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TETRAHEDRON: *ASYMMETRY*

Synthesis and conjugation of a sulfated disaccharide involved in the aggregation process of the marine sponge *Microciona prolifera*

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Abstract

The synthesis is reported of allyl (sodium 2-acetamido-2-deoxy-β-D-glucopyranosyl 3-sulfate)-(1→3)-α-L-fucopyranoside which represents an oligosaccharide fragment of the aggregation factor of the marine sponge *Microciona prolifera*. The title compound was obtained by coupling of 3-*O*-allyloxycarbonyl-2-deoxy-4,6-*O*-isopropylidene-2-phthalimido-β-D-glucopyranosyl trichloroacetimidate with allyl 2,4-di-*O*-benzoyl-α-L-fucopyranoside, followed by de-isopropylidenation, acetylation, de-allyloxycarbonylation, sulfation, de-acylation, and finally *N*-acetylation. The allyl glycoside was eventually converted into a 3-(2-aminoethylthio)propyl glycoside and then coupled to bovine serum albumin (BSA) using diethyl squarate as the bivalent linker, yielding 8 hapten molecules per molecule of BSA. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

The Ca^{2+}-dependent reaggregation of dissociated cells of the marine sponge *Microciona prolifera* is species-specific[1] and occurs through proteoglycans.[2] Cell surfaces are covered with large adhesion proteoglycans, and multiple low affinity carbohydrate–carbohydrate interactions are responsible for the interaction between the sponge cells.[3] It is suggested that similar interactions occur during embryonic development in higher animals and human beings, as well as between tumor cells. Characterization of the aggregation factors revealed the existence of pyruvylated and sulfated glycans as part of these proteoglycans.[4,5] In order to develop a model system for mimicking cellular interactions, a programme was started in our group to synthesize some of these aggregation factors.

Here, we present the synthesis of β-D-Glc*p*NAc3*S*-(1→3)-α-L-Fuc*p* as its allyl glycoside **10** (a preliminary report of **10** has appeared)[6] and the subsequent conversion into a BSA-conjugate. During

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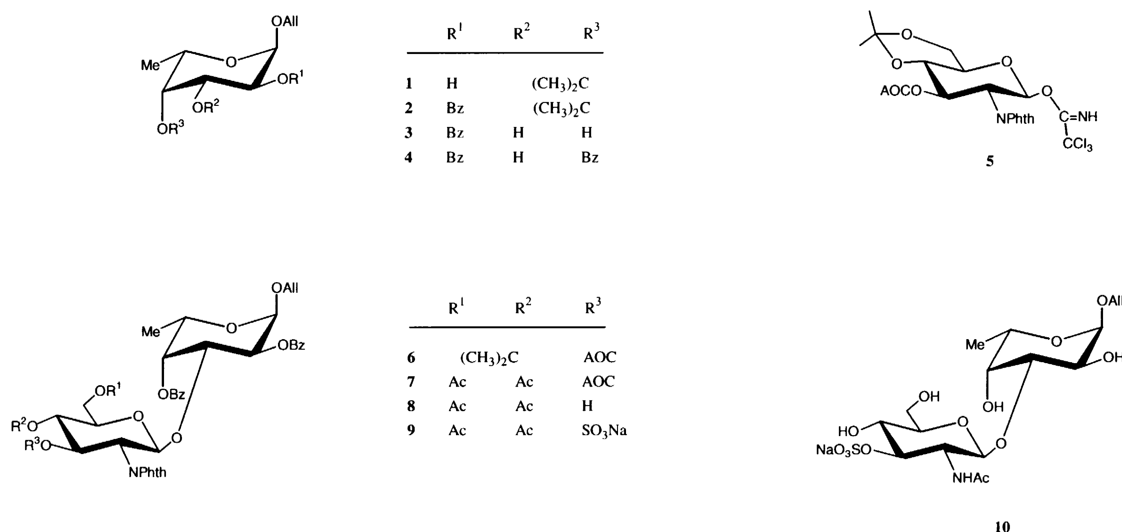
the preparation of this manuscript a study was published dealing with the synthesis of the corresponding methyl glycoside.[7] It should be noted that the anomeric configuration of fucose in the proteoglycan has not yet been determined,[5] so that the choice of the α-glycoside is arbitrary. Multivalent exposition of the synthetic material via a spacer on the surface of a suitable carrier can resemble the natural sponge surfaces.

