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Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF

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Dedicated to Professor Dr. Hans Kamerling on the occasion of his 65th birthday

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ABSTRACT

Several parasitic helminthes, such as the human parasite *Schistosoma mansoni,* express glycoconjugates that contain terminal GalNAcβ1-4(Fucα1-3)GlcNAcβ-R (LDNF) moieties. These LDNF glycans are dominant antigens of the parasite and are recognized by human dendritic cells via the C-type lectin DC-SIGN. To study the functional role of the LDNF antigen in interaction with the immune system, we have developed an easy chemoenzymatic method to synthesize multivalent neoglycoconjugates carrying defined amounts of LDNF antigens. An acceptor substrate providing a terminal *N*-acetylglucosamine was prepared by coupling a fluorescent hydrophobic aglycon, 2,6-diaminopyridine (DAP), to *N,N′*-diacetylchitobiose. By the subsequent action of recombinant *Caenorhabditis elegans* β1,4-*N*-acetylgalactosaminyltransferase and human α1,3-fucosyltransferase VI (FucT-VI), this substrate was converted to the LDNF antigen. We showed that human FucT-VI has a relatively high affinity for the unusual substrate GalNAcβ1-4GlcNAc (LDN), and this enzyme was used to produce micromolar amounts of LDNF–DAP. The synthesized LDNF–DAP was coupled to carrier protein via activation of the DAP moiety by diethyl squarate. By varying the molar glycan:protein ratio, neoglycoconjugates were constructed with defined amounts of LDNF, as was determined by MALDI-TOF analysis and ELISA using an anti-LDNF antibody.

1. Introduction

Glycan molecules linked to proteins or lipids play important roles in cellular communication, adhesion, and signaling and are key molecules in regulation of immune responses. To establish the role of individual glycans in diverse aspects of biology, the availability of neoglycoconjugates that carry defined glycan antigens is of crucial importance. Neoglycoconjugates are attractive tools to define anti-glycan responses in infection or immunization,[1,2] or to define specific carbohydrate recognition by lectin receptors that occur on many immune cells.[3] Neoglycoconjugates are also used in vaccines to elicit carbohydrate-specific antibodies that can confer protection to infection, for example, to *Neisseria meningitides* and *Streptococcus pneumoniae*.[4,5]

Helminth parasites express a variety of unusual glycan antigens that are highly antigenic in infection. In addition, recognition of helminth glycan antigens by C-type lectin receptors on dendritic cells may modulate dendritic cell function.[6,7] Of particular interest is the LDNF structure, which has been demonstrated in multiple helminth species such as *Schistosoma mansoni*, *Haemonchus contortus*, and *Dirofilaria immitis*.[1,8,9] High levels of anti-LDNF antibodies have been detected in humans and in mice infected with the trematode *S. mansoni*.[10,11] The LDNF structure has also been found capped with tyvelose on the nematode *Trichinella spiralis*.[12–14] Moreover, lambs vaccinated with excreted/secreted *H. contortus* antigens showed high serum levels of anti-LDNF IgG that correlated with protection against the parasite.[1] Interestingly, the C-type lectin DC-SIGN, a pathogen receptor on human dendritic cells, recognizes the LDNF antigen, which suggests a role for LDNF in modulating host immunity.[7,15] To study the roles of LDNF in antigenicity and immune modulation in more detail, we have developed a method to synthesize neoglycoconjugates carrying LDNF, which are not commercially available.

In a previous study we have synthesized the LDNF structure using partially purified glycosyltransferases from natural sources.[16] In this manuscript we describe a greatly improved chemoenzymatic method using recombinant glycosyltransferases, starting with the chemical modification of a commercially available acceptor with 2,6-diaminopyridine (DAP).[17] This provided a fluorescent hydrophobic aglycon to the acceptor, which facilitated detection and purification of the oligosaccharide products during

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synthesis. Coupling of the glycans to protein was performed via diethyl squarate,[18] allowing the controlled addition of defined amounts of glycan antigens to any protein carrier, which enables the study of multivalency of glycan presentation and the importance of the nature of the carrier molecule.

2. Experimental

2.1. Reagents

Acetonitrile, ammonium formate, 2,6-diaminopyridine, GDP-fucose, UDP-GalNAc, *N*,*N*′-diacetylchitobiose, Galβ1,4GlcNAc-pNp, GlcNAc-pNp, $MnCl_2$, sodium cacodylate, Tris–HCl, NaCl, $CaCl_2$, $MgCl_2$, Tween 20, diethyl squarate (3,4-diethoxy-3-cyclobutene-1,2-dione) were all obtained from Sigma–Aldrich Chemical Co. The BSA (bovine serum albumin, Fraction V) used for producing neoglycoconjugates was obtained from Calbiochem. The manufacturers from other reagents that were used are mentioned in the appropriate sections.

