Improvement of the Synthesis of Immunological Carbohydrate Vaccines Containing the Tumour Associate Antigen CaMBr1

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The simplified synthesis of a portion of Globo-H hexasaccharide, a saccharide antigen overexpressed by breast cancer cells, and its conjugation to two different immunogenic proteins are described. The conjugated vaccines, evaluated in a preclinical model, showed good immunogenic potential.

Introduction

Among the different approaches in anticancer therapy, there is increasing interest in the exploitation of the potential of the immune system through tumour vaccinology.

A constant feature of malignant cells is an aberrant glycosylation pattern, and different approaches have been explored to induce/increase immune responses against such tumour-associated carbohydrate antigens. As these oligosaccharides are recognized mainly by a T-cell-independent mechanism, a good sugar-based vaccine should contain the saccharidic antigen conjugated to an effective immunogenic protein and should be formulated with an efficacious but well tolerated adjuvant in order to promote an active specific response against cancer cells expressing the relevant antigen with minimal side effects.

We have focused on the Globo-H antigen that, although also present in some normal tissues, is overexpressed in human breast, ovary and lung carcinomas, in association with poor prognoses. The structure of this antigen, isolated from the human breast cancer cell line MCF7, was determined by Hakomori et al.[1] and immunologically defined by the murine monoclonal antibody (MAb) MBr1.

In previous papers,[2−4] through synthesis modelling and evaluation of the binding properties of some fragments of Globo-H hexasaccharide, we identified the tetrasaccharide Fucα(1→2)Galβ(1→3)GalNAcβ(1→3)Galα (ABCD) (Figure 1) as the minimal structure specifically recognized by MAb MBr1. Such a tetrasaccharide should represent a well defined and highly specific target molecule that might be exploitable for the development of a new anticancer vaccine.

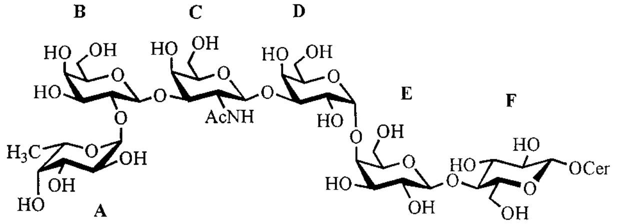

Figure 1. Structure of Globo-H antigen

Results and Discussion

Here we describe a new simplified synthetic approach for the preparation of the ABCD tetrasaccharide portion of the Globo-H antigen, its conjugation to two different immunogenic proteins (KLH and CRM197), and the immunological properties of the obtained conjugates. A different related approach has recently been described by Danishefski et al.,[5] who demonstrated that a vaccine derived from the entire Globo-H hexasaccharide conjugated to KLH is able to induce a specific humoral response in patients. Globo-H hexasaccharide has also been synthesized by other groups.[6−8]

The use of a shorter oligosaccharidic portion is obviously convenient from a practical point of view, as it shortens and simplifies the synthesis and the preparation of the vaccine, provided that conjugates derived from the shortened structure are still able to raise antibodies against the target tumour cells. The importance of having relatively simple syntheses for such compounds is attested to by a recent paper from Danishefskys' group, in which this aspect is strongly emphasized.[9]

Our previous synthesis[2] was modified with the goal of easily obtaining larger quantities of the ABCD tetrasaccharide. Modifications mainly concerned the order of assembly of the monosaccharidic units so as to reduce the

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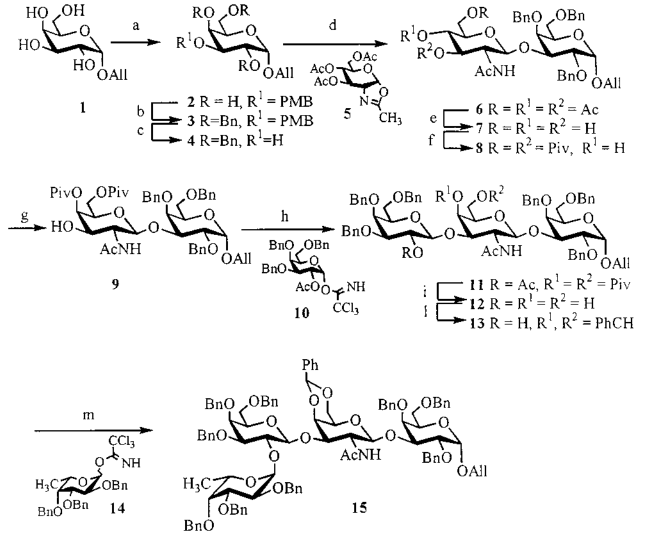
number of steps, and the glycosylation conditions. In fact, all the glycosylation reactions were performed by using trifluoromethanesulfonic acid (triflic acid, TfOH) as promoter, which gave better yields than trimethylsilyl triflate. The protected galactoside acceptor **4**,[10] corresponding to the sugar unit D, was prepared in three steps starting from allyl α-D-galactopyranoside **1**.[11] Compound **1** was selectively protected at the 3-position as the *p*-methoxybenzyl ether, using the stannylene acetal, to give compound **2**. Benzylation of **2** and removal of the PMB ether with DDQ afforded acceptor **4** in 48% overall yield (Scheme 1). The same procedure had already been described for production of either **4**,[12] but without experimental details, or **3**.[13]



