The Immunogenicity of the Tumor-Associated Antigen Lewis^y May Be Suppressed by a Bifunctional Cross-Linker Required for Coupling to a Carrier Protein

Therese Buskas, Yanhong Li, and Geert-Jan Boons*[a]

Abstract: A Lewis^y (Le^y) tetrasaccharide modified by an artificial aminopropyl spacer was synthesized by a highly convergent approach that employed a levulinoyl ester and a 9-fluorenylmethoxycarbonate for temporary protection of the hydroxy groups and a trichloroethyloxycarbonyl as an amino protecting group. The artificial aminopropyl moiety was modified by a thioacetyl group, which allowed efficient conjugation to keyhole limpet hemocyanin (KLH) modified by electrophilic 4-(maleimidomethyl)cyclohexane-1-carboxylate (MI). Mice were immunized with the KLH–MI–Le^y antigen. A detailed analysis of sera by ELISA established that a strong immunoglobulin G (IgG) antibody response was elicited against the linker region. The use of a smaller and more flexible 3-(bromoacetamido)propionate for the attachment of Le^y to KLH not only reduced the IgG antibody response against the linker but also led to a significantly improved immune response against the Le^y antigen. This study shows that highly antigenic linkers suppress antibody responses to weak antigens such as self-antigens.

Keywords: glycoconjugates · immunogenicity · oligosaccharides · tumor-associated antigens · vaccines

Introduction

Oncogenesis is often associated with the over-expression of various oligosaccharides on cell-surface glycoproteins and glycosphingolipids.[1–3] This abnormal glycosylation is an important criterion for the stage, direction, and fate of tumor progression. Numerous studies have shown that the presence of tumor-associated oligosaccharides in primary tumors is strongly correlated with the poor survival rates of patients. The Lewis antigens sialyl Lewis^x (Sle^x), Sle^x–Le^x, Le^a, and Le^y are identified as important human-tumor-associated antigens. These oligosaccharides are also ligands for the endothelial cell-surface receptors E- and P-selectin. Binding of cancer cells to the endothelium is a necessary step for metastasis. Therefore it has been established that over-expression of these Lewis antigens promotes metastasis.

Cancer vaccines are based on the elegant concept that the immunization of cancer patients with a tumor-associated antigen will raise opsonizing or cytotoxic antibodies, which recognize and eliminate circulating cancer cells and micrometastases.[4,5] The poor immunogenicity of tumor-associated saccharides presents, however, a major obstacle for the development of effective carbohydrate-based cancer vaccines. Tumor-associated oligosaccharides are autoantigens and consequently tolerated by the immune system. Furthermore, the inability of saccharides to activate helper T-lymphocytes also diminishes their usefulness for vaccine development.[6–10] For most immunogens, including saccharides, antibody production depends on the cooperative interactions of two types of lymphocytes, B-cells and helper T-cells.[8,11,12] Saccharides alone cannot activate helper T-cells and therefore have a limited immunogenicity. The formation of low-affinity immunoglobulin M (IgM) antibodies and the absence of IgG antibodies manifest this limited immunogenicity. Furthermore, saccharides alone cannot induce immunological memory and therefore do not give a booster response after repeated exposure with the antigen. Fortunately, conjugation of a saccharide to a foreign carrier protein (for example, keyhole limpet hemocyanin (KLH), detoxified tetanus toxoid) enhances their presentation to the immune system, thereby overcoming the tolerance and the helper T-cell independent properties. In this case, the carrier protein provides T-epitopes (peptide fragments of 12–15 amino acids) that can activate helper T-cells. The immune response against tumor-associated saccharide antigens can be further improved by including a potent adjuvant.

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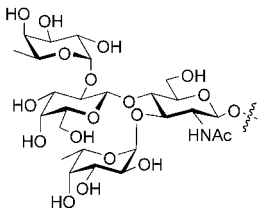
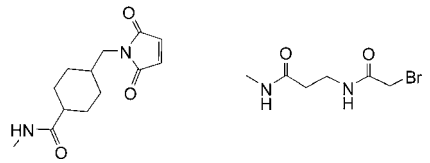
Elegant studies by Danishefsky, Livingston and co-workers at the Sloan–Kettering Institute of Cancer Research have shown that the Le^y oligosaccharide (Scheme 1) conjugated to the carrier protein KLH in the presence of the immunological adjuvant QS-21 can elicit, in mice, IgG and IgM antibody responses to naturally occurring forms of Le^y.[13] Although the antibody titers were relatively low, a phase-one clinical trial with twenty-four patients who had histologically documented ovarian, fallopian tube, or peritoneal cancer was conducted.[14] The vaccination was well tolerated with no adverse effects related to autoimmunity. The raised antibodies were capable of reacting with naturally occurring Le^y and with Le^y-expressing tumor cells. Unfortunately, the antibodies produced were mainly of the IgM class, with only three patients exhibiting detectable levels of IgG antibodies. Obviously, alternative strategies need to be developed for presenting these carbohydrate epitopes that will give a more efficient class switch to IgG antibodies and higher titers.



Scheme 1. The Le^y tetrasaccharide.

We report here that the chemical nature of a bifunctional linker greatly influences the immune response towards a synthetic Lewis^y tetrasaccharide hapten. It was found that the widely used 4-(maleimidomethyl)cyclohexane-1-carboxylate linker (Scheme 2) induces a significant immune response towards the linker itself. The application of a 3-(bromoacetamido)propionate linker not only diminished the response towards the linker but led to a considerably improved immune response towards the Le^y antigen as well.

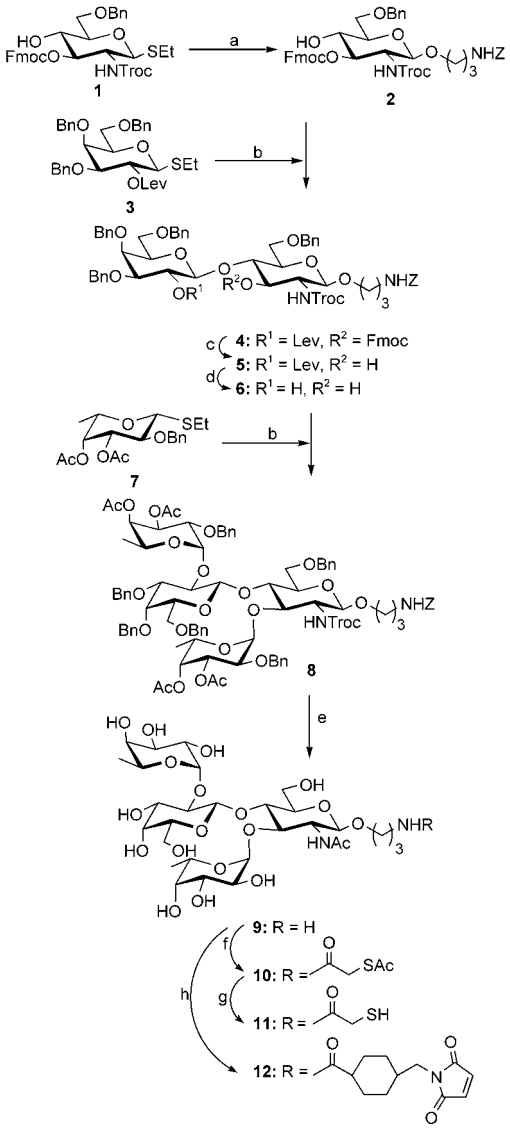


Scheme 2. 4-(*N*-Maleimidomethyl)-cyclohexane-1-carboxylate and 3-(bromoacetamido)propionate.