2.2. Derivatization of chitobiose with 2,6-diaminopyridine (DAP)

N,*N*′-Diacetylchitobiose (chitobiose) was derivatized with 2,6-diaminopyridine (DAP) using essentially the method described by Xia et al.[19] Dried pellets of chitobiose (0.85 mg, 2 μmol) were dissolved in 20 μL of DMSO, and subsequently 1 mL of a mixture of 0.27 M DAP and 0.78 M $NaCNBH_3$ in 7:3 DMSO–HOAc was added. After 16 h of incubation at 65 °C, toxic materials and free chitobiose were removed using reversed-phase HPLC cartridges, and the DAP-derivatized oligosaccharide (Chi-2-DAP) was subsequently separated from free DAP on a preparative Zorbax NH_2 PrepHT column (250 × 21.2 mm, 7 μm, Agilent) in batches of 2.1 mg, 4 μmol per run. For this purpose, a gradient was used that started with 81% acetonitrile (buffer B) and 29% 50 mM ammonium formate, pH 4.4 (buffer A) and ended after 17 min with 30% acetonitrile and 70% ammonium formate on an Äkta Explorer with a column flow of 10 mL. DAP-containing fractions were detected by measuring absorbance at 235 nm. The Chi-2-DAP was freeze dried and stored in 0.5 mg, 1 μmol pellets.

2.3. Production of recombinant β1,4-*N*-acetylgalactosaminyltransferase

For the production of recombinant *Caenorhabditis elegans* UDP-GalNAc:GlcNAc β1,4-*N*-acetylgalactosaminyltransferase (β1,4GalNAcT), HEK293T cells were cultured adherent in DMEM containing 10 mM HEPES, MEM nonessential amino acids, MEM sodium pyruvate, 10% FCS (all reagents from Gibco), and 100 U/mL penicillin–streptavidine (Lonza), in a T75 flask (Greiner) at 37 °C/5% CO_2 until the bottom was completely covered by a monolayer of cells (~6×10^6 cells/flask). Lipofectamin transfection of these cells with plasmid pCMV-SH-Ceβ4GalNAcT[20] was performed according to manufacturer's (Invitrogen) protocol with some slight modifications. The transfection mixture was prepared by mixing 600 μL of Optimem I (Gibco) containing 54 μL of Lipofectamin 2000 (Invitrogen) with 600 μL of Optimem I containing 9 μg of DNA, and subsequently incubated for 30 min at room temperature. After washing the cells twice with 9 mL of Optimem I, 4.8 mL of Optimem I was mixed with the transfection mixture and added to the cells. After incubation of the cells for 5–6 h at 37 °C/5% CO_2, 6 mL culture medium was added and the cells were cultured overnight at 37 °C/5% CO_2. Hereafter, the cells were washed with 2 mL of PBS, trypsinized with 1 mL of Trypsin (Gibco)/EDTA (containing 0.025% trypsin and 0.01% EDTA), and then cultured in 9 mL of culture medium in a new T75 flask for another 3 days. The medium containing the active enzyme was harvested daily until 7 days after transfection. The enzyme-containing medium showed an activity of approximately 200 nmol/mL/h using acceptor GlcNAc-pNp and was stored until further use at −20 °C for several months without loss of activity.

2.4. Production of recombinant human α1,3-fucosyltransferases

The human α1,3-fucosyltransferases FucT-III, FucT-IV, and FucT-VI were kindly provided by H. Kok (Organon, the Netherlands), and the expression plasmid pAMO-FucT-IX,[21] encoding cDNA from human FucT-IX,[22] was kindly provided by Dr. T. Sato and Dr. H. Narimatsu (RCG, Japan). The latter construct was transfected into HEK293T cells as described in Section 2.3, and the cell lysates were used as the enzyme source.

The plasmid ProtA-FucT-VI,[23] encoding a soluble chimaeric protein consisting of a part of Protein A fused to the catalytic domain of FucT-VI, was kindly provided by Dr. B. Macher (San Francisco State University). The transfection procedure for the plasmid was as described in Section 2.3, with the exception that Cos7 cells were used. The ProtA-FucT-VI-containing medium was harvested 2 days after transfection and stored until further use at −80 °C for several months without any loss of activity (approximately 100 nmol/mL/h measured with acceptor LDN-C_8).

2.5. Fucosyltransferase assays and enzyme kinetics

Standard fucosyltransferase assays were performed at 37 °C for 1 h in a total volume of 25 μL of 50 mM sodium cacodylate, pH 7.0, containing 1 μmol of $MnCl_2$, 2.5 nmol of GDP-[^{14}C]Fuc (4 Ci/mol, Amersham), 0.1 μmol of ATP, 0.1% Triton X-100, acceptor substrates and enzyme, essentially as described.[24] The acceptor substrates were all used at a concentration of 1 mM unless indicated otherwise. GalNAcβ1-4GlcNAcβ-O-$(CH_2)_8$-$COOCH_3$ (LDN-C_8), and Fucα1-2Galβ1-4GlcNAc-O-$(CH_2)_8$-$COOCH_3$ (H-type 2-C_8) were kind gifts from Dr. D. H. van den Eijnden (VU University, NL), and Dr. M. M. Palcic (Carlsberg Laboratory, Denmark), respectively. Galβ1-4GlcNAcβ-Nitrophenyl (LN-pNp) was from Toronto Chemicals. Products were separated from unincorporated nucleotide sugar by reversed-phase chromatography using Sep-Pak C_{18} cartridges (Waters, Milford, MA).[25] Kinetic characterization of human ProtA-FucT-VI was carried out using at least six different acceptor substrate concentrations. The apparent kinetic parameters V_{max} and K_m were obtained by fitting the data to the Michaelis–Menten equation using nonlinear regression analysis (SPSS 15 program, SPSS Inc.).

