Synthetic and Immunological Studies on Clustered Modes of Mucin-Related Tn and TF O-Linked Antigens: The Preparation of a Glycopeptide-Based Vaccine for Clinical Trials against Prostate Cancer†

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Abstract: The syntheses of two tumor-associated carbohydrate antigens, Tn and TF, have been achieved using glycal assembly and cassette methodologies. These synthetic antigens were subsequently clustered (c) and immunoconjugated to a carrier protein (KLH or BSA) or a synthetic lipopeptide (pam) for immunological study. Three Tn conjugates were used to vaccinate groups of mice, and all preparations proved to be immunogenic. The Tn(c) covalently linked to KLH (**27**−KLH) plus the adjuvant QS-21 was the optimal vaccine, inducing high median IgM and IgG titers against Tn(c) by ELISA. These antibodies were strongly reactive with the Tn(c) positive human colon cancer cell line LS-C but not the Tn(c) negative colon cancer cell line LS-B by FACS. The antibodies' reactivities with natural antigens were inhibited with synthetic Tn(c) but not with structurally unrelated compounds. On the basis of these results, vaccines containing **27**−KLH and **30**−pam plus QS-21 are being tested in patients with prostate cancer.

Introduction

The development of efficient routes for the preparation of complex oligosaccharide or carbohydrate conjugates has been our goal for some time.[1] Synthetic investigations in this area can help to provide a detailed knowledge of the structural and chemical behavior of carbohydrates and their conjugates. Furthermore, issues related to biological function of glycoconjugates can be evaluated, provided suitable quantities of informative probe structures can be constructed.

With time, we became interested in using chemistry to examine an exciting possibility. The goal would be that of recruiting the immune system to respond to malignant lesions. We were particularly drawn to the concept of inducing "active immunity" by synthetic vaccines.[2] Peptidic molecules, whose immunobiology has been studied extensively, have been used in the search for synthetic antitumor and other vaccines.[3] Such investigations had been greatly aided by preparative powers of peptide chemistry, and particularly by solid phase synthesis of peptides and recombinant technology. However, immune responses to nonpeptidic substances, such as carbohydrates, although highly abundant on surfaces of viruses, bacteria, and tumor tissues, remain poorly understood partly due to a lack of experimentation on telling probe structures. This situation reflects, in many instances, the complexity in the synthetic methodology required to reach adequate quantities of informative goal systems. In our continuing chemical studies, we have undertaken the development of synthetic methodology of general applicability for the preparation of carbohydrates, in the form of glycolipids and glycopeptides, which mimic components of the accessible cell surface of tumor cells. The disclosure herein focuses on mucin-related O-linked glycopeptides, in particular the Tn and TF antigens.

Mucins, which comprise a family of large glycoproteins expressed on cells of epithelial tissues, carry large glycodomains in clustered modes.[4] Mucin amino acid sequences possess a very high percentage of serine and threonine residues, often found in contiguous arrays ranging in number from two to five. In most cases, the details of the occupancy of such blocks of serine and threonine subunits is not known in detail.[5] Despite a large variety of mucin glycostructures, the modality wherein the first residue, an *N*-acetylgalactosamine moiety, is linked to

† Abbreviations: BSA, bovine serum albumin; DAST, diethylaminosulfurtrifluoride; DIEA, diisopropylethylamine; ELISA, enzyme-linked immunosorbent assay; FMOC, fluorenylmethoxycarbonyl; GalNAc, *N*-acetylgalactosamine; IIDQ, 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihryoquinoline; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; KLH, keyhole limpet hemacyanin; pam, palmitoylcysteine; MBS, *m*-maleimimidobenzoyl-*N*-hydroxysuccinimide ester; NHS, *N*-hydroxysuccinimide; SAMA−OPfp, *S*-acetylthioglycolic pentafluorophenyl ester; TFA, trifluoroacetic acid; Tn(c), Tn cluster.

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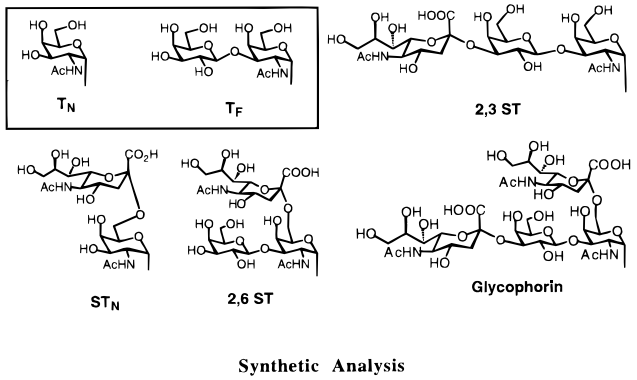

Figure 1.

a serine or threonine residue *via* an α-linkage appears to be broadly conserved (Figure 1). Interestingly, the significant changes of the glycopatterns during the malignant transformation generally result in shorter carbohydrate chains.[6] The Tn antigen represents the simplest member of the family. This antigen, as well as the related Thomsen−Friedenreich disaccharide (TF) antigen, is quite common in carcinoma malignancies, particularly of the colon and prostate.[7] Simple carbohydrate antigens have been synthesized, and their immunogenicity in conjugate vaccines has been confirmed.[8] For example, antibody titers against STn have been reported to correlate with improved prognosis in breast cancer patients.[9] Comparable studies with more complex carbohydrates have rarely been described, thus the clear interest in large clustered forms of antigens.

Given this context, we set for ourselves several goals. The first was the development of synthetic methodology to gain access to the clustered antigen motifs. We would go on from the chemistry phase to evaluate the immunogenicity of the clustered glycopeptide fragments in mice. The longer range goal would be the development and evaluation of antitumor glycopeptide-based vaccines.[10] In this paper we report the realization of these goals up to the point of the clinical trials which have just begun.

Synthetic Analysis

We have previously demonstrated that even bulky glycosyl amino acids can be efficiently incorporated into peptide backbones.[11] In light of these results, we opted to construct the glycosyl amino acids representing the Tn and TF epitopes. The amino acid domains would be protected in a fashion which anticipates the requirements of peptide assembly to form the clustered epitopes as shown in Figure 2. These constructs,

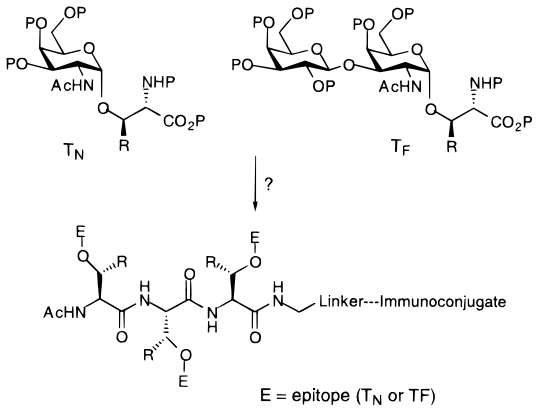

Figure 2.

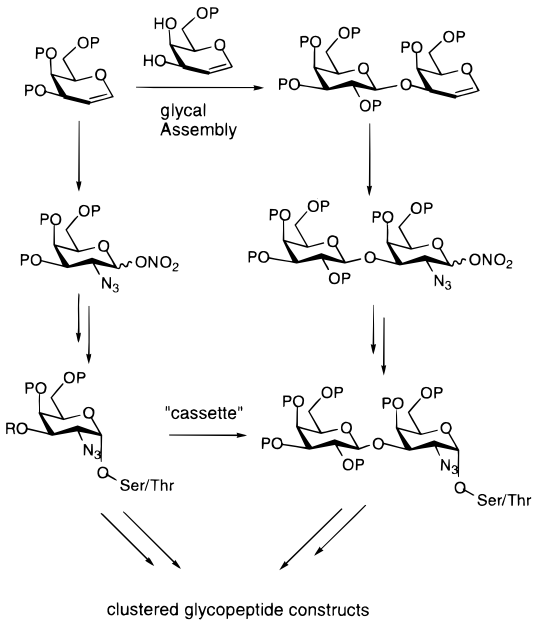

Figure 3.

suitably conjugated to immunogenic stimulants such as bacterial lipopeptides or carrier proteins, would then be evaluated in biological studies for antibody production, and eventual clinical application.

Synthetic Planning

For the synthesis of the Tn and TF antigens, we turned to our basic glycal assembly[1] logic. The key starting point would be the appropriately protected galactal, which could be used to produce both mono- and disaccharides. Azidonitration[12] of these glycals according to Lemieux might be used to introduce the 2-azido group and to produce the functionality at the anomeric carbon required for the fashioning of the prerequisite donor. Subsequent glycosylation of such a construct with the appropriately protected serine or threonine would pave the way for reaching our conjugates. An alternative and potentially more general method would involve building a shorter Tn construct as a "cassette", which would have the α-O-linked amino acid prebuilt into the GalNAc. This cassette might serve as a general acceptor to be inserted in the late stages of synthesis of virtually any O-linked glycopeptide goal structures. Such an approach, captured in Figure 3, might sidestep the serious difficulty of designing *ad hoc* methods for achieving high anomeric selectiv-

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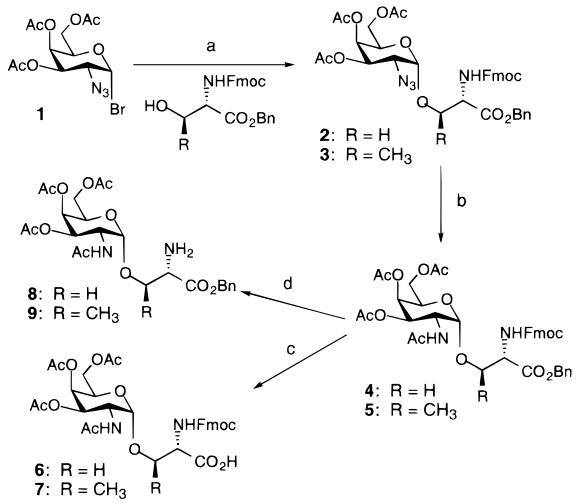
Scheme 1



Reagents: (a) $AgClO_4$, CH_2Cl_2, rt, 4:1 α:β; (b) AcSH, rt; (c) Pd/C, H_2, MeOH, H_2O, 75%; (d) 20% morpholine in DMF, 5 min.

ity in glycosylations of the side chain hydroxyls of serine and threonine for each new construct we hope to build.[13]

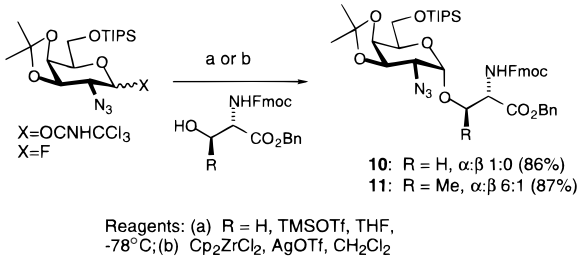
Results and Discussion

The Tn antigen had previously been prepared by a number of methods.[14] We chose a method to prepare the peracetylated form of the antigen, which is compatible with both solution and solid phase peptide chemistry. Thus, glycosylation of known bromide **1**[15] with *N*-Fmoc-serine or threonine benzyl ester afforded **2** or **3** as a separable 4:1 α/β mixture of anomers in 60% yield (Scheme 1). Reductive acetylation using neat thiolacetic acid produced fully protected Tn antigens **4** and **5**. Careful hydrogenolysis afforded the peracetylated acids **6** and **7** in 75% yield, while Fmoc removal gave **8** and **9**, which were in principle available for glycopeptide assembly.