2. Results and discussion

For the synthesis of **10** the acceptor allyl 2,4-di-*O*-benzoyl-α-L-fucopyranoside **4** and the donor 3-*O*-allyloxycarbonyl-2-deoxy-4,6-*O*-isopropylidene-2-phthalimido-β-D-glucopyranosyl trichloroacetimidate **5**[8] were selected as precursors. The allyloxycarbonyl group at O-3 of **5** was chosen in view of the sulfation of this position in a later stage of the synthesis. In the synthesis of **4**, allyl α-L-fucopyranoside[9] was isopropylidenated at O-3 and O-4 with α,α-dimethoxypropane in the presence of a catalytic amount of *p*-toluenesulfonic acid to give **1** (80%). Subsequent benzoylation with benzoyl chloride in pyridine in the presence of a catalytic amount of 4-dimethylaminopyridine (→**2**, 96%), followed by de-isopropylidenation using trifluoroacetic acid and water in dichloromethane, yielded **3** (Scheme 1). Compound **3** was selectively protected at O-4 by reaction with trimethylorthobenzoate and *p*-toluenesulfonic acid, after which the resulting 3,4-dioxolane-type acetal was opened by treatment of the residue with acetic acid–water[10] (→**4**, 71%).



Scheme 1.

Coupling of **4** with **5** in dichloromethane, using trimethylsilyl trifluoromethanesulfonate as a catalyst, gave disaccharide derivative **6** (84%). De-isopropylidenation of **6** with aqueous trifluoroacetic acid in dichloromethane, followed by conventional acetylation (→**7**, 98%), and de-allyloxycarbonylation using tetrakis(triphenylphosphine)palladium in tetrahydrofuran and morpholine afforded **8** (97%).[11,12] Sulfation of O-3 was performed using sulfur trioxide trimethylamine complex, and the product was purified on Sephadex LH-20, followed by silica gel chromatography (63%). De-phthaloylation/de-acylation was performed by stirring **9** with methylamine[13] in ethanol (8 days), followed by selective *N*-acetylation (0°C) using acetic anhydride in methanol, affording disaccharide **10** (61%). The presence

of the sulfate group on C-3′ was established by ^{1}H NMR analysis (GlcNAc H-3, δ 4.414). Other chemical shifts were in good agreement with data of Spillmann et al.[5]

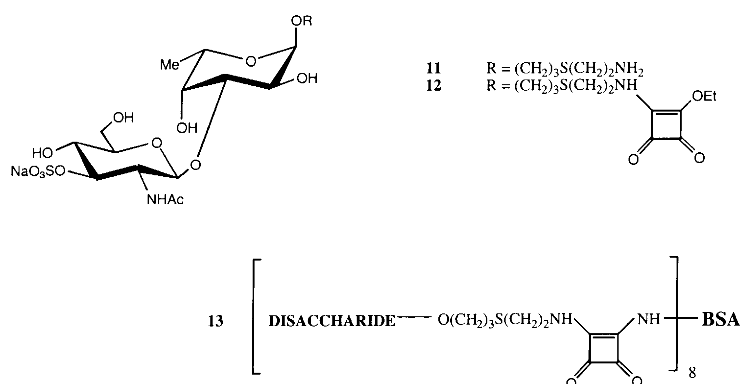
Michael addition of **10** with 2-aminoethanethiol (cysteamine) under UV-irradiation afforded the corresponding 3-(2-aminoethylthio)propyl glycoside[14,15] (→**11**, 86%). The structure of **11** was unambiguously ascertained by FAB mass spectrometry and ^{1}H (1D and 2D TOCSY) NMR analysis (Table 1).

Table 1
500 MHz ^{1}H NMR data (1D and 2D TOCSY) of compound **11**

Proton (δ_H)	Fuc	GlcNAc3*S*
H-1 ($J_{1,2}$)	4.917 (4.0)	4.821 (8.5)
H-2 ($J_{2,3}$)	4.034 (10.3)	3.817 (8.8)
H-3 ($J_{3,4}$)	3.885 (3.3)	4.424 (10.3)
H-4 ($J_{4,5}$)	4.034 (< 1)	3.621 (9.2)
H-5	n.d.[a]	3.524
H-6 ($J_{5,6}$)	1.221 (6.6)	n.d.
$NHCOCH_3$		2.01
$OCH_2CH_2CH_2S(CH_2)_2NH_2$	1.89–1.96	
$OCH_2CH_2CH_2S(CH_2)_2NH_2$	2.67–2.71	
$O(CH_2)_3S(CH_2)_2NH_2$	2.87 and 3.23	
$OCH_2(CH_2)_2S(CH_2)_2NH_2$	3.59 and 3.82	

