Design and Synthesis of Le^y-Bearing Glycopeptides that Mimic Cell Surface Le^y Mucin Glycoprotein Architecture

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Abstract: Five Lewis^y-based glycopeptide anti-cancer vaccine candidates have been designed and synthesized to target tumor-associated cell-surface glycoprotein antigens and to improve the immunizing performance in comparison to related vaccines. The peptide backbone consisted of two regions, a glycodomain AcNH-SSS- and a nonglycosylated sequence, -AVAV-. The resultant glycopeptide was conjugated, via an additional spacer, to the lipid carrier PamCysSer. In this series of totally synthetic molecular vaccine candidates, one or three of the sequentially arranged serine residues were glycosylated. Furthermore, the Le^y tetrasaccharide determinant region was kept constant while the internal glycan core was systematically varied. Glycal assembly was used to prepare the glycosyl donors, and two strategies were applied to provide the serine-*O*-linked polysaccharide domains. In the first approach, a protected serine derivative was attached directly to the fully elaborated glycan. Following this course, both α- and β-Ser derivatives were accessed. In the second route, a GalNAc-α-Ser was joined with a glycosyl donor to afford exclusively the desired α-serine-linked product. The glycopeptides were assembled using iterative solution phase peptide coupling. Following global deprotection, the lipid carrier was then coupled to the glycopeptide, resulting in the targeted constructs. The synthesis of these molecular vaccine candidates constitutes an important advance that should enable rationalization of carbohydrate-induced immune response as well as identification of optimal Le^y-based anti-cancer vaccine leads.

Background

Even following the apparent eradication of primary tumors (via surgery, chemotherapy, or radiation), the risk of recurrence in a metastatic mode can still be high. This susceptibility may well reflect persistence of microscopic disease in clinically undetectable form. As a means of reducing the likelihood of relapse, we have been pursuing a program that seeks to mobilize the formidable resources of the human immune system to target certain transformed cell types.[1,2] In principle, "circulatory immunosurveillance" could provide a nontoxic means of combating cancer at a stage of disease progression where few alternative therapies are available.[1]

For circulatory immunosurveillance to be effective against micrometastases, the target antigen should be substantially restricted to the cancer type, and be accessible for binding. Tumor-associated cell surface carbohydrates are of potential interest in this regard since they are often overexpressed in various cancer states and could well be accessible to binding by suitably elicited circulating antibodies.[1]

In pursuit of this possibility, we have prepared several carbohydrate-based vaccines. In particular, constructs based on Lewis^y (Le^y),[3] globo H,[4] TF,[5] and Tn[5] have advanced to various stages of clinical evaluation.[2b] Following the identification of a relationship between carbohydrate ensembles and cancer types through immunohistology and sequence analysis, a program

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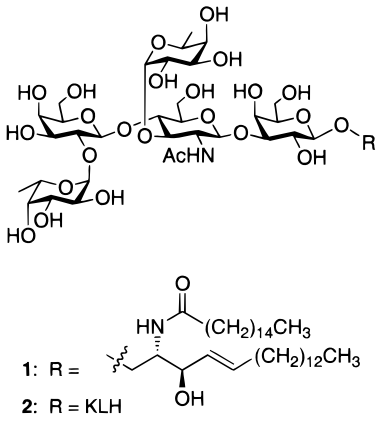

Figure 1.

directed to total synthesis can be initiated. We first focus on the core structure and proceed to conjugate the carbohydrate domain to a suitable immunogenic carrier protein. This type of conjugation may require installation of an intervening spacer. The resultant construct comprises the vaccine. Immunization of mice with synthetic vaccine allows for evaluation of the extent of the induced immune response, the preliminary assessment of specificity of serological response, and provides estimates as to safety. Given sufficiently favorable outcomes in such preclinical studies, progression toward Phase I clinical investigations is then planned.

Historically, not all carbohydrate-based antigens described in the literature as potential vaccines have proven to be productively antigenic. For example, a construct corresponding to the tumor-associated glycan GM3[6] failed to produce antibodies that exhibited promising cell surface specificity. Similarly, in 1994 Kitamura et al.[7] disclosed an immunological study with a presumed, but undocumented, Le^y-KLH conjugate. Following vaccination, antibodies were elicited against the immunizing structure; however, they did not bind well to Le^y bearing cells. This result was in contrast to vaccination with whole cells bearing Le^y, which did in fact produce cell-reactive anti-Le^y antibodies.