The mediocre yield of glycosylation and the serious inconvenience of the need for anomer separation prompted a search for an alternative method. As matters turned out, another program in our laboratory, moving forward, had been directed to a total synthesis of the F1α antigen.[16] In screening for efficient serine or threonine glycosylation reactions, it was found that compounds **10** and **11** could be prepared as shown in Scheme 2. In the case of serine-derived acceptor the glycosylation ratio apparently gave only α-product **10**. In the synthesis of the threonine product **11**, a small amount of β-product was noted. In both cases the yields were far superior to those obtained from **1**. The idea then emerged to use **10** and **11** as general inserts (cassettes) to be installed toward the end of a complex synthesis. Thus, we need only solve the very difficult O-linkage problem once for a given "reducing end" and exploit that capability for building on the desired clustered system.

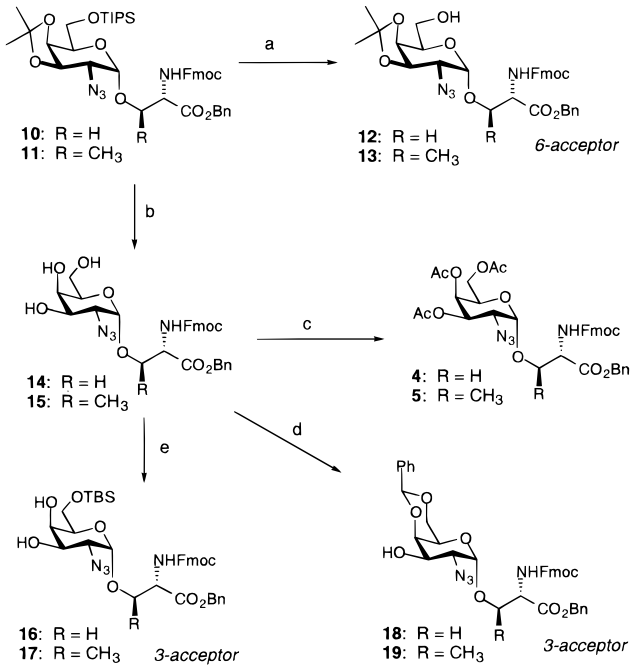
To implement this strategy, it would be necessary to fashion from the cassettes a variety of orthogonally protected modules for further use as glycosyl acceptors. In the case at hand, simple

Scheme 2



Reagents: (a) R = H, TMSOTf, THF, -78°C; (b) Cp_2ZrCl_2, AgOTf, CH_2Cl_2

Scheme 3



Reagents: (a) TBAF, AcOH, THF (94-100%); (b) I_2 / MeOH (63-81%); (c) Ac_2O, DMAP, TEA, CH_2Cl_2, (90%); (d) $PhCH(OMe)_2$, *p*-TsOH, CH_3NO_2 (73-94%); (e) TBSCl, Imidazole, DMF (64-85%).

TBAF-mediated desilylation of **10** or **11** affords position 6 acceptor **12** or **13**, respectively, in excellent yield (Scheme 3). Moreover, removal of both the TIPS and acetonide groups using I_2 in MeOH[17] gave rise to the versatile triol **14** or **15**, which could readily be converted to the peracetyl Tn precursors **4** and **5**. Alternatively the triols could be transformed, via resilylation with TBSCl, into diol 3 acceptor **16** or **17**,[18] or by benzylidene formation[19] to give specific position 3 acceptor **18** or **19**. We have used this methodology to prepare substantial quantities of the Tn antigen. In addition, we have used these intermediates for the efficient preparation of several O-linked tumor-associated antigens such as TF (vide infra) and sialosyl-Tn and sialosyl-T antigens.[20]

The amino acid sequence we chose for these clusters was to have three consecutive serines or threonines. Due to the lack of reliable information regarding which serine or threonine residues within a contiguous array constitutes an optimal epitope, we selected such trimers for the initial evaluation. The synthesis

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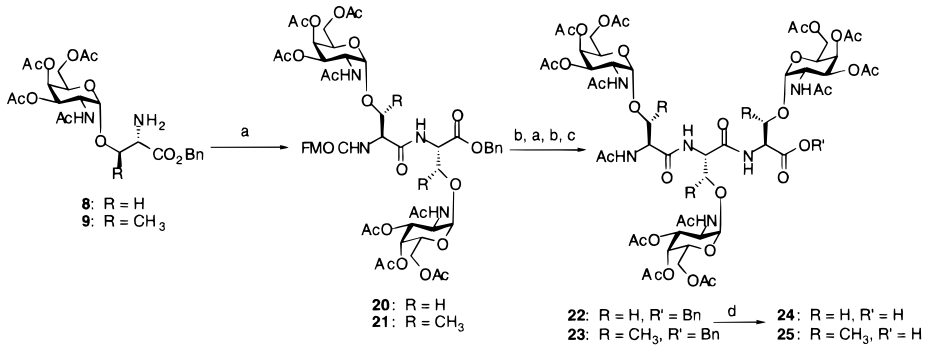
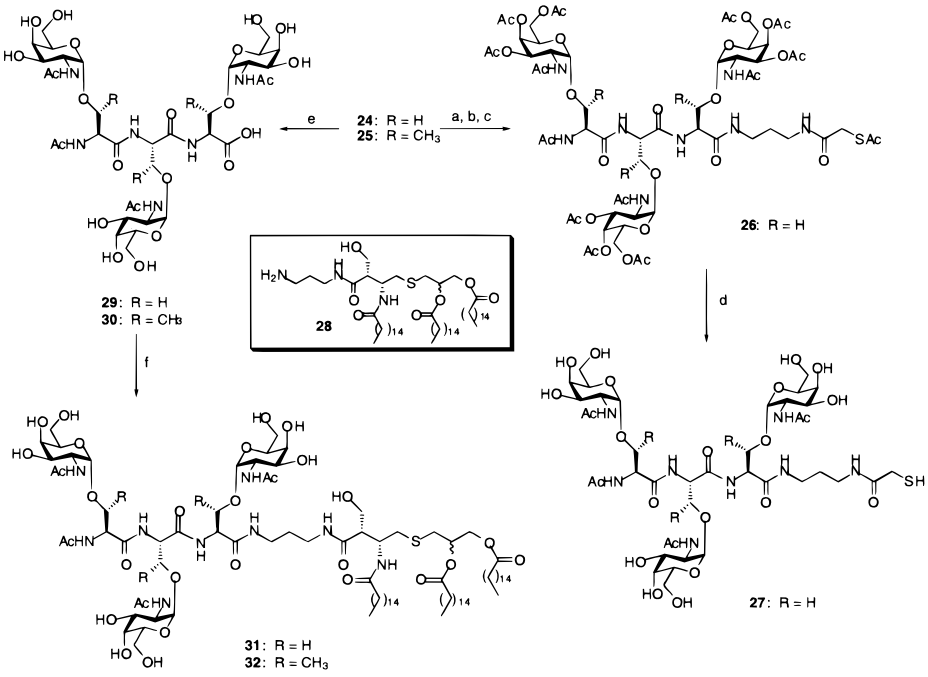
Scheme 4



Reagents: (a) **6/7**, IIDQ, CH_2Cl_2, 85-97%; (b) 20% morpholine in DMF, 90-100%; (c) Ac_2O, CH_2Cl_2, 70-76%; (d) Pd/C, H_2, MeOH, H_2O, 85-95%.

Scheme 5



Reagents: (a) $H_2N(CH_2)_3NHBoc$, IIDQ, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) SAMA-(OPfp), DIEA, CH_2Cl_2, 81%; (d) NaOMe, MeOH (degassed), 85%; (e) NaOH, MeOH, 95%; (f) **29**, NHS, EDC, DMF, DIEA or HOAt, HATu, DMF, collidine, 40%.

of the clustered Tn glycopeptides began with IIDQ-mediated coupling between acid **6** or **7** and amine **8** or **9** to afford the corresponding dipeptides (see **20** and **21**) in 97% yield (Scheme 4). The Fmoc-carbamate was then deprotected with neat morpholine, and the resulting amine was resubjected to a second IIDQ coupling. This step was followed by deprotection and acetyl capping of the N-terminus to afford the tripeptides (see **22** and **23**). The glycosylated tripeptide thus obtained was subjected to hydrogenolysis to afford acids (see **24** and **25**), which could now be further modified and conjugated to either a synthetic lipopeptide as the immunological activator or to an immunogenic carrier protein.

As shown in Scheme 5, two pathways were followed for eventual conjugation. The first involved attachment of a suitable linker to conjugate with a carrier protein. The mercaptoacetamide unit has proven to be effective for this purpose.[21] Acid **24** was coupled with *tert*-butyl-*N*-(3-aminopropyl)carbamate *via* the agency of IIDQ. This step was followed by removal of the BOC cap with TFA. The resulting amine was then coupled with *S*-acetylthioglycolic acid pentafluorophenyl ester (SAMA-OPfp, NovaBiochem) in the presence of Hunig's base in 81% two-step yield. The resulting fully protected glycopeptide **26** was then subjected to methanolysis under carefully controlled conditions (pH ~9, degassed MeOH) to give **27** in 85% yield. The latter was now ready to be conjugated to the appropriate carrier protein.

For the synthesis of a fully synthetic lipopeptide, we followed Toyokuni's method attaching tripalmitoyl-*S*-glycerylcysteinylserine (Pamcys).[22] Pamcys has proven to be a potent macrophage and B lymphocyte activator, and has been used for purposes similar to ours by Tokoyuni with one to three epitopes of serine Tn.[23] First, careful saponification of **24** or **25** with NaOMe/MeOH gave the fully deprotected glycopeptide **29** or **30** in 95% yield. Coupling with amine **28** using either the NHS or HOAt/HATU method[24] afforded glycolipid **31** or **32** in ca. 40% yield. Fortunately, the starting materials for this reaction can be recovered.

