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Synthesis and antibody-binding studies of a series of parasite fuco-oligosaccharides

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Abstract—Complex multifucosylated oligosaccharides are structural elements of glycoprotein and glycolipid subsets of larval, egg, and adult stages of *Schistosoma*, the parasitic worms that cause schistosomiasis, a serious disease affecting more than 200 million people in the tropics. The fucosylated structures are thought to play an important role in the immunology of schistosomiasis. Defined schistosomal oligosaccharides that enable immunological studies are difficult to obtain from natural sources. Therefore, we have chemically synthesized spacer-linked GlcNAc, Fucα1-3GlcNAc, Fucα1-2Fucα1-3GlcNAc, and Fucα1-2Fucα1-2Fucα1-3GlcNAc. This series of linear oligosaccharides was used to screen a library of anti-schistosome monoclonal antibodies by surface plasmon resonance spectroscopy. Interestingly, the reactive antibodies could be grouped according to their specificity for the different oligosaccharides tested, showing that these oligosaccharides form different immunological entities based on the number and linkage of the fucose residues. Subsequently, the thus defined monoclonal antibodies were used to visualize the expression of the corresponding oligosaccharide epitopes by adult *Schistosoma mansoni* worms.

1. Introduction

Schistosomes are parasitic helminths that cause a chronic, debilitating and some times fatal disease called schistosomiasis, of which at least 200 million people in the tropics suffer. Schistosomes have a complex life cycle involving a fresh water snail intermediate host and a vertebrate definitive host. Although the human definitive host develops a strong immune response after infection, this usually does not lead to elimination of the parasite or sterile immunity. It is generally accepted that in a schistosomal infection the major humoral immune response is targeted against carbohydrates (for a review see Hokke and Deelder).[1] Furthermore, several studies have provided evidence that carbohydrate structures also play a role in the pathogenesis of the disease, for instance in granuloma formation[2–4] and in the characteristic Th2 polarization of the cellular immune response (for a review see Thomas and Harn).[5] Obviously, it is desirable to determine exactly which carbohydrate structures are involved in each of these processes. This may lead to an improvement of current immunodiagnostic methods and possibly to the development of carbohydrate based therapeutics.

Numerous analytical studies, often based on sensitive mass spectrometric approaches, have revealed that schistosome glycosylation is extremely heterogeneous and complex[6] (and references cited therein). Several relatively simple, well studied tri- or tetrasaccharide elements expressed on schistosome glycoconjugates are the trisaccharide Lewis X (Le^x, Galβ1-4[Fucα1-3]GlcNAc),[7] and the fucosylated LacdiNAc (GalNAcβ1-4GlcNAc, LDN) derivatives such as GalNAcβ1-4[Fucα1-3]GlcNAc (LDNF) and GalNAcβ1-4[Fucα1-

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2Fucα1-3]GlcNAc (LDNDF),[8–10] of which the latter structure contains a characteristic Fucα1-2Fuc sequence. Using neo-glycoconjugates obtained by enzyme-assisted synthesis, it has been established that in schistosome-infected individuals or animals, an intense antibody response is elicited against LDNDF.[11,12] These neo-glycoconjugates were also used to show that LDNDF is a relatively strong inducer of IL-10 production in peripheral blood mononuclear cells.[13]

Multifucosylated structures containing the unique Fucα1-2Fuc linkage, such as ±Fucα1-2Fucα1-3-GalNAcβ1-4[±Fucα1-2Fucα1-2Fucα1-3]GlcNAc, were found on *O*-glycans from the glycocalyx of schistosomal cercariae.[14] The major egg glycolipids of *Schistosoma* (*S.*) *mansoni* contain stretches of multifucosylated structures on a repeating β1-4 linked GlcNAc backbone.[15] Using defined monoclonal antibodies (Mabs) it has been determined by immunofluorescence and blotting techniques that, as part of the LDNDF antigen, also in adult worms the Fucα1-2Fuc element occurs.[16,10]

It is extremely difficult to obtain pure compounds from complex, heterogeneous glycoconjugate mixtures of limited natural sources like schistosome larvae, adult worms, or eggs. Therefore, it is of importance to be able to obtain potentially immunogenic carbohydrates via chemical synthesis.[17,18] Well-defined synthetic carbohydrate structures allow direct, detailed studies of their immunogenicity and are useful as probes in the characterization of antibodies. Previously, we have obtained numerous schistosome-related anti-carbohydrate Mabs.[19–21] These Mabs are valuable tools in the immunodiagnosis of schistosomiasis.[22,20] In addition, anti-carbohydrate Mabs can be used to visualize the expression of their corresponding antigens throughout the different life stages of the parasite.[10,16,23–25] Using a stepwise synthetic scheme, we have now chemically synthesized a series of linearly fucosylated spacer-linked oligosaccharides containing 0, 1, or 2 of the specific α1-2-linked Fuc residues: GlcNAc(Gn **2**), Fucα1-3GlcNAc (FGn, **10**), Fucα1-2Fucα1-3GlcNAc (FFGn, **11**), and Fucα1-2Fucα1-2Fucα1-3GlcNAc (FFFGn, **15**) (Table 1). These oligosaccharides were used to screen a large library of anti-schistosomal Mabs by surface plasmon resonance (SPR) spectroscopy. Subsequently, the thus characterized Mabs served as probes to detect their corresponding antigens in adult *S. mansoni* worms by fluorescence microscopy.

Table 1. Structure and names of synthetic carbohydrates conjugated to BSA via a spacer

Name	Abbreviation		Symbol
Structures used in this screening			
GlcNAc	Gn	**2**	■
Fucα1-3GlcNAc	FGn	**10**	α1-3
Fucα1-2Fucα1-3GlcNAc	FFGn	**11**	α1-3, α1-2
Fucα1-2Fucα1-2Fucα1-3GlcNAc	FFFGn	**15**	α1-3, α1-2, α1-2
Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc	LNFPIII		β1-4, β1-4, β1-3, α1-3
Other structures discussed			
GalNAcβ1-4[Fucα1-2Fucα1-3]GlcNAc	LDNDF		β1-4, α1-3, α1-2
Fucα1-3GalNAcβ1-4GlcNAc	FLDN		β1-4, α1-3
Fucα1-3GalNAcβ1-4[Fucα1-3]GlcNAc	FLDNF		β1-4, α1-3, α1-3

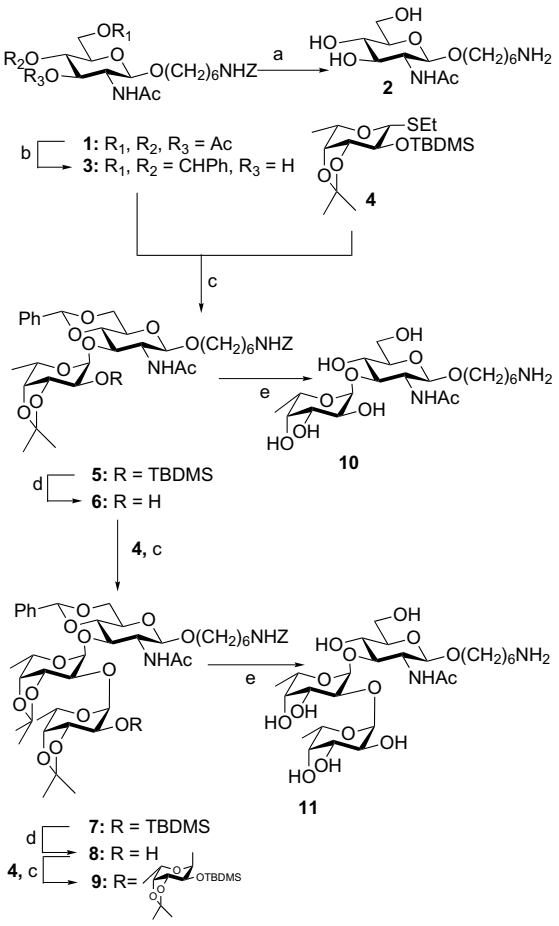
■ GlcNAc, △ Fuc, □ GalNAc, ● Gal, ○ Glc.

2. Results

2.1. Synthesis of the fucosylated carbohydrate structures

The protected oligosaccharides were assembled using a stepwise strategy. Starting from an appropriately protected glucosamine acceptor, the target di-, tri-, and tetrasaccharide can be obtained by repeated elongation with a suitable fucose donor carrying a temporary protective group at 2-OH. The selected glucosamine acceptor, 6-[*N*-(benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside was provided with an amino spacer to allow (after deprotection) conjugation to a carrier protein. The donor of choice was ethyl 2-*O*-*tert*-butyldimethylsilyl-3,4-*O*-isopropylidene-1-thio-β-D-fucopyranoside. The TBDMS group can be selectively removed in the presence of the other protecting groups, after coupling to the acceptor.

