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Chemoenzymatic synthesis of spacer-linked oligosaccharides for the preparation of neoglycoproteins

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Abstract

In the present work, the combination of chemical and enzymatic methods to obtain neoglycoproteins is described. Three bovine serum albumin (BSA)-conjugates, BSA-[GalNAcα-], BSA-[Gal(β1-3)GalNAc(α-], and BSA-[Neu5Ac(α2-3)Gal(β1-3)GalNAc(α-], were prepared. αGalNAc derivatives were galactosylated employing crude β-galactosidase from bovine testes. The use of oversaturated donor solutions (pNPβGal) enhanced the yields up to 60%. This method was verified using divalent structures as acceptors, that rendered di- and tri-galactosylated products. Further treatment of the disaccharides with CMP-Neu5Ac and α2-3 sialyltransferase from pork liver led to formation of trisaccharides. Finally, mono-, di-, and trisaccharides were coupled to BSA employing a thiolic group introduced into the protein for Michael addition to a maleinimide group in the spacer-arm of the saccharide components. The results were monitored by HPLC and MALDI-TOF. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Chemoenzymatic synthesis; Transglycosylation; β-Galactosidase; α(2→3)-Sialyltransferase; Neoglycoproteins

1. Introduction

In recent years the use of neoglycoproteins has expanded enormously [1,2]. The foremost advantage of neoglycoconjugates is that they contain carbohydrates of known structures and assured purity. Galactose-, mannose-, and *N*-acetylgalactosamine-containing neoglycoproteins are used in different fields, for example in the therapeutic area and in cancer research [3,4].

For synthesis of neoglycoproteins, many different spacer molecules and coupling methods have been employed. Oligosaccharides with an ethylene glycol spacer have been classically synthesized and exploited by Verez-Bencomo et al. [5]. Recently, we have reported syntheses of analogues of the T-antigen determinant in which 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranose is α-glycosidically linked to different diethylene glycol derivatives, using crude extracts of β-galactosidase (EC 3.2.1.23) from bovine testes [6].

Since a general and wider use of glycosidases for the synthesis of biologically active oligosaccharides is limited due to lower yields, we sought to establish an improved method by using β-galactosidase from bovine testes for the galactosylation of the spacer-linked GalNAc-derivative **1**. In this paper, the synthesis of mono-, di-, and trisaccharides by combination of chemical and enzymatic methods and the procedure for their coupling to bovine serum albumin (BSA) is described to give neoglycoproteins.

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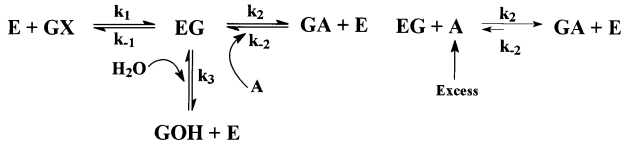
2. Results and discussion

Use of oversaturated solutions of p-*nitrophenyl β*-D-*galactopyranoside for transgalactosylation.*—Most syntheses employing glycosidases use the kinetic approach by transglycosylation. Thus, the initial formation of a glycosyl enzyme intermediate (EG) involves a nucleophilic displacement of the aglycon of the donor molecule GX.

The yield and optimal time of enzymic transglycosylation are a function of the initial concentration of reactants, and of various dissociation and catalytic rate constants for the different steps (Scheme 1, left). Thus, a high yield may be expected if the rate constant (k_1) for the formation of the glycosyl enzyme intermediate (EG) from the donor is high compared with that of the product glycoside (k_{-2}). In cases of β-galactosidase from bovine testes it was possible to use *p*- or *o*-nitrophenyl β-D-galactopyranosides, because the affinity for these donors to the active center of the enzyme is higher than the affinity for the transglycosylation products. Previously, the use of excess of acceptor was described, and proposed to shift the equilibrium of the reaction to the right side (Scheme 1, right) [7–9].

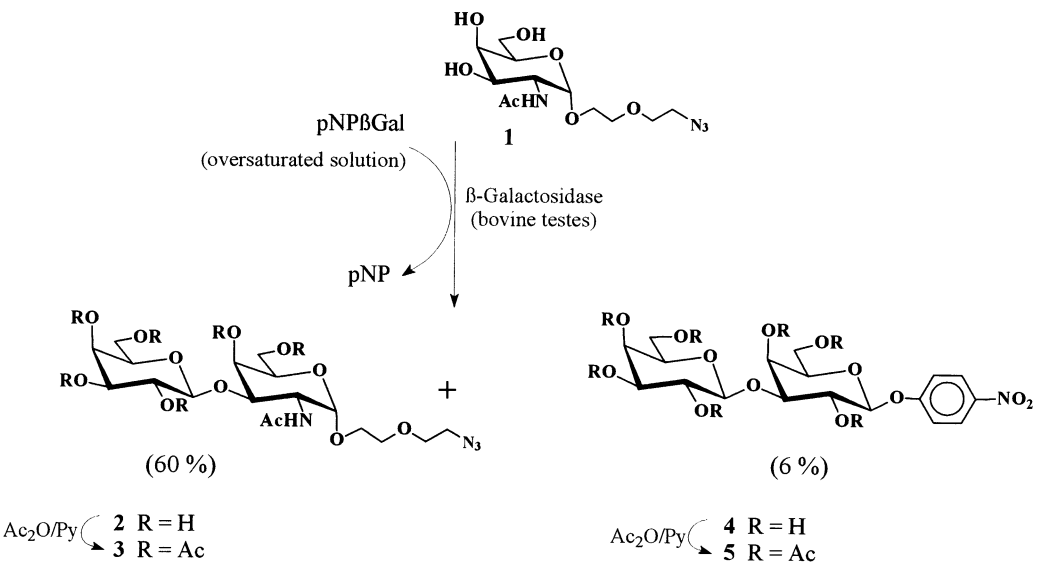


Scheme 1. E, enzyme; GX, glycosyl donor; EG, glycosyl enzyme intermediate; A, acceptor; GA, transglycosylation product; GOH, hydrolysis product.

Since the acceptor molecule **1** is very expensive, it cannot be used in high excess. Alternatively, we developed another strategy to shift this equilibrium to the right by employing an oversaturated solution of the donor molecule pNPβGal (GX). Thus, a higher concentration of the glycosyl enzyme intermediate (EG) could be achieved and this enhanced the yield of the enzymatic galactosylation considerably. In contrast to previous preparations [6], the transgalactosylation of **1** could be increased to give the disaccharide **2** in 60% yield (Scheme 2).

The HPLC chromatogram of this reaction is shown in Fig. 1. Apparently, due to the high concentration of glycosyl enzyme intermediate (EG), the donor (pNPβGal) can also act as an acceptor for β-galactosidase. Thus, by autogalactosylation of pNPβGal, the formation of the β-(1→3)-galactobioside **4** was observed in 6% yield. For NMR characterization, both these compounds were acetylated to give the corresponding heptaacetates **3** and **5**.