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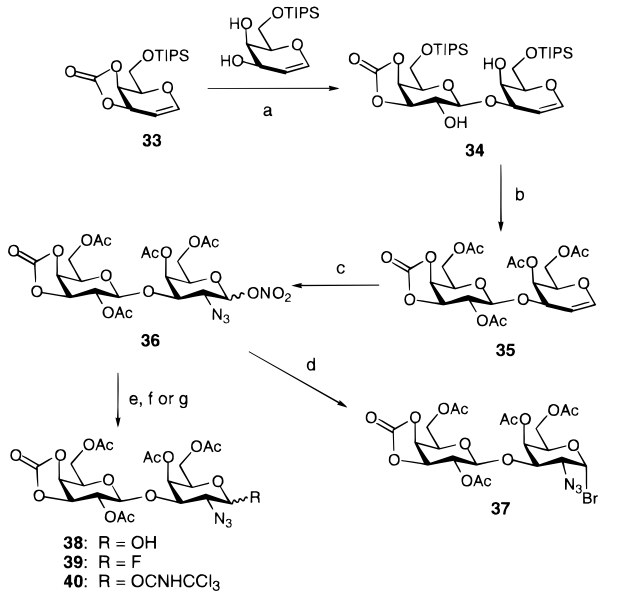
Scheme 6



Reagents: (a) i) DMDO, CH_2Cl_2, 0°C; ii) $ZnCl_2$, THF, -78°C to rt overnight, 80%; b) i) TBAF-HOAc, THF, ii) Ac_2O, DMAP, TEA, 87%; c) NaN_3, CAN, CH_3CN, -15°C, 1 hr, 67%; d) LiBr, CH_3CN, 88%; e) PhSH, DIEA, CH_3CN, 0°C, 1hr, 85%; f) K_2CO_3, Cl_3CCN, CH_2Cl_2, rt, 17h, 92%; g) DAST, THF, -40°C, 1 hr, 94%

Synthesis of Clustered TF Antigen. We then turned to the preparation of the TF disaccharide using the logic of glycal assembly based upon our experience with 1,2-anhydro sugars derived from protected galactal. The epoxide derived from treatment of **33** with DMDO in CH_2Cl_2 served as an excellent β-galactosyl donor with 6-TIPS galactal to afford disaccharide **34** in 80% yield[25] (Scheme 6). Related approaches for the synthesis of the TF antigen disaccharide have been reported using glycosyl bromides and fluorides[26] or trichloroacetimidates as the galactosyl donors.[27] At this stage the protecting groups were exchanged from triisopropylsilyl to acetate to afford **35**. The glycal underwent azidonitration[12] efficiently, but with no selectivity, to furnish **36** as a 1:1 mixture of anomers in 67% yield. Azidonitration with the TIPS group present at the 6 and 6′ positions gave a substantially lower yield (~30%) with significant byproduct formation. The azidonitrate was converted directly into the labile glycosyl bromide **37** with LiBr in CH_3CN or reduced by PhSH and DIEA in 91% yield. Treatment of **38** with DAST[28] in THF afforded glycosyl fluoride **39** in 94% yield as a 1:1 mixture of α and β isomers, and trichloroacetimidate formation with Cl_3CCN and K_2CO_3 proceeded smoothly to give **40** as a separable 3:1 mixture of α and β isomers in 92% yield.[29]

At this stage, the use of **37**, **39**, and **40** in glycosylation reactions was investigated. Results are shown in Table 1. Several interesting observations should be noted. Glycosylation with *N*-Fmoc-threonine benzyl ester and bromide **37** afforded **42**, but gave essentially no selectivity with either AgOTf or $AgClO_4$, contrary to our previous observations for the 2,6-ST case.[11] Similarly, poor selectivities have been reported for TF glycosyl bromides and chlorides.[30] Likewise, with both α and β trichloroacetimidates **40**, no meaningful selectivity was observed under a variety of conditions, but yields were generally good to excellent.[31] The glycosyl fluoride **39** also gave no selectivity and a lower yield. In the past, threonine has in many cases been observed to give a much higher α-selectivity than serine. This was not the case for the systems described here. Also to be noted that the use of BF_3 as a promoter, as well as the use of THF as solvent, afforded no product. Another observation revealed a curious substrate effect. Thus, with ether as the solvent, in the case at hand a 3.4:1 β:α ratio was observed. By contrast in the case of the seemingly similar substrate **43** (Figure 4), α-product was favored 3:1 for this LeY-related system.[32] The only positive feature of this study was that the α and β glycosylation products could be separated by careful chromatography.[33]

Plagued by this poor selectivity, the cassette route was mobilized for implementation. The idea was to use a glycosylated amino acid with the required α-O-linkage solidly in place as the glycosyl acceptor. Again, we turned to our glycal assembly methodology to simplify the construction. Thus, the epoxide derived from glycal **44** proved to be a powerful donor in reaction with **19** to afford β-linked disaccharide **45** in 97% yield (Scheme 7). The disaccharide was readily converted to **42** in high overall yield. The use of such acceptors had been previously reported by Paulsen for the synthesis of the TF antigen, using glycosyl bromides[34] or trichloroacetimidates.[35] The Paulsen method is now complemented by the highly efficient approach directly from the glycal. It should be noted that conversion from 6-TIPS to a 6-acetate on the galactal carbonate was necessary for our glycosylation reaction to proceed.

Reductive acetylation of **42** was carried out with thiolacetic acid in 87% yield to afford protected TF antigen **46** (Scheme 8). This step was followed by hydrogenolysis, leading to protected TF acid **47** in quantitative fashion. At this point, we decided to attach the protected diamine linker first, and then to proceed on to the clustering.[36] We did so because diminished yields were encountered for attachment of the linker at a late stage in the synthesis. This coupling to **48** initially failed when mediated by IIDQ, but proceeded in 84% yield with HOAt/HATU and collidine in DMF.[37]

Glycopeptide **48** was then carried through the sequence of deprotections and coupling with **46** (Scheme 9) similar to that with Tn except using the HOAt−HATU coupling method[37] was required as IIDQ was extremely sluggish for this system. In

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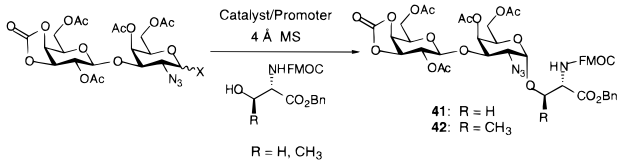
Table 1. [a]



donor X	catalyst/solvent/temp	R = H α:β (yield, %)	R = CH_3 α:β (yield, %)
37 (Br)	AgOTf, CH_2Cl_2, −78 °C to rt		1.2:1 (63)
37 (Br)	$AgClO_4$, CH_2Cl_2, −78 °C to rt		1.1:1 (73)
40 (α-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −30 °C	1:1 (80)	2:1 (80)
40 (β-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −30 °C	1:1.3 (77)	1.2:1 (72)
40 (β-TCA)	TMSOTf (0.5 equiv), CH_2Cl_2, −50 °C		1:1 (84)
40 (β-TCA)	TMSOTf (0.5 equiv), Et_2O/CH_2Cl_2 (12:1), −30 °C		1:3.4 (75)
39 (β/α-F)	Cp_2ZrCl_2, $AgClO_4$, CH_2Cl_2, −78 °C to rt		1:1 (50)

[a] Bromide: Donor **37** (1.1 equiv) was added over 30 min to acceptor, catalyst (1.5 equiv), and 4 Å molecular sieves at −78 °C and allowed to warm to rt overnight. TCA: Donor **40**, acceptor (1.5 equiv), and 4 Å molecular sieves were added in the indicated solvent, and TMSOTf was added at the noted temperatures for 1 h. Fluoride: Donor **37** (1.1 equiv) was added over 30 min to acceptor, catalysts (1.5 equiv of each), and 4 Å molecular sieves at −78 °C and allowed to warm to rt overnight. All yields and ratios are for isolated products.

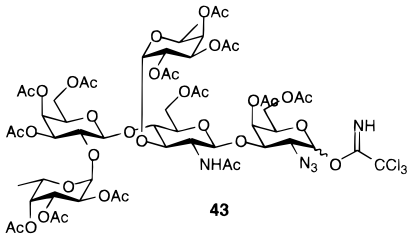
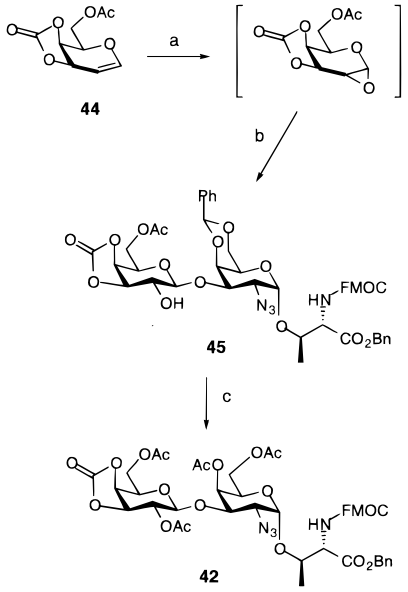


Figure 4.

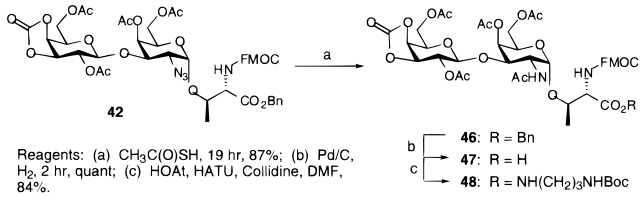
Scheme 7



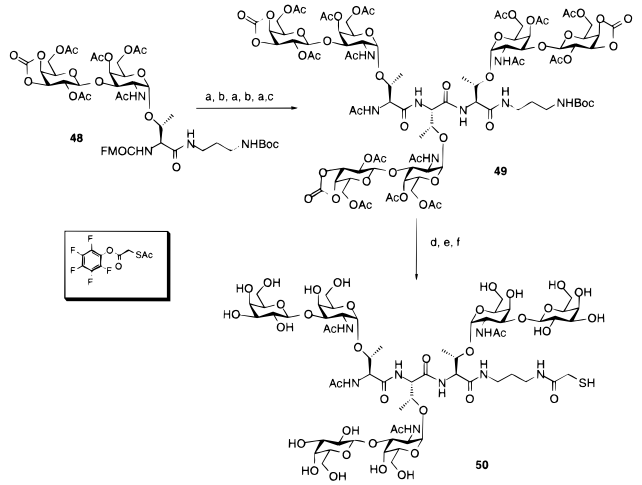
addition, deprotection of the FMOC was facilitated using KF in DMF with catalytic amount of 18-crown-6 as morpholine in DMF showed a proclivity to attack the 3,4-carbonate.[38] After removal of the Boc group of **49** with TFA, coupling with SAMA−OPfp followed by NaOMe/MeOH deprotection afforded **50** in 60% yield for the three steps.

(38) Jiang, J.; Li, W.-R.; Joullie, M. *Synth. Commun.* **1994**, *24*, 187−195.

Scheme 8



Scheme 9



Discussion of Immunological Result

The initial experiments were to evaluate the antibody response to vaccination of mice with either Tn(c) lipopeptide **30** or more conventional Tn(c)−KLH or −BSA conjugates. The preparation of these conjugates started with the previously described **27**, which was covalently linked with carrier proteins, keyhole limpet hemocyanin (KLH), or bovine serum albumin (BSA), using MBS (*m*-maleimimidobenzoyl-*N*-hydroxysuccinamide ester), a heterobifunctional reagent which cross-links thiol groups with amino groups (Scheme 10). For KLH, about 317 clusters per protein were introduced, while BSA showed only 7 clusters per protein.