In the same year that these confusing and discouraging results were reported, our laboratory described a total synthesis of Le^y-ceramide[3a] (**1**, Figure 1). The ceramide glycoside corresponds to a natural membrane-anchored mode of Le^y presentation. In addition, the same determinant is encountered in the form of cell surface glycoproteins and mucins.[8] Confident of the chemical structure and homogeneity of our fully synthetic preconjugation epitope, we advanced to mouse immunization studies. Indeed, unlike the case with the earlier Le^y studies, inoculation of mice with Le^y-glycoconjugate **2** resulted in the production of focused antibodies that reacted with Le^y-ceramide **1** in ELISA assays, as well as with Le^y-bearing cells.[3c] While we could not state with certainty why our Le^y construct was successful where the earlier product had failed, it is tempting to ascribe the successes to the more precisely defined character of our carbohydrate vaccine. The mouse immunization results formed the basis of advancing **2** into human ovarian cancer trials.[3d] At the serological level, these trials demonstrated that an anti-Le^y response that could indeed target tumor cells had been accomplished. Appropriate controls established that the response was a consequence of the fully synthetic vaccine (**2**).

Our laboratories have sought to further improve the immunizing potential of Le^y-based vaccines.[9] As mentioned above, cell surface Le^y is displayed in both glycolipid and mucin glycoprotein contexts. Vaccination of humans with **2** in the context of a limited Phase I ovarian cancer trial[3d] elicited antibodies that reacted strongly to synthetic and cell surface Le^y-glycolipids. Lower reactivity was noted, however, with mucins bearing the Le^y epitope.[3d] One could in principle interpret the difference between antibody binding reactivities to reflect subtle, but important, structural differences between mucin- and lipid-bound Le^y. The thought was that immunization with a more realistic Le^y mimic of a glycoprotein would result in still more favorable cell surface reactivity of stimulated antibodies.

Unlike the case with Le^y-ceramide, the precise structure of a tumor-associated Le^y-mucin is a complex matter.[8d,10] Aside from the obstacles to detailed characterization arising from the sheer size of the glycoprotein, the design problem is aggravated by the micro-heterogeneity of highly glycosylated mucins. Such proteins are characterized by a very high content of serine and threonine residues, and sequences of three to five of these amino acids are frequently found as adjacent residues in the primary sequence. Furthermore, most of these residues are glycosylated, and the glycan usually varies between 3 and 20 carbohydrate units. The carbohydrate directly attached to the peptide backbone is, apparently, universally an α-*O*-linked GalNAc, and a number of intervening Gal and GlcNAc spacer units may insulate this region from the nonreducing end bearing, for instance, a blood group determinant. Given this situation, our approach to a better simulation of the natural Le^y presentation envisioned synthesizing glycopeptides with Le^y-based domains projecting from α-*O*-linked motifs akin to those present on a cell surface protein.

Le^y-Mucin Vaccine Design

A generalized depiction of a mucin segment displaying Le^y as the terminal glycan is summarized in Figure 2. The model includes a cluster of sequential amino acid residues each bearing a glycan, wherein the first carbohydrate is α-*O*-linked GalNAc. This is followed by an elongation segment terminating in the Le^y determinant. This conceptualized form of a Le^y-mucin served as a guide in designing a series of mucin-mimic vaccine constructs with Le^y as the key structural element.

With a view toward facilitating immunological evaluation, and in keeping with the previous standards of our program, we set a high priority upon the construction of a homogeneous

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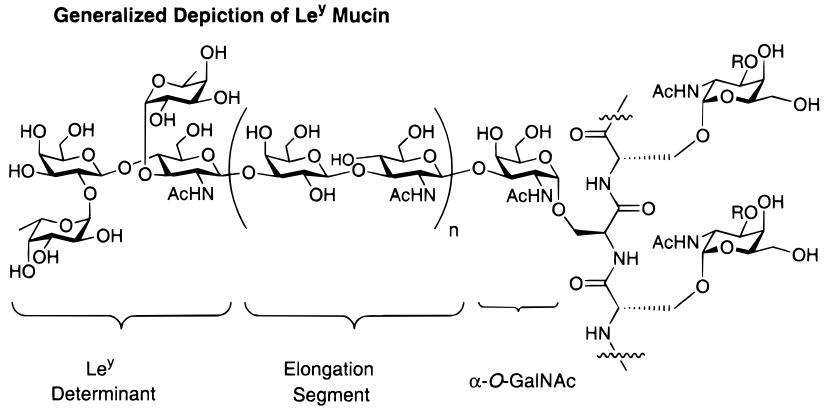

Figure 2.