2.6. Preparative enzymatic synthesis of oligosaccharides

Chi-2-DAP (2.1 mg, 4 μmol) was incubated in a volume of 400 μL of 0.1 M sodium cacodylate, pH 7.0, containing 40 mM $MnCl_2$, 10 mM UDP-GalNAc, and medium from HEK293T cells transfected with the recombinant β1,4-GalNAcT from *C. elegans*[20] as the enzyme source. The mixture was incubated at room temperature (20–24 °C) until Chi-2-DAP was completely converted to the desired end product, LDN–DAP. The product was purified by Sep-Pak C_{18} reversed-phase chromatography.[25] For the fucosylation of LDN–DAP, batches of LDN–DAP (1 μmol, 0.72 mg) were incubated each in a total volume of 200 μL of 50 mM sodium cacodylate buffer, pH 7.0, containing 5 mM $MnCl_2$, 10 mM GDP-L-fucose, and ProtA-FucT-VI as the enzyme source. The mixture was incubated at 37 °C until LDN–DAP was completely converted to the desired end product, LDNF–DAP. The product was purified by Sep-Pak C_{18} reversed-phase chromatography.[25]

2.7. Analytical HPLC analysis

During enzymatic synthesis, product formation was monitored in time by HPLC on a Surveyor (Thermo), applying 100–500 pmol of oligosaccharide in 80% acetonitrile onto a LudgerSep N1 Amide column (250 × 4.6 mm, Ludger). A 30-min gradient starting with 70% acetonitrile and 30% 50 mM ammonium formate, pH 4.4, and ending in a 50:50 ratio was run at 22 °C to separate the product from the starting oligosaccharide. The column was subsequently equilibrated with 100% ammonium formate for 10 min, followed by 70% acetonitrile and 30% 50 mM ammonium formate for 10 min. Detection by fluorescence (Waters fluorimeter 470) was done with excitation at 345 nm and measuring the emission at 400 nm.

2.8. Construction of neoglycoconjugates

LDNF–DAP (3.5 mg, 4 μmol) was dissolved completely in 1 mL of 0.1 M phosphate buffer, pH 6.95, and transferred to a glass cone-shaped vial (Wheaton). A freshly made mixture of 1 mL of ethanol (via Brunschwig Chemie from Nedalco) with 20 μL of 3,4-diethoxy-3-cyclobutene-1,2-dione, (98%, Sigma–Aldrich) was added and mixed. The sample was incubated at 22 °C for 16 h. The addition of squarate to the LDNF–DAP was checked by electrospray-ionization mass spectrometry (ESIMS) (0.5 μL of the 2 mL of reaction mixture is added to 50 μL 50% acetonitrile). Hereafter, 30 mL of Milli Q water was added to the reaction mixture that was subsequently applied to a 6 mL Sep-Pak C_{18} column for purification.

For the final conjugation step, typically 10 μL of a solution of 5 nmol of squarate-derivatized LNDF–DAP/μL of conjugation buffer (made by dissolving 1.55 g of boric acid (Gibco) and 1.31 g of KCl (Fluka) in 30 mL Milli Q water with pH adjusted to 9 with 421 mg KOH (E. Merck)) was added to 200 μg of BSA (2 μL of 0.1 mg/mL conjugation buffer) and 3 μL of conjugation buffer giving an end volume of 15 μL. This particular reaction provides a molar ratio of 17 glycans to 1 carrier molecule, but also other molar ratios were tested, varying from 17:1 to 3:1, always with the same amount of BSA in an end volume of 15 μL. The reaction mixture was incubated at 22 °C for 23 h. To remove salts, 1.1 mL Milli Q water was added to the reaction mixture, and the sample was injected in a Slide-A-Lyzer Dialysis Cassette (10.000 MWCO, Thermo Scientific) and dialyzed against 5 L of deionized water for at least 16 h at 4 °C. The neoglycoconjugates were dried in a Speedvac and stored at −20 °C.

2.9. Treatment of LDNF–DAP with α1,3/4-fucosidase

To determine the anomeric linkage of the fucose in LDNF–DAP we treated 20 nmol of this compound with 0.5 mU of α1,3/4-fucosidase (from *Xanthomonas* sp., Calbiochem) for 18 h in 20 μL 50 mM NaH_2PO_4 buffer, pH 5.5. The results were monitored by HPLC as described in Section 2.6.

2.10. Mass spectrometry

Synthesized oligosaccharide products were characterized by ESIMS on an LCQ DecaXP ion-trap mass spectrometer equipped with a nano-ES ionization source (Thermo Finnigan). The sample was loaded onto a medium NanoEs spray capillary (Proxeon). The capillary temperature was set to 200 °C. Spectra were taken in the positive-ion mode with a spray voltage of 1.0 kV and with a capillary voltage of 40.1 V.