These conjugates plus the adjuvant QS-21 or Tn(c)−pam (**30**) in intralipid and **30** in intralipid plus QS-21 were used to

Scheme 10

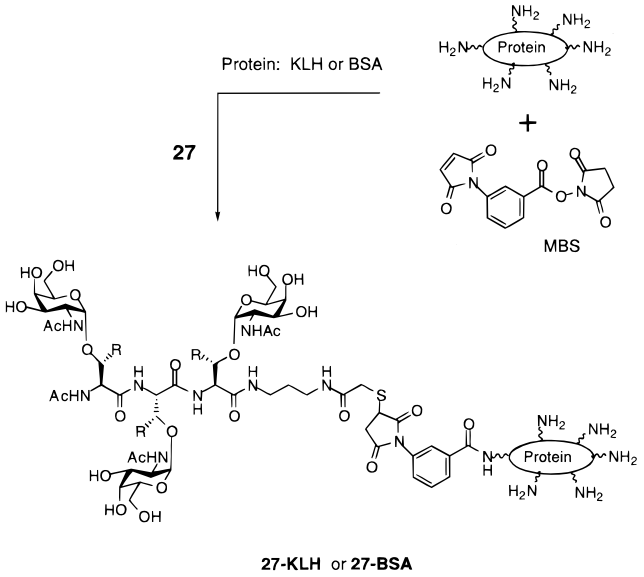


Table 2. ELISA Antibody Titers against Tn(c)[a]

	prevaccination		after third vaccination	
vaccine	IgM	IgG	IgM	IgG
30	0	0	1350	150
30 + QS-21	0	0	1350	50
27−KLH	0	0	**12150**	**450**
27−BSA	0	0	1350	150

[a] All titers are medians for groups of five mice.

vaccinate groups of five mice. All of these constructs proved to be immunogenic. There were no detectable anti-Tn(c) IgM or IgG antibodies present prior to vaccination. The median IgM and IgG ELISA titers against Tn(c)−pam in sera from the five groups of mice immunized with **27**- or **30**-conjugated vaccines at three weeks are shown in Table 2. Sera of mice immunized with 10 μg of **30** in conjunction with 10 μg of QS-21 failed to show strong reaction. In contrast to the case with **30**, construct **27** conjugated with KLH or BSA induced high IgM and moderate IgG titers. The highest titers were elicited by the KLH vaccine. IgM antibody titers remained higher than IgG titers at most time points, including the aftermath of two booster immunizations. In general, titers were no higher after the booster immunizations than after the initial immunizations.

Immunogenic protein carriers[39] have been among the most well studied approaches to the problem of increasing immunogenicity of carbohydrate antigens. Such protein conjugate vaccines have elicited high IgM and moderate IgG titer antibodies against the colon ganglioside GM2 in clinical trials.[40] The studies here with Tn(c) vaccines were patterned after previous studies with ganglioside vaccines, and the results were similar. Of the five carriers and many adjuvants tested, KLH was found to be the most effective carrier and QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria* Molina, the most effective adjuvant. KLH conjugation plus the use of QS-21 as adjuvant was also the approach found optimal previously for augmenting the immunogenicity of ganglioside GD3 and GM2.[41]

Table 3. Tn Cluster FACS Analysis: Serum Tested 11 Days Post Third Vaccination

group	IgG (tested 1/9/98) % positive cells	IgM (tested 1/12/98) % positive cells
30	46.99	39.98
30 + QS-21	12.00	46.41
27−KLH + QS-21	94.72	49.54
27−BSA + QS-21	92.14	51.89

The cell surface reactivity of anti-Tn(c) antibodies was evaluated using Tn(c) positive LS-C colon cancer cells and Tn(c) negative LS-B colon cells. Measurements involved flow cytometry assays and complement dependent cytotoxicity (CDCX) assays. The median percent positive cells by flow cytometry with sera from mice **30** or **30** with QS-21-vaccinated mice was low. However, sera from mice vaccinated with **27**−KLH or **27**−BSA with QS-21 showed clear IgM reactivity with LS-C colon cancer cells by flow cytometry (Table 3). IgG reactivity was also seen by flow cytometry.

Important characteristics of the antibody response to immunization with **27**−KLH conjugate plus QS-21 included the pattern of antibody titers and the specificity of the antibodies. IgM antibody titers were significantly higher than IgG at most time points, despite repeated booster immunizations. There was evidence for neither an IgM to IgG class switch nor a secondary antibody response, all consistent with the T cell independent antibody responses characteristic of most carbohydrate antigens. Specificity analysis of the IgM antibody responses using inhibition assays demonstrated that the response was polyclonal with antibody subpopulations recognizing several different clustered epitopes. Tn(c) expressed on cells was readily recognized by the induced antibodies, resulting in complement activation and lysis of Tn(c) positive tumor cells. More detailed study on the immunology of these vaccines will be described elsewhere.

Summary

The synthesis and clustering of Tn and Tf antigens has been achieved. The application of glycal assembly and further development of the "cassette" methodology has laid the foundation for application to more complex O-linked glycopeptide synthesis. This report confirms the immunogenicity of such fully synthetic and characterized complex synthetic carbohydrate antigens for use in vaccines. It provides a basis for the synthesis and testing of other complex carbohydrate vaccines such as TF.[42] On the basis of these observations we have initiated clinical trials with the **27**−KLH with QS-21 vaccine in patients with Tn(c) positive cancers. Early indication suggests that the synthetic vaccines are well tolerated. Immunological and clinical data will be forthcoming in due course.

Experimental Section

Materials. A cysteine group was introduced to facilitate conjugation with protein carriers. QS-21[43] was obtained from Aquila Biopharmaceutical, Inc. (Worcester, MA). Keyhole limpet hemocyanin (KLH) was obtained from PerImmune Inc. (Rockville, MD). Bovine serum

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(41) Livingston, P. O.; Wong, G. Y. C.; Adluri, S.; Tao, Y.; Padavan, M.; Parente, R.; Hanlon, C.; Calves, M. J.; Helling, F.; Ritter, G.; Oettgen, H. F.; Old, L. J. *J. Clin. Oncol.* **1994**, *12*, 1036.

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albumin (BSA) and sodium cyanoborohydride were obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibody IE3 (mAb IE3) was kindly provided by Dr. Singhal. Goat anti-mouse IgG and IgM conjugated with alkaline phosphatase and goat anti-mouse IgM fluorescence isothiocyanate (FITC) were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL). Female CB6F1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

General Procedure for Glycosylation of 1. A flame-dried flask was charged with silver perchlorate (930 mg, 2 equiv), 4.0 g of 4 Å molecular sieves, and *N*-Fmoc-L-threonine benzyl ester (1.21 g, 2.8 mmol, 1.2 equiv) in a drybox. A 30 mL sample of dry CH_2Cl_2 was added to the flask, and the mixture was stirred at rt for 10 min. Donor **1** (1.13 g, 2.87 mmol) in 16 mL of CH_2Cl_2 was added slowly over 30 min via syringe. The reaction was stirred under argon atmosphere at rt overnight. The mixture was then diluted with CH_2Cl_2 and washed twice with water. The solution was dried over Na_2SO_4 and evaporated, and the crude material (a 4:1 mixture of α/β isomers) was purified on a silica gel column (1−1.5−2−2.5% MeOH/CH_2Cl_2) to provide **3** (1.28 g, 60% yield).

Compound 2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.28−7.42 (m),), 5.94 (d, J = 8.0 Hz, 1H), 5.39 (d, J = 2.6 Hz, 1H), 5.21−5.27 (m), 4.86 (d, J = 3.4, 1H), 4.58−4.62 (m, 1H), 4.40 (d, J = 7.2 Hz, 2H), 4.24 (t, J = 7.1, 1H), 4.16 (dd, J = 3.0, 10.9 Hz, 1H), 3.50−4.08 (m), 3.58 (dd, J = 3.5, 11.2 Hz, 1H),), 2.14 (s, 3H), 1.96 (s, 3H). All data are in agreement with literature reports.[14]

Compound 3. Compound **15** (0.58 g, 0.94 mmol) was taken up in 5.0 mL of acetic anhydride, and then 1.0 mL of pyridine was added. The solution was stirred at ambient temperature for 1 h and then partitioned cautiously between 50 mL of EtOAc and 50 mL of 1 N HCl(aq). The phases were separated, and the organic phase was washed with saturated $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated. Flash chromatography of the residue (2:1 hexanes/EtOAc) furnished 0.63 g (90%) of **3** as a colorless foam: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.6 Hz, 2H), 7.27−7.42 (m), 5.66 (d, J = 9.5 Hz, 1H), 5.44 (br s, 1H),), 5.25−5.31 (m),), 5.37 (d, J = 13.2, 1/2AB, 1H), 5.20 (d, J = 12.1 Hz, 1/2AB, 1H), 4.90 (d, J = 3.6 Hz, 1H), 4.40−4.51 (m), 4.31−4.38 (m), 4.18−4.28 (m), 3.70 (d, J = 6.5 Hz, 2H), 3.58 (dd, J = 3.7, 11.2 Hz, 1H), 2.16 (s, 3H), 2.04 (s, 3H), 1.34 (d, J = 6.3 Hz, 3H). All data are in agreement with literature reports.[14]

Compound 4. To a round-bottom flask were added α-glycoside **2** (1.25 g, 1.71 mmol), pyridine (2 mL), and thiolacetic acid (2 mL) at 0 °C subsequently. The reaction was stirred from 0 °C to room temperature overnight. After evaporation of the solvent by a flow of air, the residue was separated by chromatography to give desired product (1.13 g, 88%): $[\alpha]_D^{20}$ +61.0° (*c* 0.68, $CHCl_3$); IR (film) 3346, 3018, 2954, 1748, 1732, 1715, 1668, 1557, 1538 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, J = 7.5 Hz, 2H), 7.56 (d, J = 7.5 Hz, 2H), 7.25−7.45 (m, 9H), 6.06 (d, J = 7.8 Hz, 1H), 5.74 (d, J = 8.8 Hz, 1H), 5.30 (d, J = 3.0 Hz, 1H), 5.16 (m, 2H), 5.03 (dd, J = 11.2, 3.0 Hz, 1H), 4.76 (br s, 1H), 4.59 (m, 1H), 4.49 (m, 1H), 4.40 (d, J = 6.8 Hz, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.92-4.15 (m, 5H), 2.12 (s, 3H), 1.96 (s, 3H), 1.92 (s, 3H), 1.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 170.7, 170.2, 170.1, 170.0, 168.8, 155.8, 143.6, 141.2, 134.7, 128.7, 128.3, 127.7, 127.0, 124.9, 120.0, 99.1, 69.8, 68.1, 67.6, 67.2, 61.9, 54.6, 47.6, 47.0, 23.1, 20.6, 20.5; HRMS (FAB) calcd for $C_{39}H_{43}N_2O_{13}$ $[M + H]^+$ 747.2765, found 747.2766.

Compound 5. Compound **3** (0.40 g, 0.54 mmol) was taken up in 5.0 mL of thiolacetic acid and the solution stirred for 20 h at ambient temperature. The mixture was concentrated with a N_2 flow and the residue purified by flash chromatography (1:1 hexanes/EtOAc) to give 0.31 g (76%) of **5** as a colorless oil: $[\alpha]_D^{23}$ + 49.6° (*c* 1.26, $CHCl_3$); ^{1}H NMR ($CDCl_3$, mixture of rotamers) (major rotamer) δ 7.76 (d, J = 7.4 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.33 (m, 9H), 5.78 (m, 1H), 5.60 (m, 1H), 5.36 (br s, 1H), 5.18 (d, J = 11.8 Hz, 1H), 5.06 (d, J = 11.8 Hz, 1H), 4.78 (d, J = 3.1 Hz, 1H), 4.52 (m, 1H), 4.44 (m, 3H), 4.21 (m, 3H), 4.05 (m, 3H), 2.15 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H), 1.29 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.09, 171.01, 170.53, 170.47, 156.71, 143.84, 141.45, 134.52, 129.08, 129.02, 128.73, 127.95, 127.27, 125.33, 125.21, 120.19, 100.03, 77.14, 68.56, 68.00, 67.50, 67.33, 62.36, 58.88, 47.62, 47.28, 23.37, 20.87, 20.77, 18.38; IR (neat) 3332, 2978, 1748, 1677 cm^{-1}; HRMS calcd for $C_{40}H_{44}N_2O_{13}Na$ 783.2741, found 783.2756.