Bis(galactosylation).—The use of oversaturated donor solutions (pNPβGal) as an improved method for transgalactosylation was further checked and verified using divalent acceptor structures. By reaction of the glyco-



Scheme 2.

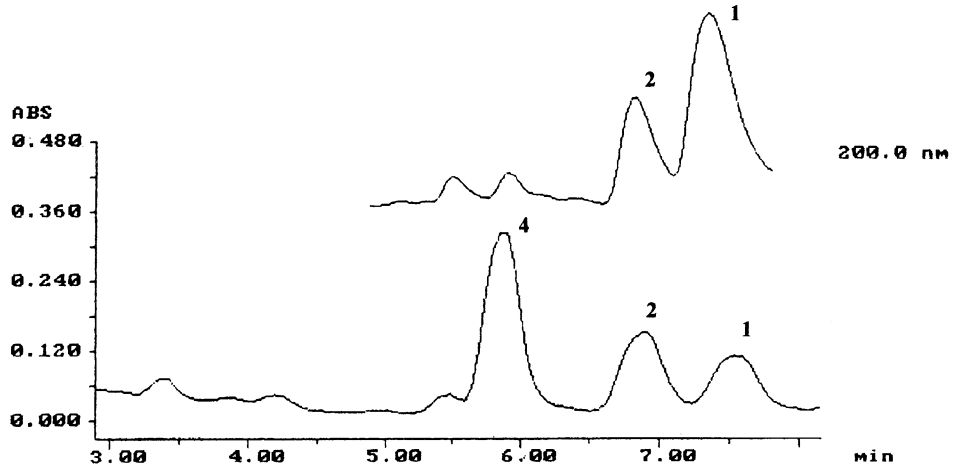
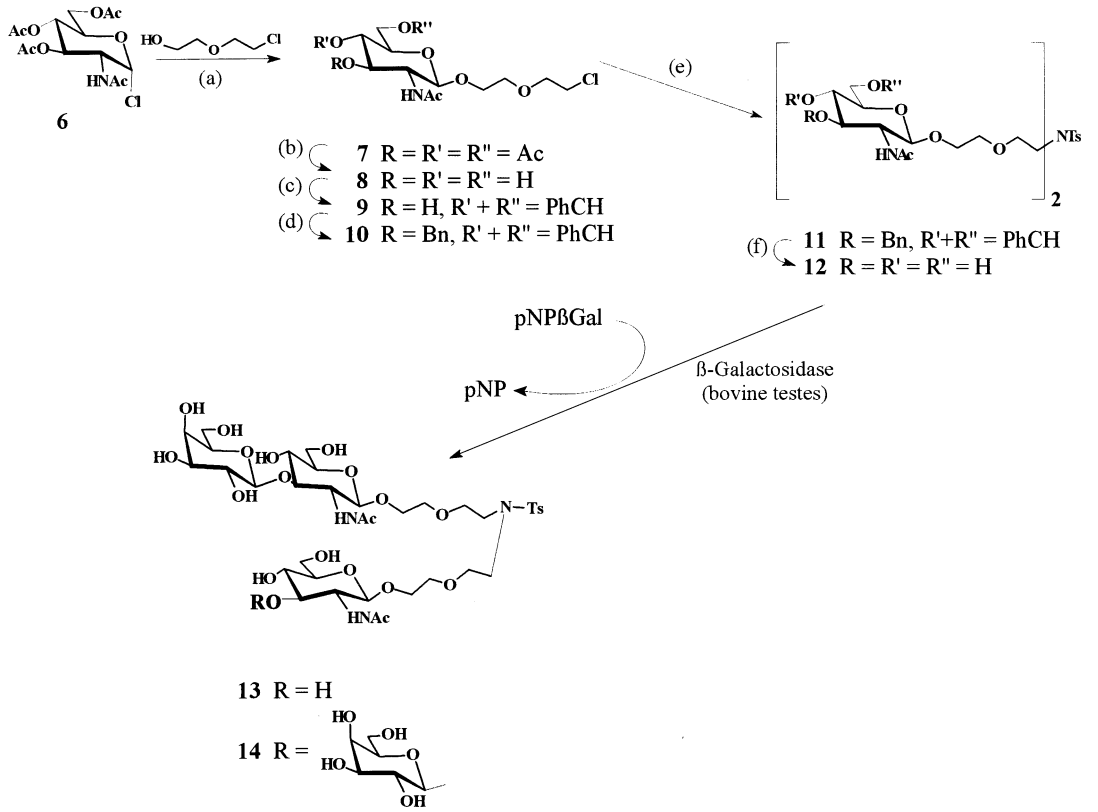


Fig. 1. HPLC of transgalactosylations of **1** with β-galactosidase employing pNPβGal in saturated (upper line) and oversaturated (lower line) solutions. The relative absorptions of the acceptor **1**, the product disaccharide **2**, and the side product disaccharide **4**, do not correlate to the yield.



Scheme 3. (a) $Hg(CN)_2$, CH_2Cl_2; (b) CH_3ONa–CH_3OH; (c) $PhCH(OCH_3)_2$, pTsOH; (d) BnBr, BaO–$Ba(OH)_2$, DMF; (e) $pTsNH_2$, Cs_2CO_3, DMF, 80 °C, 3 days; (f) H_2, 10% Pd/C.

sylchloride **6** [10] (Scheme 3) with 1-chloro-3-oxa-5-pentanol in the presence of $Hg(CN)_2$, the corresponding β-galactopyranoside **7** was obtained in 60% yield. After deacetylation to compound **8**, benzylidenation gave derivative **9**, and the subsequent benzylation led to the fully protected *N*-acetylglucosamine derivative **10**.

Employing Richman–Atkins conditions [11] to **10** and *p*-toluenesulfonamide gave the bridged component **11**, which, after hydrogenolysis with 10% Pd/C, led to the spacer-linked *N*-acetyl glucosamine-derived dimeric glycoside **12**. Its enzymatic galactosylation was performed using saturated and oversaturated