To estimate the degree of coupling of the oligosaccharides to BSA, 5–10 pmol of the neoglycoconjugates was taken up in 50% of a sinapinic acid solution (17.5 mg/mL sinapinic acid (Sigma) in 281 μL of MeOH and 422 μL of acetonitrile), applied to the target plate, and subjected to MALDI-TOFMS, using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) by measuring in Linear Mode (*m/z* range was 30,000–100,000 Da) and accumulating 5000 laser shots/MS spectrum.

2.11. ELISA

To determine the presence of the LDNF-epitope on the BSA carrier, 5 μg protein/mL coating buffer (50 mM Na_2CO_3 (E. Merck), pH 9.6) was added to each well of a Nunc Maxisorb ELISA plate and incubated at 4 °C for 16 h. After washing three times with TSM (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) containing 0.1% Tween 20, blocking solution (TSM containing 1% BSA) was added for 1 h at 37 °C. After a similar washing procedure, an anti-LDNF monoclonal antibody (SmLDNF1)[11] (kindly provided by Dr. R. D. Cummings and A. K. Nyame, Emory University, Atlanta, USA) was added to a 1:500 dilution of blocking solution and incubated at 37 °C for 1 h. After washing again, a peroxidase-conjugate Goat anti-Mouse IgG/IgM (H+L) (Jackson) was added in a 1:2500 dilution in TSM/0.1% Tween 20 and incubated for 1 h at 37 °C. The plate was developed with 3,3′,5,5′-tetramethylbenzidine (Sigma) after a final washing step. The color reaction was stopped by the addition of 50 μL of 0.8 M H_2SO_4 to each well, and absorbance was measured at 450 nm.

3. Results

3.1. Derivatization of chitobiose with 2,6-diaminopyridine (DAP)

As the starting backbone for the synthesis of LDNF, *N*,*N*′-diacetylchitobiose (chitobiose, Fig. 1) was used, providing the terminal GlcNAc required for elongation to LDN. Two micromoles of chitobiose were derivatized with 2,6-diaminopyridine (DAP) (Fig. 1, reaction a) using essentially the method described by Xia et al.[19] During this procedure the GlcNAc residue at the reducing end of chitobiose looses its ring structure (Fig. 1). Such addition of a DAP moiety provides the oligosaccharide with a hydrophobic and fluorescent aglycon spacer that greatly facilitates purification and

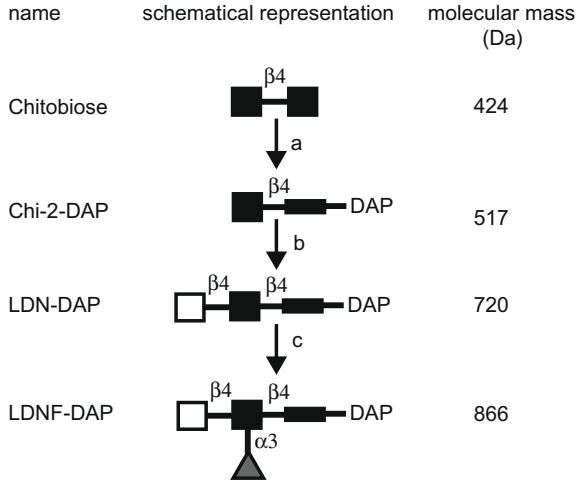

Figure 1. Oligosaccharides used in this study. A schematic representation of the oligosaccharides mentioned in this paper and their molecular mass is depicted: Filled square (*N*-acetylglucosamine), open square (*N*-acetylgalactosamine), grey triangle (fucose), rectangle (derivatized, open-chain *N*-acetylglucosamine), DAP (2,6-diaminopyridine). In addition, the short names of these oligosaccharides and their molecular masses are given.

detection of the oligosaccharides in subsequent synthesis steps. The DAP-derivatized oligosaccharide was separated from free DAP by preparative HPLC, and DAP-containing fractions were detected by UV (Fig. 2). The fractions eluting from the column after 12 min were analyzed by mass spectrometry (MS) (data not shown) to confirm the addition of DAP to Chi-2 (Fig. 2, Chi-2-DAP). The Chi-2-DAP was freeze dried in pellets of approximately 1 μmol and used for subsequent enzymatic synthesis reactions.

3.2. Enzymatic synthesis of LDNF–DAP

As a first step in the synthesis of LDNF–DAP, the produced Chi-2-DAP was converted to LDN–DAP (Fig. 1) using the recombinant β1,4-GalNAcT from *C. elegans*[20] and UDP-GalNAc. Four micromoles of Chi-2-DAP were completely converted to the desired end product, LDN–DAP, within 5 h as monitored by analytical HPLC (Fig. 3A–D). After purification of the product by C_{18} reversed-phase chromatography, the structure of the oligosaccharide was verified to be LDN–DAP by tandem MS (data not shown). We previously demonstrated by NMR the authenticity of the product LDN generated by recombinant *C. elegans* β1,4GalNAcT.[20]

