A New Model for the Presentation of Tumor-Associated Antigens and the Quest for an Anticancer Vaccine: A Solution to the Synthesis Challenge via Ring-Closing Metathesis

Insik Jeon,[†] Dongjoo Lee,[†] Isaac J. Krauss,[†] and Samuel J. Danishefsky*,[†,‡]

Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10065, and Department of Chemistry, Columbia University, Havemeyer Hall, 3000 Broadway, New York, New York 10027

Received June 26, 2009; E-mail: s-danishefsky@ski.mskcc.org

Abstract: Fully synthetic, carbohydrate-based antitumor vaccine candidates have been synthesized in highly clustered modes. Multiple copies of tumor-associated carbohydrate antigens, Tn and STn, were assembled on a single cyclic peptide scaffold in a highly convergent manner. Ring-closing metathesis-mediated incorporation of an internal cross-linker was also demonstrated. In particular, this rigidified cross-linked construct would enhance a cluster-recognizing antibody response by retaining an appropriate distance between glycans attached to the peptide platform. Details of the design and synthesis of highly clustered antigens are described herein.

Introduction

Our laboratory is seeking the development of novel, fully synthetic carbohydrate-based vaccines for the treatment of cancer. This research program is based on the finding that malignantly transformed cells often exhibit significant alteration in the nature and quantity of carbohydrates presented on their cell surfaces, either as glycosphingolipids or as glycoproteins.[1] Presumably, when properly introduced to the immune system, a tumor-associated carbohydrate-based antigen could set into motion an exploitable immune response, leading to the genera-tion of antibodies which would selectively bind to and hopefully eliminate those tumor cells which overexpress the carbohydrates in question. We are certainly not alone in this area. In particular, impressive advances of carbohydrate-based anticancer vaccines have been reported by Boons,[2] Kunz,[3] and Schmidt.[4] The viability of this carbohydrate vaccine concept has been con-firmed experimentally in our laboratory. Thus, when these tumor-associated antigens are presented to the immune system as glycoconjugates appended to immunogenic carrier molecules (such as KLH carrier protein)[5] and coadministered with an immunological adjuvant (such as QS21),[6] a carbohydrate-specific antibody response may be induced. A number of complex, fully synthetic carbohydrate-based constructs, syn-thesized in our laboratories, have shown significant promise in preclinical, and even clinical settings.[7] In designing a carbohydrate-based vaccine construct, it may be of considerable value to consider the way in which the antigen is displayed in its natural environment, i.e., on the surface of the transformed cell, to more directly mimic this natural setting in the context of the vaccine.