General Procedure B for Deprotection of Benzyl Esters. To a solution of **5** (0.46 g, 0.60 mmol) in 150 mL of methanol and 10 mL of H_2O was added 0.14 g of 5% Pd/C. The system was evacuated and purged 5× with H_2 and then placed under 1 atm of H_2 for 90 min. The suspension was gravity filtered and concentrated. Flash chromatography of the residue (10 → 15 → 20% MeOH/CH_2Cl_2) yielded 0.37 g (92%) of **7** as a colorless crystalline solid.

Compound 7. $[\alpha]_D^{23}$ + 90.6° (*c* 1.55, $CHCl_3$); ^{1}H NMR ($CDCl_3$) mixture of rotamers; spectra available in Supporting Information, confirmed by variable temperature NMR; ^{13}C NMR ($CDCl_3$, mixture of rotamers) δ 173.78, 173.51, 172.42, 171.52, 171.28, 170.58, 170.27, 170.22, 170.00, 157.73, 156.46, 143.51, 143.47, 143.21, 143.13, 141.24, 141.19, 141.16, 127.77, 127.69, 127.66, 127.12, 127.04, 126.96, 125.10, 124.77, 124.53, 124.46, 119.92, 119.85, 99.48, 98.71, 77.71, 77.20, 75.73, 68.01, 67.53, 67.26, 67.22, 66.96, 66.90, 62.15, 61.96, 58.61, 48.37, 47.77, 47.16, 46.84, 22.82, 22.12, 20.75, 20.62, 20.58, 20.54, 20.51, 18.41, 18.12; IR (neat) 3344, 2938, 1748, 1726 cm^{-1}; HRMS calcd for $C_{33}H_{38}N_2O_{13}Na$ 693.2272, found 693.2298.

General Procedure A for Deprotection of FMOC-carbamates. Compound **5** (0.18 g, 0.24 mmol) was taken up in 3.0 mL of morpholine and the solution stirred at ambient temperature for 30 min. Excess morpholine was then removed by azeotroping with dry toluene under reduced pressure (3 × 4 mL). Flash chromatography of the residue (4 → 7.5 → 10% MeOH/CH_2Cl_2) provided 0.11 g (86%) of amine **9** as a coloroless foam.

Compound 8. To a round-bottom flask were charged **4** (330 mg, 0.44 mmol) and morpholine (3 mL) at 0 °C. The reaction mixture was stirred at 0 °C to rt for 3 h. After evaporation of the solvent by a flow of air, the residue was separated by chromatography (4 → 7.5 → 10% MeOH/CH_2Cl_2) to give the desired amine (230 mg, 99%): $[\alpha]_D^{20}$ +62.0° (*c* 1.46, $CHCl_3$); IR (film) 3310, 2960, 1751, 1734, 1654, 1542 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31−7.35 (m, 5H), 5.86 (d, J = 9.8 Hz, 1H), 5.30 (d, J = 2.3 Hz, 1H), 5.15 (d, J = 11.5 Hz, 1H), 5.12 (d, J = 11.5 Hz, 1H), 5.05 (dd, J = 11.4, 3.0 Hz, 1H), 4.78 (d, J = 3.6 Hz, 1H), 4.53 (m, 1H), 4.01−4.12 (m, 3H), 3.90 (dd, J = 9.8, 3.6 Hz, 1H), 3.66−3.70 (m, 2H), 2.12 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.92 (s, 3H), 1.80 (m, 2H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 173.5, 170.8, 170.3, 170.2, 170.1, 135.2, 128.8, 128.7, 128.3, 98.8, 71.0, 68.4, 67.3, 61.9, 54.8, 47.7, 23.1, 20.7, 20.6; HRMS (FAB) calcd for $C_{24}H_{33}N_2O_{11}$ $[M + H]^+$ 525.2070, found 525.2090.

Compound 9: $[\alpha]_D^{23}$ +55.9° (*c* 1.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.34 (m, 5H), 6.01 (d, J = 9.5 Hz, 1H), 5.34 (d, J = 2.5 Hz, 1Hz), 5.16 (d, J = 12.0 Hz, 1H), 5.05 (d, J = 12.1 Hz, 2H), 4.77 (d, J = 3.7 Hz, 1H), 4.50 (m, 1H), 4.21 (m, 1H), 4.05 (m, 3H), 3.42 (br s, 1H), 2.13 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.93 (s, 3H), 1.65 (br s, 2H), 1.33 (d, J = 6.4 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 174.16, 170.68, 170.19, 134.75, 128.66, 128.43, 99.75, 78.03, 68.53, 67.28, 67.11, 67.04, 62.01, 59.06, 47.50, 22.99, 20.61, 20.49, 18.09; IR (neat) 3385, 2976, 1745, 1666 cm^{-1}; HRMS calcd for $C_{25}H_{34}N_2O_{11}Na$ 561.2060, found 561.2069.

Compound 18. To a solution of **14** (0.24 g, 0.40 mmol) in 5.0 mL of nitromethane was added benzaldehyde dimethyl acetal (0.11 mL, 0.79 mmol), followed by *p*-toluenesulfonic acid monohydrate (4.0 mg, 0.02 mmol). The mixture was stirred for 1 h at ambient temperature at which time an additional 0.11 mL of $PhCH(OMe)_2$ and 4.0 mg of TsOH were added. Stirring was continued for 2 h, and the mixture was neutralized with 5 drops of Et_3N and concentrated. Flash chromatography of the residue (2:1 hexanes/EtOAc) afforded 0.26 g (94%) of **18** as a colorless foam: $[\alpha]_D^{23}$ +79.9° (*c* 1.35, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.58 (m, 2H), 7.46 (m, 2H), 7.31−7.42 (m, 7H), 5.89 (d, J = 8.0 Hz, 1H), 5.50 (s, 1H), 5.25 (d, J = 12.1 Hz, 1H), 5.20 (d, J = 12.1 Hz, 1H), 4.90 (d, J = 3.2 Hz, 1H), 4.57 (m, 1H), 4.44 (dd, J = 10.5, 7.1 Hz, 1H), 4.32 (dd, J = 10.4, 7.3 Hz, 1H), 4.21 (app t, J = 7.2 Hz, 1H), 4.12−4.18 (m, 3H0, 3.98 (m, 2H), 3.86 (d, J = 12.7 Hz, 1H), 3.56 (br s, 1H), 3.49 (dd, J = 10.6, 3.3 Hz, 1H), 2.38 (d, J = 10.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 169.67, 155.85, 143.72, 143.58, 141.25, 137.17, 134.99, 129.31, 128.62, 128.53, 128.27, 127.74, 127.09, 127.05, 126.11, 125.07, 124.95, 120.02, 101.19, 99.97, 75.18, 69.81, 68.92, 67.77, 67.26, 66.93, 63.21, 60.41,

54.58, 46.97; IR (neat) 3423, 2921, 2109, 1721 cm^{-1}; HRMS calcd for $C_{38}H_{36}N_4O_9Na$ 715.2380, found 715.2387.

Compound 19. **19** was prepared as described for compound **18** and obtained in 73% yield from **15** as a colorless solid: $[\alpha]_D^{23}$ +90.4° (c 1.17, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.48 (m, 2H), 7.31−7.39 (m, 7H), 5.79 (d, J = 9.3 Hz, 1H), 5.54 (s, 1H), 5.23 (s, 2H), 4.93 (d, J = 3.4 Hz, 1H), 4.47 (m, 3H), 4.34 (dd, J = 10.5, 7.6 Hz, 1H), 4.23 (m, 3H), 4.09 (ddd, J = 13.3, 10.4, 3.1 Hz, 1H), 4.02 (d, J = 12.1 Hz, 1H), 3.68 (s, 1H), 3.53 (dd, J = 10.5, 3.4 Hz, 1H), 2.53 (d, J = 10.6 Hz, 1H), 1.29 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 170.06, 156.74, 143.86, 143.63, 141.24, 141.21, 137.19, 134.95, 129.35, 128.61, 128.51, 128.29, 127.67, 127.07, 127.05, 126.15, 125.16, 125.10, 119.92, 101.16, 99.20, 76.20, 75.24, 69.02, 67.70, 67.38, 67.28, 63.18, 61.03, 58.71, 47.08, 18.63; IR (neat) 3427, 2921, 2110, 1725 cm^{-1}; HRMS calcd for $C_{39}H_{38}N_4O_9Na$ 729.2537, found 729.2561.

General Procedure C for Peptide Couplings Using IIDQ. To a solution of amine **9** (0.11 g, 0.20 mmol) in 3.0 mL of CH_2Cl_2 at 0 °C was added quickly a solution of acid **7** (0.15 g, 0.23 mmol) in 3.0 mL of CH_2Cl_2. Immediately following the addition, IIDQ (74 mg, 0.25 mmol) in 1.0 mL of CH_2Cl_2 was added quickly dropwise. The mixture was allowed to warm slowly to ambient temperature and stirred for 24 h. The solvent was evaporated with an N_2 flow, and flash chromatography of the residue (EtOAc) afforded 0.23 g (97%) of dipeptide **21** as colorless crystals.

Compound 25. Benzyl ester **23** (0.21 g, 0.15 mmol) was deprotected according to general procedure B. Filtration and concentration of the crude reaction mixture provided 0.19 g (96%) of pure free acid **25** as a colorless crystalline solid: $[\alpha]_D^{23}$ +103.5° (c 1.76, $CHCl_3$); ^1H NMR (CD_3OD) δ 8.40 (d, J = 9.0 Hz, 1H), 8.31 (d, J = 9.2 Hz, 1H), 5.40 (m, 3H), 5.15 (m, 4H), 4.99 (d, J = 3.8 Hz, 1H), 4.91 (J = 3.7 Hz, 1H), 4.78 (d, J = 2.1 Hz, 1H), 4.70 (m, 1H), 4.62 (m, 1H), 4.31−4.46 (m, 10H), 4.11 (m, 6H), 2.14 (s, 3H), 2.13 (s, 3H), 2.13 (s, 3H), 2.12 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.93 (s, 3H), 1.93 (s, 3H), 1.38 (d, J = 6.2 Hz, 3H), 1.32 (m, 6H); ^{13}C NMR (CD_3OD) δ 173.83, 173.51, 173.49, 173.33, 173.16, 172.54, 172.44, 172.09, 172.06, 171.94, 101.25, 100.83, 100.45, 79.62, 77.86, 77.50, 70.45, 70.13, 69.77, 68.92, 68.87, 68.31, 68.25, 63.36, 63.14, 58.22, 58.14, 58.01, 23.41, 23.13, 23.08, 22.50, 20.74, 20.70, 20.66, 20.62, 20.58, 19.54, 19.35, 19.21; IR (neat) 3324, 2981, 1748, 1658 cm^{-1}; HRMS calcd for $C_{56}H_{82}N_6O_{32}Na$ 1373.4871, found 1373.4856.