[a] n.d. = not determined

The oligosaccharide amine **11** was treated with 0.95 equivalents of diethyl squarate in ethanol and sodium phosphate buffer, pH 7.0 (1:1 v/v).[16,17] The resulting oligosaccharide–squarate adduct **12** was left to react with bovine serum albumin (BSA) in 0.1 M sodium bicarbonate buffer (pH 9.0) at room temperature for 5 days (Scheme 2).



Scheme 2.

The degree of incorporation of oligosaccharide onto the protein was determined using matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry (MS), revealing a distribution of 7–10 hapten molecules per molecule of BSA. Monosaccharide analysis[18] showed an achieved incorporation of 52% of **12** on BSA (*n*=8). In conclusion, the conjugation method was successfully applied with only 1 mg of **12**, revealing its efficiency even on small scales. The neoglycoconjugate

will be used to test the Ca^{2+}-dependent self-binding capacity of the sulfated disaccharide in biological experiments.

3. Experimental

3.1. General procedures

Reactions were monitored by TLC on Kieselgel 60 F_{254} (Merck); compounds were visualized under UV light and by charring with aq. 50% H_2SO_4. Column chromatography was performed on Kieselgel 60 (Merck, <230 mesh). Solvents were evaporated under reduced pressure at 40°C (bath). Crystalline bovine serum albumin (BSA) was obtained from Bayer Corporation. A Hanovia UV lamp (50 W) was used for irradiations in photochemical reactions. Optical rotations were determined for solutions in $CHCl_3$, unless otherwise stated, at 20°C with a Perkin–Elmer 241 polarimeter using a 10 cm 1 mL cell. ^{1}H NMR spectra were recorded at 25°C with Bruker AC 300 or Bruker AMX 500 spectrometers. Two-dimensional double-quantum filtered ^{1}H–^{1}H correlated spectra (2D DQF ^{1}H–^{1}H COSY spectra with a mixing time of 100 ms) were recorded at 300 K using a Bruker AMX 500 spectrometer. ^{1}H chemical shifts (δ) are given in ppm relative to the signal for internal Me_4Si for solutions in $CDCl_3$ or CD_3OD, or by reference to acetone (δ 2.225) for solutions in D_2O. *J*-Values are given in Hertz. Fast-atom-bombardment mass spectrometry (FABMS) was performed on a JEOL JMS SX/SX 102A four-sector mass spectrometer using *m*-nitrobenzyl alcohol or glycerol as the matrix, operated at 10 kV accelerating voltage, equipped with a JEOL MS-FAB 10 D FAB gun operated at 10 mA emission current, producing a beam of 6 keV Xenon atoms. MALDI-TOF mass spectra were recorded on a Voyager-DE (PerSeptive Biosystems) instrument using α-cyano-4-hydroxycinnamic acid as the matrix. Proteins were analyzed in the linear mode with the acceleration voltage kept at 22.5 kV, and samples were prepared as follows: first, 1 μL matrix solution was placed on the sample plate. After evaporation of the solvent, 1 μL sample solution (7.5 pmol mL^{-1}) in acetonitrile:water (1:1) acidified with 0.1% trifluoroacetic acid was applied to the thin matrix film. Spectra were obtained by summing positive-ion signals of 183 to 188 laser shots. GLC was performed using a CP-Sil5 CB WCOT fused-silica capillary column (25 m×0.34 mm, Chrompack).