The synthesis of **2** and acceptor **3** was accomplished as follows (Scheme 1). 2-*N*-acetyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline[26] was treated with 6-*N*-benzyloxycarbonyl-hexanol in the presence of TMSOTf to afford compound **1** (79%). Subsequent de-*O*-acetylation, followed by the removal of the 6-*N*-benzyloxycarbonyl protecting group by hydrogenolysis, afforded deprotected spacer-linked **2** (Gn) in 91% yield. Alternatively, the introduction of a 4,6-*O*-benzylidene group gave glucosamine acceptor **3** in 92% yield. Fucosyl donor **4** was obtained from ethyl 3,4-*O*-isopropylidene-1-thio-β-D-fucopyranoside by silylation with TBDMSCl and imidazole (89%). Condensation of acceptor **3** with donor **4** was performed under the Ogawa conditions, which have been reported to be suitable for the selective formation of α-fucosidic linkages.[27] Thus, reactions of compound **3** with **4** promoted by copper(II) bromide and tetrabutylammonium bromide gave disaccharide **5** in 85%. Removal of the TBDMS protecting group with TBAF in THF afforded disaccharide acceptor **6** which was condensed with donor **4** under the same conditions as described above to give trisaccharide **7** (52%, α/β 12:1). In this case, the reaction was not completely stereoselective and the α/β mixture could be separated by silica gel chromatography. In analogy, trisaccharide **7** was partially deprotected to



Scheme 1. Reagents and conditions: a. (1) NaOMe, MeOH; (2) H_2, Pd/C, EtOH, HCl 1 M, rt 91 %; b. (1) NaOMe, MeOH, rt; (2) $(MeO)_2CHPh$, *p*-TsOH, CH_3CN, rt, 92%. c. $CuBr_2$, Bu_4NBr, DMF–CH_2Cl_2 (1:1), 5, 85%; 7, 52% (α/β 12:1); 9, 17%. d. TBAF, THF, rt, 6, 88%; 8, 94%. e. (1) HOAc 60%, 50 °C; (2) H_2, Pd/C, EtOH, HCl 1 M, 10, 68%; 11, 45%.

give acceptor **8**. Chain elongation of trisaccharide **8** with donor **4**, afforded tetrasaccharide **9** in only 17% yield. Treatment of compounds **6** and **7** with acetic acid 60% at 50 °C and subsequent hydrogenolysis under Pd/C in the presence of traces of acid, furnished unprotected disaccharide **10** (FGn) and trisaccharide **11** (FFGn), respectively. Unfortunately, tetrasaccharide **9** decomposed when treated with acetic acid 60% at 50 °C.

Therefore, another fucosyl donor, ethyl 2,3,4-tri-*O*-benzyl-1-thio-β-D-fucopyranoside[28] was coupled to trisaccharide **8** under Ogawa conditions (route A, Scheme 2). Under these conditions, tetrasaccharide **13** could be obtained in 16% yield. The use of other activators (for instance, Br_2, Bu_4NBr, or AgOTf) did not afford better results. Tetrasaccharide **13** could be successfully deprotected by acid treatment followed by hydrogenolysis to give compound **15** (FFFGn). In order to improve the synthesis of tetrasaccharide **13**, a 2+2 block strategy (route B, Scheme 2) was considered as alternative for the stepwise strategy. To this end, coupling of bromide **16**, prepared from thioethyl donor **12**, with ethyl 3,4-*O*-isopropylidene-1-thio-β-D-fucopyranoside **17** promoted by tetrabutylammonium bromide gave disaccharide donor **18** in 68% yield. Donor **18** and disaccharide acceptor **6** were condensed in the presence of copper(II) bromide and tetrabutylammonium bromide to give tetrasaccharide **13** in 52% as a α/β mixture (2.3:1), which could be separated by column chromatography. The overall yield of the preparation of tetrasaccharide **13** using the linear strategy was 7.2% (starting from disaccharide **6**), while the convergent strategy afforded tetrasaccharide **13** in 24.5% overall yield.

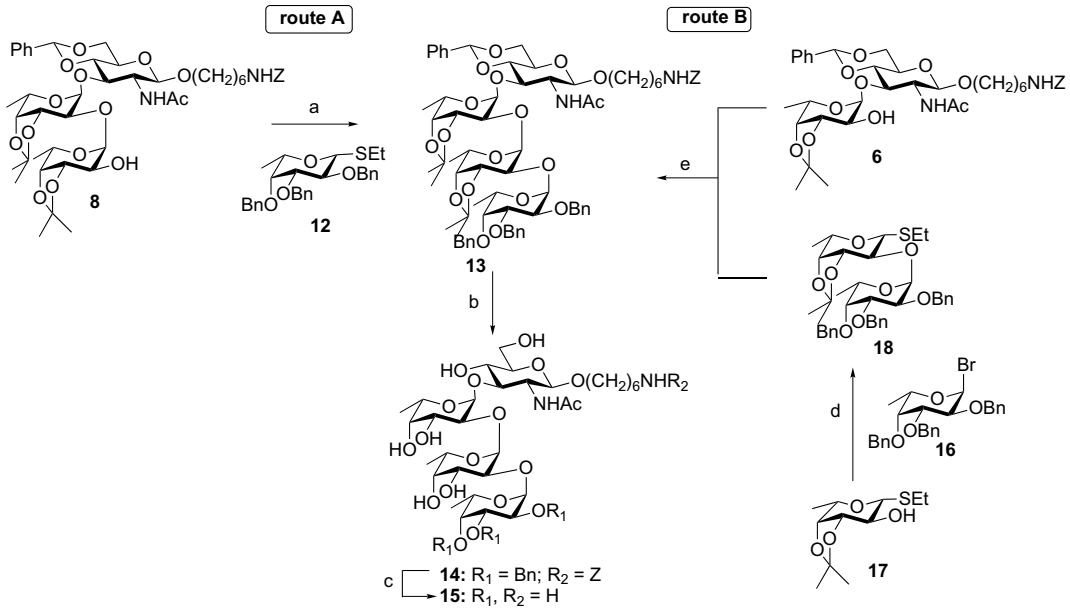
2.2. Preparation of neoglycoconjugates and coupling to a Biacore sensor chip

Glycosides **2**, **10**, **11**, and **15** were coupled to bovine serum albumin (BSA) to obtain neoglycoproteins, which could then easily be immobilized onto a SPR chip (CM5 sensor chip, Biacore). To this end, glycosides **2**, **10**, **11**, and **15** were transformed into their corresponding squaric acid amide esters by reaction with diethyl squarate in MeOH/phosphate (1:1), pH 7. The subsequent coupling to BSA proceeded in 0.1 M $NaHCO_3$, pH 9. Twenty molar equivalents of glycoside were added to BSA and the reaction was allowed to proceed for 5 days. The average coupling degree of the sugars to BSA was determined with MALDI-TOF MS (Table 2).

The neoglycoconjugates were immobilized on a Biacore CM5 sensor chip via the standard peptide chemistry method (Biacore user manual) to a level of approximately 10,000 response units (RU). BSA was conjugated on one flow channel of each of the sensor chips to serve as a control for non-specific binding and to correct for the background signal.

2.3. Binding of Mabs to BSA neoglycoconjugates by surface plasmon resonance

A panel of 831 Mabs, all previously derived from fusions with spleen cells of mice infected with schistosomes,[21] was screened for interaction with the neoglycoconjugates on the SPR sensor chip. The Mabs, which interacted with one or more of the neoglycoproteins are listed in Table 3. The concentration of each of these Mabs was determined by ELISA and responses were re-examined at a standardized concentration of 5 μg mL^{-1} to be able to compare the relative binding capacities. The positive Mabs were grouped according to their interaction with one or more neoglycoconjugates (Table 3). Mabs from group I specifically interacted with Gn, whereas Mabs from group II recognized the FGn structure. Furthermore, some Mabs were found that interacted with both structures containing the schistosome-specific Fucα1-2Fuc linkage. Finally, one Mab was found that bound only to the tri-fucosyl structure (group IV). None of the Mabs interacted with BSA (data not shown). As an additional control, each of the Mabs of group I–IV was tested for interaction with lacto-*N*-fucopentaose III (LNFPIII, Table 1), a fuco-pentasaccharide containing the Le^x trisaccharide at its terminus. None of the Mabs in Table 3 interacted with LNFPIII (data not shown).



Scheme 2. Reagents and conditions: a. $CuBr_2$, Bu_4NBr, DMF–CH_2Cl_2 (1:1), 16%. b. HOAc 60%, 50 °C, 57%. c. H_2, Pd/C, EtOH, HCl 1 M, 70%. d. (1) **12**, Br_2, CH_2Cl_2, 0 °C; (2) Bu_4NBr, DMF–CH_2Cl_2, rt, 68%. e. $CuBr_2$, Bu_4NBr, DMF–CH_2Cl_2 (1:1), 52% (α/β 2.3:1).

Table 2. Mass spectrometric data indicating the coupling degree of glycosides to BSA

Glycoside	Mass (Da) of BSA neoglycoconjugate	Average coupling degree
2	70,688	11
10	72,700	11
11	73,158	10
15	74,441	9

Table 3. Interaction of Mabs with neoglycoconjugates by surface plasmon resonance

Mab[a]	Isotype	Gn	FGn	FFGn	FFFGn
Group I					
114-2B5-A	nd[b]	++	—	—	—
114-4C1-A	IgM	+++	—	—	—
292-6G2-A	nd	+	—	—	—
294-4C4-A	IgM	+++	—	—	—
314-1G5-A	IgM	+++	—	—	—
Group II					
128-1E7-C[c]	IgM	—	+	—	—
128-3G12-A	IgG3	—	+	—	—
291-3A4-A[c]	IgM	—	+	—	—
Group III					
257-4D8	IgM	—	—	++	+
258-3E3	IgM	—	—	++	++
Group IV					
114-5B1-A[d]	IgG1	—	—	—	+++

[a] The Mabs were screened at a concentration of 5 μg/mL, + represents 100–500 RU after a 5 min dissociation period, ++ represents 500–1000 RU and +++ represents >1000 RU.
[b] Isotype of the antibody has not been determined.
[c] Also interacts with Fucα1-3GalNAcβ1-4[Fucα1-3]GlcNAc (FLDNF) (Vermeer, personal communication).
[d] Also interacts with GalNAcβ1-4[Fucα1-2Fucα1-3]GlcNAc (LDNDF).[10]

2.4. Immunofluorescence assays

Next, the identified Mabs were used to locate their corresponding natural antigens in the schistosome. To this end, frozen liver sections that contain eggs and worms, prepared from hamsters infected with *S. mansoni*, were probed with Mabs of each of the four groups in an immunofluorescence microscopy assay. Typical staining patterns for each group are presented in Figure 1. Interestingly, Mab 294-4C4-A only interacted with the tegumental outer region of the adult worm, in contrast to the other Mabs, which stained structures inside the parasite. The staining patterns for Mabs from group II and III are rather similar and resemble the duct-related patterns specified in a previous study by Thors and Linder.[29] It seems that the staining of Mab 291-3A4-A is more defined than that of Mab 258-3E3. Finally, staining with Mab 114-5B1-A resulted in completely different fluorescence patterns of canal like structures which have been previously described as part of the excretory system of the adult worms.[30]

3. Discussion

Schistosomes produce a wide variety of immunogenic fucosylated carbohydrate structures.[1] Thus far it is not well known where and when these structures are expressed and, most importantly, which elements exactly confer the different immunogenic properties to the glycoconjugates.