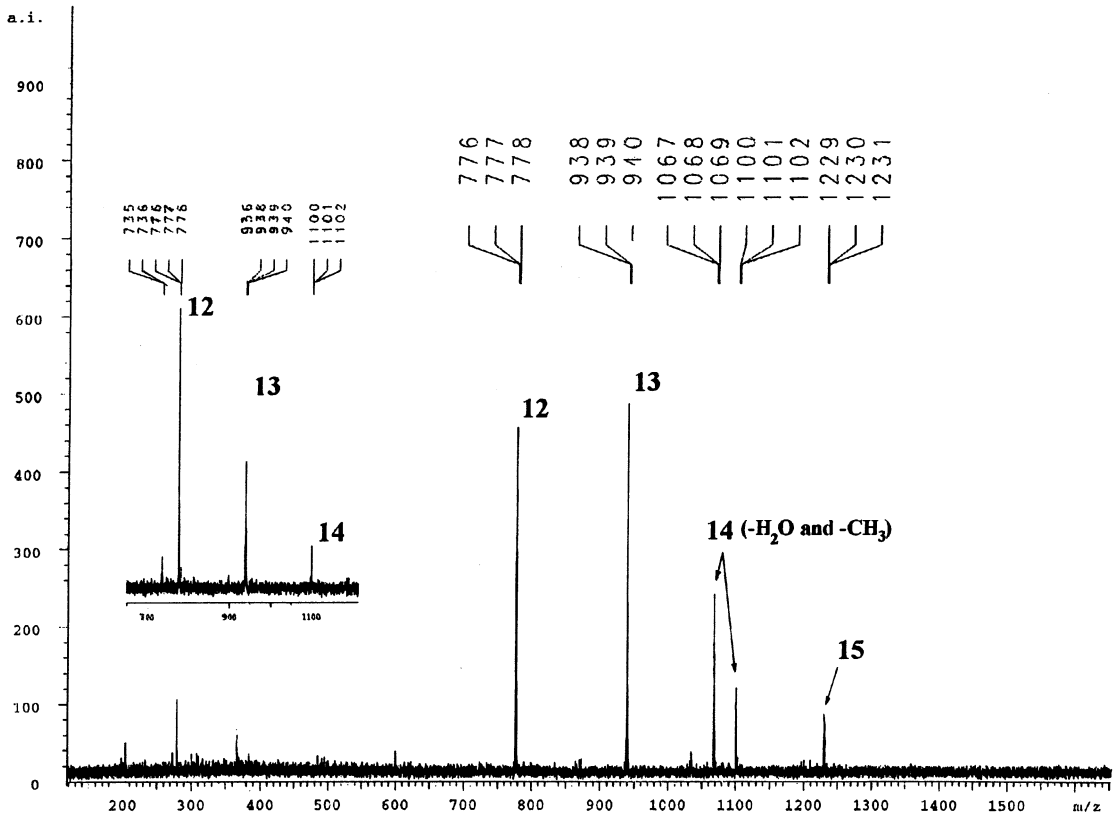


Fig. 2. MALDI-TOF spectra of mono- (**13**), bis- (**14**), and tris-galactosylated (**15**) spacer-linked dimeric GlcNAc acceptors. Upper insert: saturated conditions, lower line: oversaturated donor solutions.

solutions of pNPβGal and the results were monitored by MALDI-TOF MS as shown in Fig. 2.

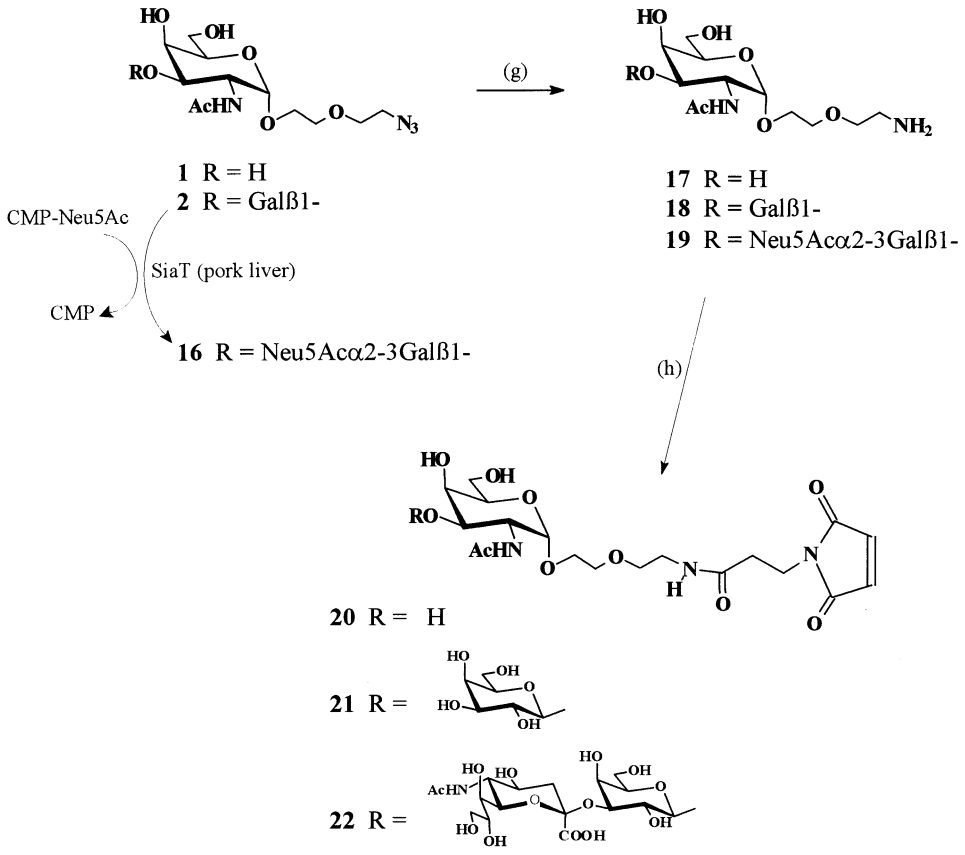
The peaks with molecular weights of 776, 938 and 1100 corresponded to the starting acceptor material **12**, the monogalactosylated compounds **13** and the bisgalactosylated derivative **14**, respectively. In fact, the use of oversaturated solutions of pNPβGal led to an increased formation of transgalactosylation products **13** and **14**. In addition to these the MALDI-TOF spectrum showed another peak at molecular weight 1229 that corresponds to a trisgalactosylated product **15**. As there were only small quantities of isolated **15**, the structure of this compound could not yet be determined unequivocally, since the third galactose residue could be interglycosidically linked to either the inner GlcNAc or the terminal Gal units.

3. Epitope synthesis and coupling to BSA

According to previous findings [12–14], disaccharide **2** could be selectively sialylated. This was done by incubation with stoichiometric amounts of CMP-Neu5Ac in the presence of sialyltransferase from pork liver for 24 h in MES-buffer pH 6.8. After HPLC purification on an RP-18 column and desalting, the trisaccharide glycoside **16** was obtained in 50% isolated yield (Scheme 4).

The subsequent reaction steps [15,16] required reduction of the azido groups in compounds **1**, **2** and **16**. With hydrogen in the presence of 5% Pd/C the corresponding mono-, di-, and trisaccharide glycosides **17**, **18**, and **19** with terminal amino groups were obtained. Further reaction of the free amines with *N*-succinimidyl-3-maleimidopropionate (NSMP) in specifically deaminated DMF afforded smoothly the activated derivatives **20**, **21** and **22** carrying the desired thiophilic substituent.

For coupling to BSA with an average of 23 amino groups per mol, the protein **23** was modified as outlined in Scheme 5. By reaction with the *N*-hydroxysuccinimide dithiopropionate (DTSP), compound **24** was obtained, which was followed by treatment with dithio-



Scheme 4. (g) H_2, 5% Pd/C; (h) NSMP, DMF.

threitol (DTT) to allow the introduction of 20 thiol groups per mol of protein to give compound **25**.