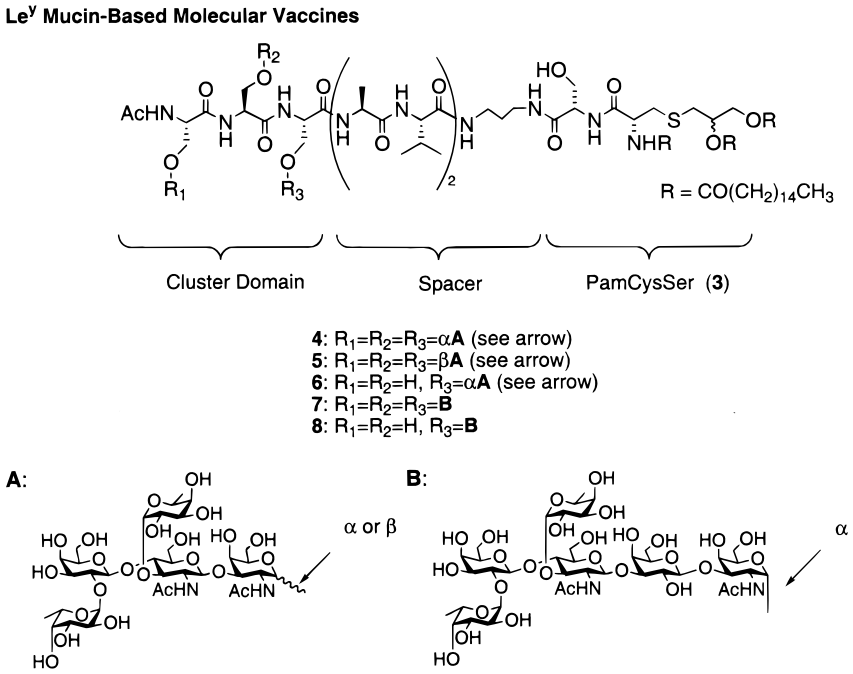

Figure 3.

vaccine.[2b] A discrete molecule allows a rigorous correlation to be established between vaccine structure and immunological properties. To this end, we first selected the lipid PamCysSer[11,12] as the *C*-terminal unit of the vaccines (see **3** in Figure 3) instead of the more typical protein conjugate KLH. In addition, PamCysSer connected by a suitable spacer unit is an established immunostimulant known to activate B-cells and macrophages.[11f] The peptide backbone consisted of two regions, a nonglycosylated sequence, -AVAV-, to further separate the glycodomain from the lipid. A segment of three contiguous glycosylated serine residues constituted the clustered carbohydrate region. As for the glycan, the determinant Le^y tetrasaccharide region was kept constant while the glycopeptide core was systematically varied.

Five clustered vaccines (**4**−**8**) were targeted for synthesis. Clustered pentasaccharide **4** satisfies the minimum requirements outlined above: the Le^y tetrasaccharide attached to GalNAc-α-*O*-Ser. An important analogue, GalNAc-*β*-*O*-Ser derivative **5**, containing unnatural *β*-linkages, was also prepared. Such a "non-self" structure might prove more immunogenic than **4** and might still elicit antibodies capable of reacting with Le^y. Another control, compound **6**, displays only a single copy of the pentasaccharide and by inference, begins to probe the importance of sequential glycosylation in a potential molecular vaccine. Compound **7** is a hexasaccharide cluster that has the natural GalNAc-α-*O*-Ser junction and a spacer Gal unit between the Le^y determinant and the first carbohydrate. This construct can be viewed as the pentasaccharide of Le^y-ceramide attached to a GalNAc-α-*O*-Ser, or as the Le^y-tetrasaccharide displayed on one of the most common mucin core structures known.[10a,d,f] Finally, construct **8** is the monovalent analogue of **7**.

Synthetic Plan

With vaccine candidates **4**−**8** identified, the plan of synthesis had to include a protecting group strategy compatible with the

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(12) It should be noted that, while PamCysSer is known to form aggregates (cf. ref 11f), the conjugates reported here were formulated as emulsions in a carrier lipid. The "pseudo-cluster" presentation of aggregated PamCysSer conjugates is not guaranteed in a lipid carrier and, regardless, cannot be expected to mimic the precise clustered form of sequentially arranged, and fully glycosylated, amino acid residues in a polypeptide.