† Sloan-Kettering Institute for Cancer Research.
‡ Columbia University.
(1) (a) Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836–863. (b) Livingston, P. O.; Zhang, S. *Cancer Immunol. Immunother.* **1997**, *45*, 1–9. (c) Livingston, P. O.; Ragupathi, G. *Cancer Immunol. Immunother.* **1997**, *45*, 10–19. (d) Toyokuni, T.; Singhal, A. K. *Chem. Soc. Rev.* **1995**, 231–242. (e) Hakomori, S. I. *Adv. Exp. Med. Biol.* **2001**, *491*, 369–402.
(2) (a) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G.-J. *Nat. Chem. Biol.* **2007**, *3*, 663–667. (b) Buskas, T.; Ingale, S.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2005**, *44*, 5985–5988.
(3) (a) Kunz, H.; Dziadek, S.; Wittrock, S.; Becker, T. *ACS Symp. Ser.* **2008**, *989*, 293–310. (b) Westerlind, U.; Hobel, A.; Gaidzik, N.; Schmitt, E.; Kunz, H. *Angew. Chem., Int. Ed.* **2008**, *47*, 7551–7556. (c) Wittrock, S.; Becker, T.; Kunz, H. *Angew. Chem., Int. Ed.* **2007**, *46*, 5226–5230. (d) Dziadek, S.; Brocke, C.; Kunz, H. *Chem.—Eur. J.* **2004**, *10*, 4150–4162. (e) Sprengard, U.; Schudok, M.; Schmidt, W.; Kretzschmar, G.; Kunz, H. *Angew. Chem., Int. Ed.* **1996**, *35*, 321–324.
(4) (a) Hermans, I. F.; Silk, J. D.; Gileadi, U.; Salio, M.; Mathew, B.; Ritter, G.; Schmidt, R.; Harris, A. L.; Old, L.; Cerundolo, V. *J. Immunol.* **2003**, *171*, 5140–5147. (b) Schmidt, R. R.; Castro-Palomino, J. C.; Retz, O. *Pure Appl. Chem.* **1999**, *71*, 729–744.
(5) (a) Helling, F.; Shang, A.; Calves, M.; Zhang, S. L.; Ren, S. L.; Yu, R. K.; Oettgen, H. F.; Livingston, P. O. *Cancer Res.* **1994**, *54*, 197–203. (b) Helling, F.; Zhang, S.; Shang, A.; Adluri, S.; Calves, M.; Koganty, R.; Longenecker, B. M.; Yao, T. J.; Oettgen, H. F.; Livingston, P. O. *Cancer Res.* **1995**, *55*, 2783–2788.
(6) (a) Kensil, C. R.; Patel, U.; Lennick, M.; Marciani, D. *J. Immunol.* **1991**, *146*, 431–437. (b) Marciani, D. J.; Press, J. B.; Reynolds, R. C.; Pathak, A. K.; Pathak, V. L.; Gundy, E.; Farmer, J. T.; Koratich, M. S.; May, R. D. *Vaccine* **2000**, *18*, 3141–3151. (c) Kim, S.-K.; Ragupathi, G.; Musselli, C.; Choi, S. J.; Park, Y. S.; Livingston, P. O. *Vaccine* **1999**, *18*, 597–603. (d) Kim, S.-K.; Ragupathi, G.; Cappello, S.; Kagan, E. P.; Livingston, O. *Vaccine* **2000**, *19*, 530–537.
(7) (a) Ragupathi, G.; Park, T. K.; Zhang, S. L.; Kim, I. J.; Graber, L.; Adluri, S.; Lloyd, K. O.; Danishefsky, S. J.; Livingston, P. O. *Angew. Chem., Int. Ed.* **1997**, *36*, 125–128. (b) Slovin, S. F.; Ragupathi, G.; Adluri, S.; Ungers, G.; Terry, K.; Kim, S.; Spassova, M.; Bornmann, W. G.; Fazzari, M.; Dantis, L.; Olkiewicz, K.; Lloyd, K. O.; Livingston, P. O.; Danishefsky, S. J.; Scher, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5710–5715. (c) Gilewski, T.; Ragupathi, G.; Bhuta, S.; Williams, L. J.; Musselli, C.; Zhang, X. F.; Bencsath, K. P.; Panageas, K. S.; Chin, J.; Hudis, C. A.; Norton, L.; Houghton, A. N.; Livingston, P. O.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3270–3275. (d) Krug, L. M.; Ragupathi, G.; Hood, C.; Kris, M. G.; Miller, V. A.; Allen, J. R.; Keding, S. J.; Danishefsky, S. J.; Gomez, J.; Tyson, L.; Pizzo, B.; Baez, V.; Livingston, P. O. *Clin. Cancer Res.* **2004**, *10*, 6094–6100. (e) Sabbatini, P. J.; Kudryashov, V.; Ragupathi, G.; Danishefsky, S. J.; Livingston, P. O.; Bornmann, W.; Spassova, M.; Zatorski, A.; Spriggs, D.; Aghajanian, C.; Soignet, S.; Peyton, M.; O'Flaherty, C.; Curtin, J.; Lloyd, K. O. *Int. J. Cancer.* **2000**, *87*, 79–85.
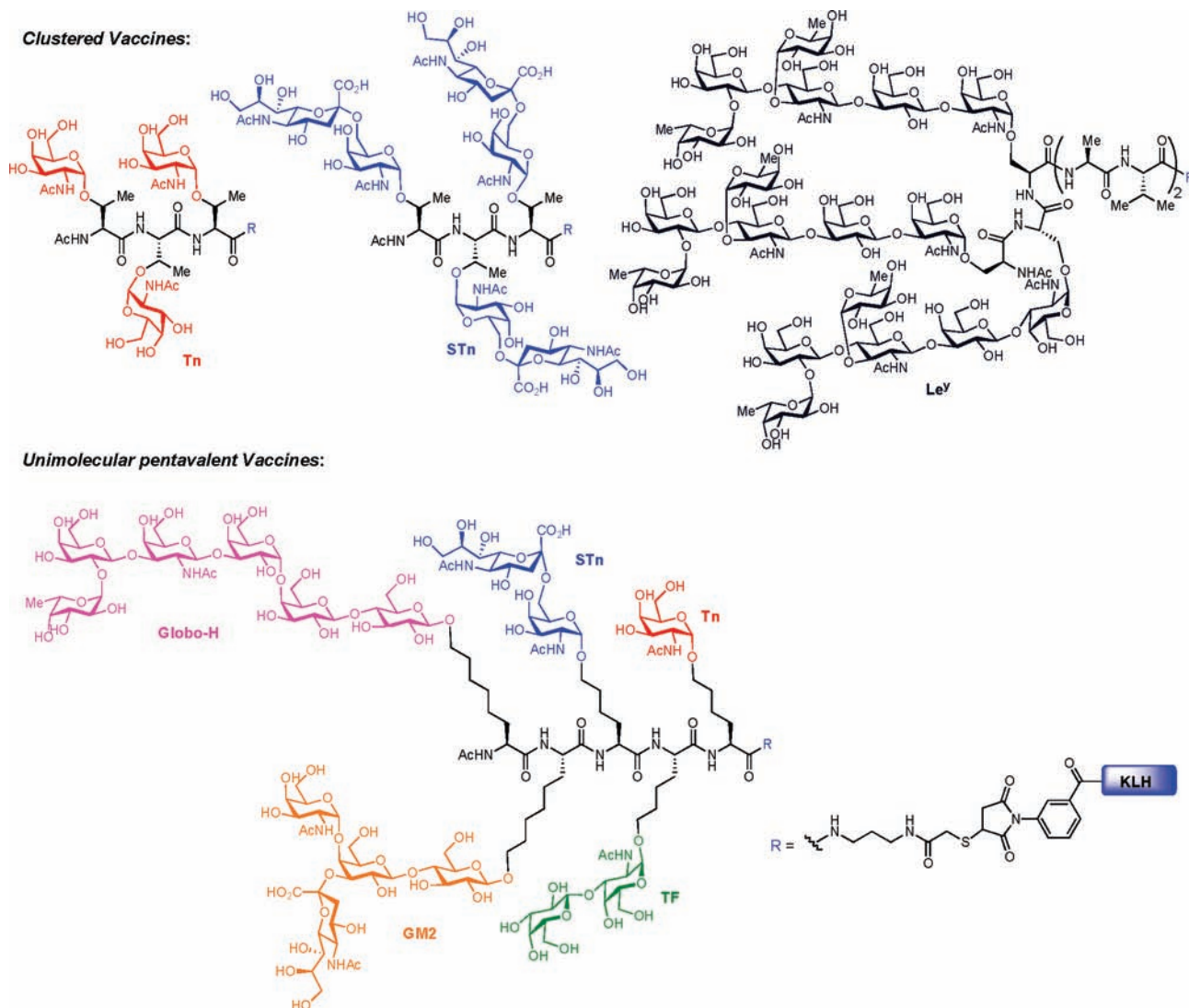
Figure 1. Representative anticancer vaccine constructs.