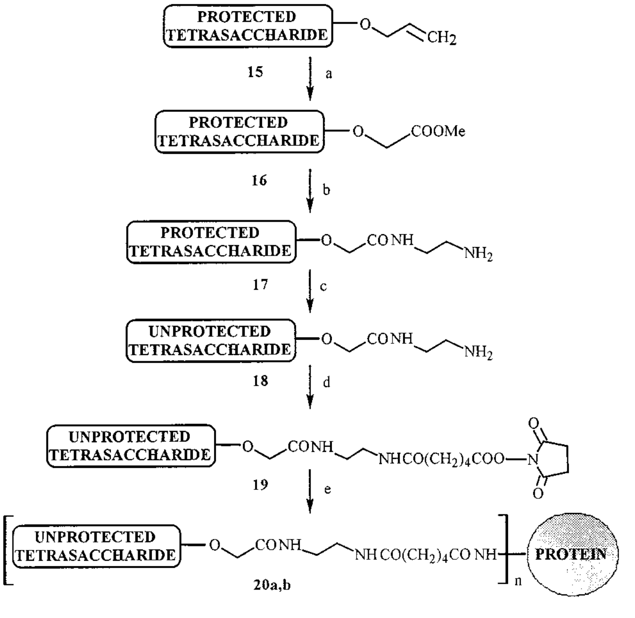
Scheme 1. Reagents and conditions: a) Bu_2SnO, Bu_4NBr, PMBCl, benzene, 24 h, 79%; b) NaH, BnBr, THF, rfx, 4 h, 86%; c) DDQ, CH_2Cl_2 H_2O, 4 h, 71%; d) TfOH, 1,2-dichloroethane, rfx, 2.5 h, 71%; e) MeONa, MeOH, room temp., 1 h, quant.; f) PivCl, CH_2Cl_2/Py, 0 °C, 2.5 h, 79%; g) Tf_2O, CH_2Cl_2/Py, −35 to 0 °C, then H_2O, rfx 1 h, room temp. overnight, 79%; h) TfOH, CH_2Cl_2, 84%; i) MeONa, MeOH, room temp., 6 h at room temp. then 6 h at 35 °C; l) 1,1-dimethoxytoluene, CSA, benzene, 84% over two steps; m) TfOH, CH_2Cl_2, −20 to 0 °C, 1 h, 81%.

Glycosylation of **4** with the acetylated oxazoline **5**[14] in 1,2-dichloroethane using triflic acid as a promoter smoothly afforded disaccharide **6** in 70% yield after crystallization. As the final product contains an *N*-acetyl galactosamine unit, the configuration at the 4′-position of **6** had to be inverted. Compound **6** was deacetylated using the Zemplén procedure and selectively pivaloylated at positions 3′ and 6′ to give **8** in 79% overall yield. We attempted the inversion using a procedure previously applied to an analogous monosaccharide.[15] Compound **8** was converted into the 4′-*O*-triflate by treatment with Tf_2O in CH_2Cl_2/pyridine. After addition of water and stirring for 12 hours we succeeded in obtaining a 79% yield of the *N*-acetyl galactosamine-containing disaccharide **9**, in which the 3′-pivaloyl group had migrated to position 4′ with inversion of configuration, leaving the 3′-position free for subsequent glycosylation. We had thus demonstrated that such a migration-inversion procedure is not limited to monosaccharidic *N*-acetyl glucosamine derivatives, but seems to be of general applicability.

Glycosylation of **9** with the donor **10**[15] was promoted by triflic acid using the inverse procedure,[16] and gave the trisaccharide **11** in 84% yield. It is worth noting that, in our hands, use of triflic acid gave a better yield than use of TMSOTf. The final acceptor **13** was obtained by removal of the ester protecting groups from **11**, and benzylidene formation between positions 4′ and 6′ in compound **12**. With respect to our previous procedure,[2] the formation of the benzylidene ring using 1,1-dimethoxytoluene and 10-camphorsulfonic acid gave a better yield (92%).

The final glycosylation between the donor **14** and the acceptor **13** was again performed by using triflic acid as a promoter with the inverse procedure, and afforded the desired tetrasaccharide **15** in 81% yield.

With the tetrasaccharide in hand, it was necessary to prepare the conjugates with immunogenic proteins, selection criteria for which are reported elsewhere.[17] We decided to use quite a long spacer (adipic acid) between the saccharide moiety and the protein, in order to leave the sugar epitope exposed on the protein surface. The presence of the allyl group at the anomeric position permitted its manipulation in order to provide an anomeric linker for the conjugation. Thus, the allyl moiety was cleaved with osmium tetraoxide/sodium periodate, the resulting aldehyde was oxidized with sodium chlorite,[18] and the carboxylic acid was converted into the methyl ester **16** with diazomethane, in 51% overall yield. Compound **16** was then dissolved in methanol and treated with an excess of ethylenediamine to give the product **17** in 93% yield. At this stage of the synthesis the protecting groups were removed from the tetrasaccharide. Hydrogenolysis of compound **17** with H_2-$Pd(OH)_2$/C gave compound **18** in almost quantitative yield (Scheme 2).



Scheme 2. Reagents and conditions: a) 1. OsO_4, $NaIO_4$, *t*BuOH; 2. $NaClO_2$, NaH_2PO_4, CH_3CN; 3. CH_2N_2, 54% overall; b) Excess of ethylenediamine, MeOH, overnight, 93%; c) H_2, $Pd(OH)_2$, HCl, MeOH, overnight, quant.; d) adipic acid bis(hydroxysuccinimidyl) ester, 1,4-dioxane/water, 5:1(v/v), 2 h; e) KLH or CRM197, phosphate buffer pH 7.4.