LDNF has been synthesized before using human milk containing different fucosyltransferases.[16] To improve the efficiency of this synthesis step, we evaluated the capacity of different recombinant human α1,3-fucosyltransferases to use LDN as an acceptor structure, using a standard radioactive fucosyltransferase assay. Among the fucosyltransferases tested (FucT-III, FucT-IV, FucT-VI, and FucT-IX), FucT-VI showed the highest catalytic activity toward LDN-C_8 as an acceptor substrate, compared to LN-pNp as an acceptor (results not shown). To define the enzymatic properties of FucT-VI on LDN as a substrate in more detail, we used the chimeric enzyme ProtA-FucT-VI, which was produced as a soluble secreted enzyme. The K_m and V values of ProtA-FucT-VI for the substrates LDN-C_8, LDN–DAP, LN-pNp and H-type2-C_8 were estimated (Table 1). Interestingly, the K_m value calculated for LDN-C_8 is in the same range as for H-type 2-C_8 and the K_m measured previously for LN-C_8,[24,26] indicating that the enzyme has a high affinity for LDN as a substrate. The K_m values of ProtA-FucT-VI for LDN–DAP or LN-pNp as acceptor appeared much higher, indicating that the type of hydrophobic spacer influences the affinity of the enzyme for the acceptor substrate.

Starting with 1 μmol LDN–DAP as an acceptor and ProtA-FucT-VI as the enzyme source, 90–95% conversion of LDN–DAP to LDNF-DAP was reached after incubation for 48 h as monitored by HPLC (Fig. 3D–F). The product was purified by C_{18} reversed-phase chromatography, followed by preparative HPLC, and the structure of the oligosaccharide was verified by tandem MS/MS (Fig. 4A). Whereas FucT-VI is known to add Fuc in an α1,3-linkage to Galβ1-4GlcNAc as acceptor, its action on LDN has not been previously verified. To establish the anomeric linkage of the Fuc in

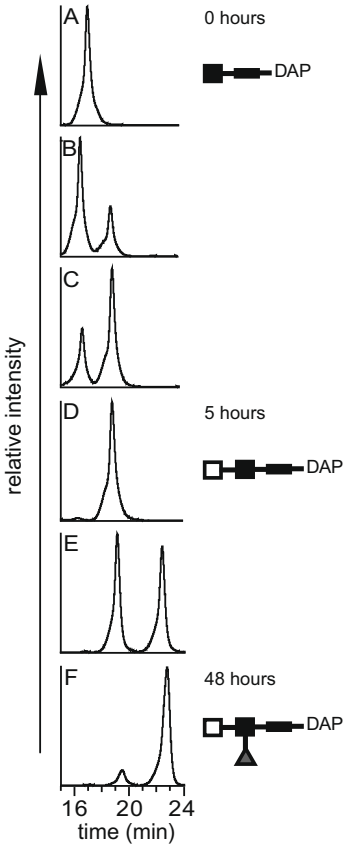

Figure 3. Product formation during enzymatic synthesis. During enzymatic synthesis samples were analyzed by analytical HPLC. The relative intensity of DAP-derivatized glycans is plotted against time of elution from the column. In panel A Chi-2-DAP is shown, which is converted with β1,4-GalNAcT in time into LDN–DAP (B–D). After isolation of LDN–DAP with reversed-phase cartridges, this compound is in turn converted in time to LDNF–DAP with ProtA-FucT-VI (E–F).

LDNF-DAP, we treated a sample of the synthesized putative LDNF-DAP with α1,3/4-fucosidase and analyzed the product by HPLC. The data in Figure 4B show that the product obtained after fucosidase treatment shows a similar retention time as LDN–DAP (see Fig. 3), thereby confirming the authenticity of the synthesized LDNF-DAP.

3.3. Construction of BSA–LDNF neoglycoconjugates via activation with diethyl squarate

To couple LDNF-DAP to a protein carrier, we first added a diethyl squarate molecule to the free amino-group of the DAP moiety (Fig. 5A). We combined the strategies described by Lefeber,[27]

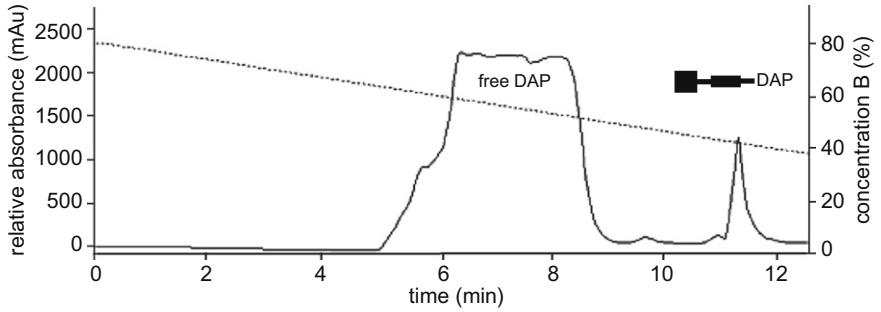

Figure 2. Purification of Chi-2-DAP. Chitobiose derivatized with DAP (Chi-2-DAP) was purified by reversed-phase chromatography to remove $NaCNBH_3$ and free chitobiose. DAP-derivatized chitobiose was separated from free DAP by preparative normal-phase HPLC. Relative absorbance of DAP was measured at a wavelength of 235 nm, and fractions containing Chi-2-DAP were collected (peak at min 11.5). The dotted line shows the concentration of buffer B (acetonitrile) in the gradient used.