This is especially true for particular multifucosylated oligosaccharides that contain Fucα1-2Fuc and Fucα1-2Fucα1-2Fuc elements that are α1-3-linked to a GlcNAc moiety, which seem to be characteristic for schistosomes.[14,15] We have synthesized a set of linear

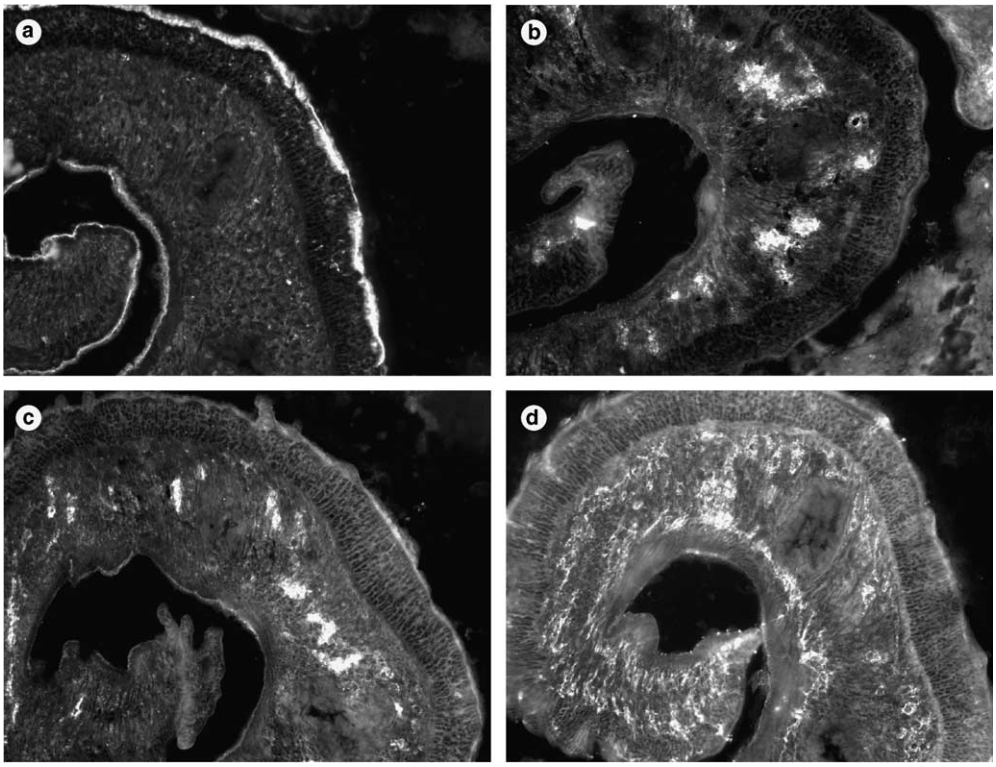


Figure 1. Immunofluorescence staining of frozen sections of *S. mansoni*-infected hamster livers incubated with Mabs from the four different groups: (a) 294-4C4-A, (b) 291-3A4-A, (c) 258-3E3, and (d) 114-5B1-A. Different structures within the worms were stained by Mabs from the different groups. Staining patterns of Mabs from group II and III are rather similar, however not identical. It cannot be determined from these pictures which exact cellular structures the two Mabs 291-3A4-A and 258-3E3 bind. In contrast, staining with Mab 114-5B1-A (group IV) resulted in a completely different fluorescence pattern. Canal like structures were stained, which have previously been described as part of the excretory system of the adult worms.[30] Incubation with fresh culture medium as a negative control did not result in fluorescence of the sections (data not shown).

oligosaccharides containing this Fucα1-2Fuc element and investigated the interaction with a large panel of anti-schistosomal Mabs.

A stepwise synthetic approach was used successfully to synthesize the desired mono- and difucosylated compounds FGn and FFGn. For the synthesis of the final target structure FFFGn, a 2+2 block synthetic strategy showed to be more efficient. In both strategies thioglycoside donors were activated with $CuBr_2/Bu_4NBr$, a method shown to be very selective for the formation of α-glycosidic linkages,[27] although in our hands this method was not always completely stereoselective.

Interestingly, for all of the tested structures one or more Mabs were found (Table 3). The Mabs could be divided into four groups. Mabs from group I selectively interacted with Gn, suggesting that terminal GlcNAc is an antigenic structure. This is somewhat surprising since GlcNAc is ubiquitously expressed in higher as well as lower organisms. In mammals however, GlcNAc usually occurs as an internal monosaccharide rather than a terminal residue. Mabs from group II bound to FGn (for an overview of the discussed carbohydrate structures see Table 1). The disaccharide Fucα1-3GlcNAc is also part of the Le^x trisaccharide antigen. However, none of the FGn-positive Mabs bound to Le^x (in the context of LNFPIII), suggesting that the additional β1-4-linked Gal in Le^x blocks the interaction of the Mabs with Fucα1-3GlcNAc. The third group of Mabs binds to oligosaccharides with at least two terminal fucoses in Fucα1-2Fuc linkage, the common element in FFGn and FFFGn. These Mabs did not interact with the Fucα1-3GlcNAc structure, indicating that in this structural context a single terminal fucose is not sufficient for binding. Moreover, the fucoses should be at a terminal position in a linear structure as these Mabs did not interact with branched LDNDF either (Vermeer, personal communication). Mab 114-5B1-A from group IV significantly interacted only with the trifucosylated FFFGn, indicating that this Mab requires two consecutive α1-2 linked fucoses.

Some of the presented Mabs have been found to bind to other carbohydrate structures as well. Mabs 128-1E7-C and 291-3A4-A from group II cross-react with FLDNF (Vermeer, personal communication), suggesting they recognize a fucose α1-3 linked to HexNAc, since both Fucα1-3GalNAc (terminal disaccharide of FLDNF) and Fucα1-3GlcNAc (FGn) are bound. However, it may be expected that clear quantitative differences in the binding of these cross-reacting Mabs to their respective antigens occur. Unfortunately, we cannot obtain quantitative SPR data with the current experimental setup, and therefore it is not possible to elaborate on the affinity and kinetic characteristics of the Mabs with respect to their different possible binding partners. However, in a direct comparison of the binding to

FLDNF- and FLDN-conjugates by SPR, 128-1E7-C gave an at least 3-fold better response for FLDNF than for FLDN, which indicates that F-LDN-F is the more accurate representation of the epitope of 128-1E7-C. This conclusion is further supported by the observation that pre-incubation with keyhole limpet haemocyanin (KLH), which expresses the F-LDN-F element,[31] inhibits the binding of 128-1E7-C to schistosome glycolipids.[16] Notably, Mab 114-5B1-A was previously shown to bind to LDNDF.[10] In the current study, Mab 114-5B1-A interacted with FFFGn. Initial binding of 114-5B1-A to FFGn could also be observed, but the SPR response returned to backgrounds levels within the 5-min equilibration period before measurement, indicating that 114-5B1-A has an insignificant, but detectable affinity for FFGn. From the primary carbohydrate sequence these characteristics are difficult to explain. Therefore we have examined the spatial orientation of these carbohydrate structures using a web-based tool (Sweet)[32] designed to construct three-dimensional models of carbohydrates. The overlay of the models of each structure (Fig. 2) suggests clearly that the terminal Fucα1-2Fuc moiety in the linear, curve-shaped FFFGn is oriented similarly as the Fucα1-2Fuc element in the branched LDNDF, thus explaining why Mab 114-5B1-A can bind both FFFGn and LDNDF. In FFGn however, this terminal Fucα1-2Fuc moiety may not be well available for binding by 114-5B1-A since the model of FFGn overlays with only the first three reducing-end residues of FFFGn (not shown).

By combining the data obtained from this and previous studies using related oligosaccharide structures, the epitopes of a number of anti-schistosomal monoclonal antibodies could be mapped and fine-tuned. It should be noted that neoglycoconjugate structures might only be limited representations of the natural glycoprotein and -lipid structures expressed by the parasites. The Mabs described in this study are however very useful tools to discriminate between different fucosylated structures with small but relevant structural and (immuno)biological differences.