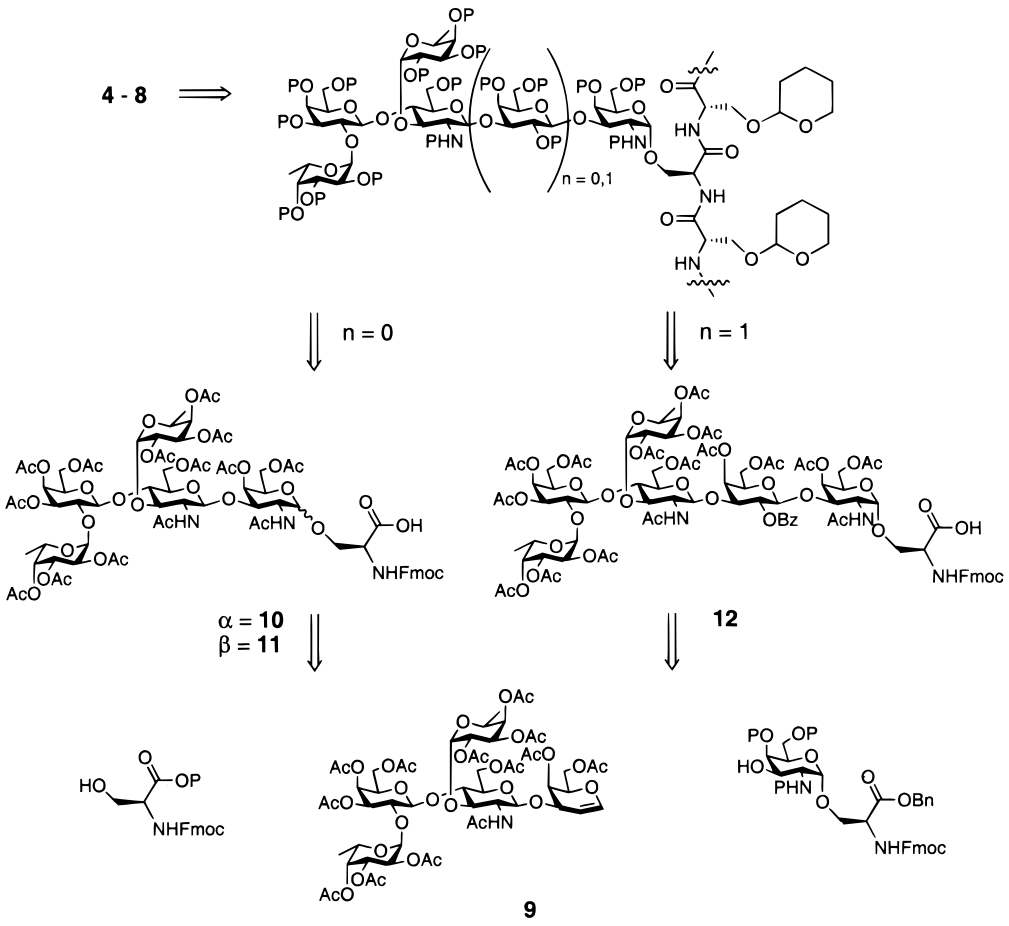

Figure 4.

incorporation of labile fucosyl units and elimination-prone glycosylated amino acids and the stereoselective formation of the glycosidic linkages. We were mindful that multiple copies of sensitive moieties could serve to amplify chemical instability. For example, compound **7** would likely be a fragile construct with its 3 glycosylated amino acids, 18 glycosidic bonds, and 45 hydroxyls, each of which would have to be created, protected, sustained, and, in time, exposed (by deprotection) at the end of the synthesis. Consequently, the route to these target structures would have to be mild, selective, and efficient, particularly in the final stages. To facilitate the synthetic end game, we planned a "global deprotection" protocol requiring only one operation to unmask the functional groups.

Two strategies were envisioned to provide the serine-*O*-linked penta- or hexasaccharide domains (Figure 4). Both routes would rely on the logic of glycal assembly[2a] to provide glycosyl donors. Pentasaccharide glycal **9**[3a,b] appeared to be a viable intermediate for conversion to *O*-linked building blocks **10**−**12**. In the first approach, an appropriately protected serine derivative would be attached directly to the fully elaborated glycan. Following this course, both α- and *β*-Ser derivatives **10** and **11** could, in principle, be accessed by careful control of the reaction conditions. We have observed that the outcome, in terms of α/*β* ratios, of direct glycosylation of a complex donor with a serine acceptor may vary with the nature of the donor and may prove quite different from that with a threonine acceptor.[2b,13] To bypass these stereochemical uncertainties, in the second route a GalNAc-α-Ser synthon would be joined with a glycan donor to afford exclusively the desired α-serine-linked product for the preparation of **12**. This cassette method has worked well for both GalNAc-α-Ser and GalNAc-α-Thr acceptors and has become our preferred approach to assemble glycoamino acids.[14] For the preparation of glycopeptides **4**−**8**, we envisioned using iterative solution phase peptide coupling to connect glycoamino acids **10**−**12** to the mucin vaccine framework.