Table 1
Apparent kinetic parameters for recombinant FucT-VI[a]

Acceptor	K_m (mM)	V_{max} (nmol/mL/h)
Galβ1-4GlcNAc-pNp[b] (LN-pNp)	3.6	41
GalNAcβ1-4GlcNAc-DAP[c] (LDN–DAP)	3.8	50
GalNAcβ1-4GlcNAc-C_8[d] (LDN-C_8)	0.27	120
Fuc-α1-2Galβ1-4GlcNAc-C_8 (H-type 2-C_8)	0.14	160
Galβ1-4GlcNAc-C_8 (LN-C_8)	0.22/0.31 K_m estimated[23,24]	

[a] The apparent kinetic parameters K_m and V_{max} were obtained by fitting the data for the transfer of fucose to the acceptor substrates at variable acceptor concentrations (for LN-pNp: 0.1–5 mM, for LDN–DAP 0.05–2 mM, for LDN-C_8 0.05–1 mM, and for H-type2-C_8 25 μM–150 μM) to the Michaelis–Menten equation using nonlinear regression analysis (SPSS 15 program, SPSS Inc.). The concentration of GDP-fucose (0.1 mM) and other assay conditions were similar.
[b] pNp = *p*-Nitrophenyl.
[c] DAP = 2,6-diaminopyridine.
[d] $C_8 = (CH_2)_8$-$COOCH_3$.

and Hou,[18] and using ESIMS, we confirmed the formation of the desired molecule with an expected molecular mass of 990 Da (Fig. 5C). No free LDNF-DAP was detected, indicating that squarate was coupled to LDNF-DAP with high efficiency.

After purification of the squarate-modified LDNF-DAP using C_{18} reversed-phase chromatography, the oligosaccharides were coupled to BSA (Fig. 5B) essentially as described.[18] Different ratios of glycan to protein were used to create different numbers of glycans per carrier molecule. In this case, we used either a 17 times (high) or a 3 times (low) molar excess of oligosaccharide to BSA. The degree of coupling of the oligosaccharides to BSA was determined by MALDI-TOFMS analysis (Fig. 6B and C). This revealed for the high coupling ratio an average peak shift of around 7.3 kDa, implicating an average of 7.4 LDNF epitopes per BSA molecule. For the reaction with a low coupling ratio, the average peak shift was around 1 kDa, indicating that the BSA molecules contained on average one LDNF epitope. To investigate whether the LDNF moieties were intact, the binding of an anti-LDNF-antibody to the BSA–LDNF neoglycoconjugates was evaluated by ELISA. The data showed a strong binding of the antibody to both BSA–LDNF preparations (Fig. 6D, showing the antibody binding to BSA with the low LDNF content), but not to BSA (Fig. 6D), confirming the structural integrity of the LDNF moiety.

4. Discussion

Ongoing research efforts in many labs aim at the development of improved methods to synthesize bioactive oligosaccharides to study, modulate, or inhibit processes that are dependent on interactions with carbohydrates. We have described here an easy method to synthesize milligrams of neoglycoconjugates, containing the LDNF epitope, by a combination and optimization of different existing methodologies. The advantages in using DAP to derivatize oligosaccharides have been outlined before by Xia et al.[19] The linkage of DAP to acceptor substrates destined for enzymatic synthesis has not been described before and appears to have great advantages above other linker adaptors. The use of DAP provides a hydrophobic aglycon to the acceptor, allowing easy purification of the product after enzymatic reactions, and its fluorescent properties facilitate sensitive detection during the synthesis. In principle, each acceptor structure can be easily derivatized with DAP, facilitating enzymatic synthesis prior to coupling to carrier molecules without the need of specific spacer-linked acceptor substrates.

The study of the biological properties of particular helminth glycan antigens highly depends on the availability of neoglycocon-