Results and Discussion

Synthesis: As part of a program to develop a fully synthetic anticancer vaccine, we required substantial quantities of the tumor-associated antigen Le^y. Furthermore, as a reference antigen, a conjugate of the saccharide to a carrier protein was required. The target compound, Le^y derivative **9** (Scheme 3), was selected as it has an artificial aminopropyl spacer at the anomeric center for selective conjugation to a carrier protein.

An efficient solution-phase synthesis for **9** was developed, which employs the building blocks **1**,[15] **3**,[16] and **7**.[17] Thus, coupling of **1** with 3-[(*N*-benzyloxycarbonyl)amino]propanol[18] in the presence of NIS/TESOTf gave, after purification by silica gel column chromatography, **2** with an excellent yield of 83%. This product was immediately used in the next NIS/TESOTf-mediated glycosylation with galactosyl donor **3** to give disaccharide **4**. The Fmoc protecting group of **4** was cleaved by treatment with Et_3N in CH_2Cl_2. The re-



Scheme 3. Synthesis of the Le^y tetrasaccharide. a) $HO(CH_2)_3NH$-Z, NIS, TESOTf, CH_2Cl_2, 0°C, 83%; b) NIS, TESOTf, CH_2Cl_2, 0°C, 76%; c) Et_3N/CH_2Cl_2 (1:1 *v*/*v*), 84%; d) NH_2NH_2·HOAc, MeOH, CH_2Cl_2, 87%; e) 1. Zn, HOAc; 2. Ac_2O, pyridine; 3. MeONa, MeOH, pH 10; 4. Pd/C, H_2, HOAc/EtOH (5:1 *v*/*v*), 52% over four steps; f) SAMA-OPfp, Et_3N, DMF; g) 7% NH_3 (g)/DMF, H_2O; h) sulfo-SMCC, 0.1 M sodium phosphate buffer (pH 6.2). Fmoc = 9-fluorenylmethoxycarbonyl, Bn = benzyl, Troc = 2,2,2-trichloroethyloxycarbonyl, Z = benzyloxycarbonyl, Lev = levulinoyl, NIS = *N*-iodosuccinimide, TES = triethylsilyl, Tf = triflate = trifluoromethanesulfonyl, SAMA-OPfp = *S*-acetylthioglycolic acid pentafluorophenyl ester, DMF = *N*,*N*-dimethylformamide, sulfo-SMCC = sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

sulting compound **5** was subjected to hydrazine buffered with acetic acid to remove the Lev group to give diol **6**. Coupling of **6** with 3.8 equivalents of fucosyl donor **7** resulted in clean and stereoselective glycosylation at the C-3 and C-2′ hydroxy groups and the fully protected tetrasaccharide **8** was isolated with a yield of 61%. The fucosylation proceeded with complete α selectivity, as confirmed by $^3J_{H,H}$ couplings ($J = 3.5$ Hz). Deprotection of **8** could easily be accomplished by a four-step procedure involving removal of the Troc group by treatment with activated Zn followed by acetylation of the resulting amine with acetic anhydride. Next, the acetyl esters were saponified by treatment with NaOMe in methanol. Finally, the benzyl ethers and the benzyloxycarbonyl moiety were removed by catalytic hydrogenation over Pd/C in a mixture of ethanol and acetic acid to give, after purification by P2 Bio-gel size-exclusion column chromatography, target compound **9**. The amino functionality of **9** was derivatized with an acetyl thioacetic acid moiety by treatment with SAMA-OPfp to give **10** and a maleimide group by reaction with sulfo-SMCC to give **12** in good yield. Derivatives **10** and **12** may be employed for glycosylation of proteins that are modified by electrophilic or nucleophilic moieties, respectively.

Preparation of carbohydrate–protein conjugates and immunizations: In order to obtain an Le^y–carrier protein conjugate, we selected, in the first instance, maleimide–thiol ligation methodology involving compound **10** and maleimide-activated keyhole limpet hemocyanin (KLH–MI). This strategy was thought to be attractive because 1) conjugation of expensive thiol-modified antigens to KLH in a high-yielding and reproducible manner is relatively easy, 2) KLH–MI has been successfully used as a carrier for various tumor antigens in immunological studies, and 3) maleimide-activated KLH may conveniently be obtained in lyophilized form from Pierce Endogen as part of an antibody production kit.[19] This kit also contains maleimide-activated bovine serum albumin (BSA–MI) for the purpose of selective antibody detection by ELISA.

Prior to the conjugation, the *S*-acetyl group of tetrasaccharide **10** was cleaved by treatment with ammonia in DMF under an argon atmosphere to prevent formation of the corresponding disulfide. Next, the thiol **11** was added to KLH–MI in a phosphate buffer (pH 7.2) containing sodium azide and ethylenediaminetetraacetate (EDTA). After a reaction time of 2 h, the glycoprotein was purified by using a centrifugal filter device with a nominal molecular-weight limit of 10 KDa. The number of Le^y copies conjugated to KLH was determined by using Dubois' phenol–sulfuric acid assay[20] and Lowry's protein-concentration test.[21] The conjugation efficiency was typically around 80%, based on the number of available maleimide groups, which correlated to an average of 620 Le^y copies per KLH. For the ELISA assay, a BSA–MI–Le^y conjugate was prepared by using a similar procedure but starting from BSA activated with maleimide groups.

Groups of five mice were immunized with the KLH–MI–Le^y conjugate together with the immunoadjuvant QS-21 (Antigenics Inc., Lexington, MA). The immunizations were repeated four times and sera were collected seven days after the last boost. Titers of anti-Le^y antibodies (Abs) were determined by ELISA whereby microtiter plates were coated with BSA–MI–Le^y and serial dilutions of sera added. An anti-mouse IgG (heavy-chain specific) antibody labeled with alkaline phosphatase were employed as a secondary antibody for detection purposes, for which the measured titers are listed in Table 1. A surprisingly high IgG antibody titer was determined (Table 1, entry 1) compared to previously reported immunizations with Le^y–KLH conjugates.[13,22] The two different proteins KLH and BSA had purposely been used in the immunizations and ELISA experiments, respectively, and consequently, anticarrier antibodies could not account for the high titers. It was realized that antibodies raised against the maleimide linker might cause the high titers. To correct for this possible false positive result, a second conjugate was prepared. In this conjugate, the Le^y derivative **11** was conjugated to BSA that had been activated with electrophilic bromoacetyl groups. This functionality also reacts with sulfhydryl groups at an appreciable rate at pH values above 8.0. Thus, BSA was activated by incubation with succinimidyl 3-(bromoacetamido)propionate (SBAP) in a sodium phosphate buffer at pH 8.0. The conjugate was purified by centrifugal filter devices. Subsequently, the Le^y antigen **11** was treated with the bromoacetyl-activated protein through thioether bond formation in a sodium phosphate buffer (pH 8.0) containing EDTA; this reaction gave a glycoconjugate with an Le^y/BSA ratio of 9:1. As expected, when the BSA–BrAc–Le^y conjugate was used for coating ELISA plates, much lower titers of IgG antibodies were measured (Table 1, entry 2). These results clearly demonstrate that the convenient use of a commercial preactivated protein kit led to false positive results and mainly antibodies against the maleimide linker were detected.