(13) See refs 5a and 13a and references therein.

Synthesis

The common precursor to compounds **10**−**12** (the peracetate-protected pentasaccharide glycal, **9**)[3a,b] was prepared from lactal (Scheme 1). After the necessary protection steps, lactal **13** was subjected to 2-fold glycosylation to introduce the two α-fucose residues of the Le^y determinant. The resulting glycal underwent iodosulfonamidation followed by "direct rollover"[15] to yield the pentasaccharide glycal **14**, which in turn was converted to **9** in anticipation of global deprotection.

With ample quantities of the pentasaccharide in hand, building blocks for the synthesis of **4**−**8** could be accessed as follows

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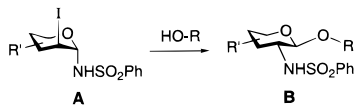
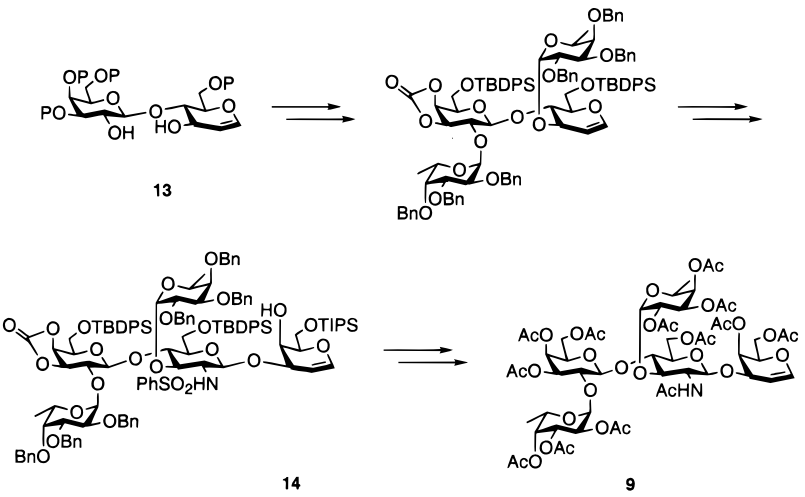
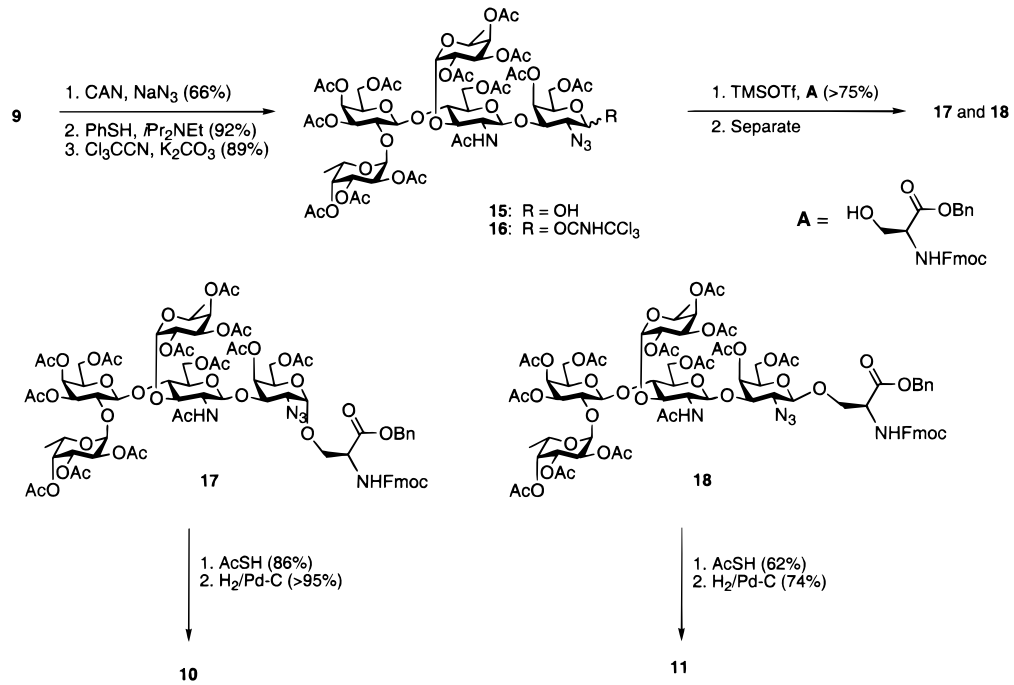
(15) 2-Iodo-1-sulfonamidoglycosides (**A**) can be converted, in a reaction referred to as "direct-rollover", to the 2-sulfonamide glycoside products (**B**) (cf. refs 2a and 3a and references therein).