Compound 26. Boc-protected 1,3-diaminopropane (12.1 mg, 0.07 mmol) in 1 mL of CH_2Cl_2 (anhydrous) was added to a flask charged with glycopeptide **24** (70 mg, 0.05 mmol). While the solution was being stirred, IIDQ (21.2 mg, 0.07 mmol) in 0.8 mL of CH_2Cl_2 was added at 0 °C. After 5 min the reaction was removed from the bath and stirred for 48 h. Longer reaction times are required for this coupling. The reaction mixture was purified directly on a silica column (7% MeOH/CH_2Cl_2). The product was dissolved in 0.2 mL of CH_2Cl_2, and 0.6 mL of CF_3COOH was added. The reaction was complete in 30 min. The solvent was removed with a stream of nitrogen, and the material was dried *in vacuo*. The crude product was dissolved in a 1:1 mixture of tBuOH/AcOH and lyophilized. To a solution of the crude ammonium salt (27 mg, 0.026 mmol) was added pentafluorophenyl acetylmercaptoacetate (9.1 mg, 1.5 equiv) in 0.7 CH_2Cl_2 and $(iPr)_2NEt$ (7.3 μL, 0.04 mmol). The mixture was purified by a silica column (3−4−5−6−7% MeOH, CH_2Cl_2) to yield pure product **26** (27 mg, 70% yield): ^1H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, J = 8.3 Hz), 7.72 (d, J = 7.9 Hz), 7.39−7.59 (m), 7.22 (d, J = 9.5 Hz), 7.10 (d, J = 8.9 Hz), 6.70−6.82 (m), 5.32−5.40 (m), 5.08−5.17 (m), 5.03 (d, J = 2.9 1H), 4.99 (d, J = 3.0 Hz, 1H), 4.97 (d, J = 3.3 Hz, 1H), 4.51−4.70 (m), 4.13−3.91 (m), 3.65−3.72 (m), 3.60 (d, J = 15.0 Hz), 1.95−2.08 (14 acetates), 1.42 (d, J = 7.14), 1.39 (d, J = 6.6 Hz); MS (ES) [M + Na] 1503.6, calcd for $C_{56}H_{84}N_8O_3Na$ 1503.6.

Compound 27. The protected glycopeptide **26** was dissolved in anhydrous MeOH (deoxygenated with a stream of nitrogen/argon in order to prevent oxidative dimerization!). The pH was adjusted to 9−10 with a 25% solution of NaOMe in MeOH, and the mixture was stirred under argon overnight. Acidic Amberlyst resin was then added until the solution became neutral or mildly acidic (pH 5−6). The solvent was removed, and the crude was purified by RP chromatography (C18 silica gel, eluted with H_2O). The solution was lyophilized to yield glycopeptide **27** (85%) as a white solid. Further analysis was renounced to avoid dimerization of the thiol, and the material was stored under Ar at −78 °C: MS (ES) [M + Na] 1083.4, calcd for $C_{40}H_{68}N_8O_{23}SNa$ 1083.4.

Compound 29. To a stirred solution of peracetylated cluster **24**[14] (0.19 g, 0.14 mmol) in 10.0 mL of methanol was added sodium methoxide (25 wt % solution in MeOH) until the pH of the solution reached 9 (ca. 10 drops). Stirring was continued for 16 h, at which time Amberlyst-15 was added to lower the pH of the mixture to ca. 4. The solution was separated from the resin *via* pipet, the resin was rinsed with methanol (3 × 5 mL), and the combined extracts were concentrated. Purification of the residue on LiChroprep RP-18 using 1:1 MeOH/H_2O as eluant furnished 0.13 g (95%) of tripeptide **29** as a colorless crystalline solid. $[\alpha]_D^{23}$ + 160.7° (*c* 1.30, $CHCl_3$); ^1H NMR (CD_3OD) δ 8.32 (J = 8.5 Hz, 1H), 8.24 (d, J = 8.8 Hz, 1H), 7.53 (m, 2H), 7.38 (m, 2H), 4.98 (d, J = 3.2 Hz, 1H), 4.81 (d, J = 3.5 H, 1H), 4.72 (br s, 1H), 4.66 (br s, 1H), 4.48 (s, 1H), 4.16−4.29 (m, 6H), 3.92 (m, 6H), 3.79 (m, 3H), 3.71 (m, 6H), 2.09 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.31 (m, 6H), 1.22 (d, J = 6.3 Hz, 3H); ^{13}C NMR (CD_3OD) δ 174.33, 174.24, 173.79, 172.69, 171.85, 101.09, 100.79, 78.24, 77.66, 77.37, 72.87, 72.81, 70.81, 70.43, 70.31, 62.76, 62.66, 58.58, 58.31, 57.92, 51.68, 51.59, 51.47, 23.48, 23.43, 23.25, 22.49, 19.32, 19.07, 19.03; IR (neat) 3372, 2933, 1633 cm^{-1}; LRMS calcd for $C_{38}H_{64}N_6O_{23}Na$ 995.3920, found 995.3886.

Compound 31. **31** was prepared as described for **32** in 40% yield: MS (ES) [M + Na] 1988.5, calcd for $C_{95}H_{172}N_{10}O_{30}S$ 1988.5. Data are in agreement with literature description.[14]

Compound 32. To a slurry of tripeptide **30** (52 mg, 0.053 mmol) and amine acetate **28** (100 mg, 0.090 mmol) in 4.0 mL of dry DMF at ambient temperature were added HATU (81 mg, 0.21 mmol), HOAt (14.5 mg, 0.11 mmol), and then diisopropylethylamine (37 μL, 0.21 mmol). The mixture was stirred at ambient temperature for 16 h, at which time the DMF was removed *in vacuo*. The residue was rinsed with CH_2Cl_2 (3 × 0.5 mL). The remainder of the residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH/H_2O, 65:25:4) to afford 40 mg (38%) of the lipid conjugate as a colorless amorphous solid. ^1H NMR ($CDCl_3/CD_3OD/D_2O$, 65:25:4) δ 8.37 (d, J = 9.3 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H), 8.03 (d, J = 7.1 Hz, 3H), 7.92 (m, 1H), 7.72 (app t, J = 8.2 Hz, 1H), 7.66 (d, J = 8.8 Hz, 1H), 7.32 (d, J = 9.2 Hz, 1H), 5.19 (m, 1H), 4.87 (d, J = 3.6 Hz, 1H), 4.83 (d, J = 3.6 Hz, 1H), 4.80 (d, J = 3.7 Hz, 1H), 4.67 (m, 2H), 4.57 (m, 2H), 4.13 (m, 6H), 3.89 (br s, 7H), 3.74 (m, 1H), 3.19 (m, 4H), 3.04 (m, 1H), 2.88 (m, 1H), 2.77 (m, 1H), 2.31 (m, 6H), 2.11 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.61 (m, 9H), 1.27 (s, 90H), 1.19 (d, J = 6.1 Hz, 3H), 0.88 (t, J = 6.6 Hz, 9H); LRMS calcd for $C_{98}H_{178}N_{10}O_{30}S$ 2007.2, found 2007.2.

Compound 36. To a solution of protected galactal **35** (1.01 g, 2.01 mmol) in 11 mL of anhydrous CH_3CN at −20 °C was added a mixture of NaN_3 (392 mg, 6.03 mmol) and CAN (3.30 g, 6.03 mmol). The reaction mixture was vigirously stirred at −15 °C for 1 h. Then the reaction mixture was diluted with diethyl ether, and washed with cold water and brine subsequently. Finally, the solution was dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by chromatography on silica gel (50−75% EtOAc/hexanes). A mixture of α and β anomers **36** (2.17 g, 67% yield) was obtained. The ratio of α and β anomers was almost 1:1 based on ^1H NMR: IR (film) 3476, 2963, 2119, 1813, 1747, 1660, 1372, 1227, 1051 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) 6.25 (d, J = 4.2 Hz, 1H, H-1, α anomer), 5.40 (d, J = 8.6 Hz, 1H, H-1, β anomer); MS (EI) calcd 629.2, found 629.2 (M + Na). Copies of ^1H and ^{13}C NMR spectra are available in the Supporting Information.

Bromide 37. A solution of compound **36** (220 mg, 0.363 mmol) in 1.0 mL of dry acetonitrile was mixed with lithium bromide (140 mg, 1.6 mmol, 5 equiv) and stirred at rt for 3 h in the dark. The heterogeneous mixture was diluted with dichloromethane, the solution was washed twice with water and dried over magnesium sulfate, and the solvent was evaporated without heating. After rapid flash chromatography on predried silica gel (EtOAc), α-bromide **37** (200 mg, 88%) was isolated and stored under argon atmosphere at −80 °C to

prevent hydrolysis unitl use: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.16 (d, J = 3.6 Hz, 1H), 5.63 (d, J = 2.8 Hz, 1H), 5.19−5.15 (m, 2H), 4.90−4.81 (m, 2H), 4.41−3.96 (m, 18H); CI (NH_3) MS calcd for (M + H) $C_{21}H_{27}N_3BrO_{14}$ 629, found 629.

Compound 38. To a solution of a mixture of azidonitrates **36** (390 mg, 6.64 mmol) in 3 mL of anhydrous CH_3CN at 0 °C were slowly added Et(*i*-Pr)$_2$N (111 μL, 0.64 mmol) and PhSH (198 μL, 1.9 mmol) subsequently. The reaction mixture was stirred at 0 °C for 1 h, and then the solvent was blown off by an argon flow. The residue was purified by chromatography on silica gel (EtOAc) to give a 1:1 mixture of α and β anomers of **38** (318 mg, 85%) as a white foam: ^{1}H NMR (400 MHz, $CDCl_3$) 5.35 (d, J = 3.4 Hz, 1H, H-1 α anomer), 4.86 (d, J = 8.4 Hz, 1H, H-1, β anomer); MS (EI) calcd 562.2, found 562.2 (M + H); FAB HRMS calcd for (M + Na) $C_{21}H_{27}N_3O_{15}Na$ 584.1340, found 584.1319. Copies of ^{1}H and ^{13}C NMR spectra are available in the Supporting Information.

Fluoride 39. To a solution of **38** (53 mg, 0.081 mmol) in 1 mL of THF was added 20 mL (0.12 mmol, 1.5 equiv) of DAST at −40 °C, and the reaction was allowed to warm to rt over 1 h. The reaction was then quenched with 0.5 mL of MeOH and purified by chromatography on silica gel (50−80% EtOAc/hexane) to afford 25 mg (47%) of **39**α and 26 mg of **39**β (47%). These were used immediately to prevent degradation of the donor. **39**α: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.68 (dd, J = 55, 2.5 Hz, 1H), 5.59 (d, J = 6.4 Hz, 1H), 5.12−5.10 (m, 2H), 4.86 (dd, J = 8.5, 1.2 Hz, 1H), 4.79 (dd, J = 8.5 Hz, 1.2 Hz, 1H), 4.39−4.17 (m, 4H), 4.13 (br t, J = 5.7 Hz, 1H), 4.06 (dd, J = 10.5, 2.9 Hz, 1H), 3.96−3.87 (m, 2H), 2.20 (s, 3 H), 2.15 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H); ^{19}F NMR (421 MHz, $CDCl_3$) δ 14.2 (dd, J = 55, 26.5, 1F). **39**β: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (br t, 1H), 5.13−5.06 (m, 2H), 4.94 (d, J = 7.6 Hz, 1H), 4.87 (dd, J = 8.6, 1.6 Hz, 1H), 4.80 (dd, J = 8.6, 1.6 Hz, 1H), 4.40−4.20 (m, 4H), 4.10 (dt, J = 6.5, 1.4 Hz, 1H), 3.98 (dd, J = 11.7, 1.6 Hz, 1H), 3.86−3.79 (m, 2H), 3.56 (dd, J = 10.3, 3.6 Hz, 1H), 2.21 (s, 3H), 2.18 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H); ^{19}F NMR (421 MHz, $CDCl_3$) δ 23.5 (dd, J = 54.8, 14.2, 1F): CI (NH_3) MS calcd for (M + H) $C_{21}H_{26}FN_3O_{14}$ 564, found 564.