Table 1. ELISA antibody titers[a] against Le^y after four immunizations with KLH–MI–Le^y.

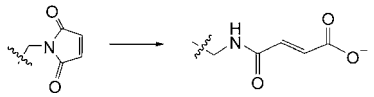
Entry	Coating	Titers
1	BSA–MI–Le^y	358928
2	BSA–BrAc–Le^y	*120*
3	BSA–MI–ME	171969
4	BSA–MI–Le^y RA	459509

[a] All titers are medians for a group of five mice. Titers were determined by regression analysis, with $\log_{10}$ dilution plotted versus absorbance. The titers were calculated to be the highest dilution that gave three times the absorbance of normal saline mouse sera diluted 1:120. The real antibody titer against Le^y is highlighted in italics.

Intrigued by this remarkable discrepancy, we were compelled to investigate this linker effect further. Quenching the maleimide groups of BSA–MI with 2-mercaptoethanol (ME) and coating ELISA plates with the obtained linker-modified protein allowed the detection of only the antibodies against the maleimide cross-linker. As can be seen in entry 3 of Table 1, significant IgG antibody titers were measured, a result demonstrating that the cyclohexylmethyl maleimide linker is highly immunogenic.[23–26]

It is well known that a maleimide moiety is susceptible to hydrolysis even at slightly basic conditions. The irreversible

hydrolysis results in the corresponding open form, maleamic acid, which is unreactive towards sulfhydryl groups (Scheme 4). It has been shown that the rate of reactivity of the maleimide moiety with sulfhydryl groups increases with increasing pH values.[27] However, raising the pH value above 7.5 also significantly increases the rate of hydrolysis. To overcome the sensitivity towards hydrolysis, the maleimide functionality is commonly stabilized by a neighboring cyclohexane moiety,[28,29] as in the SMCC linker used by Pierce Endogen in the preactivated protein kits. Despite the induced stability, it has been established that considerable hydrolysis of the maleimide groups of the cyclohexylmethyl maleimide linker occurs even after only 2 h at pH 7.0.[29]



Scheme 4. Hydrolysis of the maleimide.

We reasoned that the anti-linker response might be directed only against terminal hydrolyzed maleimides and not against maleimides connecting the saccharides to the protein, that is, internal maleimides (Scheme 5). In this respect, it is important to realize that only 80% of the maleimides are functionalized by saccharides and the rest are hydrolyzed. To distinguish between the two different modes of interaction, microtiter plates were coated with BSA that was thiolated by incubation with Traut's reagent in a triethanolamine buffer (pH 8.0) containing EDTA and then treated with maleimide-activated Le^y **12**. This reversely activated conjugate (BSA–MI–Le^y RA) displays only maleimide moieties that are linked to saccharides. As a consequence, anti-linker antibodies that only recognize terminal hydrolyzed maleimides should not be detected. As can be seen in Table 1 (entry 4), this conjugate gave a titer just as high as the one for BSA–MI–Le^y (entry 1) and, thus, it can be concluded that the anti-linker antibodies recognize both internal and terminal hydrolyzed maleimide residues.

The high immunogenicity of the linker region may well suppress the formation of IgG antibodies against the Le^y epitope. Furthermore, although the anti-linker antibodies recognized both internal and external cyclohexylmethyl maleimide moieties, it was thought that the terminal moieties are much more immunogenic. Therefore, immunizations with a KLH–Le^y conjugate that does not contain any hydrolyzed maleimides may give a more targeted immune response towards the Le^y antigen. Such a conjugate can be obtained by a reverse-activation protocol whereby KLH is thiolated with Traut's reagent and subsequently treated with **12**, Le^y modified with a cyclohexylmethyl meleimide moiety.[30] This conjugate (KLH–MI–Le^y RA) could conveniently be prepared as described above for BSA and gave a remarkably good incorporation of the saccharide epitope (Le^y/KLH 965/1) as determined by Dubois' total-carbohydrate assay and the Lowry protein assay.

A more targeted immune response may also be obtained by employing a linker that is smaller, more flexible, and hence less immunogenic, such as an aliphatic 3-(bromoacetamido)propionate spacer. To investigate the immunological properties of such a conjugate, Le^y **11** was treated with KLH that was activated with SBAP at pH 8.0 to give KLH–BrAc–Le^y. In this case, the ratio of Le^y/KLH was determined to be 692:1.

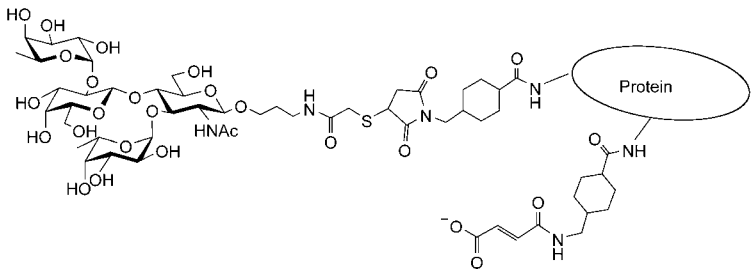
Groups of five mice were immunized with the two conjugates four times at weekly intervals and sera were collected seven days after the last immunization. Gratifyingly, the KLH–MI–Le^y RA conjugate gave a dramatically improved titer against Le^y, as can be seen from the ELISA assay in which BSA–BrAc–Le^y was employed as a coating (Table 2, entry 1). The measured titer is the best reported thus far. A significant antibody response against the cyclohexylmethyl maleimide linker was raised (entries 2 and 3) but the response was less profound than for immunizations with KLH–MI–Le^y (Table 1, entry 1). The anti-linker antibodies recognized both internal and external epitopes since similar titers were measured with coatings of Le^y linked to BSA by the direct (entry 2) and reverse maleimide activation procedure (entry 3).

Table 2. ELISA antibody titers[a] against Le^y after four immunizations with KLH–MI–Le^y RA.

Entry	Coating	Titers
1	BSA–BrAc–Le^y	*10066*
2	BSA–MI–Le^y	116960
3	BSA–MI–Le^y RA	103699

[a] All titers are medians for a group of five mice. Titers were determined by regression analysis, with $\log_{10}$ dilution plotted versus absorbance. The titers were calculated to be the highest dilution that gave three times the absorbance of normal saline mouse sera diluted 1:120. The real antibody titer against Le^y is highlighted in italics.