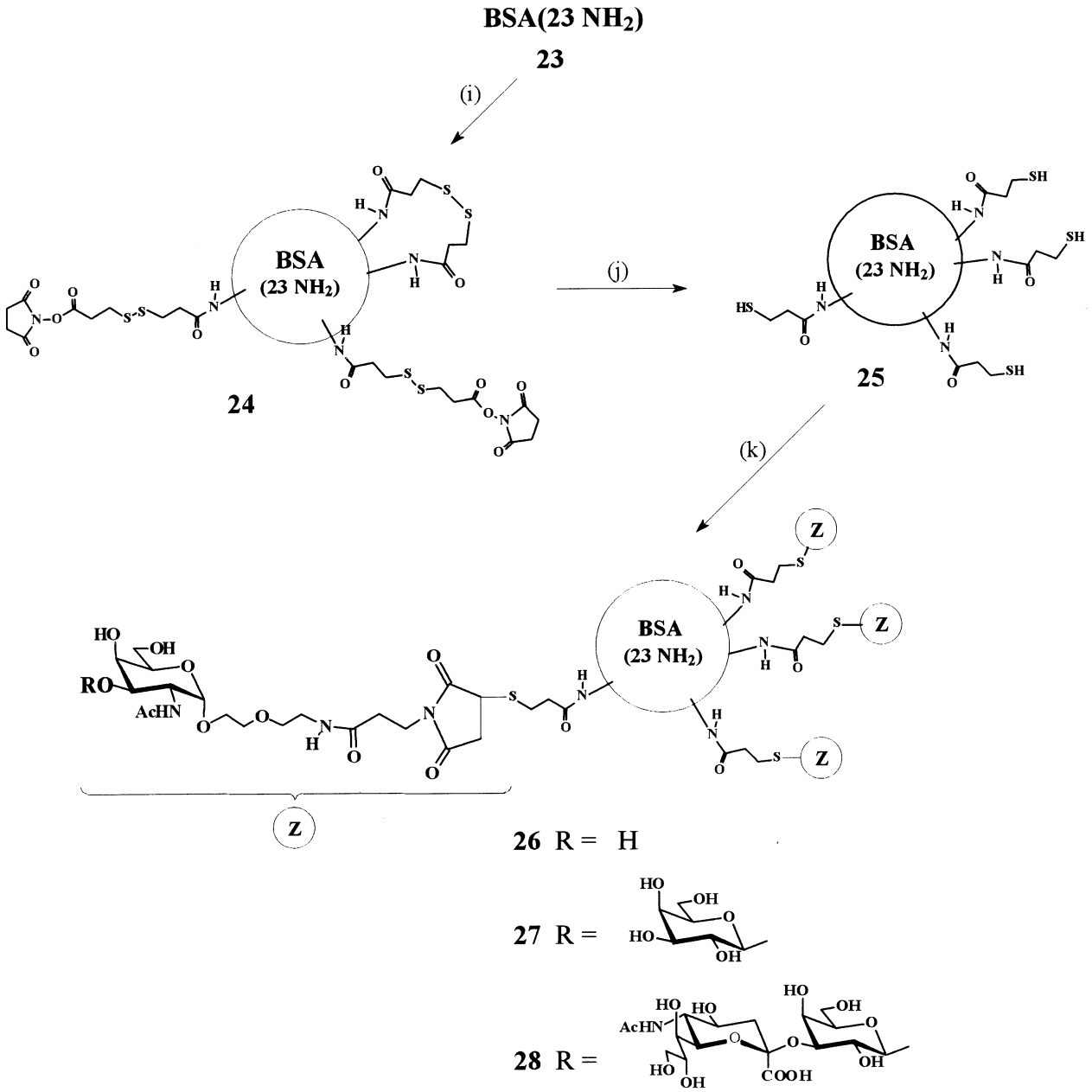
The final coupling reaction was performed between derivative **25** and the corresponding sugar epitopes carrying the maleinimide function **20**, **21** and **22** in PBS-buffer at pH 7.2. By this Michael addition of the thiol groups in the protein to a maleimide group in the spacer-armed sugar derivatives, the corresponding neoglycoproteins **26**, **27** and **28** were obtained in almost quantitative yields.

For closer determination of the structures of these neoglycoproteins, several different methods had to be applied. As observed by MALDI-TOF spectra, the neoglycoprotein having the GalNAc monosaccharide epitope **26** contained 18 mol of GalNAc per mol protein **23**. Again 18 mol of Galβ(1-3)GalNAc disaccharide epitope per mol protein **23** were determined for the conjugate **27** by use of the phenol–sulfuric acid method [17]. Finally, for compound **28** carrying the trisaccharide epitope 15 mol of Neu5Acα(2-3)Galβ-(1-3)GalNAc per mol protein, **23** could be measured by the TBA assay [18].

As shown in Fig. 3, MALDI-TOF spectra of BSA **23**, the two functionalized BSA components **24** and **25**, and two sugar-modified neoglycoconjugates **26** and **28**, were compared. The molecular weights nicely reflected the progress of the functionalization process and the formation of neoglycoproteins. The novel neoglycoproteins of the Tn, T, and sT epitope type are now being subjected to formation of monoclonal antibodies for further investigations.

4. Experimental

General procedures.—Specific rotations were determined with a Perkin–Elmer polarimeter, ^1H and ^{13}C NMR spectra were recorded at 400 or 500 MHz (100.6 or 125.7 MHz) with a Bruker AMX-400 or DRX-500 spectrometer. FAB spectra were recorded with VG 70-250S. MALDI-TOF spectra were recorded with a Bruker Biflex. Analytical and



Scheme 5. (i) DTSP, buffer pH 8; (j) DTT, buffer pH 8; (k) **20**, **21**, or **22**, buffer pH 7.2.

preparative HPLC was performed on silica gel LiChrosorb RP-18 columns. Protein concentrations were determined by the method of Bradford [19]. Thiol group concentrations were determined by the method of Ellman [20]. Sialic acid concentrations in glycoconjugtgates were determined by the TBA assay [18]. Galactose concentrations in glycoconjugates were determined using the phenol–sulfuric acid method [17].

*5-Azido-3-oxapentyl 2-acetamido-2-deoxy-3-*O*-(β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**2**).—(a) For preparation of **2** using a saturated solution of pNPβGal, see Ref. [6].