Scheme 1



Scheme 2



(Schemes 2 and 3). As shown in Scheme 2, after subjecting glycal **9** to Lemeiux azidonitration,[16] the nitrate ester was reduced to give the anomeric alcohol **15**. Conversion of **15** to a Schmidt-type trichloroacetimidate[17] smoothly furnished donor **16**, appropriate for coupling with a serine acceptor. In the coupling event, FmocSerOBn was found to undergo promotion with TMSOTf to afford a separable mixture of the desired *O*-linked glycoamino acids in a ratio of 2.6:1, α(**17**):*β*(**18**). By altering the reaction conditions, the ratio of **17**:**18** was reversed to 1:1.7. The azide functions of **17** and **18** were converted directly to the corresponding acetamides.[18] Hydrogenolysis of the benzyl esters afforded acids **10** and **11**, primed for peptide coupling.

As anticipated, access to the *O*-linked constructs **10** and **11** had suffered from rather poor stereocontrol in the key glycosylation steps. Since both derivatives were desired, this nonspecificity proved to be awkward, but of some utility. For the *O*-linked hexasaccharide, however, we turned to our cassette strategy,[13] in which the desired serine-α-*O*-linkage is preinstalled (Scheme 3). Thus acceptor **19** was prepared from galactal **20**, which was coupled to FmocSerOBn in excellent yield and high selectivity to give the desired GalNAc-α-*O*-Ser **21**. This glycosylation reaction has been successfully employed on large scale without deterioration in selectivity. Further manipulation of the protecting groups provided acceptor **19** ready for coupling to a suitable donor.

To prepare the requisite pentasaccharide donor peracetate, glycal **9** was subjected to the action of DMDO. The resultant glycal 1,2-epoxide was converted to either the thioethyl donor **22**[19] or the Fraser-Reid type[20] pentenyl donor **23**. Thus, treatment of the epoxy adduct with EtSH and catalytic TFA afforded **22**, while treatment with 4-penten-1-ol and $ZnCl_2$ gave rise to the

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(17) Schmidt, R. R. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 212. Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 84.
(18) Rosen, T.; Lico, I. M.; Chu, D. T. W *J. Org. Chem.* **1988**, *53*, 1580.
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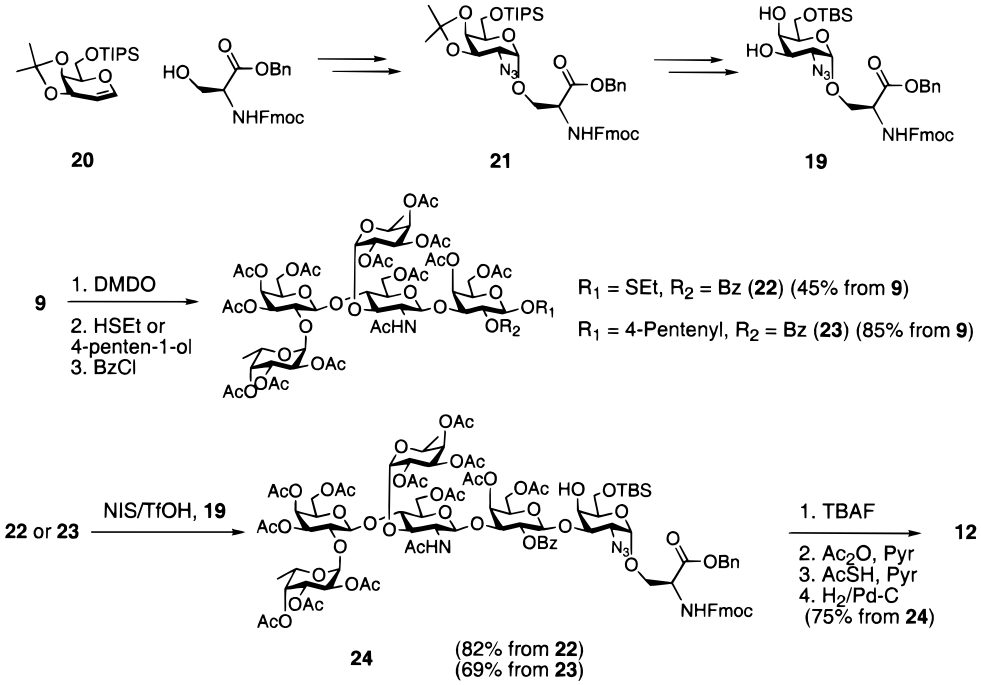
Scheme 3



pentenyl glycoside, **23**. The 2-hydroxy glycosides were then protected as benzoate esters. While the sequence leading to **22** was moderately efficient, **9** was best converted to **23** without isolation of the intermediate alcohol and in an excellent overall yield.