Linkage with the immunogenic proteins was achieved by using the bis-hydroxysuccinimidyl ester of adipic acid as a doubly activated linker.[19,20] Treatment of the deprotected compound **18** with 10 molar equivalents of the adipic acid derivative gave the monosubstituted compound **19**, which was separated from the excess of adipic acid bis-hydroxysuccinimidyl ester by washing the crude reaction mixture with water. The activated ester remained undissolved, while compound **19** was recovered from the water solution by lyophilization, and then directly used for the conjugation. Treatment of compound **19** with the relevant protein (KLH or CRM197) in phosphate buffer (pH 7.4) gave the conjugates **20a** and **20b** after dialysis. The obtained conjugates had tetrasaccharide-protein ratios of 248:1 and 11:1 for the KLH- and the CRM197-conjugates, respectively. The complete immunogenic evaluation is described in detail elsewhere.[17] In summary, the tetrasaccharide moiety was recognized by the Mab MBr1 in a dose-dependent manner with both conjugates. Such result indicates that the conjugation process neither compromises proper presentation of the epitope, nor modifies its conformation. The spacer can thus be considered a good substitute for the lactose unit present in the natural compound. Mice (Balb/c x C57BL/6) vaccinated with a formulation containing the conjugates and a suitable adjuvant produced high titres of *anti*-CaMBr1 IgG antibodies, indicating that T-cell activation had occurred and had enabled the generation of a memory long-lasting response. Moreover, although KLH,[21,22] seemed to be a more potent carrier, CRM197[23] conjugate gave a more specific response and a dose/response effect. Finally, the sera of immunized mice exerted a complement-mediated cytotoxic activity on the human CaMBr1 positive carcinoma cell line MCF7.

Conclusion

In this work we report the synthesis of the tetrasaccharide ABCD, which constitutes the minimal antigenic structure of Globo-H (Figure 1), and the conjugates **20a** and **20b** with two different immunogenic proteins, each through a long spacer. Our results simplify the synthesis of the saccharidic antigen, while maintaining its immunogenic properties. The comparison of our immunological results with those obtained using the whole Globo-H hexasaccharide conjugated to KLH suggest that the vaccine formulation based on such minimal antigenic structure and a simpler and structurally better defined carrier (CRM197) might result in more efficient stimulation of the immune response in humans.

Experimental Section

List of Abbreviations: DDQ: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, TfOH: trifluoromethanesulfonic acid, Piv: 2,2-dimethylpropanoyl, PMB: *p*-methoxybenzyl.

General Remarks: Reagents and dry solvents were added by oven-dried syringes through septa. Thin layer chromatography (TLC): Merck 60 F_{254} silica gel plates; detection by spraying with 5% H_2SO_4 in methanol followed by heating. Flash column chromatography: Merck 60 silica gel (230−400 mesh). M.p. Büchi apparatus; uncorrected. Specific optical rotations: Perkin−Elmer-241 polarimeter at 20 °C. − ^1H and ^{13}C NMR Spectra: Bruker-AC-300 instrument.

Allyl 3-*O*-(4-Methoxybenzyl)-α-D-galactopyranoside (2): Allyl α-D-galactopyranoside (**1**, 5.00 g, 22.7 mmol) and dibutyltin oxide (5.80 g, 27.2 mmol, 1.2 equiv.) in benzene (400 mL) were refluxed in a flask equipped with a Dean−Stark apparatus. When the water evolution ceased (about 6 h), the volume of the solution was reduced to 250 mL and the mixture was allowed to cool to room temp. Tetrabutylammonium bromide (11.20 g, 34 mmol, 1.5 equiv.) and 4-methoxybenzyl chloride (4.7 mL, 34 mmol, 1.5 equiv.) were then added in one portion and the solution was stirred for 24 h at room temp. The solvent was removed under reduced pressure. Flash chromatography (EtOAc/CH_2Cl_2 6:4 → EtOAc/CH_2Cl_2/MeOH 12:8:1) gave 6.1 g of **2** as a colourless oil (79% yield). M.p. 61−63 °C; (ref.[13] 62−63.6 °C). − $[\alpha]_D^{20} = +125.2$ (c = 1, $CHCl_3$) [ref.[13] +127.4(c = 1, $CHCl_3$)]. − ^1H NMR (300 MHz, $CDCl_3$): δ = 2.2 (br. s, 3 H, 3 OH), 3.68−3.81 (m, 4 H, OCH_3 and H-3), 3.7−4.2 (m, 5 H, H-4, H-5, 2 H-6, $CH-CH=CH_2$), 4.20 (m, 1 H, $CH-CH=CH_2$), 4.63 and 4.68 (2d, 2 H, J = 11.4 Hz, $PhCH_2$), 5.00 (d, $J_{1,2}$ = 3.9 Hz, H-1), 5. 19−5.31 (2 br d, 2 H, $CH=CH_2$), 5.93 (m, 1H, $CH=CH_2$), 6.92 (d, J = 8.8 Hz, 2 H, ArH), 7.30 (d, J = 8.8 Hz, 2 H, ArH). − $C_{17}H_{24}O_7$ (340.17): calcd. C 59.99, H 7.11; found C 59.74, H 6.89. To confirm the position of attachment of the *p*-methoxybenzyl group, the product was acetylated under standard conditions. − ^1H NMR (300 MHz, $CDCl_3$): δ = 2.15−2.05 (3 s, 9 H, Ac), 3.78 (s, 3 H, CH_3O), 3.98−4.17 (m, 5 H, H-5, 2H-6, $CH_2-CH=CH_2$), 4.38 and 4.61 (2d, 2 H, J = 11.0 Hz, $PhCH_2$), 5.03 (dd, $J_{1,2}$ = 3.8 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 5.12 (d, $J_{1,2}$ = 3.8 Hz, H-1), 5.17−5.29 (2 br d, 2 H, $CH=CH_2$), 5.53 (br. d, $J_{3,4}$ = 3.2 Hz, H-4), 5.85 (m, 1 H, $CH=CH_2$), 6.82 (d, J = 8.8 Hz, 2 H, ArH), 7.20 (d, J = 8.8 Hz, 2 H, ArH).