For the KLH–BrAc–Le^y conjugate, IgG antibody titers against Le^y and the linker region were determined by coating microtiter plates with BSA–MI–Le^y, BSA–BrAc–Le^y, and BSA–BrAc quenched with 2-mercaptoethanol (BSA–BrAc–ME). In this case, a significantly improved anti-Le^y IgG antibody response was also obtained (Table 3, entry 1) compared with the KLH–MI–Le^y conjugate. There was only a very weak response towards the linker region, as can be seen from the experiments with the BSA–BrAc–Le^y (entry 2) and BSA–BrAc–ME (entry 3) coat-



Scheme 5. Internal and terminal maleimides.

ings. In this respect, the BSA–BrAc–Le^y conjugate measures a combined titer for Le^y and linker whereas a coating with BSA–BrAc–ME only determines a response for the linker region.

Table 3. ELISA antibody titers[a] against Le^y after four immunizations with KLH–BrAc–Le^y.

Entry	Coating	Titers
1	BSA–MI–Le^y	*3294*
2	BSA–BrAc–Le^y	3957
3	BSA–BrAc–ME	824[b]

[a] All titers are medians for a group of four mice (one mouse died). Titers were determined by regression analysis, with $\log_{10}$ dilution plotted versus absorbance. The titers were calculated to be the highest dilution that gave three times the absorbance of normal saline mouse sera diluted 1:120. The real antibody titer against Le^y is highlighted in italics. [b] One mouse gave a strong response that was far out of the range of values for the rest of the group and was consequently omitted.

A remarkable finding is that although KLH–MI–Le^y RA (obtained by the reverse-activation protocol) gives a significantly higher anti-linker antibody response than the KLH–BrAc–Le^y conjugate, it also elicited a stronger anti-Le^y antibody response. An important difference between the two constructs is that the reverse-activation protocol gave a much higher incorporation of the Le^y epitopes, which may be relevant for the improved immunogenicity.

Our studies have shown that the selection of appropriate conjugation technology is of critical importance in eliciting IgG antibodies for weakly immunogenic epitopes such as for tumor-associated carbohydrate antigens. In particular, reducing the immunogenicity of the linker region and optimizing epitope loading are critical factors. In this respect, Danishefsky and co-workers have shown that conjugation of a Le^y to KLH by reductive amination gave a significantly better immune response than the use of a construct that was obtained by coupling of a thiolated saccharide derivative with KLH modified with maleimides. Probably, this difference in immunogenicity arises from a linker effect.

Conclusion

Conjugation of a carbohydrate antigen to a carrier protein is commonly used to overcome its T-cell independence. A wide variety of coupling methods utilizing various heterobifunctional cross-linking reagents have been described for this purpose. Here, we demonstrate that the choice of linker is of critical importance for the induction of a strong immune response against a synthetic Lewis^y tumor-associated antigen. The widely used 4-(maleimidomethyl)cyclohexane-1-carboxylate linker proved to be highly immunogenic and suppressed an IgG antibody response against the Le^y epitope. Detailed studies established that the anti-linker antibodies recognize terminal hydrolyzed maleimides as well as internal maleimides connecting the saccharides to the protein. The use of the smaller and more flexible 3-(bromoacetamido)propionate cross linker resulted in a significant reduction of antibodies against the linker region. However, more importantly, a considerable improvement of immune response towards the Le^y antigen was observed. A reverse-conjugation protocol in which the Le^y epitope was modified by a maleimide moiety and then conjugated to thiolated KLH also gave suppression of anti-linker antibodies, a result indicating that terminal hydrolyzed linker residues are more immunogenic than internal ones. The anti-linker IgG antibody titers were, however, significantly higher than for the KLH–BrAc–Le^y conjugate. The conjugate obtained by the reverse-activation method also elicited higher IgG antibody titers against Le^y. This high titer is a result of the reduced response against the linker region and a higher incorporation of Le^y epitopes into KLH. The studies reported here call for an awareness of possible false positives stemming from the use of commercial kits in which the same linker is employed for conjugation of a hapten to different proteins for antibody production and detection.

Experimental Section

General: Succinimidyl 3-(bromoacetamido)propionate (SBAP), sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), 2-iminothiolane (Traut's reagent), keyhole limpet hemocyanin (KLH), maleimide-activated mariculture KLH (mcKLH–MI), and bovine serum albumin (BSA–MI) were purchased from Pierce Endogen, Rockford, IL. BSA was purchased from Sigma. *N*-Iodosuccinimide (NIS) was purchased from Fluka and recrystallized from dioxane/CCl_4. All other chemicals were purchased from Aldrich, Acros, or Fluka and used without further purification. Molecular sieves were activated at 145 °C for 10 h. All solvents employed were of reagent grade and dried by refluxing over appropriate drying agents. All the reactions were performed under anhydrous conditions and monitored by TLC on Kieselgel 60 F_{254} (Merck) plates, with detection by UV light (254 nm) and/or by charring with 8% sulfuric acid in ethanol. Column chromatography was performed on silica gel (Merck, mesh 70–230). Size-exclusion column chromatography was performed on Sephadex LH-20 or Sephadex G10 gel (Pharmacia Biotech AB, Uppsala, Sweden). Extracts were concentrated under reduced pressure at $\leq$40 °C (water bath). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Inova300 spectrometer and a Varian Inova500 spectrometer equipped with Sun workstations. ^{1}H spectra recorded in $CDCl_3$ were referenced to residual $CHCl_3$ at $\delta = 7.26$ ppm or to tetramethylsilane at $\delta = 0$ ppm; ^{13}C spectra were referenced to the central peak of $CDCl_3$ at $\delta = 77.0$ ppm. Assignments were made by using standard 1D and gCOSY, gHSQC, and TOCSY 2D experiments. Positive-ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded by using an HP-MALDI instrument with gentisic acid as a matrix. Centrifugal filter devices were purchased from Millipore Inc. The immunoadjuvant QS-21 was a gift from Antigenics Inc., Lexington, MA. Immulon II Hb ELISA plates were purchased from Fisher Scientific Inc.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-{[(2,2,2-trichloroethoxy)carbonyl]amino}-3-*O*-(9-fluorenylmethoxycarbonyl)-β-D-glucopyranoside (2): A mixture of thioglycoside **1** (100 mg, 0.14 mmol) and 3-[(*N*-benzyloxycarbonyl)amino]propanol (33 mg, 0.17 mmol) in dry dichloromethane (10 mL) was dried azeotropically with toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves for 30 min, whereafter the mixture was cooled to 0 °C and treated with NIS (35 mg, 0.16 mmol) and TESOTf (3 μL, 0.01 mmol). After the reaction mixture was stirred for 10 min at 0 °C, TLC showed full conversion of the donor. The solution was diluted with dichloromethane (60 mL) and the molecular sieves were removed by filtration through a plug of celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL) and brine, then dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel, hexane/EtOAc 2:1) to give the product **2** as a white