Trichloroacetimidate 40. To a solution of **38** (327 mg, 0.58 mmol) in 5 mL of CH_2Cl_2 at 0 °C were added K_2CO_3 (300 mg) and Cl_3CCN (0.58 mL, 5.8 mmol). The reaction mixture was stirred at 0 °C to room temperature overnight. The suspension was filtered through a pad of Celite and washed with CH_2Cl_2. The filtrate was evaporated, and the residue was purified by chromatography on silica gel (40−80% EtOAc/hexane) to give α-trichloroacetimidate **40**α (94 mg, 22%) and β-trichloroacetimidate **40**β (270 mg 72%) as white foams. **40**β: $[\alpha]_{20}^{D}$ +36.8° (*c* 0.28, $CHCl_3$); IR (film) 3318, 2962, 2116, 1750, 1679, 1370, 1224, 1071 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (br s, 1H), 5.58 (d, J = 8.6 Hz, 1H), 5.47 (d, J = 3.2 Hz, 1H), 5.10−5.05 (m, 2H), 4.85−4.80 (m, 2H), 4.37 (dd, J = 10.2, 6.7 Hz, 1H), 4.28 (dd, J = 10.0, 6.6 Hz, 1H), 3.99−3.89 (m, 7H), 3.62 (dd, J = 10.3, 3.3 Hz, 1H), 2.21 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0, 170.3, 170.2, 169.9, 168.6, 160.6, 152.9, 100.1, 96.4, 90.1, 79.5, 73.0, 72.6, 72.3, 68.3, 67.6, 67.5, 62.1, 61.6, 60.2, 20.8, 20.6, 20.5, 14.0. **40**α: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (br s, 1H), 6.45 (br s, 1H), 5.61 (br s, 1H), 5.13 (br t, 2H), 4.87 (d, J = 8.5 Hz, 1H), 4.85 (d, J = 8.5 Hz, 1H), 4.37−4.08 (m, 16H), 3.86 (dd, J = 8.2, 4.1 Hz, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.07 (s, 3H), 1.99 (s, 3H); FAB HRMS calcd for (M + Na) $C_{23}H_{27}N_4O_{15}C_{l3}Na$ 727.0436, found 727.0446.

General Procedure for Glycosylation with Glycosyl Bromide. A flame-dried flask was charged with silver perchlorate (185 mg, 0.896 mmol), 300 mg of 4 Å molecular sieves, and *N*-Fmoc-L-threonine benzyl ester (283 mg, 0.672 mmol, 1.5 equiv) in a glovebag. Then, 2.5 mL of CH_2Cl_2 was added to the flask, and the mixture was stirred at rt for 10 min. Donor **37** (280 mg, 0.448 mmol) in 2.5 mL of CH_2Cl_2 was added slowly over 1 h. The reaction was stirred under argon atmosphere at rt for 2 h. The mixture was then diluted with CH_2Cl_2 and filtered through Celite. The precipitate was thoroughly washed with CH_2Cl_2, the filtrate was evaporated, and the crude material was purified on silica gel chromatography (50−80% EtOAc/hexane) to provide **42**α (180 mg, 39%) and **42**β (161 mg, 34%).

General Procedure for Glycosylation with Glycosyl Trichloroacetimidates. A flame-dried flask was charged with donor **40** (98 mg, 0.139 mmol), *N*-Fmoc-L-threonine benzyl ester (70 mg, 0.167 mmol), and 200 mg of 4 Å molecular sieves in a glovebag. The mixture was dissolved in 6 mL of dry CH_2Cl_2. The reaction mixture was cooled to −30 °C, trimethylsilyl triflate (14 μL, 0.5 equiv) was added, and the mixture was stirred until completion judged by TLC and then quenched with TEA. The mixture was then directly separated by flash chromatography on silica gel (50−80% EtOAc/hexane) to yield α-product **42**α (56 mg, 42%) and β-product **42**β (57 mg, 42%).

General Procedure for Glycosylation with Glycosyl Fluorides. A flame-dried flask was charged with *N*-Fmoc-L-threonine benzyl ester (28 mg, 1.5 equiv), Cp_2ZrCl_2 (12.9 mg, 0.044 mmol), $AgClO_4$ (18.2 mg, 0.088 mmol), and 200 mg of 4 Å molecular sieves in a glovebag. The mixture was cooled to −30 °C, and 1 mL of CH_2Cl_2 was added. Then, donor **39** (25 mg, 0.044 mmol) in 1 mL of CH_2Cl_2 was added dropwise, and the reaction was stopped after judged complete by TLC (1 h). Purification by silica gel chromatography yielded α-product **42**α (10 mg, 25%) and β-product **42**β (10 mg, 25%).

Compound 42α: $[\alpha]^{20}_D$ +61.44° (*c* 0.5, $CHCl_3$); IR (film) 3434, 3362, 3065, 2956, 2114, 1815, 1746, 1514, 1371, 1077 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, 2H), 7.67 (d, 2H), 7.47−7.33 (m, 9H), 5.78 (d, J = 9.4 Hz, 1H), 5.51 (d, J = 2.0 Hz, 1H), 5.24 (d, J = 12.3 Hz, 1H), 5.20 (d, J = 12.3 Hz, 1H), 5.12 (br s, 1H), 4.86−4.76 (m, 3H), 4.49−4.10 (m, 6H), 3.98 (dd, J = 10.4, 2.6 Hz, 1H), 3.88 (dd, J = 13.0, 6.9 Hz, 1H), 3.69 (dd, J = 10.6, 3.5 Hz, 1H), 2.26 (s, 3H), 2.18 (s, 3H), 2.16 (s, 3H), 2.02 (s, 3H), 1.29 (d, J = 7.0 Hz, 3H); ^{13}C NMR 100 MHz, $CDCl_3$) δ 170.2, 170.1, 169.8, 168.4, 156.5, 152.8, 143.7, 143.5, 141.0, 134.7, 132.4, 128.5, 128.4, 128.3, 127.5, 126.9, 124.9, 119.8, 100.2, 98.7, 77.1, 76.4, 72.2, 69.1, 68.1, 68.0, 67.6, 67.5, 67.1, 62.7, 62.1, 59.1, 58.6, 53.3, 46.9, 20.5, 20.4, 18.2. **42**β: $[\alpha]^{20}_D$ + 17.3° (*c* 0.64, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, 2H), 7.61 (d, 2H), 7.41−7.28 (m, 9H), 5.71 (d, J = 9.2 Hz, 1H), 5.35 (d, J = 2.9 Hz, 1H), 5.19 (d, J = 12.3 Hz, 1H), 5.11 (d, J = 12.3 Hz, 1H), 5.08 (t, J = 3.2 Hz, 1H), 5.00 (t, J = 3.4 Hz, 1H), 4.83−4.79 (m, 3H), 4.50−4.09 (m, 10H), 3.89 (dd, J = 11.4, 6.9 Hz, 1H), 3.62 (br t, 1H), 3.56 (t, J = 5.2 Hz, 1H), 3.36 (d, J = 10.4, 3.3 Hz, 1H), 2.19 (s, 3H), 2.18 (s, 3H), 2.12 (s, 3H), 2.03 (s, 3H), 1.32 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 170.4, 170.0, 169.8, 168.6, 156.8, 143.9, 143.7, 141.2, 135.3, 128.6, 128.4, 128.2, 127.6, 127.0, 125.2, 125.1, 120.0, 100.1, 99.9, 79.1, 74.9, 72.7, 72.4, 71.4, 68.4, 68.2, 67.7, 67.3, 62.5, 62.3, 61.9, 58.4, 47.1, 20.7, 20.6, 16.8; FAB HRMS calcd for (M + Na) $C_{47}H_{50}N_4O_{19}Na$ 997.2967, found 997.2961.

Compound 45. Glycal **44** (65 mg, 0.303 mmol) was dissolved in 1 mL of CH_2Cl_2 and treated with 6.06 mL of DMDO (0.06 M in acetone, 0.364 mmol) at 0 °C for 30 min. The solvent was removed *in vacuo* and placed under high vacuum for 1 h. The residue was dissolved in 2 mL of THF and cooled to −78 °C, and acceptor **19** (177 mg, 0.155 mmol) in 3 mL of THF was added followed by dropwise addition of 0.155 mL of $ZnCl_2$ (1.0 M in Et_2O, 0.155 mL). The reaction was allowed to warm to room temperature and stirred overnight. The reaction was quenched with saturated $NaHCO_3$, extracted with Et_2O, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by chromatography on silica gel (50−75% EtOAc/hexanes) to afford 128 mg (97%) of **45** as a white film: [**a**]$^{20}_D$ +61.84° (*c* 0.25, $CHCl_3$); IR (film) 3425, 3064, 2920, 2112, 1809, 1740, 1513, 1237, 1042 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 9.0 Hz, 2H), 7.61 (d, J = 8.1 Hz, 2H), 7.55 (d, J = 8.0 Hz, 2H), 7.43−7.30 (m, 12H), 5.86 (d, J = 9.4 Hz, 1H), 5.56 (s, 1H), 5.21 (s, 2H), 4.93 (d, J = 3.3 Hz, 1H), 4.80 (d, J = 5.7 Hz, 1H), 4.48−4.44 (m, 2H), 4.25−4.23 (m, 3H), 4.25−4.03 (m, 9H), 3.90 (m, 2H), 3.61 (br s, 1H), 3.01 (br s, 1H), 2.12 (s, 3H), 1.30 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 170.1, 156.7, 153.5, 143.8, 143.6, 141.2, 137.4, 134.8, 129.0, 128.6, 128.5, 128.4, 128.1, 127.7, 127.0, 126.9, 126.4, 125.0, 199.9, 101.8, 100.8, 98.8, 76.1, 75.5, 75.4, 73.3, 69.2, 68.9, 68.7, 67.7, 67.2, 63.4, 62.2, 59.2, 58.7, 47.0, 20.6, 18.6; FAB HRMS calcd for (M + Na) $C_{48}H_{48}N_4O_{16}Na$ 959.2963, found 959.2927.