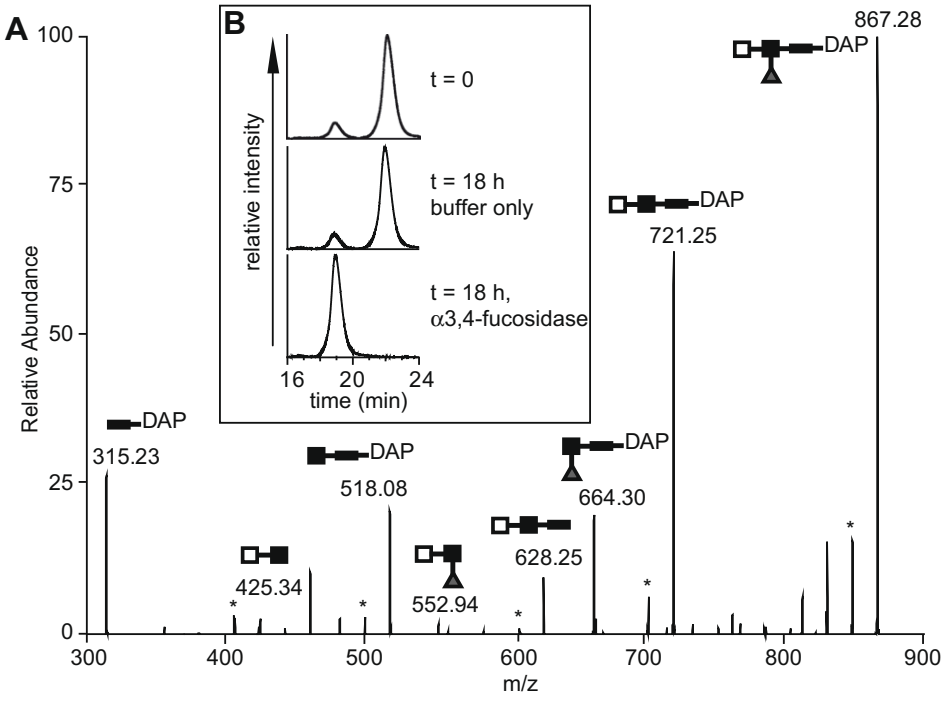

Figure 4. Characterization of LDNF–DAP by tandem mass spectrometry and α1,3/4-fucosidase treatment. (A) Oligosaccharide products were characterized in the positive-ion mode by ESIMS. The expected molecular mass of the protonated LDNF–DAP ion (*m/z* 867.28) was found, and by fragmentation using tandem MS/MS the structure was confirmed. Schematic figures of the oligosaccharides representing the found fragments are depicted above the peaks. The asterisks above detected peaks indicate ions that are similar to the structures shown, but that lack a water molecule (−18 Da). (B) (insert) To characterize the anomeric linkage of the fucose, we treated LDNF-DAP in an acidic phosphate buffer without (middle panel) or with α1,3/4-fucosidase (lower panel) for 18 h, as described in Section 2.8, and subsequently subjected the mixture to HPLC analysis as in Section 2.6. The upper panel ($t = 0$) shows the starting material before fucosidase treatment.

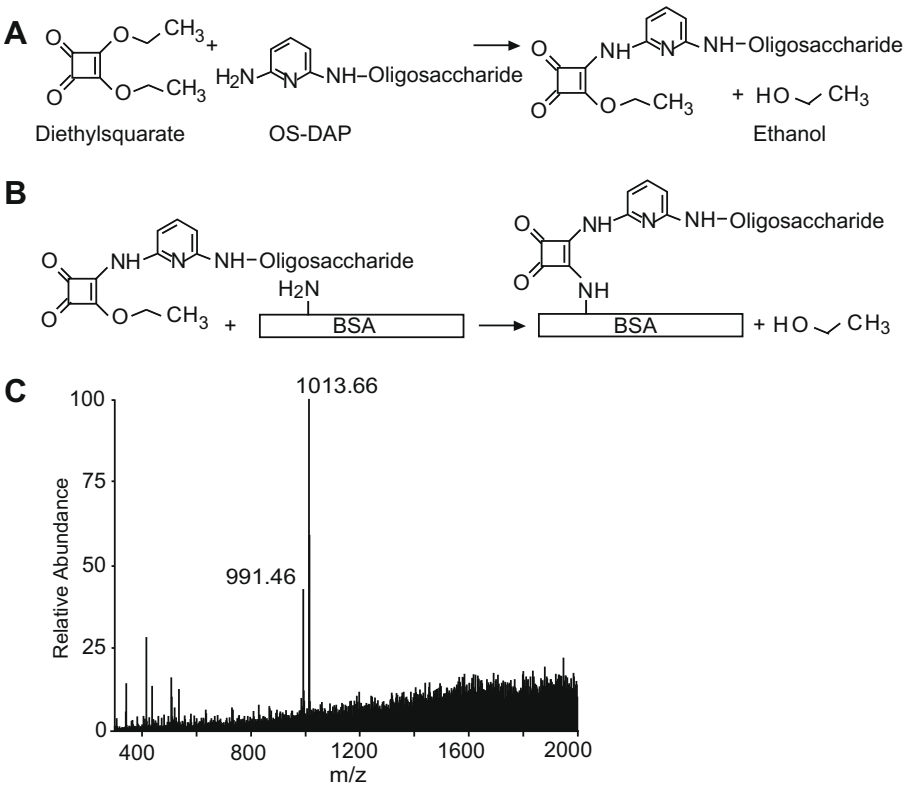

Figure 5. Diethyl squarate coupling and analysis. (A) Chemical reaction schemes for the coupling of diethyl squarate to DAP-derivatized oligosaccharides. (B) Coupling of the derivatized oligosaccharide to protein carrier BSA. (C) ESIMS analysis of the product after derivatization with diethyl squarate of LDNF–DAP. Both the protonated (*m/z* 991.46) and sodiated molecular ion (*m/z* 1013.66) were measured.