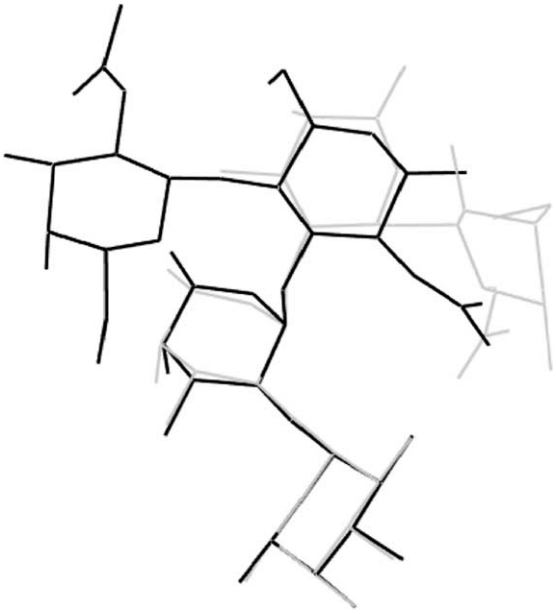


Figure 2. Overlay of modeled structures of FFFGn (gray) and LDNDF (black). The Fucα1-2Fuc Moiety of LDNDF overlays almost perfectly with the terminal Fucα1-2Fuc moiety of FFFGn, this might be the explanation for the binding of Mab 114-5B1-A to both these structures. The picture was created using PyMol Molecular Graphics System (DeLano, W.L. 2002, http://www.pymol.org).

The expression of the fucosylated carbohydrate structures within the adult worms was studied by immunofluorescence assays, similar to previous studies on other sets of related fucosylated and non-fucosylated glycoconjugates.[30,23,33,16,10] The results of the current microscopic study show major differences between the staining patterns obtained with Mabs from each of the groups, except group II and III (Fig. 1). The Mabs may for instance be applied as immuno-affinity tools to purify glycoproteins or glycolipids carrying predefined glycosylation patterns from crude schistosome extracts. In conclusion, the presented results provide a good starting point for further studies to assess the role of these carbohydrate structures in the immunology of schistosomiasis, utilizing both the synthesized structures and the corresponding Mabs.

4. Experimental section

4.1. General

TLC analysis was performed on Merck Alumina silica gel F_{254} plates, with detection by means of UV absorption (254 nm) and/or charring with ammonium molybdate (25 g/L) and ceric ammonium sulfate (10 g/L) in 10% aq H_2SO_4 or a ninhydrine solution or 0.2% orcinol in 20% methanolic H_2SO_4. Column chromatography was performed with Fluka Silica Gel (230–400 mesh). Unless otherwise indicated, all the reagents were purchased from commercial sources and used without further purification. EtOAc and Pet. Ether 60–80 were of technical grade and distilled before used. CH_2Cl_2 was distilled over CaH_2, the rest of the solvents were of p.a. grade (Baker) and stored over molecular sieves. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (200 and 50 MHz), Bruker AV 400 (400 and 100 MHz) or Bruker DMX 600 (600 and 150 MHz) as indicated. Optical rotations were measured on a Propol automatic polarimeter (sodium D line $\lambda = 589$ nm). High resolution mass spectra were recorded on a Q-star Applied Biosystems Q-TOF instrument (TOF-section). MALDI-TOF mass spectrometric analyses were performed on a Reflex III mass spectrometer or an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics).

4.2. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (1)

A mixture of known[26] 2-methyl-(3,4,6-tri-*O*-acetyl-1.2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (4.67 g, 14.2 mmol) and 6-*N*-(benzoyloxycarbonylamino)-1-hexanol (5.3 g, 21.11 mmol) was coevaporated three times

with 1,2-dichloroethane, and then dissolved in dry CH_2Cl_2 (170 mL). Molecular sieves 4 Å were added and the mixture stirred at room temperature for 15 min. Then TMSOTf (0.52 mL, 2.82 mmol) was added and stirring was continued for 40 h. The reaction mixture was then neutralized with triethylamine and washed with aq satd $NaHCO_3$ and water, dried ($MgSO_4$), filtered, and evaporated. The crude was purified by column chromatography (Pet. Ether–EtOAc 1:1) to give **1** (6.53 g, 79%). R_f (CH_2Cl_2–MeOH 10:1): 0.65. $[\alpha]_D$ −13.4 (*c* 2.5, CH_2Cl_2). Lit. $[\alpha]_D$ −12.7 (*c* 1, CH_3Cl). ^1H NMR (400 MHz, $CDCl_3$): δ 7.35–7.28 (m, 5H, Ar), 6.28 (d, 1H, J = 8.7 Hz, NHAc), 5.29 (t, 1H, J = 9.9 Hz, H-3), 5.15–5.05 (m, 4H, CH_2Ph, H-4, NHZ), 4.63 (d, J = 8.2 Hz, H-1), 4.24 (dd, 1H, J = 4.6 Hz, J = 12.3 Hz, H-6), 4.10 (br d, 1H, J = 12.1 Hz, H-6), 3.83 (m, 2H, CH_2O, H-2), 3.63 (m, 1H, H-5), 3.46 (m, 1H, CH_2O), 3.18 (m, 2H, CH_2NH), 2.07 (s, 3H, Ac), 2.01 (s, 6H, 2 Ac), 1.92 (s, 3H, Ac), 1.53 (m, 4H, $(CH_2)_2$), 1.32 (m, 4H, $(CH_2)_2$). ^{13}C NMR ($CDCl_3$): δ 170.5, 170.2, 169.2 (CO), 156.4 (CONH), 136.4, 128.2, 127.7, 127.6 (Ar), 100.2 (C-1), 77.2, 71.3, 68.5 (C-3, C-4, C-5), 69.0, 66.2 (C-6, CH_2Ph), 61.9 (OCH_2), 54.3 (C-2), 40.3 (CH_2NH), 29.4, 28.7, 25.7, 24.9 (($CH_2)_4$), 22.9 (Ac), 20.4 (Ac).

4.3. 6-Aminohexyl-2-acetamido-2-deoxy-β-D-glucopyranoside (2)

Compound **1** (25 mg, 0.043 mmol) was treated with NaOMe in MeOH (0.033 M, 3 mL) at rt for 15 h. The mixture was then neutralized with Amberlite-$H^{\oplus}$, filtered, and evaporated. The crude was dissolved in EtOH 90% in water (1.5 mL) and HCl 1 M (38 μL), Pd/C 10% (36 mg) was added and the reaction mixture was stirred overnight under H_2 atmosphere. The reaction was filtered over Hyflo® and evaporated. The crude was purified by Gel permeation chromatography on a Superdex Peptide PE 7.5/300 column (NH_4HCO_3 0.025 M in water) to give **2** (11 mg, 73%). ^1H NMR (600 MHz, D_2O): δ 4.48 (d, J = 8.5 Hz, 1H, H-1 GlcNAc), 3.91–3.86 (m, 2H, H-6, OCH_2), 3.72–3.70 (m, 1H, H-2), 3.69 (dd, 1H, J = 5.2 Hz, J = 9.8 Hz, H-6 GlcNAc), 3.60 (t, 1H, J = 8.7 Hz, H-3), 3.55 (m, 1H, OCH_2), 3.48 (t, 1H, J = 8.7 Hz, H-4), 3.42 (m, 1H, H-5 GlcNAc), 2.95 (t, 2H, J = 7.6 Hz, CH_2NH), 1.99 (s, 3H, Ac) 1.52 (m, 2H, CH_2), 1.45 (m, 2H, CH_2), 1.30 (m, 4H, $(CH_2)_2$). ^{13}C NMR (200 MHz, D_2O): δ 175.2 (CO), 102.6 (C-1 GlcNAc), 77.6, 75.7, 71.8 (C-3, C-4, C-5 GlcNAc), 70.2 (CH_2O), 62.5 (C-6 GlcNAc), 57.1 (C-2 GlcNAc), 40.4 (CH_2NH), 29.9, 28.2, 26.8, 26.4 (($CH_2)_4$), 22.8 (Ac). MALDI-TOF *m/z*: 321. 3 $[M+H]^+$, 343.2 $[M+Na]^+$.

4.4. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (3)

Compound **1** (6.53 g, 11.25 mmol) was treated with NaOMe in MeOH (0.033 M, 300 mL) at rt for 15 h. The mixture was then neutralized with Amberlite-$H^{\oplus}$, filtered, and evaporated. The crude was dissolved in CH_3CN (8.5 mL) and benzaldehyde dimethyl acetal (0.7 mL, 4.64 mmol) and *p*-TsOH (90 mg, 0.49 mmol) were added. The reaction mixture was stirred overnight at room temperature and then neutralized with triethylamine and evaporated. The crude was purified by column chromatography (CH_2Cl_2–MeOH 30:1) to give **3** (0.86 g, 92%). R_f (CH_2Cl_2–MeOH 10:1): 0.65. $[\alpha]_D$ −50.2 (*c* 1, DMF). Lit. $[\alpha]_D$ −49.2 (*c* 1.5, DMF). ^1H NMR (400 MHz, $CDCl_3$): δ 7.51–7.31 (m, 10H, Ar), 5.55 (s, 1H, CHPh), 5.55 (br s, 1H, NHAc), 5.08 (br s, 2H, CH_2Ph), 4.61 (d, 1H, J = 8.3 Hz, H-1 GlcNAc), 4.32 (dd, 1H, J = 4.9 Hz, J = 10.4 Hz, H-6), 3.92–3.37 (m, 7H, OCH_2, H-2, H-3, H-4, H-5, H-6), 3.16 (m, 2H, CH_2NH), 1.99 (s, 3H, Ac), 1.59–1.48 (m, 4H, $(CH_2)_2$), 1.34 (m, 4H, $(CH_2)_2$). ^{13}C NMR ($CDCl_3$–CD_3OD 10:1): δ 136.8, 128.9, 128.2, 127.9, 127.8, 127.6, (Ar), 101.5 (CHPh), 101.1 (C-1), 81.4, 70.9, 65.9 (C-3, C-4, C-5), 70.0 (C-6), 68.4 (CH_2Ph), 66.3 (OCH_2), 57.1 (C-2), 40.5 (CH_2NH), 29.4, 28.6, 25.9, 22.5 (–$(CH_2)_4$–), 22.5 (CH_3).