Allyl 2,4,6-Tri-*O*-benzyl-3-*O*-(4-methoxybenzyl)-α-D-galactopyranoside (3): NaH (1.20 g, 50 mmol, previously washed twice with hexane and dried under vacuum) was added in portions to **2** (3.0 g, 8.81 mmol) in dry THF (25 mL) under N_2, and the mixture was stirred for 20 min. Benzyl bromide (3.5 mL, 29 mmol) was then slowly added and the solution was warmed to reflux. After 4 h, methanol (1 mL) was added and the solvent was evaporated. The residue was dissolved in CH_2Cl_2, washed with water and dried (Na_2SO_4), and the solvent was evaporated. Flash chromatography (hexane/EtOAc, 1:1) afforded 4.6 g of **3** (86% yield) as a colourless oil. $[\alpha]_D^{20} = +16.5$ (c = 1, $CHCl_3$). NMR spectroscopic data were identical to those reported in ref.[13]. − $C_{38}H_{42}O_7$ (610.29): calcd. C 74.73, H 6.93; found C 74.56, H 6.89.

Allyl 2,4,6-Tri-*O*-benzyl-α-D-galactopyranoside (4): Water (5 mL) and DDQ (2.25 g, 9.73 mmol, 1.1 equiv.) were added in one portion to a solution of **3** (5.40 g, 8.85 mmol) in CH_2Cl_2 (90 mL), and the mixture was stirred for 4 h at room temp. After filtration through Celite, the solution was washed with $NaHCO_3$ (5%) and water and dried on Na_2SO_4, and the solvent was evaporated. The residue was purified by flash chromatography (hexane/EtOAc, 8:2) and the product was crystallized from Et_2O to give 3.08 g of **4** (71% yield). White solid; m.p. 64−65 °C. − $[\alpha]_D^{20} = +61.9$ (c = 1, $CHCl_3$) [ref.[10] +61.5 (c = 1, $CHCl_3$)]. − $C_{30}H_{34}O_6$ (490.24): calcd. C 73.45, H 6.99; found C 73.56, H 6.79.

Allyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (6): Compounds **4** (6.09 g, 12.42 mmol) and **5** (11.7 g, 2.8 equiv.) were dissolved in dry 1,2-dichloroethane under N_2, and the solution was cooled to 0 °C. TfOH (0.2 equiv.) was added, and the reaction mixture was heated at reflux for 2.5 h. The reaction mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ and dried with Na_2SO_4, and the solvents were evaporated. The crude mixture was crystallized from EtOAc to give 7.23 g of compound **6** (71% based on the acceptor) as a white solid. M.p. 154−155 °C. − $[\alpha]_D^{20} = +2.2$ (c = 1, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$, COSY): δ = 1.67 (s, 3H, AcNH), 2.08, 2.02, 2.01 (3 s, 9 H, 3 Ac), 3.41−3.52 (m, 2 H, 2 H-6), 3.69 (m, 1 H, H-5′), 3.92−3.99 (m, 3 H, H-2, H-5, C*H*−CH=CH$_2$), 4.07−4.18 (m, 5 H, H-2′, H-3, H-4, H-6′b, C*H*−CH=CH$_2$), 4.25 (dd, $J_{6'a,6'b}$ = 12.3 Hz, $J_{5',6'a}$ = 4.9 Hz, H-6′a), 4.37 and 4.41 (2 d, 2 H, PhCH$_2$ J = 11.8 Hz), 4.53 (d, J = 11.6 Hz, 1 H, PhCH), 4.61 (s, 2 H, PhCH$_2$), 4.79 (d, $J_{1',2'}$ = 8.5 Hz, H-1′), 4.82 (d, $J_{1,2}$ = 4.1 Hz, H-1), 4.91 (d, J = 11.6 Hz, 1 H, PhCH), 5.00 (dd app. as t, $J_{2',3'} = J_{3',4'}$ = 9.5 Hz, H-3′), 5.09 (dd app. as t, $J_{3',4'} = J_{4',5'}$ = 9.5 Hz, H-4′), 5.15 (d, J = 9.2 Hz, 1 H, N*H*Ac), 5.17 (br. d, 1 H, J = 10.4 Hz, CH$_2$−CH=C*H*$_b$), 5.27 (br. d, 1 H, J = 17.4 Hz, CH$_2$−CH=C*H*$_a$), 5.90 (m, 1 H, C*H*=CH$_2$), 7.24−7.42 (m, 15 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$, APT): δ = 20.6 (*q*, 4 C, Ac), 54.3 (d, C-2′), 62.1 (t), 68.3 (t), 68.5 (d), 69.1 (t), 69.5 (d), 71.8 (d), 72.6 (t), 73.2 (d), 73.4 (t), 74.8 (t), 76.4 (d), 76.9 (d), 78.9 (d), 95.8 (d, C-1), 102.4 (d, C-1′), 118.1 (t, CH=*C*H$_2$), 127.4 (d), 127.7 (d), 128.2 (d), 128.4 (d), 128.6 (d), 128.7 (d), 133.8 (d, *C*H=CH$_2$), 138.4 (s), 138.8 (s), 169.2 (s, CO), 169.3 (s, CO), 169.7 (s, CO), 170.6 (s, CO). − $C_{44}H_{53}NO_{14}$ (819.89): calcd. C 64.46, H 6.52, N 1.71; found C 64.78, H 6.22, N 1.69.