powder (100 mg, 83%). $[\alpha]_D = -32.2$ ($c = 1.0$ in CH_2Cl_2); $R_f = 0.48$ (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.80$ (d, $J = 7.5$ Hz, 2H, Ar-H), 7.72–7.20 (m, 16H, Ar-H), 5.74 (d, $J = 8.3$ Hz, 1H, NH), 5.20 (s, 1H, $CH_2NHCOOCH_2Ph$), 5.07 (s, 2H, $CH_2NHCOOCH_2Ph$), 4.86 (t, $J = 9.4$ Hz, 1H, H-3), 4.65–4.51 (m, 4H, $ArCH_2$, Troc), 4.48–4.37 (m, 3H, H-1, Fmoc-CH_2), 4.29 (t, $J = 6.3$ Hz, 1H, Fmoc-CH), 3.90–3.71 (m, 5H, H-2, H-4, H-6a, $CH_2CH_2CH_2NHCOOCH_2Ph$), 3.52–3.41 (m, 3H, H-5, H-6b, $CH_2CH_2CH_2^aNHCOOCH_2Ph$), 3.26–3.18 (m, 1H, $CH_2CH_2CH_2^bNHCOOCH_2Ph$), 1.72 (m, 2H, $CH_2CH_2CH_2NHCOOCH_2Ph$) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 156.9 ($NHCOOCH_2Ph$), 155.9 ($CHCH_2COO$), 154.8 ($NHCO$), 142.5–120.3 (24C, Ar-C), 101.4 (C-1), 95.7 (CCl_3), 79.4 (C-3), 74.6, 74.1 (2C, OCH_2Ph, OCH_2CCl_3), 73.9 (C-5), 70.9 ($OCH_2CH_2CH_2$), 70.7 ($CHCH_2CO$, Fmoc), 70.2 (C-4), 67.5 (C-6), 66.9 ($COOCH_2Ph$), 56.2 (C-2), 46.8 ($CHCH_2CO$, Fmoc), 38.0 ($OCH_2CH_2CH_2$), 29.9 ($OCH_2CH_2CH_2$) ppm; HR MALDI-TOF MS: m/z: calcd for $C_{42}H_{43}Cl_3N_2O_{11}$: 856.1932; found: 879.1941 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-{[(2,2,2-trichloroethoxy)carbonyl]amino}-3-*O*-(9-fluorenylmethoxycarbonyl)-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-levulinoyl-β-D-galactopyranosyl)-β-D-glucopyranoside (4): A mixture of acceptor **2** (95 mg, 0.11 mmol) and thiogalactoside **3** (80 mg, 0.13 mmol) in dry dichloromethane (10 mL) was dried azeotropically with toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves for 30 min. The mixture was cooled to 0°C and treated with NIS (33 mg, 0.15 mmol) and TESOTf (3 μL, 0.01 mmol). After 30 min, TLC indicated that the reaction was complete and the mixture was diluted with dichloromethane (60 mL) and filtered through celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (silica gel, hexane/EtOAc 2:1) to give the product **4** as a white powder (110 mg, 76%). $[\alpha]_D = -48.9$ ($c = 1.0$ in CH_2Cl_2); $R_f = 0.41$ (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 7.76 (d, $J = 3.0$ Hz, 2H, Ar-H), 7.74–7.08 (m, 31H, Ar-H), 5.62 (d, $J =$ 8.3 Hz, 1H, NH), 5.25 (t, $J = 6.5$ Hz, 1H, H-2′), 5.20 (s, 1H, $CH_2NHCOOCH_2Ph$), 5.07 (s, 2H, $CH_2NHCOOCH_2Ph$), 4.93 (t, $J =$ 9.5 Hz, 1H, H-3), 4.85–4.61 (m, 6H, $2\times ArCH_2$, Troc), 4.58–4.17 (m, 8H, H-1, H-1′, Fmoc-CH_2, $2\times ArCH_2$), 4.20 (t, $J = 6.3$ Hz, 1H, Fmoc-CH), 3.90–3.83 (m, 5H, H-4, H-4′, H-6, H-6′a), 3.80–3.62 (m, 4H, H-2, H-5′, H-6′b, $CH_2CH_2CH_2NHCOOCH_2Ph$), 3.60–3.41 (m, 2H, H-5, $CH_2CH_2CH_2^aNHCOOCH_2Ph$), 3.39–3.15 (m, 1H, H-3′), 3.26–3.18 (m, 1H, $CH_2CH_2CH_2^bNHCOOCH_2Ph$), 2.81–2.15 (m, 4H, $OCOCH_2CH_2$, Lev), 2.12 (s, 3H, CH_2COCH_3, Lev), 1.72 (m, 2H, $CH_2CH_2CH_2NHCOOCH_2Ph$) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 171.5 ($OCOCH_2CH_2$, Lev), 156.8 ($NHCOOCH_2Ph$), 154.9 ($CHCH_2COO$), 154.7 ($NHCO$), 143.7–120.1 (42C, Ar-C), 101.4 (C-1′), 101.2 (C-1), 95.7 (CCl_3), 80.5 (C-3′), 77.3 (C-3), 75.5 (C-4), 74.9 (2C, C-5, C-5′), 74.6, 73.8, 73.6, 72.0 (5C, $4\times OCH_2Ph$, OCH_2CCl_3), 73.4 (C-5), 72.5 (C-4), 72.1 (C-2), 70.1 ($CHCH_2CO$, Fmoc), 68.1, 67.9, 67.4, 66.8 (4C, C-6, C-6′, $OCH_2CH_2CH_2$, $COOCH_2Ph$), 56.4 (C-2), 46.8 ($CHCH_2CO$, Fmoc), 38.0 (2C, $OCOCH_2CH_2$, (Lev), $OCH_2CH_2CH_2$), 30.1 (CH_2COCH_3, Lev), 29.9 ($OCH_2CH_2CH_2$), 28.1 ($OCOCH_2CH_2$, Lev) ppm; HR MALDI-TOF MS: m/z: calcd for $C_{74}H_{77}Cl_3N_2O_{18}$: 1386.4237; found: 1409.4187 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-levulinoyl-β-D-galactopyranosyl)-β-D-glucopyranoside (5): Compound **4** (100 mg, 0.072 mmol) was dissolved in a solution of triethylamine in dichloromethane (5 mL, 1:1 *v*/*v*). The reaction mixture was stirred at ambient temperature under argon for 18 h and then concentrated to dryness under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/EtOAc 2:1) to give the product **5** as a white powder (60 mg, 84%). $[\alpha]_D = -71.2$ ($c = 1.0$ in CH_2Cl_2); $R_f = 0.21$ (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.39$–7.26 (m, 25H, Ar-H), 5.40 (d, $J = 8.3$ Hz, 1H, NH), 5.28 (t, $J = 6.5$ Hz, 1H, H-2′), 5.12 (s, 1H, $CH_2NHCOOCH_2Ph$), 5.08 (s, 2H, $CH_2NHCOOCH_2Ph$), 4.92–4.30 (m, 10H, $4\times ArCH_2$, Troc), 4.41 (m, 1H, H-1′), 4.36 (m, 1H, H-1), 3.90–3.83 (m, 2H, H-4, H-6′a), 3.60–3.50 (m, 7H, H-3, H-4, H-6, H-5′, $CH_2CH_2CH_2NHCOOCH_2Ph$,), 3.49–3.35 (m, 5H, H-2, H-5, H-3′, H-6′a, $CH_2CH_2CH_2^aNHCOOCH_2Ph$), 3.26–3.18 (m, 1H, $CH_2CH_2CH_2^bNHCOOCH_2Ph$), 2.78–2.19 (m, 4H, $OCOCH_2CH_2$, Lev), 2.12 (s, 3H, CH_2COCH_3, Lev), 1.75 (m, 2H, $CH_2CH_2CH_2NHCOOCH_2Ph$) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 171.6 ($OCOCH_2CH_2$, Lev), 156.8 ($NHCOOCH_2Ph$), 154.8 ($NHCO$), 138.6–127.1 (30C, Ar-C), 101.6 (C-1′), 101.3 (C-1), 95.9 (CCl_3), 81.2 (C-4), 80.5 (C-3′), 74.8, 74.7, 73.9, 73.7, 72.4 (5C, $4\times OCH_2Ph$, OCH_2CCl_3), 73.4 (C-5), 74.0 (C-5′), 72.6 (C-3), 72.4 (C-4′), 71.6 (C-2′), 68.4, 67.3, 67.4, 66.8 (4C, C-6, C-6′, $OCH_2CH_2CH_2$, $COOCH_2Ph$), 57.7 (C-2), 38.1 ($OCOCH_2CH_2$, Lev), 37.9 ($OCH_2CH_2CH_2$), 30.1 (CH_2COCH_3, Lev), 29.8 ($OCH_2CH_2CH_2$), 29.0 ($OCOCH_2CH_2$, Lev) ppm; HR MALDI-TOF MS: m/z: calcd for $C_{59}H_{67}Cl_3N_2O_{16}$: 1164.3556; found: 1187.3429 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl-β-D-galatopyranosyl)-β-D-glucopyranoside (6): A solution of hydrazine acetate (3 mL, 0.5M in methanol) was added dropwise to a stirred mixture of compound **5** (50 mg, 0.043 mmol) in dichloromethane (10 mL). The reaction was kept at room temperature for 2 h, quenched by addition of acetonylacetone (0.2 mL), and diluted by dichloromethane (40 mL). The organic phase was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (silica gel, hexane/EtOAc 2:1) to give the product **6** as a white powder (40 mg, 87%). $[\alpha]_D = -40.7$ ($c = 1.0$ in CH_2Cl_2); $R_f = 0.31$ (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.39$–7.28 (m, 25H, Ar-H), 5.45 (d, $J = 8.3$ Hz, 1H, NH), 5.22 (s, 1H, $CH_2NHCOOCH_2Ph$), 5.08 (s, 2H, $CH_2NHCOOCH_2Ph$), 4.90–4.31 (m, 10H, $4\times ArCH_2$, Troc), 4.61 (m, 1H, H-1), 4.26 (d, $J = 6.3$ Hz, 1H, H-1′), 3.99–3.83 (m, 3H, H-2′, H-4′, H-6′a), 3.86–3.65 (m, 6H, H-3, H-6, H-5′, $CH_2CH_2CH_2NHCOOCH_2Ph$), 3.59–3.35 (m, 6H, H-2, H-4, H-5, H-3′, H-6′a, $CH_2CH_2CH_2^aNHCOOCH_2Ph$), 3.26–3.18 (m, 1H, $CH_2CH_2CH_2^bNHCOOCH_2Ph$), 1.75 (m, 2H, $CH_2CH_2CH_2NHCOOCH_2Ph$) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): $\delta =$ 156.7 ($NHCOOCH_2Ph$), 154.8 ($NHCO$), 138.4–127.9 (30C, Ar-C), 104.5 (C-1′), 101.3 (C-1), 95.9 (CCl_3), 83.3 (C-4), 82.1 (C-3′), 74.8, 74.7, 73.7, 73.6, 72.8 (5C, $4\times OCH_2Ph$, OCH_2CCl_3), 74.2 (C-5), 73.9 (C-5′), 72.8 (C-3), 72.7 (C-4′), 71.3 (C-2′), 69.6, 68.6, 67.4, 66.8 (4C, C-6, C-6′, $OCH_2CH_2CH_2$, $COOCH_2Ph$), 57.6 (C-2), 38.1 ($OCH_2CH_2CH_2$), 29.8 ($OCH_2CH_2CH_2$) ppm; HR MALDI-TOF MS: m/z: calcd for $C_{54}H_{61}Cl_3N_2O_{14}$: 1066.3188; found: 1089.3102 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-[[(2,2,2-trichloroethoxy)carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside (8): A solution of compound **6** (50 mg, 0.05 mmol) and compound **7** (72 mg, 0.19 mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves for 30 min. The mixture was cooled to 0°C and NIS (46 mg, 0.20 mmol) and TESOTf (4 μL, 0.01 mmol) were added. The reaction mixture was stirred at 0°C for 30 min, diluted by dichloromethane (60 mL), and filtered through celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL) and brine, dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (silica gel, hexane/EtOAc 2:1) to give the product **8** as a white powder (45 mg, 61%). $[\alpha]_D = -87.0$ ($c = 1.0$ in CH_2Cl_2); $R_f = 0.31$ (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.39$–6.82 (m, 35H, Ar-H), 5.54 (d, $J = 3.5$ Hz, 1H, H-1‴), 5.22 (s, 1H, NH), 5.39–4.13 (m, 5H, H-1″ (d, $J = 3.5$ Hz), H-3″, H-3‴, H-4″, H-4‴), 5.11–4.86 (m, 2H, H-1, H-5‴), 4.82–4.65 (m, 3H, H-3, H-4, H-5″), 4.49 (d, 1H, H-1′), 4.28–3.86 (m, 8H, H-2′, H-2″, H-2‴, H-4′, H-6, H-6′), 3.39–3.05 (m, 5H, H-5′, H-5, H-3′, $CH_2CH_2CH_2NHCOOCH_2Ph$), 3.02–2.86 (m, 1H, H-2), 2.07, 2.06, 1.97, 1.96 (s, 4H, $4\times CH_3CO$), 1.69 (s, 1H, $OCH_2CH_2CH_2$), 1.15 (d, $J = 6.7$ Hz, 3H, H-6‴), 0.93 (d, $J = 6.5$ Hz, 3H, H-6″) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 170.8$, 170.6, 170.5, 170.3 (4C, $4\times CH_3CO$), 156.7 ($NHCOOCH_2Ph$), 153.8 ($NHCO$), 143.9–120.1 (42C, Ar-C), 99.7 (C-1′), 99.4 (C-1), 98.4 (C-1″), 97.6 (C-1‴), 95.7 (CCl_3), 83.7 (C-3′), 75.9 (C-5), 75.3 (C-3), 74.7, 74.0, 73.3, 73.0, 72.8, 71.9, 71.6, 71.2, (8C, $6\times OCH_2Ph$, OCH_2CCl_3, $COOCH_2Ph$), 74.6, 74.1, 73.7, 72.6, 72.4, 72.1 (7C, C-2′, C-2″, C-2‴, C-4, C-4′, C-4″, C-4‴), 73.7 (C-5′), 70.9 (C-3‴), 70.0 (C-3″), 68.0, 65.0, 64.8 (C-6, C-6′, $OCH_2CH_2CH_2$), 67.3 (C-5‴), 66.7 (C-5″), 60.0 (C-2), 35.2 ($OCH_2CH_2CH_2$), 25.0 ($OCH_2CH_2CH_2$), 21.2, 21.1, 20.9, 20.8 (4C, $4\times CH_3CO$), 15.7 (C-6″), 15.5 (C-6‴) ppm; HR MALDI-TOF MS: m/z: calcd for $C_{80}H_{95}Cl_3N_2O_{24}$: 1572.5340; found: 1595.5398 $[M+Na]^+$.