Having avoided the potentially problematic direct glycosylation of serine, the final coupling between donors **22** and **23** with acceptor **19**[21] was all that remained to construct building block **12**. The outcome of this glycosylation was difficult to predict as the acceptor exhibited only moderate reactivity in other glycosylation reactions. Furthermore, anomeric onium type intermediates are presumably less likely to arise from deactivated peracylated donors.[22] Apparently, transient cationic carbohydrate species are destabilized by the cumulative electron withdrawing effects of ester protecting groups. *In the event, however, NIS/TfOH-promoted* [23] *coupling of either donor **22** or **23** with acceptor **19** cleanly afforded the desired* α*-O-serine hexasaccharide **24**.* In an alternative coupling procedure, promotion of the thioethyl donor via the action of methyl triflate did achieve glycosylation in low yield. This reaction was accompanied by methylation of the acetamide functionality to afford the methyl imidate. Attempts to hydrolyze the imidate linkage under acidic conditions were unsuccessful, leading instead to decomposition. This phase of the synthesis was completed by removal of the silyl ether followed by acetylation, reductive acylation of the azide, and benzyl ester cleavage. Thus acid **12**, suitably protected for peptide assembly, was in hand.

The vaccines were assembled using the Carpino HOAt/HAtU peptide coupling methodology (Scheme 4).[24] The three clustered targets derived from monomeric units **10**, **11**, and **12** were prepared in analogous fashion. The glycoamino acid was coupled to H_2N-AVAV-OBn. The Fmoc group was subsequently removed, and a second acid unit was introduced. This process was repeated to give the trimer. The terminal Fmoc group was cleaved and the free amine was capped with Ac_2O. The benzyl ester was removed by hydrogenolysis to afford the fully protected clustered glycopeptide acids **25**−**27**.

To prepare the precursors of control compounds **6** and **8**, each of which only bears one glycan, FmocSer(OAc)Ser(OAc)-OH was coupled instead of the last two units of **10** and **12**. The amino-termini were converted to the acetamide and the benzyl ester was cleaved to furnish glycopeptides **28** and **29**, respectively.

To conjugate the glycopeptides to PamCysSer,[11d] it was first necessary to effect removal of the acyl groups from the carbohydrate residues, in the context of global deprotection (Scheme 4). In the case of the pentasaccharide clusters **25**, **26**, and **28**, the acetate group was readily removed as designed by mild saponification (aq NaOH/MeOH, pH 10).

However, with the hexasaccharide-derived glycopeptides **27** and **29**, extensive optimization of the deprotection conditions was required to achieve removal of the benzoyl esters, while preserving the base-labile α-*O*-linkage. The C2-benzoate function resisted the protocol used in the pentasaccharide series, as well as well as the actions of LiOH, LiOOH, and Zemplen procedures. Fortunately, careful treatment with hydrazine hydrate in methanol[25] cleanly removed the acetate and benzoate protection to afford the fully deprotected glycopeptides. Finally, the lipid amine was coupled to the acid terminus of each heptapeptide to afford the synthetic vaccine constructs **4**−**8**.

Discussion

Having constructed the target molecules, we are in a position to evaluate their immunostimmulatory capacity. While a full account of the immunological properties of **4**−**8** will appear in due course, several remarkable observations have already been registered in murine preclinical models. *Specifically, clustering the glycodomain was found to be crucial for anti-Le^y antibody production.* Furthermore, the clustered variants proved im-

(21) See ref 13a for the preparation of this acceptor and refs 5a, 9, and 13b−h for related couplings.

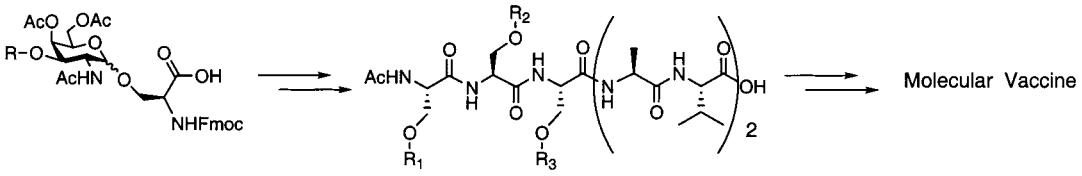
(22) Andrews, C. W.; Rodebaugh, R.; Fraser-Reid, B. *J. Org. Chem.* **1996**, *61*, 5280 and references therein.