3.2. Allyl 3,4-O-isopropylidene-α-L-fucopyranoside **1**

To a solution of allyl α-L-fucopyranoside[9] (1.0 g, 4.9 mmol) in dry DMF (17 mL) was added α,α-dimethoxypropane (7.5 mL) and *p*-toluenesulfonic acid monohydrate (16.7 mg). The mixture was stirred for 3 h at 20°C, when TLC (CH_2Cl_2:MeOH 97:3) showed a complete conversion into **1** (R_f=0.66). After quenching with NEt_3 (0.8 mL) and concentration, the residue was dissolved in CH_2Cl_2 (25 mL) and the solution was extracted with aq. 5% NaCl (3×10 mL), dried ($MgSO_4$), filtered, and concentrated. Column chromatography (CH_2Cl_2:MeOH 98:2) of the residue gave **1**, isolated as a colorless syrup (0.9 g, 80%); $[\alpha]_D = -120$ (c=1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.272 (d, 3H, $J_{5,6}$=6.7 Hz, H-6,6,6), 1.313 and 1.470 (2 s, each 3H, $C(CH_3)_2$), 4.014 and 4.199 (2 m, each 1H, $OCH_2CH{=}CH_2$), 4.819 (d, 1H, $J_{1,2}$=3.9 Hz, H-1), 5.166 and 5.258 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.872 (m, 1H, $OCH_2CH{=}CH_2$); FABMS (positive): calcd for $C_{12}H_{20}O_5$ 244.2, found *m/z* 245.1 ($[M+H]^+$). Elemental analysis: found C, 58.74%; H, 8.35%; calcd C, 59.00%; H, 8.25%.

3.3. Allyl 2-O-benzoyl-3,4-O-isopropylidene-α-L-fucopyranoside **2**

To a solution of **1** (0.92 g, 3.8 mmol) in pyridine (14 mL) were added benzoyl chloride (0.55 mL, 4.9 mmol) and a catalytic amount of 4-dimethylaminopyridine. The solution was stirred overnight, when TLC (CH_2Cl_2:acetone 95:5) showed the complete disappearance of **1** and the formation of a new spot (R_f=0.80). The mixture was diluted with CH_2Cl_2 (150 mL) and the solution was extracted with aq. 5% $NaHCO_3$ (50 mL). The organic phase was washed with aq. 5% $NaHCO_3$ (50 mL) and water (50 mL), dried ($MgSO_4$), filtered, concentrated, and co-concentrated with toluene, EtOH and CH_2Cl_2 (each 3×75 mL). Column chromatography (CH_2Cl_2:acetone 98:2) of the residue gave **2**, isolated as a colorless syrup (1.28 g, 96%); $[\alpha]_D = -110$ (*c*=1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.402 (d, 3H, $J_{5,6}$=6.7 Hz, H-6,6,6), 1.364 and 1.568 (2 s, each 3H, $C(CH_3)_2$), 3.985 and 4.175 (2 m, each 1H, $OCH_2CH{=}CH_2$), 4.149 (dd, 1H, $J_{3,4}$=5.4, $J_{4,5}$=2.6 Hz, H-4), 4.520 (dd, 1H, $J_{2,3}$=8.2 Hz, H-3), 5.074 (d, 1H, $J_{1,2}$=3.6 Hz, H-1), 5.115 and 5.260 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.168 (dd, 1H, H-2), 5.825 (m, 1H, $OCH_2CH{=}CH_2$), 7.40–8.17 (m, 5H, Bz); FABMS (positive): calcd for $C_{19}H_{24}O_6$ 348.3, found *m/z* 349.1 ($[M+H]^+$), 371.1 ($[M+Na]^+$).