Allyl 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (7): Compound **6** (5.00 g, 6.1 mmol) was dissolved in sodium methoxide in methanol (0.15 M, 100 mL). The solution was stirred for 20 min and the product **7** was recovered in quantitative yield (4.23 g) by neutralization with Amberlite IR 120, filtration and evaporation of the solvent. Colourless oil. − $[\alpha]_D^{20} = +23.5$ (c = 1, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$, COSY): δ = 1.67 (s, 3H, *Ac*NH), 3.31 (m, 1 H, H-3′), 3.4−3.7 (m, 5 H, H-2′, 2 H-6, 2 H-6′), 3.8−4.2 (m, 8 H, H-2, H-3, H-4, H-4′, H-5, H-5′, C*H*$_2$−CH=CH$_2$), 4.44 and 4.49 (2 d, 2 H, J = 11.8 Hz, PhCH$_2$), 4.55 (d, J = 11.6 Hz, 1 H, PhCH), 4.63 (m, 3 H, H-1′, PhCH$_2$), 4.8−4.9 (m, 2 H, H-1, PhCH), 5.17 (br. d, 1 H, J = 10.5 Hz, CH$_2$−CH=C*H*$_b$), 5.27 (br. d, 1 H, J = 16.9 Hz, CH$_2$−CH=C*H*$_a$), 5.88 (m, 1 H, C*H*=CH$_2$), 6.38 (d, J = 4.4 Hz, 1 H, N*H*Ac), 7.40−7.19 (m, 15 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.8 (*q*, Ac), 58.1 (d, C-2′), 62.3 (t), 68.4 (t), 68.9 (t), 69.4 (d), 71.3 (d), 71.8 (t), 73.3 (t), 74.6 (t), 75.3 (d), 75.9 (d), 79.6 (d), 95.5 (d, C-1), 102.9 (d, C-1′),118.3 (t, CH=*C*H$_2$), 127.4 (d), 127.6 (d), 128.1 (d), 128.3 (d), 128.6 (d), 133.6 (d, *C*H=CH$_2$), 137.8 (s), 138.6 (s), 173.0 (s, CO). − $C_{38}H_{47}NO_{11}$ (693.78): calcd. C 65.79, H 6.83, N 2.02; found C 65.52, H 6.62, N 2.11.

Allyl 2-Acetamido-2-deoxy-3,6-di-*O*-pivaloyl-β-D-glucopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (8): Compound **7** (4.15 g, 5.99 mmol) was dissolved under N_2 in 20 mL of a 1:1 mixture of CH_2Cl_2/pyridine and then cooled to 0 °C. PivCl (2.20 mL, 3 equiv.) was added and the mixture was stirred at 0 °C for 2.5 h. The reaction mixture was diluted with CH_2Cl_2, washed with 5% HCl, water, 5% $NaHCO_3$ and water, dried with Na_2SO_4, and the solvent was evaporated. Flash chromatography (EtOAc/hexane, 6:4) afforded 4.08 g of **8** (79%). Colourless oil. − $[\alpha]_D^{20} = -2.2$ (c = 1, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.20 (s, 18 H, Piv), 1.72 (s, 3 H, *Ac*NH), 3.19 (br. s, 1 H, OH), 3.3−3.6 (m, 4 H, 2 H-6, H-2′, H-5′), 3.9−4.0 (m, 4 H, H-2, H-5, H-6′b, C*H*$_b$−CH=CH$_2$), 4.0−4.2 (m, 4 H, H-3, H-4, H-6′a, C*H*$_a$−CH=CH$_2$), 4.3−4.5 (m, 3 H, H-4′, PhCH$_2$), 4.50 (d, J = 11.6 Hz, 1 H, PhCH), 4.58 and 4.67 (2 d, 2 H, J = 12.4 Hz, PhCH$_2$), 4.7−4.8 (m, 2 H, H-1, H-1′), 4.89 (m, 1 H, H-3′), 4.95 (d, J = 11.6 Hz, 1 H, PhCH), 5.15 (br. d, 1H, J = 10.2 Hz, CH$_2$−CH=C*H*$_b$), 5.25 (br. d, J = 17.7 Hz, CH$_2$−CH=C*H*$_a$), 5.50 (d, J = 9.6 Hz, 1 H, AcN*H*), 5.90 (m, 1 H, C*H*=CH$_2$), 7.4−7.2 (m, 15 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$): δ = 23.2 (*q*, *Ac*), 27.1 (*q*), 39.5 (s), 39.8 (s), 53.8 (d, C-2′), 63.3 (t), 68.3 (t), 69.3 (t), 69.4 (d), 69.6 (d), 72.9 (t), 73.4 (t), 74.2 (d), 74.9 (t), 75.5 (d), 76.1 (d), 77.3 (d), 79.1 (d), 96.1 (d, C-1), 103.0 (d, C-1′), 118.0 (t, CH$_2$−CH=*C*H$_2$), 127.5 (d), 128.0 (d), 128.1 (d), 128.3 (d), 128.6 (d), 133.9 (d, CH$_2$-*C*H=CH$_2$), 138.1 (s), 138.4 (s), 139.5 (s), 169.7 (s, CO), 178.2 (s, CO), 179.1 (s, CO). − $C_{48}H_{63}NO_{13}$ (862.01): calcd. C 66.88, H 7.37, N 1.62; found C 66.68, H 7.47, N 1.74.