(b) 5-Azido-3-oxapentyl 2-acetamido-2-deoxy-α-D-galactopyranoside [6] (**1**) (25 mg, 0.074 mmol) was dissolved in sodium phosphate–citrate buffer (50 mM, pH 4.3, 0.5 mL). The reaction mixture was incubated with β-galactosidase (crude extract) from bovine

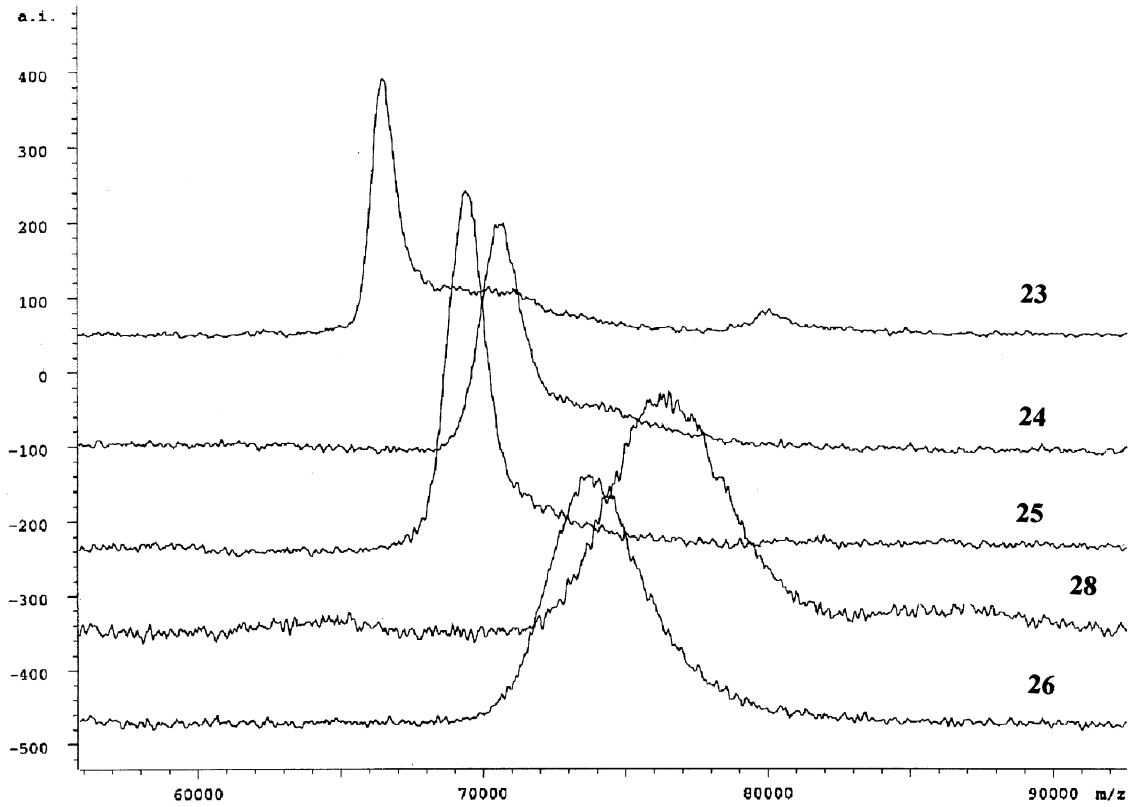


Fig. 3. MALDI-TOF spectra of bovine serum albumin (BSA-23 NH_2, **23**); the masked thiol-functionalized BSA compound **24**; the thiol-functionalized BSA **25**, and the neoglycoproteins, with GalNAc monosaccharide epitopes **26** or with Neu5Acα2-3Galβ-1-3GalNAc trisaccharide epitopes **28**.

testes (10 mg, 0.5 U) at 37 °C. An oversaturated solution of pNPβGal (1.5–2 equiv) in phosphate–citrate–buffer was injected, and the reaction mixture was further incubated for 60 h. The reaction was terminated by heating for 5 min at 90 °C. The desired product was purified by HPLC on an RP-18 column with water/acetonitrile to give **2** (22.6 mg, 61%); $[\alpha]_D + 143°$ (*c* 1.0, water); R_f 0.35 2:1 EtOAc–MeOH, ^1H NMR (400 MHz, D_2O): δ 4.93 (d, 1 H, $J_{1,2}$ 3.5, H-1), 4.46 (d, 1 H, $J_{1,2}$ 8.1, H-1′); ^{13}C NMR (250 MHz, D_2O): δ 105.1 (C-1′), 97.8 (C-1), 48.9 (C-2), 50.6 (CH_2–N_3). Anal. Calcd for $C_{18}H_{32}N_4O_{12}$: 496.48. Found: *m*/*z* 497 $[M + 1]^+$ (FABMS).

*5-Azido-3-oxapentyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-α-*D*-galactopyranoside* (**3**).—Treatment of **2** (100 mg, 0.22 mmol) with pyridine and Ac_2O gave **3** (145 mg, 96%); $[\alpha]_D + 178°$ (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 5.73 (d, 1 H, $J_{2,NH}$ 9.6, NH), 5.30 (d, 1 H, $J_{3,4}$ 3.0, H-4), 5.28 (d, 1 H, $J_{3,4}$ 3.0, H-4′), 5.03 (dd, 1 H, $J_{1,2}$ 7.6, H-2′), 4.87 (dd, 1 H, $J_{2,3}$ 10.1, H-3′), 4.82 (d, 1 H, $J_{1,2}$ 3.5, H-1), 4.48 (d, 1 H, $J_{1,2}$ 7.6, H-1′), 4.43 (ddd, 1 H, $J_{1,2}$ 3.5, H-2), 4.11–4.01 (m, 1 H, H-5), 3.88 (dd, 1 H, $J_{2,3}$ 11.1, H-3), 3.72–3.68 (m, 9 H, H-5′), 3.66–3.59 (m, 6 H, H-spacer), 3.30 (t, 2 H, H-spacer), 2.22–1.81 (m, 21 H, 6 CH_3CO, CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 100.4 (C-1′), 97.8 (C-1), 72.5 (C-3), 70.3 (C-3′), 70.2 (C-5′), 68.4 (C-4), 68.2 (C-2′), 67.0 (C-5), 66.3 (C-4′), 62.2, 60.4 (C-6, C-6′), 48.6 (C-2), 50.1 (CH_2-N_3). Anal. Calcd for $C_{30}H_{44}N_4O_{18}$: 748.52. Found: *m*/*z* 749 $[M + 1]^+$ (FABMS).

p-*Nitrophenyl 3-*O*-(β-*D*-galactopyranosyl)-β-*D*-galactopyranoside* (**4**).—This compound was isolated as a side-product during formation of **2**. Based on the amount of pNPβGal, the yield was 6%; $[\alpha]_D + 166°$ (*c* 1.0, H_2O); R_f 0.37 2:1 EtOAc–MeOH; ^1H NMR (400 MHz, D_2O): δ 8.13 and 7.16 (d, 2 H, *J* 9.6, H-arom), 5.07 (d, 1 H, $J_{1,2}$ 7.6, H-1), 4.27 (d, 1 H, $J_{1',2'}$ 7.1, H-1′), 3.98 (m, 1 H, H-3); ^{13}C NMR (250 MHz, D_2O): δ 161.9 (OPh), 142.8 (C–NO_2),

126.5, 116.5 (C-arom), 106.5 (C-1′), 100.2 (C-1). Anal. Calcd for $C_{18}H_{25}NO_{13}$: 463.25. Found: m/z 464 $[M+1]^+$ (FABMS).