(23) (a) Veeneman, G. H.; van Leeuwen, S. H.; van Boom, J. H. *Tetrahedron Lett.* **1990**, *31*, 1331. (b) Konradsson, P.; Udodong, U. E.; Fraser-Reid, B. *Tetrahedron Lett.* **1990**, *31*, 4313.

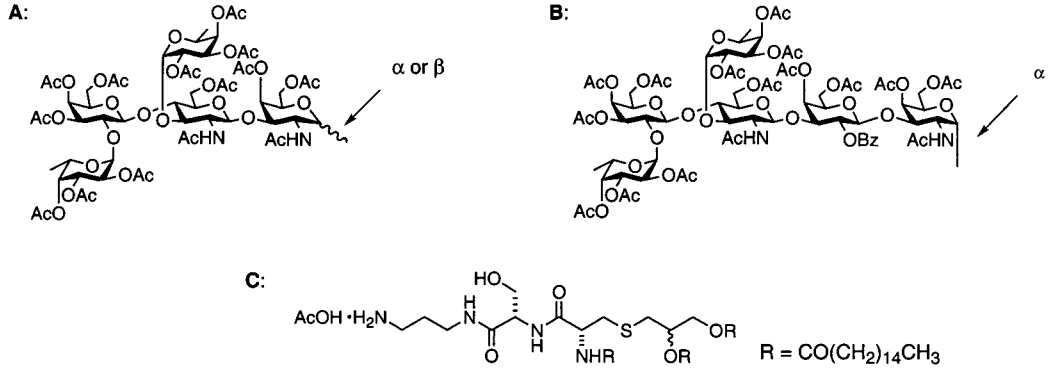
(24) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.

(25) Kunz, H.; Birnbach, S.; Wernig, P. *Carbohydr. Res.* **1990**, *202*, 207.

Scheme 4[a]



10 (α-penta)	a,b,c,b, c,b,d,e	**25**: $R_1 = R_2 = R_3 =$ αA (see arrow)	i,j	**4**
11 (β-penta)	a,b,f,b, f,b,d,e	**26**: $R_1 = R_2 = R_3 =$ βA (see arrow)	i,j	**5**
10 (α-penta)	a,b,h, b,d,e	**28**: R_1 = Ac, R_2 = Ac, R_3=αA (see arrow)	k,j	**6**
12 (α–hexa)	a,b,g,b, g,b,d,e	**27**: $R_1 = R_2 = R_3$ = B	i,j	**7**
12 (α–hexa)	a,b,h, b,d,e	**29**: R_1 = Ac, R_2 = Ac, R_3 = B	k,j	**8**



[a] Conditionas: (a) NH_2-AVAV-OBn, HOAt, HAtU. (b) Morpholine. (c) **10**, HOAt, HAtU. (d) Ac_2O, Pyr. (e) H_2/Pd-C. (f) **11**, HOAt, HAtU. (g) **12**, HOAt, HAtU. (h) FmocSer(OAc)Ser(OAc)-OH, HOAt, HAtU. (i) NaOH/MeOH, pH 10. (j) **C**, HOAt, HAtU. (k) N_2H_4, MeOH. (The yield of each reaction is reported in the Experimental Section.)

munogenic and immunoreactive with natural Le^y. For example, **7** was found to elicit an immune response and the antibodies so stimulated recognized both Le^y-ceramide and, importantly, Le^y-mucin glycoproteins. Since compound **7** is the most structurally realistic mucin-mimic, and as the synthetic route is both selective and efficient, we have chosen a construct based on **7** for conjugation to KLH and comparison in preclinical trials. From these results the most promising candidate will be advanced to clinical evaluation.

In summary, we undertook to construct the simplest, most realistic molecules that might serve as mimics for mucins bearing the Le^y epitope. Given the uncertainties regarding glycoprotein structure, it is difficult to design with total confidence molecules that will stimulate an effective immune response which will subsequently identify and target cancer cell surface mucin glycoprotein. Success in this demanding goal requires fashioning and implementing the synthetic chemistry to build a new type of SAR database to rationalize immune response. The results described herein constitute an important advance toward this long-term goal and are already suggestive of the value of glycopeptide domains for mimicking cell surface architecture.

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Supporting Information Available: Experimental procedures for the preparation of each synthetic intermediate, analytical characterization, including ^{1}H NMR, ^{13}C NMR of select compounds (37 experimental (1−37) and 59 spectral (S1−S59)) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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