Allyl 2-Acetamido-2-deoxy-4,6-di-*O*-pivaloyl-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (9): A stirred solution of **8** (4.00 g, 4.65 mmol) in dry CH_2Cl_2 (30 mL) and pyridine (4 mL) under N_2 was cooled to −35 °C and Tf_2O (0.84 mL, 5.12 mmol) was added. The solution was allowed to warm to room temp. and stirred until the starting material disappeared. Water (3 mL) was then added and the reaction mixture was warmed at reflux for 1 h and then stirred overnight at room temp. The reaction mixture was diluted with CH_2Cl_2 and washed with 5% HCl, water, 5% $NaHCO_3$ and water, and dried with Na_2SO_4. The solvents were evaporated. Flash chromatography (EtOAc/hexane, 6:4) gave 3.16 g of compound **9** (79% yield). Foam. − $[\alpha]_D^{20} = +8.3$ (c = 1, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$, COSY): δ = 1.16 (s, 9 H, Piv), 1.27 (s, 9 H, Piv), 1.62 (s, 3H, *Ac*NH), 3.27 (dd, J = 9.6 Hz, $J_{5,6b}$ = 5.6 Hz, H-6b), 3.48 (dd, $J_{6a,6b}$ = 9.6 Hz, $J_{5,6a}$ = 6.6 Hz, H-6a), 3.73 (dd, $J_{2,3}$ = 10.4 Hz, $J_{2,1}$ = 3.3 Hz, H-2), 3.8−4.2 (m, 9 H, C*H*$_2$−CH=CH$_2$, H-2, H-4, H-5, 2 H-6′, H-2′, H-5′), 4.33 and 4.43 (2 d, 2 H, J = 11.9, Hz PhCH$_2$), 4.63 (d, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.5−4.7 (m, 3 H, PhCH), 4.9−5.0 (m, 2 H, H-1, PhCH), 5.1−5.3 (m, 3 H, H-4′, CH=C*H*$_2$), 5.8−5.9 (m, 1 H, C*H*=CH$_2$), 6.03 (d, 1 H, J = 3.5 Hz, AcN*H*), 7.4−7.2 (m, 15 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.6 (*q*), 27.0 (*q*), 27.2 (*q*), 38.7 (s), 39.2 (s), 56.3 (d, C-2′), 62.2 (t), 68.0 (d), 68.4 (t), 69.3 (t), 69.6 (d), 71.5 (t), 73.3 (t), 73.6 (d), 74.4 (t), 75.9 (d), 76.4 (d), 80.3 (d), 95.2 (d, C-1), 103.1 (d, C-1′), 118.3 (t, CH$_2$−CH=*C*H$_2$), 127.2 (d), 127.6 (d), 127.7 (d), 128.2 (d), 128.5 (d), 128.8 (d), 133.6 (d, CH$_2$-*C*H=CH$_2$), 137.7 (s), 138.1 (s), 138.3 (s), 174.0 (s, CO), 177.4 (s, CO), 177.9 (s, CO). − $C_{48}H_{63}NO_{13}$ (862.01): calcd. C 66.88, H 7.37, N 1.62; found C 66.71, H 7.33, N 1.56.

Allyl 2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-4,6-di-*O*-pivaloyl-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (11): A solution of **10** (2.53 g, 2 equiv.) in dry CH_2Cl_2 (10 mL) was added dropwise over 20 min, to a solution of **9** (2.04 g, 2.37 mmol) and TfOH (0.1 equiv.) in dry CH_2Cl_2 under N_2 at 0 °C (14 mL), during which the mixture was allowed to warm to room temp. The reaction mixture was neutralized with TEA and the solvent evaporated. Flash chromatography (EtOAc/hexane, 1:1) of the residue gave 2.66 g of compound **11** in 84.0% yield. − $[\alpha]_D^{20}$ = + 31.2 (c = 1, $CHCl_3$) [ref.[2] +30.5 (c = 1.5, $CHCl_3$)].

Allyl 3,4,6-Tri-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (13): Compound **11** (2.55 g, 1.91 mmol) was dissolved in a solution of sodium methoxide in methanol (0.15 M, 70 mL). The solution was stirred for 6 h at room temp., and a white precipitate appeared. The mixture was then warmed to 30−35 °C to dissolve the precipitate and stirred for another 6 h. The mixture was neutralized with Amberlite IR-120, the resin was filtered off, and the solvent was evaporated. Crude compound **12** was dissolved in 25 mL of dry benzene and treated at room temp. with 1,1-dimethoxytoluene (260 μL) and a catalytic amount of camphorsulfonic acid. The reaction mixture was stirred at room temp. for 2 h, diluted with EtOAc, washed with $NaHCO_3$ and water, and dried with Na_2SO_4. The solvent was evaporated. Flash chromatography (EtOAc/hexane, 7:3→9:1) gave 1.95 g of compound **13** (84% yield from **11**). − $[\alpha]_D^{20}$ = +32 (*c* = 1.0, $CHCl_3$) [ref.[2] +30.3 (*c* = 1.0, $CHCl_3$)].

Allyl 2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranoside (15): Compound **13** (915 mg, 0.75 mmol) and a catalytic amount of TfOH were dissolved under N_2 in dry CH_2Cl_2, and the solution was cooled to −20 °C. A solution of **14**[24] (750 mg, 2.1 equiv.) in dry CH_2Cl_2 was added to the mixture over 20 min. At the end of the addition, the solution was allowed to warm to 0 °C. After neutralization with TEA and evaporation of the solvent, the product was purified by flash chromatography (hexane/EtOAc, 6:4) to give 991 mg of compound **15** (81% yield). − $[\alpha]_D^{20}$ = − 29.1 (*c* = 1.0, $CHCl_3$) [ref.[2] −27.7 (*c* = 1.0, $CHCl_3$)].