Aminopropyl 2-deoxy-2-acetamido-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-L-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (9): Zinc (10 mg, 0.15 mmol, nanosize powder) was added to a stirred solution of tetrasaccharide **8** (40 mg, 0.02 mmol) in acetic acid (2 mL). After 20 min, the zinc was removed by filtering through celite and the filtrate was concentrated to dryness. The residue was dissolved in pyridine (2 mL) and acetic anhydride (1 mL) and the mixture was stirred at room temperature overnight, whereafter it was quenched by addition of methanol (2 mL). The solution was diluted by dichloromethane (60 mL) and was washed successively with 1 M HCl solution, aqueous sodium hydrogencarbonate (15%), and brine. The organic layer was dried over $MgSO_4$ and concentrated. The obtained residue was dissolved in methanol (5 mL), then sodium methoxide (1 M in methanol) was added until the pH value reached 10. The solution was stirred at room temperature for 24 h, neutralized with Dowex 50 H^+ resin, diluted with methanol (50 mL), filtered, and concentrated. The residue was purified by column chromatography (silica gel, EtOAc/methanol 10:1). The obtained compound was dissolved in acetic acid (5 mL) and ethanol (1 mL). The mixture was hydrogenated over Pd/C (10%, 20 mg) at ambient temperature. After 24 h, the mixture was filtered through celite to remove the catalyst and concentrated to dryness under reduced pressure. The residue was purified by size-exclusion column chromatography (Biogel P2 column, eluted with H_2O containing 1% *n*BuOH) to give the product **9** as a white powder (9 mg, 52%). $[\alpha]_D = -62.9$ ($c = 1.0$ in MeOH); ^{1}H NMR (500 MHz, D_2O, 30 °C): $\delta = 5.20$ (d, $J = 3.0$ Hz, 1H), 5.02 (d, $J = 3.9$ Hz, 1H), 4.79 (q, $J = 6.4$ Hz, 1H), 4.42 (d, $J = 8.0$ Hz, 2H), 4.19 (q, $J = 6.4$ Hz, 1H), 3.95–3.58 (br s, 31H), 3.36 (br s, 1H), 3.24 (m, 1H), 3.08–3.02 (m, 2H), 1.98–1.79 (m, 5H), 1.16 (d, $J = 6.4$ Hz, 3H), 1.12 (d, $J = 6.4$ Hz, 3H) ppm; ^{13}C NMR (500 MHz, D_2O, 30 °C): data of anomeric carbons: $\delta = 101.3$, 100.4, 99.6, 98.8 ppm; HR MALDI-TOF MS: *m*/*z*: calcd for $C_{29}H_{52}N_2O_{19}$: 732.3164; found: 755.2945 $[M+Na]^+$.