jugates, which in contrast to many mammalian-type glycans, are not commercially available. Organic synthesis has been used successfully for the synthesis of typical helminth glycan antigens, as has been reported, for example, for fuco-oligosaccharides of *Schistosoma* spp.,[28] or the mono- or dimethylated glycan antigens from *Toxocara canis*.[29] Alternatively, enzymatic approaches may be used, using glycosyltransferases, which offer significant advantages as the reactions are fast and combine a high regio- and stereospecificity with the potential availability of many different glycosidic linkages. A drawback for the enzymatic synthesis of unusual glycans such as helminth glycans is that not many recombinant parasite-type glycosyltransferases are available. Previously, we have synthesized LDNF antigen using β1,4GalNAcT from the albumen gland of the snail *Lymnaea stagnalis* and partially purified α1,3-FucT from human milk.[16] More recently, the cloning of a few helminth-type glycosyltransferases has been reported, including the β1,4GalNAcT from *C. elegans* that we have used here to synthesize the LDN structure.[20,30–32] In this report we show that the human α1,3-fucosyltransferase FucT-VI is a useful catalyst in the synthesis of the LDNF structure. Our data demonstrate that FucT-VI has a relatively high affinity for LDN as an acceptor. The estimated K_m of 0.27 mM of FucT-VI for LDN-C_8 closely resembles the K_m values of 0.22 mM and 0.32 mM estimated previously for the conventional acceptor LN-C_8,[23,24] which contains the same C_8 aglycon structure. These data indicate that LDN is as good an acceptor as LN, whereas the K_m value observed for H-type 2 acceptor is slightly lower, as observed previously by De Vries et al.[24,26] These data support the earlier findings that modification at the C-2 of galactose does not hinder, or even may enhance the affinity of FucT-VI for its acceptor substrate. Remarkably, our data show that the nature of the hydrophobic spacer greatly affects the K_m of FucT-VI for its substrate, as demonstrated by the 10-fold increase in K_m value for both the substrates LDN–DAP and LN-pNp. Whereas both the latter substrates contain a hydrophobic aglycon, a long hydrophobic stretch directly connected to the reducing end of GlcNAc is lacking in these acceptors, which may explain the reduced affinity for the enzyme FucT-IV.

The observation that human α1,3fucosyltransferases are capable of efficiently synthesizing LDNF suggests that these epitopes could be present in human cells when FucT-VI is co-expressed with one of the human β1,4GalNAcTs described.[33,34] There are only a few reports that describe the presence of LDN or LDNF in human glycoproteins, suggesting that their presence is very limited (reviewed in Ref. 35). The most likely explanation of this limited presence seems a limited activity or expression of the human β1,4GalNAcTs responsible for synthesis of the LDN structure, which has to be present before FucT-VI can act.

Multivalency of glycoconjugates is an important property that may greatly influence the biological activity of specific glycan molecules. It has been reported previously[19] that the linkage of DAP to oligosaccharides allows the subsequent coupling to maleimide-activated proteins. We showed here that linkage of diethyl squarate to DAP-derivatized oligosaccharides prior to linkage to protein offers an attractive alternative coupling strategy. An advantage is that the protein does not need to be activated prior to use, so any protein can be used as a carrier. The procedure could be controlled by varying the amount of oligosaccharides to protein, allowing the coupling of defined amounts of oligosaccharides to the carrier protein.

In summary, we have described a straightforward method to synthesize the neoglycoconjugate BSA–LDNF, and the methodology described here can be easily applied for the synthesis of other neoglycoconjugates. The availability of neoglycoconjugates with different average numbers of glycans per carrier allows the evaluation of glycan density and importance of the carrier molecule for biological activity of the neoglycoconjugates.

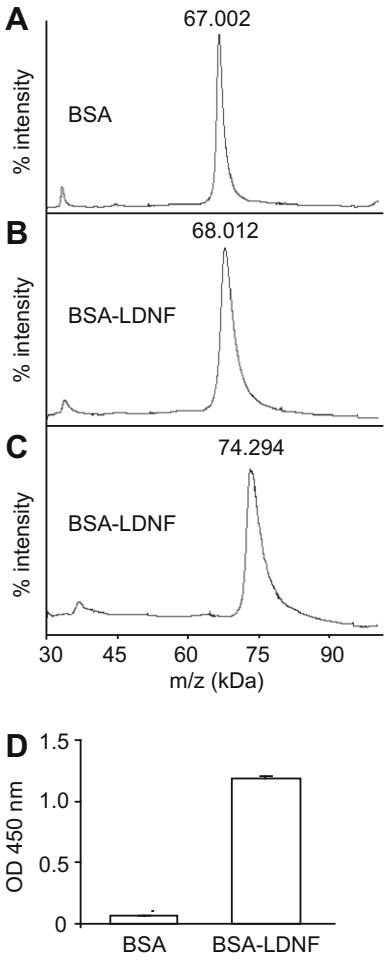

Figure 6. Analysis of BSA–LDNF with MALDI-TOFMS and ELISA. BSA–LDNF neoglycoconjugates that were produced with different molar ratios of sugar to the carrier molecule BSA, were analyzed by MALDI-TOFMS, and the molecular masses (B and C) were compared to those of untreated BSA (A). LDNF epitopes were detected on the BSA–LDNF with low ligand density using an anti-LDNF antibody in ELISA (D). Untreated BSA was used as a negative control.

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