4.5. Ethyl 2-*O*-*tert*-Butyldimethylsilyl-3,4-*O*-isopropylidene-1-thio-β-L-fucopyranoside (4)

A solution of ethyl 3,4-*O*-isopropylidene-1-thio-β-D-fucopyranoside[34] (1.71 g, 6.89 mmol), imidazole (1.09 g, 16.18 mmol) and TBDMSCl (1.62 g, 10.75 mmol) in DMF (10 mL) was stirred at room temperature for 16 h and then evaporated. The residue was dissolved in EtOAc (200 mL) and washed with aq satd $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated. The crude was purified by column chromatography (Pet. Ether–EtOAc 30:1) to give **4** (2.24 g, 89%). R_f (Pet. Ether–EtOAc 20:1): 0.55. $[\alpha]_D$ +18.7 (*c* 1.1, CH_2Cl_2). ^1H NMR (200 MHz, $CDCl_3$): δ 4.27 (d, 1H, J = 9.5 Hz, H-1), 4.02 (dd, 1H, J = 5.8 Hz, J = 2.2 Hz, H-3), 3.93 (m, 1H, H-4), 3.80 (m, 1H, H-5), 3.54 (q, 1H, J = 9.5 Hz, J = 5.8 Hz, H-2), 2.64 (m, 2H, CH_2SEt), 1.50 (s, 3H, $CH_3{}^iPr$), 1.38–1.27 (m, 9H, $CH_3{}^iPr$, CH_3Fuc, CH_3SEt). ^{13}C NMR (50 MHz, $CDCl_3$): δ 109.1 ($C_q{}^iPr$), 84.7 (C-1), 80.3, 76.3, 73.5, 72.01 (C-2, C-3, C-4, C-5), 27.8, 26.2 ($CH_3{}^iPr$), 25.7 ($CH_3{}^tBuSi$), 24.0 (CH_2SEt), 17.9 ($C_q{}^tBuSi$), 16.6 (CH_3Fuc), 14.6 (CH_3SEt).

4.6. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(2-*O*-*tert*-butyldimethylsilyl-3,4-*O*-isopropylidene-α-L-fucopyranosyl)-β-D-glucopyranoside (5)

Acceptor **3** (334 mg, 0.63mmol) and donor **4** (280 mg, 0.77 mmol) were coevaporated together three times with toluene, dissolved in DMF–CH_2Cl_2 (1:1, 8 mL) and stirred at room temperature in the presence of activated molecular sieves 4 Å for 1 h. Bu_4NBr (656 mg, 2.04 mmol) was added and 1 h later $CuBr_2$ (456 mg, 2.04 mmol), and stirring was continued for 48 h at room temperature. The reaction mixture was diluted with CH_2Cl_2, filtered over Hyflo, washed three times with aq satd $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and concentrated. The crude was purified by column chromatrography (Pet. Ether–EtOAc 20:1→1:1) to give **5** (454 mg, 85 %). R_f (DCM–MeOH 40:1): 0.5. $[\alpha]_D$ −85.6 (*c* 0.53, CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ 7.49–7.33 (m, 10H, Ar), 5.93 (d, 1H, J = 7.5 Hz,

NHAc), 5.50 (s, 1H, CHPh), 5.10 (s, 2H, CH_2Ph), 4.98 (d, 1H, $J = 2.9$ Hz, H-1 Fuc), 4.86 (m, 1H, NHZ), 4.73 (d, 1H, $J = 8.0$ Hz, H-1 GlcNAc), 4.40 (m, 1H, H-5 Fuc), 4.32 (m, 2H, H-6 GlcNAc, H-3 GlcNAc), 4.08 (t, 1H, $J = 6.5$ Hz, H-3 Fuc), 3.91 (dd, 1H, $J = 2.5$ Hz, $J = 5.7$ Hz, H-4 Fuc), 3.83 (m, 1H, OCH_2), 3.73 (m, 2H, H-6 GlcNAc, H-2 Fuc), 3.59 (m, 2H, H-2 GlcNAc, H-4 GlcNAc), 3.46 (m, 2H, OCH_2, H-5 GlcNAc), 3.18 (m, 2H, CH_2N), 1.98 (s, 3H, Ac), 1.49 (m, 4H, $(CH_2)_2$), 1.46 (s, 3H, $CH_3{}^iPr$), 1.32 (s, 3H, $CH_3{}^iPr$), 1.30 (s, 3H, CH_3), 1.08 (d, 1H, $J = 6.6$ Hz, CH_3Fuc), 0.92 (s, 9H, ^{t}BuSi), 0.14 (s, 3H, CH_3Si), 0.11 (s, 3H, CH_3Si). ^{13}C NMR ($CDCl_3$): δ 170.1 (CO), 137.1, 128.7, 128.3, 127.9, 127.8, 125.8 (Ar), 108.4 (Cq^iPr), 101.1 (CHPh), 100.9 (C-1), 97.8 (C-1′), 80.6, 76.1, 75.8, 74.3, 71.9, 65.9, 63.6, (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′) 57.1 (C-2), 69.4, 68.6 (CH_2Ph, C-6), 66.2 (OCH_2), 40.5 (CH_2NH), 28.9 (CH_2), 25.7 (^{t}BuSi, $CH_3{}^iPr$), 25.1 (CH_2), 19.5 (Ac), 17.9 (C_qSi), 16.3 (CH_3Fuc). HRMS *m/z*: calcd for $C_{44}H_{67}N_2O_{12}Si$ $[M+H]^+$: 843.4463; found 843.4464.

4.7. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(3,4-*O*-isopropylidene-α-L-fucopyranosyl)-β-D-glucopyranoside (6)

Disaccharide **5** (372 mg, 0.44 mmol) was treated with TBAF (1 M in THF, 0.9 mL) under N_2 for 16 h at rt. The reaction mixture was then evaporated, the residue dissolved in EtOAc and washed with $NaHCO_3$ and brine, dried ($MgSO_4$), filtrated, and evaporated. The crude was purified by column chromatography (EtOAc–MeOH 30:1→15:1) to give **6** (285 mg, 88%). R_f (DCM–MeOH 25:1): 0.25. $[\alpha]_D$ −105.6 (*c* 1.2, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47–7.26 (m, 10H, Ar), 6.75 (d, 1H, $J = 8.3$ Hz, NHAc), 5.46 (s, 1H, CHPh), 5.11 (m, 2H, CH_2Ph), 4.95 (br s, 1H, NHZ), 4.87 (d, 1H, $J = 3.3$ Hz, H-1 Fuc), 4.68 (d, 1H, $J = 8.1$ Hz, H-1 GlcNAc), 4.32 (m, 2H, H-5 Fuc, H-6 GlcNAc), 4.06 (m, 2H, H-3 Fuc, H-3 GlcNAc), 3.87–3.73 (m, 4H, OCH_2, H-2 GlcNAc, H-4 Fuc, H-6 GlcNAc), 3.57 (m, 2H, H-2 Fuc, H-4 GlcNAc), 3.48–3.42 (m, 2H, H-5 GlcNAc, OCH_2), 3.19 (m, 2H, CH_2NH), 1.98 (s, 3H, Ac), 1.50 (m, 4H, $(CH_2)_2$), 1.46 (s, 3H, $CH_3{}^iPr$), 1.32 (m, 4H, $(CH_2)_2$), 1.29 (s, 3H, $CH_3{}^iPr$), 0.79 (d, 1H, $J = 6.6$ Hz, CH_3Fuc). ^{13}C NMR ($CDCl_3$): δ 170.6, 156.5 (CO), 137.0, 129.1, 128.4, 128.1, 127.9, 127.7, 126.2, 126.1 (Ar), 108.7 ($C_q{}^iPr$), 101.9 (CHPh), 100.3 (C-1 GlcNAc), 99.7 (C-1 Fuc), 80.1, 77.4, 76.4, 75.9, 70.2, 66.1, 63.3 (C-3, C-5, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc), 69.0, 68.7 (C-6, CH_2Ph), 66.5 (OCH_2), 56.8 (C-2 GlcNAc), 40.4 (CH_2NH), 29.6, 28.7, 25.6, 24.8 ($(CH_2)_4$), 28.1, 26.1 ($CH_3{}^iPr$), 23.2 (Ac), 15.5 (CH_3Fuc). HRMS *m/z*: calcd for $C_{38}H_{53}N_2O_{12}$ $[M+H]^+$: 729.3598; found 729.3640.