3.4. Allyl 2,4-di-O-benzoyl-α-L-fucopyranoside **4**

To a solution of **2** (1.25 g, 3.6 mmol) in CH_2Cl_2 (58 mL) were added CF_3CO_2H (3.4 mL, 44 mmol) and water (0.43 mL, 22.5 mmol). After 30 min TLC (CH_2Cl_2:acetone 9:1) indicated the de-isopropylidenation to be completed, showing a new spot with R_f=0.12 (**3**). The mixture was concentrated and toluene, EtOH, and CH_2Cl_2 (each 3×100 mL) were evaporated from the residue. To a solution of **3** (1.02 g, 3.3 mmol) in dry CH_2Cl_2 (23 mL) were added trimethylorthobenzoate (3.2 mL, 18.9 mmol) and *p*-toluenesulfonic acid monohydrate (pH 3). The solution was stirred overnight, after which TLC (CH_2Cl_2:acetone 9:1) showed the formation of a new product (R_f=0.88). Then, the solution was concentrated to about 15 mL, and HOAc (8 mL) and water (15 mL) were added. The resulting mixture was stirred for 15 min at 40°C, when TLC (CH_2Cl_2:acetone 9:1) showed the formation of **4** (R_f=0.72). After cooling to room temperature, the mixture was diluted with EtOAc (200 mL), then washed with aq. 10% $NaHCO_3$ (100 mL) and water (50 mL), and the organic layer was dried ($MgSO_4$), filtered, concentrated, and co-concentrated with toluene, EtOH and CH_2Cl_2 (3×75 mL). Column chromatography (CH_2Cl_2:acetone 95:5) of the residue gave **4**, isolated as a white foam (0.96 g, 71%); $[\alpha]_D = -160$ (*c*=1); ^{1}H NMR data (300 MHz, $CDCl_3$): δ 1.224 (d, 3H, $J_{5,6}$=6.6 Hz, H-6,6,6), 4.050 and 4.221 (2 m, each 1H, $OCH_2CH{=}CH_2$), 4.267 (m, 1H, H-5), 4.518 (dd, 1H, $J_{2,3}$=10.4, $J_{3,4}$=3.6 Hz, H-3), 5.163 and 5.311 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.249 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 5.374 (dd, 1H, H-2), 5.553 (dd, 1H, $J_{4,5}$=1.2 Hz, H-4), 5.873 (m, 1H, $OCH_2CH{=}CH_2$), 7.40–8.15 (m, 10H, 2 Bz). Elemental analysis for $C_{23}H_{24}O_7$: found C, 66.29%; H, 6.06%; calcd C, 66.98%; H, 5.87%.

3.5. Allyl (3-O-allyloxycarbonyl-2-deoxy-4,6-O-isopropylidene-2-phthalimido-β-D-glucopyranosyl)-(1→3)-2,4-di-O-benzoyl-α-L-fucopyranoside **6**

A solution of 3-*O*-allyloxycarbonyl-2-deoxy-4,6-*O*-isopropylidene-2-phthalimido-β-D-glucopyranosyl trichloroacetimidate[8] (**5**; 0.45 g, 0.77 mmol) and **4** (0.24 g, 0.59 mmol) in dry CH_2Cl_2 (6.5 mL), containing molecular sieves (4 Å, 0.79 g), was stirred for 30 min under Ar. Then, trimethylsilyl trifluoromethanesulfonate (4.9 μL, 26 μmol) was added, and the mixture was stirred for 10 min, when TLC (*n*-hexane:EtOAc 3:2) showed the formation of a new spot (**6**; R_f=0.26). The reaction was quenched by the addition of NEt_3 until neutral pH, and the mixture was diluted with CH_2Cl_2 (50 mL),

filtered, washed with aq. 5% NaCl, dried ($MgSO_4$), filtered, and concentrated. Column chromatography (*n*-hexane:EtOAc 3:2) of the residue yielded **6**, isolated as a colorless syrup (0.4 g, 84%); $[\alpha]_D = -124$ (c=0.5); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.052 (d, 3H, $J_{5,6}$=6.5 Hz, H-6,6,6), 1.353 and 1.364 (2 s, each 3H, $C(CH_3)_2$), 4.095 (dd, 1H, $J_{1',2'}$=8.3, $J_{2',3'}$=10.2 Hz, H-2′), 4.515 (dd, 1H, $J_{2,3}$=10.3, $J_{3,4}$=3.5 Hz, H-3), 4.896 and 4.988 (2 m, each 1H, $C(O)OCH_2CH{=}CH_2$), 5.142 and 5.323 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.232 (d, 1H, $J_{1,2}$=3.8 Hz, H-1), 5.342 (d, 1H, H-4), 5.373 (dd, 1H, $J_{3',4'}$=9.0 Hz, H-3′), 5.392 (dd, 1H, H-2), 5.509 (m, 1H, $C(O)OCH_2CH{=}CH_2$), 5.587 (d, 1H, H-1′), 5.844 (m, 1H, $OCH_2CH{=}CH_2$), 7.23–8.16 (m, 14H, Phth and 2 Bz); FABMS (positive): calcd for $C_{44}H_{45}O_{15}N$ 827.4, found *m/z* 828.2 ($[M+H]^+$), 850.1 ($[M+Na]^+$).