Fully Protected Threonine TF Antigen 46. Compound **45** (120 mg, 0.123 mmol) was treated with thiolacetic acid (5 mL, distilled three times) for 19 h at rt. The thiolacetic acid was removed with a stream of nitrogen, followed by toluene evaporation (three times). The crude product was purified by flash chromatography (80−100% EtOAc/hexane) to yield 103 mg (87%): $[\alpha]^{20}_D$ +90.18° (*c* 0.055, $CHCl_3$); IR

(film) 3324, 3064, 2954, 1815, 1747, 1674, 1527, 1372, 1228, 1043 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H), 7.65 (d, 2H), 7.43−7.32 (m, 7H), 5.90 (br d, J = 9.3 Hz, 1H), 5.76 (br d, 1H), 5.44 (br s, 1H), 5.20 (d, J = 12.0 Hz, 1H), 5.07 (d, J = 12.0 Hz, 1H), 4.97 (br d, J = 7.7 Hz, 2H), 4.81−4.76 (m, 3H), 4.48−4.41 (m, 4H), 4.18−3.90 (m, 8H), 2.20 (s, 3H), 2.16 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.29 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 170.5, 170.3, 170.1, 168.8, 156.5, 152.9, 143.6, 143.5, 141.1, 134.4, 128.7, 128.3, 127.6, 126.9, 124.9, 124.5, 119.9, 99.9, 75.0, 72.9, 72.3, 68.8, 68.2, 67.9, 67.5, 66.9, 62.9, 62.1, 60.2, 58.6, 48.7, 47.0 ,23.2, 20.8, 20.6, 20.5, 18.2, 14.0; FAB HRMS calcd for (M + Na) $C_{49}H_{54}N_2O_{20}Na$ 1013.3158, found 1013.3201.

Glycopeptide 48. Compound **47** (30 mg, 0.03 mmol) and 10 mg of 10% Pd/C were dissolved in 3 mL of MeOH under a hydrogen atmosphere (balloon) for 4 h. After consumption of starting material by TLC, the solution was filtered through Celite and concentrated to afford 27 mg (100%) of **48** as a white film: $[\alpha]^{20}_D$ +69.05° (*c* 0.2, $CHCl_3$); IR (film) 3333, 3064, 2938, 1817, 1746, 1642, 1528, 1372, 1226, 1044 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_6) δ 7.70 (d, 2H), 7.64 (t, 2H), 7.35 (t, 2H), 7.26−7.30 (m, 2H), 4.88−5.00 (m, 4H), 4.59−4.65 (m, 2H), 4.15−4.47 (m, 11H), 4.00 (dd, J = 11.0, 8.1 Hz, 1H), 3.92 (dd, J = 11.2, 3.0 Hz, 1H), 2.07 s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.95 (s, 3H), 1.18 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, MeOH-d_6) δ 173.6, 173.5, 172.4, 172.3, 172.2, 170.6, 159.1, 159.0, 155.2, 145.4, 145.2, 142.6, 128.9, 128.3, 128.1, 126.2, 126.1, 125.1, 121.1, 101.9, 100.9, 77.6, 76.7, 76.0, 74.9, 71.2, 70.6, 69.1, 67.7, 64.4, 63.5, 60.0, 59.9, 50.0, 48.5, 23.3, 21.0, 20.9, 20.8, 19.2; FAB HRMS calcd for (M + Na) $C_{42}H_{48}N_2O_{20}Na$ 923.2598, found 923.2692.

General Procedure D for Peptide Coupling with HOAt/HATU. Glycosyl amino acid **14** or **15** (1.0 equiv) and the peptide with a free amino gruop (1.2 equiv) were dissolved in CH_2Cl_2 (22 mL, 1 mmol). The solution was cooled to 0 °C, and IIDQ (1.15−1.3 equiv) was added (1 mg in ca. 20 mL of CH_2Cl_2). The reaction was then stirred at rt for 1−2 h. The mixture was then purified directly by chromatography on silica gel (3−5% MeOH/CH_2Cl_2) and subsequently deprotected.

General Procedure E for FMOC Deprotection with KF. A substrate (1 mmol in 36 mL of DMF) was dissolved in anhydrous DMF followed by addition of KF (10 equiv) and 18-crown-6 (catalytic amount). The mixture was then stirred for 48−60 h at rt until complete by TLC. Evaporation of DMF *in vacuo* was followed by flash chromatography on silica gel (7−10% MeOH/CH_2Cl_2). The resulting amine was then used directly in peptide coupling or capping with Ac_2O.

Glycopeptide 48. $[\alpha]^{20}_D$ +52.33° (*c* 0.7, $CHCl_3$); IR (film) 3249, 2942, 2865, 1748, 1684, 1370, 1243, 1034 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, 2H), 7.68 (t, 2H), 7.42−7.30 (m, 4H), 5.43 (d, J = 2.8 Hz, 1H), 4.92−4.83 (m, 3H), 4.57 (dd, J = 10.8, 6.3 Hz, 1H), 4.48 (dd, J = 10.8, 6.3 Hz, 1H), 4.39−4.09 (m, 11H), 3.94−3.89 (m, 2H), 3.21−3.15 (m, 2H), 3.02 (br t, 2H), 2.09 (br s, 6H), 2.06 (s, 3H), 2.00 (br s, 6H), 1.58 (t, J = 6.5 Hz, 2H), 1.45 (s, 9H), 1.20 (d, J = 6.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 170.5, 170.4, 169.9, 168.8, 157.0, 156.9, 152.8, 143.8, 143.7, 141.3, 127.7, 127.1, 125.1, 125.0, 120.0, 100.6, 100.1, 79.7, 77.7, 76.7, 72.6, 72.3, 69.0, 68.3, 68.2, 67.2, 63.0, 62.3, 58.7, 49.3, 47.2, 37.1, 36.0, 30.1, 28.4, 23.4, 20.8, 17.7; FAB HRMS calcd for (M + Na) $C_{50}H_{64}N_4O_{21}Na$ 1079.3961, found 1079.3955.

Glycopeptide 50. Compound **49** (70 mg, 0.03 mmol) was treated with 1.5 mL of TFA in 0.5 mL of CH_2Cl_2 for 30 min at rt. The reaction was concentrated and lyophilized with AcOH/*tert*-butyl alcohol (1:5). To the resulting white solid was added 18.3 mg (2.0 equiv, 0.06 mmol) of SAMA−OPfp in 2.5 mL of CH_2Cl_2 followed by the dropwise addition of DIEA (10.6 mL, 0.06 mmol). After 1 h at rt, the reaction mixture was concentrated and purified by silica gel chromatography (7−10% MeOH/CH_2Cl_2) to afford 63 mg of white film. This material was dissolved in a degassed solution of NaOMe in MeOH (pH ∼10) under Ar for 2 h. The reaction was treated with Amberlyst-15 resin until pH ∼5, and then filtered, concentrated, and purified via chromatography on RP-18 silica gel (degassed H_2O) to afford 36 mg (60%) of **50** as a fluffy solid after lyophilization. Further analysis was renounced to avoid dimerization of the thiol, and the material was stored under Ar at −78 °C: ^1H NMR (400 MHz, D_2O) δ 4.89−3.12 (m, ∼60H), 2.13 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.69 (br m, 2H), 1.32 (d, J = 6.2 Hz, 3H), 1.29 (d, J = 5.8 Hz, 3H), 1.23 (d, J = 5.7 Hz, 3H); CI (NH_3) MS calcd for (M + Na) $C_{21}H_{27}N_3BrO_{14}Na$ 1611.8, found 1611.8.

Vaccine Preparation. **27**−KLH and **27**−BSA vaccines were prepared as described previously for the MUC1−KLH conjugate vaccine.[45] The yield of this procedure was generally around 10%. Unreacted Tn(c) was removed by a molecular cutoff filter (MW 30 000, Centriprep, Amicon Inc., Beverly, MA). The samples was filtered through a 0.22 μm filter under sterile conditions. The protein content was determined using the BioRad protein assay and the carbohydrate content by a HPAEC-PAD assay. The epitope ratio of **27**−KLH and −BSA was 317:1 and 7:1, respectively. The resulting more effective conjugation to KLH versus BSA may reflect uncertainties in the quality of the reagents employed for the latter. The aliquoted vaccine was stored at 4 °C.

Immunization Protocol. Groups of mice (female CB6F1 mice, Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously five times (weeks 0, 1, 2, 7, and 20) with 10 μg of **30** alone dissolved in intralipid, 10 μg of **30** alone dissolved in intralipid plus 20 μg of QS-21, and **27**−KLH or **27**−BSA (containing 3 μg of synthetic Tn(c) trisaccharide) plus 20 μg of QS-21. Mice were bled 10 days after the third, fourth, and fifth vaccinations and, sera were separated and stored at −30 °C.

Serological Analysis. The serological response was analyzed by several serological methods.

(1) ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to determine the titer of antibodies against Tn(c)−pam as described previously.[44] Serially diluted antiserum was added to wells coated with antigen (0.1 μg) and incubated for 1 h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase served as secondary antibodies. Absorbance was measured at 414 nm. The antibody titer was defined as the highest serum dilution showing an absorbance of 0.1 or greater than that of normal mouse sera.

(2) Flow Cytometry. Cell surface reactivity of these antibodies was assayed by flow cytometry on Tn(c) positive LS-C cells and Tn(c) negative LS-B cells.[45] Single cell suspensions of 2×10^5 cells/tube were washed in PBS with 3% fetal calf serum and 0.01 M NaN_3 and incubated with 20 μL of 1:20 diluted antisera or mAb IE3 for 30 min on ice. After the cells were washed twice with 3% FCS in PBS, 20 μL of 1:15 goat anti-mouse IgM labeled with fluorescein−isothiocyanate (FITC) was added. The solution was mixed and incubated for 30 min. After the cells were washed, the positive population and mean fluorescence intensity of stained cells were analyzed by flow cytometry (EPICS-Profile II, Coulter, Co., Hialeah, FL) as described.[46]

(3) Complement Dependent Cytotoxicity (CDC). The ability of these sera to mediate complement lysis was assessed by a chromium release complement dependent cytotoxicity assay against LS-C cells (6). Complement dependent cytotoxicity was assayed at a serum dilution of 1:10 with LS-C cells by a 4 h chromium-release assay as previously described. All assays were performed in triplicate. Controls included cells incubated only with culture medium, complement, antisera, or mAb IE3. Spontaneous release was the europium released by target cells incubated with complement alone. Percent cytolysis was calculated according to the formula specific release (%) = experimental release − spontaneous release/maximum release − spontaneous release × 100

Inhibition Assay. Antisera at 1:1500 dilution or mAb IE3 at 0.1 μg/mL were mixed with various concentrations of structurally related and unrelated carbohydrate antigens. The mixture was incubated at room temperature for 30 min, and transferred to an ELISA plate coated with Tn(c)−pam. ELISAs were performed as described above. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum.

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ELISAs were also performed with sera that had been inhibited (absorbed) by incubation with LS-C or LS-B cells. For this assay 5×10^5 cells were incubated with sera for 1 h and the cells removed by centrifugation. ELISA was performed as described above.

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Supporting Information Available: ^{1}H and ^{13}C spectra for compounds **5**, **7**, **9**, **14**−**19**, **21**, **23**, **25**, **30**, **35**−**38**, **40**β, **41**α, **42**α, and **45**−**47**, ^{1}H spectra for compounds **32**, **39**α,β, **40**α, and **50**, and experimental details for compounds **14**−**17**, **20**−**23**, **35**, **41**α, **41**β, and **49** (60 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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