p-*Nitrophenyl 2,4,6-tri*-O-*acetyl-3*-O-*(2,3,4,-6-tetra*-O-*acetyl-β*-D-*galactopyranosyl)-β*-D-*galactopyranoside* (**5**).—Treatment of **4** (100 mg, 0.21 mmol) with pyridine and acetic anhydride gave **5** (155 mg, 95%); $[\alpha]_D + 177°$ (*c* 1.0, chloroform); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 and 7.01 (d, 2 H, *J* 9.6, H-arom), 5.34 (d, 1 H, $J_{3',4'}$ 3.5, H-4), 5.29 (d, 1 H, $J_{3,4}$ 3.5, H-4), 5.22 (dd, 1 H, $J_{1,2}$ 8.1, $J_{2,3}$ 10.2, H-2), 5.02 (dd, 1 H, $J_{1',2'}$ 8.1, $J_{2',3'}$ 10.1, H-2′), 4.92 (d, 1 H, $J_{1,2}$ 8.2, H-1), 4.58 (d, 1 H, $J_{1',2'}$ 8.1, H-1′), 4.40 (dd, 1 H, $J_{3,4}$ 3.5, $J_{2,3}$ 10.2, H-3), 4.22 (dd, 1 H, J_{gem} 12.2, $J_{5,6}$ 4.8, H-6a), 4.11–4.02 (m, 3 H, H-6b, H-6′a/b), 3.84 (m, 2 H, H-5, H-5′), 2.16–1.92 (m, 21 H, 7 CH_3CO); ^{13}C NMR (250 MHz, $CDCl_3$): δ 161.7 (OPh), 142.5 (C–NO_2), 128.9, 116.4 (C-arom), 101.6 (C-1′), 100.6 (C-1). Anal. Calcd for $C_{32}H_{39}NO_{20}$: 757.23. Found: m/z 758 $[M+1]^+$ (FABMS).

3-Oxa-5-chloro-pentyl 2-acetamido-3,4,6-tri-O-*acetyl-2-deoxy-β*-D-*glucopyranoside* (**7**).—To a solution of **6** [10] (1.88 g, 5.7 mmol) and 5-chloro-3-oxapentanol (1.40 g, 5.7 mmol) in CH_2Cl_2 (40 mL) was added $CaSO_4$ (4.2 g, 0.03 mol), and the mixture was stirred for 30 min. $Hg(CN)_2$ (1.44 g, 5.7 mmol) was then added and stirring was continued for 48 h. The mixture was diluted with CH_2Cl_2, washed with 10% KI solution, with satd $NaHCO_3$ solution and water. The organic phase was separated and concentrated. The residual syrup was chromatographed on a column of silica gel with 4:1 CH_2Cl_2–acetone to afford **7** (1.8 g, 60%); $[\alpha]_D + 82°$ (*c* 1.0, $CHCl_3$); R_f 0.52 3:2 petroleum ether–EtOAc; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.30 (d, 1 H, $J_{2,NH}$ 9.2, NH), 5.05 (t, 1 H, *J* 9.5, H-3), 4.80 (t, 1 H, *J* 9.5, H-4), 4.60 (d, 1 H, $J_{1,2}$ 9.1, H-1), 4.00 (dd, 1 H, J_{gem} 12.2, $J_{5,6}$ 7.0, H-6a), 3.88 (dd, 1 H, J_{gem} 12.2, $J_{5,6}$ 2.05, H-6b), 3.70–3.41 (m, 10 H, H-2, H-spacer, H-5), 1.81, 1.75, 1.68 (s, 12 H, CH_3CONH, 3 CH_3CO); ^{13}C NMR (250 MHz, $CDCl_3$) δ 171.0, 170.9, 170.8, 169.7 (3 CO, CONH), 101.2 (C-1), 73.0 (C-3), 72.0 (C-4), 69.0 (C-5), 70.8, 70.0, 68.8 (C-spacer), 62.6 (C-6), 54.5 (C-2), 43.6 (CH_2–Cl), 23.5 (CH_3CONH), 21.2, 21.0, 20.9 (CH_3CO); Anal. Calcd for $C_{18}H_{28}ClNO_{10}$ (453.87): C, 47.63; H, 6.17; N, 3.08. Found: C, 47.75; H, 6.26; N, 3.02.

5-Chloro-3-oxapentyl 2-acetamido-4,6-O-*benzylidene-2-deoxy-β*-D-*glucopyranoside* (**9**).—Deacetylation of **7** (100 mg, 0.33 mmol) in $NaOCH_3$–CH_3OH at pH 9 gave 5-chloro-3-oxapentyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**8**). (70 mg, 98%); $[\alpha]_D + 55°$ (*c* 1.0, water); ^{1}H NMR (400 MHz, D_2O): δ 4.52 (d, 1 H, $J_{1,2}$ 8.6, H-1), 3.95 (m, 1 H, H-spacer), 3.88 (dd, 1 H, J_{gem} 12.2, $J_{5,6a}$ 1.0, H-6a), 3.79–3.63 (m, 9 H, H-spacer, H-5, H-2), 3.50 (t, 1 H, *J* 9.1, H-3), 3.41 (m, 2 H, H-4, H-6b), 2.00 (s, 3 H, CH_3CONH); ^{13}C NMR (250 MHz, D_2O): δ 174.9 (CONH), 101.3 (C-1), 76.2, 74.2 (C-3, C-4), 71.2, 69.2, 69.9 (C-spacer), 70.2 (C-5), 61.1 (C-6), 55.8 (C-2), 43.6 (CH_2–Cl), 22.6 (CH_3CONH).

To a solution of **8** (1.0 g, 3.0 mmol) in DMF (20 mL) were added benzaldehyde dimethylacetal (1.85 mL, 12.2 mmol) and *p*-toluenesulfonic acid (catalytic amount). The mixture was stirred overnight and terminated with a satd solution of $NaHCO_3$. After work-up and concentration, the material was crystallized from ethyl acetate to give **9** (900 mg, 71%); $[\alpha]_D + 44°$ (*c* 1.0, $CHCl_3$); R_f 0.42 4:1 CH_2Cl_2–acetone; mp: 239–240 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, 1 H, $J_{2,NH}$ 8.8, NH), 7.7–7.6 (m, 5 H, H-arom), 5.82 (s, 1 H, H-benzylidene), 5.51 (d, 1 H, *J* 5.6, OH), 4.76 (d, 1 H, $J_{1,2}$ 8.6, H-1), 4.43 (dd, 1 H, $J_{5,6}$ 5.0 J_{gem} 10.1, H-6a), 4.01 (m, 1 H, H-spacer), 3.97–3.86 (m, 7 H, H-6b, H-spacer), 3.86–3.71 (m, 3 H, H-3, H-2, H-spacer), 3.65 (t, 1 H, *J* 9.1, H-4), 2.05 (s, 3 H, CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 169.4 (CONH), 138.1 (C–CHO_2), 129.2–126.7 (CH-arom), 101.8 (C-benzylidene), 101.0 (C-1), 81.6 (C-4), 70.8 (C-3), 70.9, 69.7, 68.5 (C-spacer), 68.2 (C-6), 66.3 (C-5), 56.4 (C-2), 43.9 (CH_2–Cl), 23.4 (CH_3CONH); Anal. Calcd for $C_{19}H_{26}ClNO_7$ (415.5): C, 54.87; H, 6.25; N, 3.36. Found: C, 54.91; H, 6.32; N, 3.15.