3.6. *Allyl (4,6-di-*O*-acetyl-3-*O*-allyloxycarbonyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-(1→3)-2,4-di-*O*-benzoyl-α-*L*-fucopyranoside* **7**

To a solution of **6** (0.83 g, 1.02 mmol) in CH_2Cl_2 (15.8 mL) was added CF_3CO_2H (0.9 mL) and water (0.1 mL). The mixture was stirred for 30 min when TLC (CH_2Cl_2:acetone 75:25) revealed the de-isopropylidenation to be completed, showing a new spot at R_f=0.43. After concentration and co-concentration with toluene, EtOH, and CH_2Cl_2 (each 3×50 mL), Ac_2O (10 mL) and pyridine (10 mL) were added, and the mixture was stirred overnight. Then, TLC (CH_2Cl_2:acetone 95:5) showed the formation of **7** (R_f=0.54). After concentration and co-concentration with toluene, EtOH, and CH_2Cl_2 (each 3×50 mL), column chromatography (CH_2Cl_2:acetone 95:5) of the residue yielded **7**, isolated as a colorless syrup (0.64 g, 98%); $[\alpha]_D = -120$ (c=1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.076 (d, 3H, $J_{5,6}$=6.6 Hz, H-6,6,6), 1.997 and 2.016 (2 s, each 3H, 2 Ac), 4.212 (dd, 1H, $J_{1',2'}$=8.4, $J_{2',3'}$=10.5 Hz, H-2′), 4.588 (dd, 1H, $J_{2,3}$=10.4, $J_{3,4}$=3.4 Hz, H-3), 4.908 and 4.993 (2 m, each 1H, $C(O)OCH_2CH{=}CH_2$), 5.035 (dd, 1H, $J_{3',4'}$=9.3, $J_{4',5'}$=10.0 Hz, H-4′), 5.108 and 5.257 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.249 (d, 1H, $J_{1,2}$=3.7 Hz, H-1), 5.382 (dd, 1H, H-2), 5.407 (dd, 1H, $J_{4,5}$<1 Hz, H-4), 5.500 (m, 1H, $C(O)OCH_2CH{=}CH_2$), 5.552 (dd, 1H, H-3′), 5.585 (d, 1H, H-1′), 5.812 (m, 1H, $OCH_2CH{=}CH_2$), 7.19–8.16 (m, 14H, Phth and 2 Bz); FABMS (positive): calcd for $C_{45}H_{45}O_{17}N$ 871.4, found *m/z* 872.2 ($[M+H]^+$), 894.1 ($[M+Na]^+$). Elemental analysis: found C, 61.36%; H, 4.90%; calcd C, 61.98%; H, 5.20%.

3.7. *Allyl (4,6-di-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-(1→3)-2,4-di-*O*-benzoyl-α-*L*-fucopyranoside* **8**

To a solution of **7** (0.59 g, 0.68 mmol) in THF (9.8 mL) and morpholine (0.35 mL) was added tetrakis(triphenylphosphine)palladium (114 mg). The mixture was stirred and boiled under reflux until the de-allyloxycarbonylation was complete (TLC, CH_2Cl_2:acetone 9:1; new spot at R_f=0.50). Then, the mixture was diluted with CH_2Cl_2 (50 mL), washed with aq. 5% NaCl (25 mL), dried ($MgSO_4$), filtered, and concentrated. Column chromatography (CH_2Cl_2:acetone 9:1) of the residue yielded **8**, isolated as a syrup (0.52 g, 97%); $[\alpha]_D = -123$ (c=1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.079 (d, 3H, $J_{5,6}$=6.5 Hz, H-6,6,6), 2.005 and 2.041 (2 s, each 3H, 2 Ac), 4.032 (dd, 1H, $J_{1',2'}$=8.4, $J_{2',3'}$=10.5 Hz, H-2′), 4.593 (dd, 1H, $J_{2,3}$=10.5, $J_{3,4}$=3.4 Hz, H-3), 4.765 (t, 1H, $J_{3',4'}$=$J_{4',5'}$=9.6 Hz, H-4′), 5.104 and 5.257 (2 m, each 1H, $OCH_2CH{=}CH_2$), 5.248 (d, 1H, $J_{1,2}$=3.8 Hz, H-1), 5.379 (dd, 1H, H-2), 5.420 (dd, 1H, $J_{4,5}$=0.8 Hz, H-4), 5.458 (d, 1H, H-1′), 5.812 (m, 1H, $OCH_2CH{=}CH_2$), 7.17–8.16 (m, 14H, Phth and 2 Bz); FABMS (positive): calcd for $C_{41}H_{41}O_{15}N$ 787.4, found *m/z* 788.2 ($[M+H]^+$), 810.1 ($[M+Na]^+$). Elemental analysis: found C, 61.78%; H, 5.33%; calcd C, 62.51%; H, 5.25%.

