl C).

Analytical data: calculated for $C_{16}H_{30}O_{12}$, C 46.4, H 7.30, O 46.3; mass deviation found, M + Na 4.0 mmu.

8-O-(β-D-Lactopyranosyl)-3,6-dioxaoctanoic acid (**10**). $[\alpha]_D$ +7° (*c* 0.5, water). ^{13}C-NMR data (^2H_2O, $p^2H = 2$): δ 61.0, 61.8 (C-6,6'), 68.5–79.3 (C-2,2',3,3',4,4',5,5', 5 × OCH_2), 102.9, 103.1 (C-1,1'), 175.2 (carbonyl C).

Analytical data: calculated for $C_{18}H_{32}O_{15}$, C 44.3, H 6.60, O 49.1; mass deviation found, M + H 2.3 mmu.

14-(β-D-Lactopyranosyl)-3,6,9,12-tetraoxatetradecanoic acid (**11**). $[\alpha]_D$ +10° (*c* 1.0, water). ^{13}C-NMR data ($^2H_2{}^0$, $p^2H = 2$): δ 61.0, 61.9 (C-6,6'), 68.5–79.3 (C-2,2'3,3',4,4',5,5'), 9 × OCH_2), 103.0, 103.8 (C-1,1'), 175.2 (carbonyl C).

Analytical data: calculated for $C_{22}H_{40}O_{17}$, C 45.8, H 6.99, O 47.2; mass deviation found, M + H 3.8 mmu.

N-*Pentyl 14-O-(β-D-glycopyranosyl)-3,6,9,12-tetraoxatetradecanamide* (**12**)

N,N,N',N''-Tetramethyl(succinimido)uronium tetrafluoroborate (TSTU) (59 mg, 0.20 mmol) was added at room temperature to a stirred solution of **9** (51 mg, 0.12 mmol)

and triethylamine (17.1 µl, 0.12 mmol) in 2 ml dioxane:water (4:1 by vol). After 10 min, the reaction mixture was added to a solution of pentylamine hydrochloride (0.15 mmol) in 0.5M borate buffer (1 ml, pH 8.5). The resulting mixture was stirred for an additional 10 min, whereafter filtration through Dowex H^+ resins (to remove excess of pentylamine), concentration and purification on a silica gel column (chloroform:methanol:25% aqueous ammonia, 70:30.5 by vol) gave **11** (38 mg, 64%), $[\alpha]_D$ −10° (*c* 1.0, methanol). ^{13}C-NMR data (^2H_2O): δ 14.2 (CH_3), 22.5, 29.0, 29.2, 39.8 (CH_2), 61.6 (C-6), 69.5–76.8 (C-2,3,4,5,9 × OCH_2), 103.1 (C-1), 173.0 (carbonyl C).

Analytical data: calculated for $C_{21}H_{41}O_{11}N$, C 52.2, H 8.55, O 36.4, N 2.90; mass deviation found, M + H 1.0 mmu.

General procedure for coupling of the oligosaccharide derivaties **8** *and* **9** *to bovine serum albumin (BSA)*

To a solution of the spacer oligosaccharide **8** or **9** (23–30 mg, 73 µmol) and triethylamine (10.2 µl, 73 µmol) in 2 ml dioxane:water (4:1 by vol), TSTU (35 mg, 117 µmol) was added and the reaction stirred at room temperature for 10 min. The reaction mixture was then added, via a pipette, to a solution of BSA (Sigma, No A-4503, Fraction V Powder) (30 mg) in 0.1M sodium borate buffer (6 ml, pH 8.5). The coupling reaction was monitored by TLC (ethyl acetate:methanol:acetic acid:water, 12:3:3:2 by vol) and a charring baseline product was formed immediately. The reaction was maintained at room temperature for 30 min (15 min to 18 h) and then transferred to dialysis tubing and dialysed extensively against distilled water (4 × 1 l). Freeze drying of the product gave the oligosaccharide–BSA conjugate (30–70 glucose units per BSA molecule).

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