4.8. 6-Aminohexyl-2-acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-β-D-glucopyranoside (10)

Disaccharide **5** (25 mg, 0.044 mmol) was treated with acetic acid 60% (10 mL) at 50 °C for 6 h. The reaction mixture was then concentrated coevaporating with toluene. The crude was dissolved in EtOH 90% in water (1.5 mL) and HCl 1 M (38 μL), Pd/C 10% (36 mg) was added and the reaction mixture was stirred overnight under H_2 atmosphere. The reaction was filtered over Hyflo® and evaporated. The crude was purified by HW-40 (NH_4HCO_3 0.15 M in water) to give **10** (14 mg, 68%). ^{1}H NMR (600 MHz, D_2O): δ 4.96 (d, $J = 4.1$ Hz, 1H, H-1 Fuc), 4.48 (d, $J = 8.6$ Hz, 1H, H-1 GlcNAc), 4.30 (m, 1H, H-5 Fuc), 3.91–3.86 (m, 2H, H-6 GlcNAc, OCH_2), 3.81–3.76 (m, 3H, H-3 Fuc, H-4 Fuc, H-2 GlcNAc), 3.72 (dd, 1H, $J = 5.7$ Hz, $J = 12.2$ Hz, H-6 GlcNAc), 3.67 (dd, 1H, $J = 4.1$ Hz, $J = 10.4$ Hz, H-2 GlcNAc), 3.60 (t, 1H, $J = 8.7$ Hz,, H-3 GlcNAc), 3.55 (m, 1H, OCH_2), 3.48 (t, 1H, $J = 8.7$ Hz, H-4 GlcNAc), 3.44 (m, 1H, H-5 GlcNAc), 2.64 (t, 2H, $J = 7.1$ Hz, CH_2NH), 1.99 (s, 3H, Ac) 1.52 (m, 2H, CH_2), 1.45 (m, 2H, CH_2), 1.30 (m, 4H, $(CH_2)_2$), 1.13 (d, $J = 6.6$ Hz, 3H, CH_3Fuc). ^{13}C NMR (D_2O): δ 175.2 (CO), 101.7 (C-1 GlcNAc), 100.7 (C-1 Fuc), 81.2, 76.6, 72.6, 70.3, 69.4, 68.8, 67.7 (C-2, C-3, C-4, C-5 Fuc, C-3, C-4, C-5 GlcNAc), 71.3 (CH_2O), 61.6 (C-6 GlcNAc), 56.1 (C-2 GlcNAc), 41.7 (CH_2NH), 29.3, 26.4, 25.6 ($(CH_2)_4$), 23.0 (Ac), 15.9 (CH_3Fuc). MALDI-TOF *m/z*: 467.25 $[M+H]^+$, 489.26 $[M+Na]^+$.

4.9. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[3,4-*O*-isopropylidene-2-*O*-(2-*O*-*tert*-butyldimethylsilyl-3,4-*O*-isopropylidene-α-L-fucopyranosyl)-α-L-fucopyranosyl]-β-D-glucopyranoside (7)

Acceptor **6** (430 mg, 0.59 mmol) and donor **4** (278 mg, 0.77 mmol) were coevaporated together three times with toluene, dissolved in DMF–CH_2Cl_2 (1:1, 7.5 mL) and stirred at room temperature in the presence of activated molecular sieves 4 Å for 1 h. Bu_4NBr (643 mg, 1.99 mmol) was added and 1 h later $CuBr_2$ (444 mg, 1.99 mmol), and stirring was continued for 4 days at room temperature. The reaction mixture was diluted with CH_2Cl_2, filtered over Hyflo, washed three times with aq satd $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and concentrated. The crude was purified by column chromatrography (Pet. Ether–EtOAc 7:1→0:1) to give **α-7** (291 mg), **β-7** (24 mg), yield 52%, α/β = 12:1. R_f (DCM–MeOH 40:1): 0.45. $[\alpha]_D$ −96.5 (*c* 1.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.5–7.2 (m, 10H, Ar), 6.0 (br s, 1H, NHAc), 5.5 (s, 1H, CHPh), 5.09 (s, 2H, CH_2Ph), 5.03 (d, 1H, $J = 2.7$ Hz, H-1 Fuc), 4.94 (d, 1H, $J = 3.3$ Hz, H-1 Fuc), 4.74 (d, 1H, $J = 8.3$ Hz, H-1 GlcNAc), 4.50 (m, 1H, H-5 Fuc), 4.45 (m, 1H, H-5 Fuc), 4.33 (m, 1H, H-6 GlcNAc), 4.19–4.09 (m, 4H, H-3 Fuc, H-3 GlcNAc, H-4 Fuc, F-4 Fuc), 3.95 (m, 1H, H-4 Fuc), 3.85–3.74 (m, 4H, OCH_2, H-6, H-2 Fuc, H-2 Fuc), 3.64 (m, 1H, H-2 GlcNAc), 3.53 (m, 1H, H-4 GlcNAc), 3.46 (m, 2H, H-5 GlcNAc, OCH_2), 3.18 (m, 2H, CH_2NH), 2.01 (s, 3H, Ac), 1.49–1.30 (m, 15H, $4CH_3{}^iPr$, CH_3Fuc), 1.08 (d, 3H, $J = 6.6$ Hz, CH_3Fuc), 0.89 (s, 3H, ^{t}BuSi), 0.14 (s, 6H, CH_3Si). ^{13}C NMR (50 MHz, $CDCl_3$): δ 170.6, 156.1 (CO), 137.1, 128.8, 128.4, 128.0, 127.9, 126.0 (Ar), 108.6, 108.4 ($C_q{}^iPr$), 101.2 (CHPh, C-1 GlcNAc), 97.3 (C-1 Fuc), 97.1 (C-1 Fuc), 81.0, 76.4, 75.9, 74.5, 72.1, 66.0, 63.7, 63.4 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc, C-2, C-3, C-4, C-5 Fuc), 69.7, 68.6 (CHPh, C-6), 66.0

(OCH_2), 56.8 (C-2 GlcNAc), 40.7 (CH_2NH), 29.6, 29.1, 25.3, (($CH_2)_2$), 28.2 ($CH_3{}^iPr$), 26.1 ($CH_3{}^iPr$), 23.6 (Ac), 18.4 ($C_q{}^t$BuSi), 16.1, 16.0 (CH_3Fuc), −3.8 (CH_3Si), −4.2 (CH_3Si). HRMS *m/z*: calcd for $C_{53}H_{84}N_3O_{16}Si$ $[M+NH_4]^+$: 1046.5620; found 1046.5668.

4.10. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[3,4-*O*-isopropylidene-2-*O*-(3,4-*O*-isopropylidene-α-L-fucopyranosyl)-α-L-fucopyranosyl]-β-D-glucopyranoside (8)

Trisaccharide **7** (52.8 mg, 0.05 mmol) was treated with TBAF (1 M in THF, 100 μl) under N_2 for 6 h at rt The reaction mixture was then evaporated, the residue dissolved in EtOAc and washed with $NaHCO_3$ and brine, dried ($MgSO_4$), filtrated, and evaporated. The crude was purified by column chromatography (Pet. Ether–EtOAc 1:4) to give **8** (42 mg, 94%). R_f (DCM–MeOH 20:1): 0.5. $[\alpha]_D$ −128.9 (*c* 0.7, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46–7.34 (m, 10H, Ar), 5.47 (s, 1H, CHPh), 5.10 (br s, 2H, CH_2Ph), 5.03 (br s, 1H, H-1 Fuc), 4.89 (s, 1H, H-1 Fuc), 4.58 (m, 1H, H-5 Fuc), 4.43 (m, 2H, H-5 Fuc, H-1 GlcNAc), 4.33 (m, 1H, H-6 GlcNAc), 4.22 (m, 1H, H-3 Fuc), 4.12 (m, 3H, OCH_2, H-3 Fuc, H-4 Fuc), 3.89–3.73 (m, 7H, H-2 Fuc, H-2 Fuc, H-4 Fuc, H-3 GlcNAc, H-4 GlcNAc, H-6 GlcNAc, OCH_2), 3.52 (m, 2H, H-2 GlcNAc, H-5 GlcNAc), 3.20 (m, 2H, CH_2NH), 1.95 (s, 3H, Ac), 1.49–1.25 (m, 23H, $4CH_3{}^iPr$, CH_3-Fuc, $(CH_2)_2$), 0.91 (d, 3H, *J* = 5.1 Hz, CH_3-Fuc). ^{13}C NMR (50 MHz, $CDCl_3$): δ 170.5, 156.5 (CO), 137.1, 128.9, 128.4, 128.1, 128.0, 127.9, 126.1 (Ar), 108.8, 108.4 ($C_q{}^iPr$), 101.5 (CHPh), 99.9 (C-1 GlcNAc), 95.7 (C-1 Fuc), 95.3 (C-1 Fuc), 80.7, 76.4, 76.1, 75.9, 74.5, 74.1, 73.6, 71.0, 65.7, 62.7 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc, C-2, C-3, C-4, C-5 Fuc), 69.7, 68.8 (CHPh, C-6), 66.6 (OCH_2), 58.7 (C-2 GlcNAc), 40.5 (CH_2NH), 29.5, 28.9, 25.7, 25.1 (($CH_2)_2$), 28.3 ($CH_3{}^iPr$), 26.3 ($CH_3{}^iPr$), 23.3 (Ac), 15.9, 15.6 (CH_3Fuc). HRMS *m/z*: calcd for $C_{47}H_{67}N_2O_{16}$ $[M+H]^+$: 915.4490; found 915.4450.