*3.8. Allyl (sodium 4,6-di-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl 3-sulfate)-(1→3)-2,4-di-*O*-benzoyl-α-*L*-fucopyranoside* ***9***

A solution of **8** (0.46 g, 0.59 mmol) and SO_3–NMe_3 complex (3.26 g, 23.4 mmol) in abs. DMF (27 mL) was stirred at 55°C overnight. Then, TLC (CH_2Cl_2:acetone 9:1) showed the sulfation to be complete (new spot at R_f=0.29). After cooling to room temperature and quenching with MeOH, the suspension was concentrated and co-concentrated with toluene, EtOH, and CH_2Cl_2 (each 3×60 mL). A solution of the residue in MeOH was stirred for 30 min with Dowex-Na^+ resin. After filtration and concentration, the residue was purified on Sephadex LH-20 (CH_2Cl_2:MeOH 1:1), followed by silica gel column chromatography (CH_2Cl_2:acetone 9:1) to give **9**, isolated as a white solid (0.32 g, 63%); $[\alpha]_D$= −114 (*c*=1); ^{1}H NMR (300 MHz, CD_3OD): δ 1.038 (d, 3H, $J_{5,6}$=6.5 Hz, H-6,6,6), 1.985 and 2.010 (2 s, each 3H, 2 Ac), 3.963 (dd, 1H, $J_{1',2'}$=8.5, $J_{2',3'}$=10.4 Hz, H-2′), 4.718 (dd, 1H, $J_{2,3}$=10.6, $J_{3,4}$=3.3 Hz, H-3), 4.855 (dd, 1H, $J_{3',4'}$=8.9, $J_{4',5'}$=10.1 Hz, H-4′), 5.098 (dd, 1H, H-3′), 5.098 and 5.279 (2 m, each 1H, OCH_2CH=CH_2), 5.184 (d, 1H, $J_{1,2}$=3.8 Hz, H-1), 5.352 (dd, 1H, H-2), 5.451 (d, 1H, H-4), 5.594 (d, 1H, H-1′), 5.854 (m, 1H, OCH_2CH=CH_2), 7.22–8.17 (m, 14H, Phth and 2 Bz); FABMS (positive): calcd for $C_{41}H_{40}O_{18}NSNa$ 889.4, found *m/z* 890.1 ($[M+H]^+$), 912.0 ($[M+Na]^+$).

*3.9. Allyl (sodium 2-acetamido-2-deoxy-β-*D*-glucopyranosyl 3-sulfate)-(1→3)-α-*L*-fucopyranoside* ***10***

A solution of **9** (38.4 mg, 44 µmol) in ethanolic 30% NH_2Me (5 mL) was stirred for 5 days, when TLC (*n*-butanol:EtOH:H_2O:HOAc 4:2:2:1) showed an incomplete conversion of **9** into an intermediate amino compound (R_f=0.56). Ethanolic 30% NH_2Me (5 mL) was again added, and the mixture was stirred for 3 days, when TLC showed the de-acylation to be completed. After concentration, a solution of the residue in MeOH (2 mL) and Ac_2O (77 µL) was stirred for 2 h at 0°C, then concentrated and co-concentrated with toluene and MeOH (1:1, 3×2 mL). The remaining oil was purified on Bio-Gel P-2 (H_2O) followed by silica gel column chromatography (CH_2Cl_2:methanol 8:2) to afford **10**, isolated, after lyophilization, as an amorphous white powder (12 mg, 61%); $[\alpha]_D$= −106 (*c*=0.4, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 1.215 (d, 3H, $J_{5,6}$=6.7 Hz, H-6,6,6), 2.012 (s, 3H, NAc), 3.824 (dd, $J_{1',2'}$=8.6, $J_{2',3'}$=10.4 Hz, H-2′), 3.892 (dd, 1H, $J_{2,3}$=10.4 Hz, H-2), 4.046 (dd, 1H, $J_{2,3}$=10.4, $J_{3,4}$=3.4 Hz, H-3), 4.414 (dd, 1H, $J_{3',4'}$=8.8 Hz, H-3′), 4.805 (d, 1H, H-1′), 4.964 (d, 1H, $J_{1,2}$=3.9 Hz, H-1), 5.266 and 5.362 (2 m, each 1H, OCH_2CH=CH_2), 5.973 (m, 1H, OCH_2CH=CH_2); FABMS (positive): calcd for $C_{17}H_{28}O_{13}NSNa$ 509.2, found *m/z* 532.0 ($[M+Na]^+$).