3-(*S*-Acetylthioglycolylamido)-propyl 2-deoxy-2-acetamido-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-L-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (10): Compound **9** (6 mg, 0.01 mmol) was dried under vacuum overnight. The sugar was slurried in dry DMF. SAMA-OPfp (5.3 mg, 0.02 mmol) was added. TEA (2.3 μL, 0.02 mmol) was added dropwise into the mixture. After stirring at room temperature for 2 h, the mixture was evaporated and the residue was purified by size-exclusion chromatography (Biogel P2 column, eluted with H_2O containing 1% *n*BuOH) to give thioacetate **10** as a white powder (4 mg, 58%). ^{1}H NMR (300 MHz, D_2O, 30 °C): $\delta = 5.21$ (d, $J = 3.0$ Hz, 1H), 4.95 (d, $J = 3.9$ Hz, 1H), 4.78 (q, $J = 6.4$ Hz, 1H), 4.40 (d, $J = 8.0$ Hz, 2H), 4.11 (q, $J = 6.4$ Hz, 1H), 3.85–3.51 (br s, 33H), 3.36 (br s, 1H), 3.24 (m, 1H), 3.08–3.02 (m, 2H), 1.98–1.79 (m, 8H), 1.17 (d, $J = 6.4$ Hz, 3H), 1.12 (d, $J = 6.4$ Hz, 3H) ppm; HR MALDI-TOF MS: *m*/*z*: calcd for $C_{33}H_{56}N_2O_{21}S$: 848.3096; found: 871.2983 $[M+Na]^+$.

3-(Mercaptoacetamido)-propyl 2-deoxy-2-acetamido-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-L-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (11): 7% NH_3 (g) in DMF solution (50 μL) was added to a solution of thioacetate **10** (1 mg) in H_2O (15 μL) and the mixture was stirred under argon atmosphere. The reaction was monitored by MALDI-TOF MS, which showed the product peak of $[M+Na]^+$. After 45 min, the mixture was evaporated under reduced pressure and coevaporated twice with toluene. The thiol was dried under high vacuum for 30 min and then used immediately for conjugation without further purification.

3-[4-(*N*-Maleimidomethyl)cyclohexane-1-carbonylamino]-propyl 2-deoxy-2-acetamido-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-L-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (12): A mixture of tetrasaccharide **9** (1.2 mg, 1.64 μmol) and sulfo-SMCC (1.07 mg, 2.46 μmol) in 0.1 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.2, 600 μL) was stirred at ambient temperature for 2 h. The compound was purified by size-exclusion chromatography on a Sephadex G10 column equilibrated in 0.1 M sodium phosphate buffer containing 5 mM EDTA (pH 6.2). Fractions containing compound **12**, as determined by TLC and MALDI-TOF MS ($[M+Na]^+$ 976.5), were pooled and used immediately without further characterization for conjugation to thiolated BSA or KLH.

Conjugation of Le^y derivative 11 to mcKLH–MI and BSA–MI: The conjugations were performed as suggested by Pierce Endogen. In short, thiol **11** (2.5 equiv excess to available MI groups on the protein), deprotected just prior to conjugation as described above, was dissolved in ddH_2O (100 μL) and added to a solution of maleimide-activated protein (2 mg) that had been restored with ddH_2O (200 μL) to give the protein in the conjugation buffer (sodium phosphate buffer (pH 7.2) containing EDTA and sodium azide). The mixture was incubated for 2 h at room temperature and then purified by use of a Millipore centrifugal filter device with a 10000 Da molecular cut-off. All centrifugations were made at 15 °C for 20 min, with spinning at 13 *g*. The reaction mixture was centrifuged off and the filter was washed with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (pH 6.5, 3×200 μL). The filtrates were checked for the presence of carbohydrate by TLC. The conjugate was retrieved and taken up in 10 mM HEPES buffer (pH 6.5, 1 mL). The average number of copies of Le^y attached to mcKLH and BSA was determined to be 620 and 9, respectively, according to Dubois' phenol–sulfuric acid total-carbohydrate assay and Lowry's protein-concentration test.