4.11. 6-Aminohexyl-2-acetamido-2-deoxy-3-*O*-[2-*O*-(α-L-fucopyranosyl)-α-L-fucopyranosyl]-β-D-glucopyranoside (11)

Compound **8** (38 mg, 0.04 mmol) was treated with AcOH 60% (2.4 mL) at 50 °C for 8 h, and then evaporated to dryness with the aid of toluene. The crude was dissolved in EtOH 90% in water (2.7 mL), and HCl 1 M (60 μL), Pd/C 10% was added and the reaction mixture was stirred under H_2 atmosphere for 4 h, filtrated over Hyflo, and evaporated. The residue was purified by gel filtration chromatography HW-40 (NH_4HCO_3 0.15 M in H_2O) to give **11** (11 mg, 45%). $[\alpha]_D$ −126.4 (*c* 0.5, CH_3OH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.25 (d, 1H, *J* = 2.7 Hz, H-1 Fuc), 4.94 (d, 1H, *J* = 3.1 Hz, H-1 Fuc), 4.45 (d, 1H, *J* = 8.3 Hz, H-1 GlcNAc), 4.32 (m 1H, H-5 Fuc), 4.27 (m, 1H, H-5 Fuc), 3.88–3.39 (m, 14H, H-2, H-3, H-4, H-4, 2H-6 GlcNAc, H-2, H-3, H-4 Fuc, H-2, H-3, H-4 Fuc), 2.90 (t, 1H, *J* = 7.4 Hz, CH_2NH), 2.00 (s, 3H, Ac), 1.63 (m, 2H, CH_2), 1.54 (m, 2H, CH_2), 1.39 (m, 4H, $(CH_2)_2$), 1.17 (m, 6H, $2CH_3$Fuc). ^{13}C NMR (50 MHz, CD_3OD): δ 173.6 (CO), 102.3 (C-1 GlcNAc), 97.4 (C-1 Fuc), 97.3 (C-1 Fuc), 80.3, 77.5, 73.9, 73.6, 73.5, 71.6, 71.0, 70.1, 69.2, 68.1, 67.4, 62.4 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc, C-2, C-3, C-4, C-5 Fuc), 69.7 (C-6 GlcNAc), 62.4 (OCH_2), 56.6 (C-2 GlcNAc), 40.5 (CH_2NH), 29.8, 28.1, 26.7, 26.4 (($CH_2)_2$), 23.2 (Ac), 16.3 (CH_3-Fuc). MALDI-TOF *m/z* 613.30 $[M+H]^+$, 635.32 $[M+Na]^+$.

4.12. Ethyl 3,4-*O*-isopropylidene-2-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-1-thio-β-L-fucopyranoside (18)

Bromine (45 μL, 0.88 mmol) was added to a solution of ethyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranosyl (382 mg, 0.8 mmol) in CH_2Cl_2 (7.7 mL) at 0 °C. After 10 min, the mixture was evaporated, coevaporating twice with CH_2Cl_2 to give crude bromide **16**. Acceptor **17** was coevaporated with toluene (three times), dissolved in DMF (3.7 mL) under N_2, and stirred in the presence of activated molecular sieves 3 Å for 15 min at rt. Bu_4NBr (159 mg, 0.49 mmol) was added followed by the crude bromide dissolved in CH_2Cl_2 (2 mL), and stirring was continued overnight at rt. The mixture was filtrated, and the filtrated washed with aq satd $NaHCO_3$, water, and brine, dried ($MgSO_4$), and evaporated. The crude was purified by column chromatography Pet. Ether–EtOAc 6:1→3:1 (1% Et_3N), to give **18** (178 mg, 68%). $[\alpha]_D$ −90 (*c* 1.1, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42–7.26 (m, 15H, Ar), 5.51 (d, 1H, *J* = 3.6 Hz, H-1′), 4.99–4.62 (m, 6H, 3 CH_2Ph), 4.43 (d, 1H, *J* = 10.2 Hz, H-1), 4.24 (br q, 1H, *J* = 6.1 Hz, *J* = 12.7 Hz, H-5), 4.15 (t, *J* = 5.7 Hz, H-3), 4.08 (dd, 1H, *J* = 3.7 Hz, *J* = 10.2 Hz, H-2′), 4.01 (dd, 1H, *J* = 1.9 Hz, *J* = 5.4 Hz, H-4), 3.96 (dd, 1H, *J* = 2.7 Hz, *J* = 10.2 Hz, H-3′), 3.81–3.70 (m, 3H, H-2, H-4′, H-5′), 2.70 (m, 2H, CH_2SEt), 1.54–1.11 (m, 15H, $2CH_3{}^iPr$, $2CH_3$Fuc, CH_3SEt). ^{13}C NMR ($CDCl_3$): δ 138.9, 138.6, 138.4 (C-*ipso*), 128.2, 128.0, 127.3, 127.2 (Ar), 109.3 ($C_q{}^iPr$), 96.7 (C-1′), 83.5 (C-1), 79.1, 78.8, 77.7, 76.1, 74.1, 72.9, 72.1, 66.3 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 74.6, 73.1 (CH_2Ph), 29.5 (CH_2SEt), 26.4, 24.1 ($CH_3{}^iPr$), 16.7, 16.3 (CH_3Fuc, CH_3Fuc′), 14.5 (CH_3SEt). HRMS *m/z*: calcd for $C_{38}H_{52}NO_8S$ $[M+NH_4]^+$: 682.3413; found 682.3460.

4.13. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-{3,4-*O*-isopropylidene-2-*O*-[3,4-*O*-isopropylidene-2-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-α-L-fucopyranosyl]-α-L-fucopyranosyl}-β-D-glucopyranoside (13)

Route A: Acceptor **8** (60 mg, 0.065 mmol) and donor **12** (47 mg mg, 0.097 mmol) were coevaporated together three times with toluene, dissolved in DMF–CH_2Cl_2 (1:1, 1 mL) and stirred at room temperature in the presence of activated molecular sieves 4 Å for 1 h. Bu_4NBr (81.3 mg, 0.25 mmol) was added and 1 h later $CuBr_2$ (58.8 mg, 0.25 mmol), and stirring was continued for 48 h at room temperature. The reaction mixture was diluted with CH_2Cl_2, filtered over Hyflo, washed three times with aq satd $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and concentrated. The crude was purified by

column chromatrography (Pet. Ether–EtOAc 2:1→1:1) to give **13** (14 mg, 16%) and recovered acceptor **8** (75%).

Route B: Acceptor **6** (42 mg, 0.057 mmol) and donor **18** (50 mg, 0.075 mmol) were coevaporated together three times with toluene, dissolved in DMF–CH_2Cl_2 (1:1, 710 μL) and stirred at room temperature in the presence of activated molecular sieves 4 Å for 1 h. Bu_4NBr (62 mg, 0.19 mmol) was added and 1 h later $CuBr_2$ (43 mg, 0.19 mmol), and stirring was continued for 5 days at room temperature. The reaction mixture was diluted with CH_2Cl_2, filtered over Hyflo, washed three times with aq satd $NaHCO_3$ and brine, dried ($MgSO_4$), filtered, and concentrated. The crude was purified by column chromatrography (Pet. Ether–EtOAc 3:1→1:1) to give **α-13** (27 mg, 36%), **β-13** (12 mg, 16%), yield 52%, α/β = 2.25:1. $[\alpha]_D$ −89.0 (*c* 0.2, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.35–7.16 (m, 25H, Ar), 6.64 (d, 1H, *J* = 7.2 Hz, NHAc), 5.38 (s, 1H, CHPh), 5.19 (br s, 1H, H-1 Fuc), 5.11–5.07 (m, 4H, H-1 Fuc, H-1 Fuc, CH_2Ph), 4.93–4.61 (m, 8H, 3CH_2Ph, H-1 GlcNAc, NHZ), 4.56 (m, 1H, H-3 GlcNAc), 4.45–4.43 (m, 2H, 2H-5 Fuc), 3.20 (m, 1H, H-3 Fuc), 4.25 (dd, 1H, *J* = 10.4 Hz, *J* = 4.6 Hz), 4.14 (m, 4H, H-2 Fuc, H-3 Fuc, H-4 Fuc, H-5 Fuc), 3.95 (dd, 1H, *J* = 10.1 Hz, *J* = 2.7 Hz, H-3 Fuc), 3.83 (m, 1H, H-4 Fuc), 3.79–3.64 (m, 5H, H-2 Fuc, H-2 Fuc, H-6 GlcNAc, H-4 Fuc, OCH), 3.48–3.43 (m, 2H, H-5 GlcNAc, H-4 GlcNAc), 3.33 (m, 2H, H-2 GlcNAc, OCH), 3.12 (m, 2H, CH_2NH), 1.97 (s, 3H, Ac), 1.60–1.21 (m, 23H, $(CH_2)_4$, 4$CH_3{}^i$Pr, CH_3Fuc), 1.13 (d, 1H, *J* = 6.4 Hz, CH_3 Fuc), 0.95 (d, 1H, *J* = 6.6 Hz, CH_3Fuc). ^{13}C NMR ($CDCl_3$): δ 171.0 (CO), 138.6, 138.4, 138.3, 137.1, 128.9, 128.3, 128.2, 128.0, 127.6, 127.3, 127.0, 126.1 (Ar), 108.7, 108.4 ($C_q{}^i$Pr), 101.6 (CHPh), 100.1 (C-1 GlcNAc), 95.8 (C-1 Fuc), 95.9 (C-1 Fuc), 93.7 (C-1 Fuc), 81.1, 79.1, 75.9, 75.7, 75.2, 74.6, 74.2, 74.1, 66.7, 65.9, 62.2 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc1, 2, 3), 72.5, 72.3, 69.2, 68.7 (CH_2Ph, C-6), 60.2 (OCH_2) 58.0 (C-2 GlcNAc), 40.7 (CH_2NH), 29.5, 29.1, 26.1 (CH_2), 28.3, 28.1, 25.2 ($CH_3{}^i$Pr), 23.6 (Ac), 16.0, 15.7, 14.0 (CH_3Fuc). HRMS *m/z*: calcd for $C_{74}H_{95}N_2O_{20}$ $[M+H]^+$: 1331.6478; found 1331.6511.