Methyl [2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyloxy]acetate (16): Compound **15** (200 mg, 0.12 mmol) was dissolved in *t*BuOH (20 mL) and acetone (15 mL), and distilled water was added until the solution became cloudy. OsO_4 (1 mL of a 5 mg/mL solution in *t*BuOH) and $NaIO_4$ (260 mg) were added and the solution was stirred overnight. The solvent was evaporated and the residue was diluted with CH_2Cl_2, washed with an aqueous solution of $K_2S_2O_5$ (5%) and water, and dried with Na_2SO_4. The solvent was evaporated. ^{1}H NMR analysis showed a signal at δ = 9.69, confirming the presence of the aldehyde group. The product was unstable and therefore it was oxidized immediately. The compound was dissolved in acetonitrile (12 mL), NaH_2PO_4 solution (1.25 M, 0.73 mL) and distilled water (0.5 mL), and $NaClO_2$ (148 mg) was added. The solution was stirred vigorously for 1 h, then washed with a 5% aqueous solution of $K_2S_2O_5$ and extracted with CH_2Cl_2. The organic phase was dried with Na_2SO_4 and the solvents were evaporated. The residue was dissolved in methanol. Diazomethane was added until the solution became slightly yellow, then the excess of diazomethane was destroyed with acetic acid. After evaporation of the methanol, the residue was dissolved in EtOAc and washed with $NaHCO_3$ and water. The organic phase was dried with Na_2SO_4 and evaporated, and 110 mg of the product **16** were obtained (54% overall yield) after purification by flash chromatography (hexane/EtOAc, 1:1). White foam. − $[\alpha]_D^{20}$ = −17.8 (*c* = 1, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$, COSY): δ = 0.82 (d, *J* = 6.3 Hz, 2 H, H-6′′′), 1.71 (s, 3 H, *Ac*NH), 3.38 (m, H-4′, 1 H),3.4−3.6 (m, 2 H-6, H-3′′, H-4′′′, 2 H), 3.69 (s, 3 H, CH_3O), 3.87 (m, 2 H, H-3′′′, H-4′′), 3.9−4.0 (m, 3 H, H-2, H-5, H-2′′′), 4.05 (m, 1 H, H-2′), 4.10 (d, *J* = 16.5 Hz, 1 H, C*H*$_b$COOCH$_3$), 4.1−4.3 (m, H-4, H-3′, H-2′′, H-5′′′, C*H*$_a$COOCH$_3$, 2 H), 4.3−4.5 (m, 6 H, H-1′′, PhC*H*), 4.52 (s, 2 H, PhC*H*$_2$), 4.6−4.9 (m, 9 H, PhC*H*), 4.88 (d, $J_{1,2}$ = 3.6 Hz, H-1), 5.05 (d, $J_{1',2'}$ = 7.9 Hz, H-1′), 5.16 (d, *J* = 11.8 Hz, 1 H, PhC*H*), 5.51 (s, 1 H, PhC*H*), 5.60 (d, $J_{1''',2'''}$ = 3.5 Hz, H-1′′′), 5.85 (d, *J* = 7.9 Hz, 1 H, AcN*H*), 7.1−7.5 (m, 50 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.3 (*q*, C-6′′′), 23.6 (*q*, *Ac*NH), 51.8 (*q*, OCH$_2$COO*Me*), 53.4 (d, C-2′), 63.6 (t, O*C*H$_2$COOMe), 66.5 (d), 66.8 (d), 69.0 (t), 69.2 (t), 69.5 (t), 70.1 (d), 71.9 (t), 72.4 (t), 72.5 (t), 73.2 (d), 73.3 (t), 73.5 (t), 73.7 (d), 74.5 (t), 74.9 (t), 75.3 (d), 75.7 (d), 76.0 (d), 77.2 (d), 78.1 (d), 79.0 (d), 79.3 (d), 83.8 (d), 96.9 (d), 97.4 (d), 101.1 (d), 102.7 (d), 103.0 (d), 126.6−128.7 (cluster of d, ArC), 138.0 (s), 138.1 (s), 138.5 (s), 138.6 (s), 139.1 (s), 170.4 (s, CO), 170.4 (s, CO). − $C_{99}H_{107}NO_{22}$ (1662.90): calcd. C 71.51, H 6.49, N 0.84; found C 71.34, H 6.22, N 0.78