5-Chloro-3-oxapentyl 2-acetamido-3-O-*benzyl-4,6*-O-*benzylidene-2-deoxy-β*-D-*glucopyranoside* (**10**).—To a solution of **9** (900 mg, 2.1 mmol) in DMF (20 mL) were added BaO (900 mg, 5.8 mmol), $Ba(OH)_2 \times 8H_2O$ (290 mg, 0.9 mmol) and benzyl bromide (0.6 mL, 4.9 mmol). The reaction mixture was stirred for 2

h at rt, then filtered through Celite and the filtrate washed with 2% aq HCl solution, satd $NaHCO_3$ solution, and water. The organic phase was separated and concentrated, diethyl ether added, and compound **10** (980 mg, 92%) was isolated as white solid; $[\alpha]_D + 32°$ (*c* 1.0, $CHCl_3$); R_f 0.75 4:1 CH_2Cl_2–acetone; mp 225–228 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.55–7.30 (m, 10 H, H-arom), 5.67 (d, 1 H, $J_{2,NH}$ 7.1, NH), 5.62 (s, 1 H, H-benzylidene), 5.02 (d, 1 H, $J_{1,2}$ 8.6, H-1), 4.79 (AB, 2 H, *J* 11.7, $PhCH_2$), 4.39 (dd, 1 H, $J_{5,6}$ 5.0 J_{gem} 10.6, H-6a), 4.18 (t, 1 H, *J* 9.1, H-3), 3.95 (m, 1 H, H-spacer), 3.85–3.65 (m, 9 H, H-5, H-4, H-spacer), 3.52 (m, 2 H, H-6b, H-2), 1.95 (s, 3 H, CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 170.7 (CONH), 138.8 (C–CHO_2), 137.7 (C–CH_2O), 129.3–126.4 (CH-arom), 101.6 (C-benzylidene), 101.3 (C-1), 83.0 (C-4), 77.1 (C-3), 74.8 (CH_2Ph), 71.6, 71.1, 69.2 (C-spacer), 69.1 (C-6), 66.4 (C-5), 57.6 (C-2), 43.5 (CH_2–Cl), 23.9 (CH_3CONH); Anal. Calcd for $C_{26}H_{32}ClNO_7$ (505.5): C, 61.72; H, 6.33; N, 2.77. Found: C, 61.62; H, 6.44; N, 2.69.

*Bis-1,11-[2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-*D*-glucopyranosyl]-(6-tosyl-3,9-dioxa-6-aza-undecane)* (**11**).—To a solution of **10** (1.0 g, 1.9 mmol) and *p*-toluene-sulfonamide (0.95 mmol) in DMF (10 mL) was added Cs_2CO_3 (10 mmol). The reaction mixture was stirred for 3 days at 80 °C, then filtered through Celite/silica gel and concentrated. The residual syrup was chromatographed on a column of silica gel with EtOAc to afford **11** (1.8 g, 85%); $[\alpha]_D + 56°$ (*c* 1.0, $CHCl_3$); R_f 0.55 10:1 EtOAc–MeOH; mp 214–216 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.70–7.20 (m, 20 H, H-arom), 6.44 (d, 2 H, $J_{2,NH}$ 8.1, 2 × NH), 5.47 (s, 2 H, 2 × H-benzylidene), 4.94 (d, 2 H, $J_{1,2}$ 8.6, 2 × H-1), 4.74 (AB, 4 H, *J* 11.7, 2 × $PhCH_2$), 4.31 (dd, 2 H, $J_{5,6}$ 4.5 J_{gem} 10.1, 2 × H-6a), 4.14 (t, 2 H, *J* 9.6, 2 × H-3), 3.86 (m, 4 H, H-spacer), 3.73 (t, 2 H, *J* 10.1, 2 × H-6b), 3.66 (m, 2 H, 2 × H-4), 3.52 (m, 2 H, 2 × H-2), 3.50 (m, 2 H, 2 × H-5), 3.32 (t, 4 H, *J* 5.5, 2 × CH_2NTs), 2.42 (s, 3 H, CH_3Ph), 1.89 (s, 6 H, 2 × CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 170.6 (CONH), 143.5 (C–SO_2), 138.5 (C–CHO_2), 137.4 (C–CH_2O), 136.2 (C–CH_3), 129.7–126.0 (C-arom), 101.0 (C-benzylidene), 100.9 (C-1), 82.5 (C-4), 77.5 (C-3), 74.7 (CH_2Ph), 70.3, 70.0 (C-spacer), 68.7 (C-6), 68.6 (C-spacer), 65.9 (C-5), 56.8 (C-2), 49.3(CH_2–NTs), 23.4 (CH_3CONH), 21.5 (CH_3Ph); Anal. Calcd for $C_{59}H_{71}N_3O_{16}S$ (1109.87): C, 63.84; H, 6.40; N, 3.78. Found: C, 63.32; H, 6.51; N, 3.64.

*Bis-1,11-[2-acetamido-2-deoxy-β-*D*-glucopyranosyl]-(6-tosyl-3,9-dioxa-6-aza-undecane)* (**12**).—Compound **11** (200 mg, 0.19 mmol) was hydrogenolyzed for 2 days in methanol (10 mL) in the presence of 10% Pd/C (catalytic amount). The catalyst was filtered off, the filtrate was concentrated, and the residue chromatographed on a column of silica gel with CH_2Cl_2–MeOH to give **12** (110 mg, 85%); $[\alpha]_D + 22°$ (*c* 1.0, water); R_f 0.15 10:10:2:1 EtOAc–MeOH–water–AcOH; ^1H NMR (400 MHz, D_2O): δ 7.48 and 7.19 (d, 2 H, H-arom), 4.26 (d, 2 H, $J_{1,2}$ 8.6, 2 × H-1), 3.64 (m, 4 H, 2 × H-3, 2 × H-6a), 3.50–3.20 (m, 2 × (H-2, H-4, H-6b, and H-spacer)), 3.17 (m, H-spacer, 2 × H-5), 2.16 (s, 3 H, CH_3Ph), 1.79 (s, 6 H, 2 × CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 177.3 (CONH), 148.0 (C-SO_2), 137.9 (C–CH_3), 133.0, 129.9 (C-arom), 104.0 (C-1), 78.8 (C-3), 76.8 (C-4), 74.5 (C-5), 72.9, 72.5, 71.4 (C-spacer), 63.7 (C-6), 58.2 (C-2), 50.6 (CH_2–NTs), 25.2 (CH_3CONH), 23.7 (CH_3Ph).