4.14. 6-[*N*-(Benzyloxycarbonylamino)hexyl]-2-acetamido-2-deoxy-3-*O*-{2-*O*-[2-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-α-L-fucopyranosyl]-α-L-fucopyranosyl}-β-D-glucopyranoside (14)

Compound **13** (16 mg, 0.01 mmol) was treated with AcOH 60% (700 μL) at 50 °C for 8 h, and then evaporated to dryness with the aid of toluene. The crude was purified by column chromatography (CH_2Cl_2–MeOH 25:1→15:1) to give **14** (8 mg, 57%). $[\alpha]_D$ −92 (*c* 0.16, MeOH). ^{1}H NMR (600 MHz, CD_3OD): δ 7.85–7.22 (m, 20H, Ar), 5.42 (s, 1H, H-1 Fuc), 5.34 (s, 1H, *J* = 3.5 Hz, H-1 Fuc), 5.31 (s, 3H, *J* = 3.1 Hz, H-1 Fuc), 5.06 (br s, 2H, CH_2Ph), 4.81–4.52 (m, 7H, 3CH_2Ph, H-1 GlcNAc), 4.41 (m, 1H, H-5 Fuc), 4.20 (m, 1H, H-5 Fuc), 4.09–3.35 (m, 17H, H-2, H-3, H-4, H-5, 2H-6 GlcNAc, 3H-2, H-3, F-4 Fuc, OCH_2), 3.07 (m, 2H, CH_2NH), 1.91 (s, 3H, Ac), 1.68–0.94 (m, 17H, $(CH2)_2$, 3CH_3-Fuc). ^{13}C NMR (CD_3OD): δ 129.5, 129.1, 128.9, 128.5, 128.3 (Ar), 101.7 (C-1 GlcNAc), 96.4 (C-1 Fuc), 94.9 (C-1 Fuc), 94.0 (C-1 Fuc), 79.9, 79.1, 77.6, 76.8, 73.3, 73.0, 70.5, 69.6, 69.5, 68.1, 68.0 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc1, 2, 3), 75.9, 73.7, 70.5, 67.0 (CH_2Ph, C-6), 62.7 (OCH_2), 57.7 (C-2 GlcNAc), 41.5 (CH_2NH), 30.6, 30.2, 27.2, 26.5 (CH_2), 23.3 (Ac), 16.7, 16.2 (CH_3Fuc). HRMS *m/z*: calcd for $C_{61}H_{83}N_2O_{20}$ $[M+H]^+$: 1163.5539; found 1163.5562.

4.15. 6-Aminohexyl-2-acetamido-2-deoxy-3-*O*-{2-*O*-[2-*O*-(α-L-fucopyranosyl)-α-L-fucopyranosyl]-α-L-fucopyranosyl}-β-D-glucopyranoside (15)

Compound **14** (8 mg, 0.007 mmol) was dissolved in EtOH 90% in water (0.5 mL), and HCl 1 M (12 μL), Pd/C 10% was added and the reaction mixture was stirred under H_2 atmosphere for 4 h, filtrated over Hyflo, and evaporated to give **15** (3.7 mg, 70%). ^{1}H NMR (600 MHz, D_2O): δ 5.50 (d, *J* = 3.6 Hz, 1H, H-1 Fuc), 5.18 (d, *J* = 3.8 Hz, 1H, H-1 Fuc), 5.14 (d, *J* = 3.4 Hz, 1H, H-1 Fuc), 4.40 (d, *J* = 8.4 Hz, 1H, H-1 GlcNAc), 4.30 (br q, 1H, H-5 Fuc), 4.25 (br q, 1H, H-5 Fuc), 4.06 (br q, 1H, H-5 Fuc), 4.00 (m, 2H, H-6 GlcNAc, OCH_2), 3.93–3.78 (m, 11H, C-2, C-3 GlcNAc, C-2, C-3, C-4 Fuc 1, 2, 3), 3.58 (t, 1H, *J* = 9.1 Hz, H-4 GlcNAc), 3.51 (m, 1H, OCH_2), 3.44 (m, 1H, H-5), 2.95 (t, 2H, *J* = 7.5 Hz, CH_2NH), 2.00 (s, 3H, Ac), 1.61 (m, 2H, CH_2), 1.51 (m, 2H, CH_2), 1.31 (m, 4H, CH_2), 1.19 (m, 9H, 3CH_3Fuc). ^{13}C NMR (D_2O): δ 174.9 (CO), 102.3 (C-1 GlcNAc), 96.9 (C-1 Fuc), 93.6 (C-1 Fuc), 92.4 (C-1 Fuc), 76.3, 75.8, 72.9, 72.7, 72.4, 71.1, 70.6, 68.9, 68.8, 68.6, 68.5, 67.8, 67.7, 67.5 (C-3, C-4, C-5 GlcNAc, C-2, C-3, C-4, C-5 Fuc1, 2, 3), 71.2.(C-6), 61.5 (OCH_2), 56.2 (C-2 GlcNAc), 40.2 (CH_2NH), 29.2, 27.5, 26.1, 25.5 (CH_2), 23.2 (Ac), 16.1, 16.1, 16.0 (CH_3Fuc). MALDI-TOF *m/z* 759.4 $[M+H]^+$, 781.4 $[M+Na]^+$.

4.16. General procedure for the derivatization of glycosides with diethyl squarate

Spacer containing **2**, **10**, **11**, and **15** (1–7 mg) were dissolved a 1:1 solution of 0.1 M phosphate buffer pH 7.0 and MeOH (~250 μL total volume). Diethyl squarate (1.1 equiv) was added and the reaction was allowed to stand until TLC analysis (*n*-BuOH–MeOH–water–HOAc 4:2:2:0.5) indicated complete conversion of the glycoside into a faster moving product. After concentration, the crude residue was dissolved in water (0.5 mL) and loaded onto a C-18 Sep-Pak cartridge. The column was washed with water (3 × 0.5 mL), then the product was eluted with water/MeOH (75/25→25/75 v/v). After TLC analysis, the fractions containing the squarate adduct were pooled, and evaporated.

4.17. General procedure for the preparation of the bovine serum albumin (BSA) neoglycoconjugates

BSA was dissolved in 0.2 M carbonate buffer pH 9 (25 mg mL^{-1}) and stirred for 30 min. Then the diethyl squarate adduct, dissolved in water (0.5 mg mL^{-1}), was added to the BSA solution (20 mol equiv based on

BSA) and the reaction was allowed to proceed for 5 days. The BSA neoglycoconjugates were purified using gel permeation chromatography. The average degree of incorporation of the glycosides onto BSA was determined by MALDI-TOF MS (Table 2). The LNFPIII-HSA neoglycoconjugate was a kind gift of Dr. B. J. Appelmelk (Amsterdam, The Netherlands). The neoglycoconjugate was originally purchased from Isosep AB (Tullinge, Sweden) and contained on average 22 mol LNFPIII per mole HSA.

4.18. Immobilization of BSA neoglycoconjugates on CM5 sensor chips

The Biacore 3000 instrument, CM5 sensor chips and an amino coupling kit were purchased from Biacore AB (Uppsala, Sweden). All buffers were filtered and degassed under vacuum before use. Immobilization of the neoglycoconjugates was performed using the standard amino coupling procedure according to the instructions of the manufacturer at a flow rate of 5 μL min^{-1}. In short, the carboxymethylated surface of the CM5 sensor chip was activated by a 7 min pulse of a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-Ethyl-*N*′-(dimethylaminopropyl)carbodiimide, followed by several injections of the neoglycoconjugate at a concentration of 25 μg mL^{-1} in 10 mM sodium acetate pH 4.5 until the desired level of immobilization was achieved. The BSA neoglycoconjugates were immobilized to a level of approximately 10,000 response units (RU). After immobilization unreacted groups were blocked by the injection of 300 μL 1 M ethanolamine-HCl, pH 8.5. The same protocol was followed for the LNFPIII-HSA neoglycoconjugate. Unconjugated BSA was coupled on one flow channel as a control.

4.19. Binding analysis of monoclonal antibodies to fucosylated glycosides by SPR

Hybridoma culture supernatant (sup) containing the Mab of interest was injected on the different CM5 sensor chip channels containing immobilized neoglycoconjugates during 2 min with a flow of 20 μL s^{-1}. After a dissociation period of 5 min following the end of the injection, the response unit (RU) value was measured. Subsequently, the surface was regenerated with 20 μL 20 mM HCl. At the start of each new cycle, a 3-min equilibration period preceded injection of the sup, during this period 5 μL HBS buffer was injected as a control for the surface. After subtraction of the response units of the BSA control channel, the binding pattern of each Mab is represented in Table 3 as + (100–500 RU), ++ (500–1000 RU), or +++ (>1000 RU).

4.20. Immunofluorescence assay (IFA)

IFAs were carried out on 6 μm-thick cryostat liver sections of infected hamsters. These sections were incubated with Mabs (Fig. 1), washed and then incubated with an FITC labeled conjugate of RaM immunoglobulin antibodies (Nordic Immunological Laboratories) diluted 1:50 in PBS (0.035 M phosphate, 0.15 M NaCl, pH 7.8) containing 0.1 mg/mL Evan's blue. The slides were observed with a Leica DM RBE fluorescence microscope with the appropriate filter combination for FITC fluorescence. A negative control consisted of incubation with fresh culture medium followed by staining with the FITC labeled conjugate. Fluorescence was interpreted visually (Fig. 1).

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