***N*-(2-Aminoethyl)-2-[2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyloxy]acetamide (17):** Ethylenediamine (250 μL) was added to a solution of **16** (100 mg, 60.2 μmol) in 5 mL of methanol and the solution was stirred overnight. Evaporation of the solvent and flash chromatography (EtOAc/MeOH, 8:2) gave 95 mg of **17** (93% yield). White foam. $[\alpha]_D^{20}$ = −16.9 (*c* = 1.5, $CHCl_3$). − ^{1}H NMR (300 MHz, $CDCl_3$, COSY): δ = 0.78 (d, *J* = 6.2 Hz, 2 H, H-6′′′), 1.79 (s, 3 H, *Ac*NH), 3.5−3.7 (m, H-4′′′, C*H*$_2$N, 6 H), 3.69 (s, 3 H, CH_3O), 3.8−4.1 (m, H-2, H-2′′′, 4 H), 4.4−4.1 (m, H-5′′′, C*H*$_2$COOCH$_3$, 5 H), 4.4−4.6 (m, 8 H, H-1′′, PhC*H*), 4.6−4.9 (m, 11 H, H-1, H-1′, PhC*H*), 5.22 (d, *J* = 11.8 Hz, 1 H, PhC*H*), 5.34 (d, *J* = 7.6 Hz, 1 H, AcN*H*), 5.49 (s, 1 H, PhC*H*), 5.64 (d, $J_{1''',2'''}$ = 3.1 Hz, H-1′′′), 7.1−7.5 (m, 50 H, ArH). − ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1 (*q*, C-6′′′), 23.7 (*q*, *Ac*NH), 36.7 (t, *C*H$_2$NH), 39.8 (t, *C*H$_2$NH$_2$), 53.7 (d, C-2′), 62.8 (t, O*C*H$_2$CON), 66.4 (d), 66.7 (d), 68.2 (d), 68.9 (t), 69.1 (t), 70.3 (d), 71.7 (t), 72.2 (t), 72.5 (t), 72.8 (d), 73.1 (t), 73.3 (t), 73.5 (t), 74.6 (t), 74.8 (t), 75.7 (d), 76.1 (d), 78.0 (d), 79.1 (d), 79.3 (d), 80.6 (d), 84.0 (d), 96.8 (d), 98.8 (d), 101.1 (d), 103.0 (d), 103.2 (d), 126.6−129.7 (cluster of d, ArC), 137.7 (s), 137.9 (s), 138.2 (s), 138.4 (s), 139.1 (s), 171.2 (s, CO), 171.6 (s, CO). − $C_{100}H_{111}N_3O_{21}$ (1690.96): calcd. C 71.03, H 6.62, N 2.48; found C 70.78, H 6.43, N 2.59.

***N*-(2-Aminoethyl)-2-[α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactoyranosyl-(1→3)-α-D-galactopyranosyloxy]acetamide (18):** A catalytic amount of Pd(OH)$_2$/C was added to a solution of compound **17** (99.7 mg, 59.0 μmol) and HCl (1.1 equiv.) in methanol (5 mL). The mixture was stirred overnight under H_2. Once TLC (MeOH/H$_2$O/AcOH, 8:2:0.15) showed the formation of a single product, the mixture was filtered through Celite and the solvent was evaporated. The product was dissolved in water and lyophilised to give 49 mg of **18** as a white foam (quant. yield). $[\alpha]_D^{20}$ = +21.7 (*c* = 0.68, H_2O). − ^{1}H NMR (300 MHz, D_2O, COSY): δ = 1.28 (d, 3 H, H-6′′′), 2.11 (s, 3 H, *Ac*NH), 3.27 (br. t, 2 H, CH$_2$N), 3.6−4.1 (m, 23 H), 4.1−4.3 (m, OC*H*$_b$CON, 1 H), 4.4−4.6 (m, 3 H, OC*H*$_a$CON, H-5′′′, H-1′), 4.69 (d, $J_{1'',2''}$ = 7.6 Hz, H-1′′), 5.05 (d, $J_{1,2}$ = 3.7 Hz, H-1), 5.31 (d, $J_{1''',2'''}$ = 3.6 Hz, H-1′′′). − ^{13}C NMR (75 MHz, D_2O): 18.2 (*q*, C-6′′′), 25.2 (*q*, *Ac*NH), 39.3 (t, *C*H$_2$N), 42.1 (t, *C*H$_2$N), 54.5 (d, C-2′),63.9 (t), 69.2 (t), 69.6 (d), 70.2 (d), 70.9 (d), 71.3 (d), 72.0 (d), 72.2 (d), 72.4 (d), 73.8 (d), 74.7 (d), 76.4 (d), 77.5 (d), 77.9 (d), 78.9 (d), 79.2 (d), 81.1 (d), 101.9 (d), 102.1 (d), 104.9 (d), 106.7 (d), 175.8 (s, CO), 177.1 (s, CO). − $C_{30}H_{53}N_3O_{21}$ (791.75): calcd. C 45.51, H 6.75, N 5.31; found C 45.26, H 6.74, N 5.33.

5-{2-[2-(α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→3)-α-D-galactopyranosyloxy)acetylamino]ethylcarbamoyl}pentanoic Acid *N*-Hydroxy–succinimidyl Ester (19): Compound **18** (72 mg, 91.1 μmol) and adipic acid bis(succinimidyl) ester (310 mg, 0.91 mmol, 10 equiv.) were dissolved in 1,4-dioxane/water (5:1, v/v, 15 mL) and the reaction mixture was stirred for 2 h. The mixture was then lyophilized and the residue was washed with water. The mixture was filtered to eliminate the undissolved excess of reagent, and the solution was lyophilised to give 79 mg of the tetrasaccharide derivative

19. The crude compound was not purified because of the instability of the succinimidyl ester group and was stored at −20 °C.

Conjugation to Proteins KLH and CRM197: Crude **19** (93.88 mg) was added to a solution of KLH (100 mg, MW = 2000 kDalton) in phosphate buffer (pH 7.4, 0.1 M, 40 mL) and the mixture was stirred overnight. The solution was lyophilized and the solid was dialysed to give 118 mg of conjugate.

In the same way, crude **19** (28.3 mg) and CRM197 (47.34 mg, MW = 58 kDalton) in phosphate buffer (pH 7.4, 10 mL) gave 51 mg of the conjugate.

Tetrasaccharide−protein ratios were determined for each conjugate by determination of free lysine amino groups before and after conjugation by titration with trinitrobenzenesulfonic acid.[25]

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