*Bis-1-[2-acetamido-2-deoxy-3-*O*-(β-*D*-galactopyranosyl)-β-*D*-glucopyranosyl]-11-[2-acetamido-2-deoxy-β-*D*-glucopyranosyl]-(6-tosyl-3,9-dioxa-6-aza-undecane)* (**13**) *and bis-1,11-[2-acetamido-2-deoxy-3-*O*-(β-*D*-galactopyranosyl)-β-*D*-glucopyranosyl]-(6-tosyl-3,9-dioxa-6-azaundecane)* (**14**).—Compound **12** (25 mg, 0.033 mmol) was dissolved in sodium phosphate–citrate buffer, (50 mM, pH 4.3, 0.5 mL). The reaction mixture was incubated with β-galactosidase (crude extract) from bovine testes (10 mg, 0.5 U) at 37 °C. An oversaturated solution of pNPβGal (1.5–2 equiv) in phosphate–citrate buffer was injected and the reaction mixture was further incubated for 5 days. The reaction was terminated by heating for 5 min at 90 °C. The desired products were purified by HPLC on a RP-18 column with water–acetonitrile to give **13** (10 mg, 30%) and **14** (5 mg, 15%).

Compound **13**: $[\alpha]_D + 46°$ (*c* 1.0, H_2O); Anal. Calcd for $C_{37}H_{61}N_3O_{21}S$: 915.38. Found *m/z* 938 $[M + Na]^+$ (MALDI-TOF).

Compound **14**: $[\alpha]_D + 165°$ (*c* 1.0, H_2O); ^1H NMR (400 MHz, D_2O): δ 7.73 and 7.42 (d, 2 H, H-arom), 4.58 (d, 2 H, $J_{1',2'}$ 8.3, 2 × H-1′), 4.38 (d, 2 H, $J_{1,2}$ 8.0, 2 × H-1), 3.79 (m, 2 H, H-2′), 3.47 (m, 2 H, H-2), 2.39 (s, 3 H, CH_3Ph), 1.96 (s, 6 H, 2 × CH_3CONH); ^{13}C NMR (250 MHz, $CDCl_3$): δ 175.0 (CONH), 147.5 (C–SO_2), 137.6 (C–CH_3), 133.4, 129.0 (C-arom), 103.9 (C-1), 101.2 (C-1′), 50.3 (CH_2–NTs), 25.0 (CH_3CONH), 23.2 (CH_3Ph). Anal. Calcd for $C_{43}H_{71}N_3O_{26}S$: 1077.43. Found *m*/*z* 1100 $[M + Na]^+$ (MALDI-TOF).

5-Azido-3-oxapentyl 3-O-*{(5-acetamido-3,5-dideoxy-α*-D-glycero-D-galacto-*2-nonulopyranosonate)-3*-O-*(β*-D-*galactopyranosyl)}-2-acetamido-2-deoxy-α*-D-*galactopyranoside* (**16**).—A solution of **2** (50 mg, 0.10 mmol) and CMP-Neu5Ac (45 mg, 0.075 mmol) in MES-buffer (pH 6.8, 2.5 mL) was incubated at 37 °C with α-(2→3) sialyltransferase from pork liver (crude extract, 30–50 mU) for 12 h. At this time, more CMP-Neu5Ac (45 mg) was added and incubation was continued for another 12 h. HPLC then showed the formation of 98% of **16**. The mixture was centrifuged and freeze-dried. The desired product was purified by HPLC on a preparative RP-18 column with phosphate buffer acetonitrile. After evaporation of solvents the residue was applied to a G-15 column, equilibrated and eluted with water, to afford **16** (40 mg, 50%); $[\alpha]_D + 32°$ (*c* 1.0, in water); R_f 0.12; 7:3 1-propanol–water; ^1H NMR (500 MHz, D_2O): δ 4.83 (d, 1 H, $J_{1,2}$ 3.6, H-1), 4.43 (d, 1 H, $J_{1,2}$ 7.6, H-1′), 4.25 (dd, 1 H, $J_{2,3}$ 10.6, H-2), 4.14 (d, 1 H, $J_{3,4}$ 3.1, H-4), 3.97 (dd, 2 H, $J_{2,3}$ 10.6, $J_{3,4}$ 3.1, H-3, H-3′), 3.90 (t, 2 H, H-5, H-5′), 3.84 (d, 1 H, $J_{3,4}$ 3.05, H-4′), 2.66 (dd, 1 H, $J_{3,4}$ 4.5, $J_{3,3}$ 12.2, H-3″eq), 1.69 (t, 1 H, $J_{3,3}$ 12.2, H-3″ax), 1.93 (s, 6 H, 2 CH_3CONH); ^{13}C NMR (250 MHz, D_2O): δ 174.0, 175.0 (CO, CONH), 104.9 (C-1′), 100.0 (C-2″), 97.7 (C-1), 77.8 (C-3), 76.0 (C-3′), 62.8 (C-9″), 61.5, 61.3 (C-6, C-6′), 52.0 (C-5″), 50.5 (CH_2–N_3), 48.8 (C-2), 40.1 (C-3″), 22.4 (2 CH_3CONH). Anal. Calcd for $C_{29}H_{49}N_5O_{20}$: 787.24. Found *m*/*z* 786 $[M - 1]^-$ (MALDI-TOF).

Hydrogenolysis of the azido group.—Compounds **1**, **2** or **16** (0.05 mmol) were hydrogenolyzed for 12 h in MeOH (5 mL) in the presence of 5% Pd/C (catalytic amount) [16]. The catalyst was filtered off and the filtrate was concentrated to afford **17**, **18** or **19** (0.048 mmol, 95%).

Introduction of the maleimide group.—To a solution of the saccharides **17**, **18** or **19** (4 μmol) in DMF (distilled in vacuo and pumped for 30 min before use, 0.5 mL), was added NSMP (1.3 mg, 5 μmol). After 2 h, the solution was concentrated and dried in vacuo. The residue was resuspended in D_2O (0.5 mL) and centrifuged to remove the excess of reagent. The maleimide derivatives **20**, **21** and **22** were obtained and could be stored in a lyophilized form at 0 °C, or directly used in the coupling reaction; ^1H NMR (D_2O): δ 6.89 (s, 2 H, HC=CH), 3.42 (t, 2 H, CH_2N), 2.52 (t, 2 H, CH_2CO) [16].

Preparation of neoglycoproteins.—Compounds **20**, **21** and **22** were added to a solution of **25** [15] in PBS buffer (pH 7.2, 0.4 mL). After 2 h, the process was complete as evidenced by a negative Ellman test [20]. The resulting solution was diafiltrated against PBS buffer (pH 7.2) to afford neoglycoproteins **26**, **27** or **28**. The carbohydrate content was determined by the Bradford method [19], phenol–sulfuric acid method [17], TBA assay, and MALDI-TOF analysis.

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