*3.10. 3-(2-Aminoethylthio)propyl (sodium 2-acetamido-2-deoxy-β-*D*-glucopyranosyl 3-sulfate)-(1→3)-α-*L*-fucopyranoside* ***11***

To a solution of **10** (5.0 mg, 10.3 µmol) in water (0.5 mL) was added cysteamine hydrochloride (6.4 mg, 56.6 µmol), and the mixture was irradiated for 2 h in a quartz vial using a Hanovia UV lamp. Then, TLC (*n*-butanol:H_2O:HOAc 2:1:1) showed a complete conversion of **10** into a lower moving spot (R_f=0.18). The mixture was loaded directly on a Toyopearl HW-40SO column yielding, after lyophilization, **11** as an amorphous white powder (5.7 mg, 86%); $[\alpha]_D$= −112 (*c*=0.6, H_2O); ^{1}H NMR data (500 MHz) are given in Table 1; FABMS (positive): calcd for $C_{19}H_{35}O_{13}N_2S_2Na$ 586.2, found *m/z* 587.3 ($[M+Na]^+$).

*3.11. 3-[2-*N*-(3,4-Dione-2-ethoxycyclobutene)aminoethylthio]propyl (sodium 2-acetamido-2-deoxy-β-*D*-glucopyranosyl 3-sulfate)-(1→3)-α-*L*-fucopyranoside **12***

To a solution of compound **11** (1.0 mg, 2.1 μmol) in 0.75 mM sodium phosphate buffer (pH 7.0; 100 μL) was added a solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (diethyl squarate; 0.28 μL, 2.0 μmol) in EtOH (100 μL). The mixture was stirred for 2.5 h when TLC (CH_2Cl_2:H_2O:MeOH 120:12:7) showed complete conversion to a higher moving spot (R_f=0.73). After concentration, a solution of the crude residue in water (1 mL) was loaded on a C-18 Sep-Pak cartridge. The column was washed with water (3×2 mL), then the product was eluted with MeOH (3×2 mL). The MeOH phase was evaporated and a solution of the residue in water (2 mL) was lyophilized to yield **12** as a white powder. The material was used directly for the preparation of neoglycoconjugate **13**.

3.12. Pretreatment of bovine serum albumin

BSA was stirred in 0.1 M NaOAc buffer (pH 4.5; 40 mg/mL) containing 10 mM $NaIO_4$ for 1.5 h at rt to oxidize carbohydrate of glycoprotein contaminants.[19] Excess of periodate was destroyed by adding glycerol to a final concentration of 10 mM. The solution was dialyzed against water (three changes; Milli Q), followed by lyophilization. After treatment of the material with an aq. solution of $NaBH_4$ (catalytic amount) for 1 h at rt, the solution was diluted with water (1 mL) and neutralized with 4 M HOAc. After lyophilization, the quality of pretreated BSA was checked by SDS–polyacrylamide gel electrophoresis. To verify the complete removal of sugar contaminants with GLC, a small amount of the protein material (1 mg) was subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C) followed by re-*N*-acetylation and trimethylsilylation.[18]

*3.13. Preparation of the BSA-glycoconjugate **13***

Pretreated BSA (9.1 mg) was dissolved in 0.1 M $NaHCO_3$ buffer (pH 9.0; 400 μL) and stirred for 30 min. Then, a solution of **12** in 0.1 M $NaHCO_3$ buffer (pH 9.0, 400 μL) was added and the mixture was stirred for 5 days at rt, when TLC (CH_2Cl_2:H_2O:MeOH 120:12:7) showed complete transfer of **12** onto BSA (R_f=0.0). The mixture was purified by HiTrap gel filtration (aq. 5% NH_4HCO_3) to afford, after lyophilization, neoglycoconjugate **13**. The incorporation degree of **12** onto BSA was determined by MALDI-TOF MS, revealing an average incorporation of 7–9 hapten molecules per molecule BSA. The incorporation degree was also determined by GLC; a small amount of the neoglycoconjugate (1 mg) was subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C) followed by re-*N*-acetylation, and trimethylsilylation,[18] revealing the presence of 8 hapten molecules per molecule BSA.

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