Conjugation of Le^y derivative 11 to BSA–BrAc: A solution of SBAP (5 mg) in DMSO (40 μL) was added to a solution of BSA (2 mg) in 0.1 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM EDTA (200 μL). The mixture was slowly stirred for 1 h at room temperature and then purified by using centrifugal filters with a molecular cut-off of 10000 Da. All centrifugations were performed at 15 °C for 20 min, with spinning at 13 *g*. The reaction mixture was centrifuged off and the filter was washed with conjugation buffer (2×200 μL). The activated protein was retrieved by spinning at 13 *g* for 20 min at 15 °C and taken up in 0.1 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM EDTA (200 μL). A solution of thiol derivative **11** (1.2 mg) in the conjugation buffer (100 μL) was added to the activated protein and the mixture was incubated at room temperature overnight. Purification was achieved by using the centrifugal filters as described above for the KLH/BSA–MI–Le^y conjugates. This gave a glycoconjugate with nine Le^y residues per BSA as determined by the phenol–sulfuric acid total-carbohydrate assay and Lowry's protein-concentration test.

BSA–MI–ME and BSA–BrAc–MI: A solution of 2-mercaptoethanol in DMF (37 μL, 1:100 *v*/*v*) was added to 3-(bromoacetamido)propionate-activated BSA (2 mg) prepared as described above or (maleimidomethyl)-cyclohexane-1-carboxylate-activated BSA (2 mg) from Pierce Endogen. The mixture was incubated at room temperature overnight. The mercaptoethanol conjugates were purified by using spin filters as described above.

Conjugation of Le^y derivative 11 to KLH–BrAc: A solution of KLH (2.3 mg) in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (200 μL) was added to a solution of SBAP (1 mg) in DMSO (40 μL). The mixture was slowly stirred for 1 h at room temperature and then purified by using centrifugal filters with a molecular cut-off of 10000 Da. All centrifugations were performed at 15 °C for 20 min, with spinning at 13 *g*. The reaction mixture was centrifuged off and the filter was washed with conjugation buffer (2×200 μL) The activated protein was retrieved by spinning at 13 *g* for 20 min at 15 °C and taken up in 0.1 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM EDTA (200 μL). A solution of thiol derivative **11** (0.6 mg) in the conjugation buffer (100 μL) was added to the activated protein and the mixture was incubated at room temperature overnight. Purification was achieved by using the centrifugal filters as described above for the KLH/BSA–MI–Le^y conjugates. This gave a glycoconjugate with 692 Le^y residues per KLH molecule as determined by the phenol–sulfuric acid total-carbohydrate assay and Lowry's protein-concentration test.

Thiolation of BSA: BSA (1.2 mg, 0.015 μmol) in thiolation buffer containing 50 mM triethanolamine, 0.15 M NaCl, and 5 mM EDTA (pH 8.0, 200 μL) was incubated for 1.5 h with Traut's reagent (1.08 mg, 7.83 μmol). The activated protein was purified on a D-salt dextran column (Pierce Endogen) preequilibrated with 0.1 M sodium phosphate buffer containing 0.15 M NaCl and 0.1 M EDTA (pH 7.2). The first fractions positive to Ellman's reagent (total of approximately 0.560 μmol of thiol) were pooled and used directly.

BSA–MI–Le^y (RA): The pooled fractions of Le^y derivative **12** and thiol-activated BSA were mixed and incubated overnight under an argon atmosphere. The conjugate was purified by using centrifugal filters with a molecular cut-off of 10 KDa. All centrifugations were performed at 13 *g* for 30 min. The reaction mixture was centrifuged and the filter was washed with 10 mM HEPES buffer (pH 6.5, 2×200 μL). The glycoconjugate was retrieved (15 min at 13 *g*) and taken up in 10 mM HEPES buffer (pH 6.5). The glycoconjugate had 9 copies of Le^y per BSA as determined

by using Dubois' carbohydrate assay and Lowry's protein-concentration test.

Thiolation of KLH: KLH (11.5 mg, 1.44 nmol) in thiolation buffer containing 50 mM triethanolamine, 0.15 M NaCl, and 5 mM EDTA (pH 8.0, 1.0 mL) was incubated with Traut's reagent (2 mg, 15 μmol) for 2 h. The thiolated protein was purified by using a D-salt dextran column (Pierce Endogen) that had been preequilibrated with 0.1 M sodium phosphate buffer containing 0.15 M NaCl and 0.1 M EDTA (pH 7.2). The first fractions positive to Ellman's reagent were pooled and used directly in a conjugation reaction with **12**.

KLH–MI–Le^y RA: Pooled fractions of Le^y derivative **12** (4.92 μmol), prepared and purified as described above, and thiol-activated KLH were mixed and incubated overnight under an argon atmosphere. The conjugate was purified by using centrifugal filters with a nominal molecular cut-off of 10 KDa. All centrifugations were performed at 13 *g* for 30 min. The reaction mixture was centrifuged and the filter was washed with 10 mM HEPES buffer (pH 6.5, 2×200 μL). The glycoconjugate was retrieved (15 min at 13 *g*) and taken up in 10 mM HEPES buffer (pH 6.5). The glycoconjugate had 965 copies of Le^y per KLH as determined by using Dubois' carbohydrate assay and Lowry's protein-concentration test.

Immunizations: Groups of five mice (female, BALB/c, 8–10 weeks) were immunized subcutaneously (3 different sites with a total of 150 μL) on days 0, 7, 14, and 28 with 20 μg of carbohydrate and 10 μg of adjuvant QS-21 in each boost. The mice were bled on day 35 (leg vein) and the sera were tested for the presence of anti-Le^y antibodies.

ELISA: 96-well ELISA plates were coated overnight at 4 °C with BSA–MI–Le^y, BSA–BrAc–Le^y, BSA–MI–ME, BSA–BrAc–ME, or BSA–MI–Le^y (RA) (2.5 μg mL^{-1}) in 0.2 M borate buffer (pH 8.5) containing 75 mM NaCl (100 μL per well). The plates were washed three times with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer containing 0.5% Tween 20 and 0.02% sodium azide. Blocking was achieved by incubating the plates for 1 h at room temperature with 1% BSA in 0.01 M phosphate buffer containing 0.14 M NaCl. Next, the plates were washed and then incubated for 2 h at room temperature with serum dilutions in phosphate-buffered saline containing 0.02% sodium azide and 0.5% Tween 20. Excess antibody was removed and the plates were washed three times. The plates were incubated with rabbit anti-mouse IgG Fcγ fragment specific alkaline-phosphatase-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 h at room temperature. Then, after the plates were washed, enzyme substrate (*p*-nitrophenyl phosphate) was added and allowed to react for 30 min before the enzymatic reaction was terminated by adding 3 M aqueous NaOH and the absorbance was read at dual wavelengths of 405 and 490 nm. Antibody titers were determined by regression analysis, with $\log_{10}$ dilution plotted versus absorbance. The titers were calculated to the $\log_{10}$ highest dilution that gave three times the absorbance of normal mouse sera diluted 1:120.

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