Along these lines, we have taken note of the mucin-bound carbohydrate antigens, Tn and STn, which are overexpressed on the surfaces of a variety of epithelial cancers, such as prostate, breast, colon, and ovarian.[8] On the tumor cell surface, Tn and STn are presented in broadly conserved "clusters" of 2−4 carbohydrate units, O-linked to the mucin peptide through serine or threonine residues.

Approaches using monomeric Tn or STn antigen, in which one glycan unit is covalently appended to an immunogenic carrier protein, have proven beneficial.[9] However, it has been shown that[10] clustered vaccines—wherein multiple copies of the carbohydrate are incorporated on a peptide backbone (Figure 1)—induce higher titers against some carbohydrate epitopes.[10a] Indeed, recent studies with the antitumor monoclonal antibody (mAb) B72.3 revealed that it preferentially recognized clustered STn in comparison with monovalent STn.[9a,10b,c]

To some extent, the lowered entropic penalty associated with the increase in valency seems to enhance carbohydrate−protein

interactions. However, the choice of template for multivalent carbohydrate display may be crucial. Excessively flexible scaffolds will permit attached glycans to remain far apart in most conformations, thereby perhaps decreasing effective clustering of the antigen. In considering a template for the presentation of the clustered carbohydrates, we have been attracted to the model, depicted in Figure 2, upon which the clustered glycans would be displayed in a well-defined orientation. Our design, clearly inspired by Dumy[11] and Robinson,[12] is amenable to variations in the number and type of carbohydrates displayed, as well as the spacing of the carbohydrate domains. Moreover, the promise of such templates is evidenced by recent studies demonstrating their use for clustered antigen

(8) (a) Springer, G. F. *Science* **1984**, *224*, 1198–1206. (b) Itzkowitz, S. H.; Yuan, M.; Montgomery, C. K.; Kjeldsen, T.; Takahashi, H. K.; Bigbee, W. L.; Kim, Y. S. *Cancer Res.* **1989**, *49*, 197–204. (c) Kim, Y. S.; Gum, J.; Brockhausen, I. *Glyconjugate J.* **1996**, *13*, 693–707. (d) Springer, G. F. *J. Mol. Med.* **1997**, *75*, 594–602. (e) Springer, G. F. *Crit. Rev. Oncogenesis* **1995**, *6*, 57–85.

(9) (a) Adluri, S.; Helling, F.; Ogata, S.; Zhang, S.; Itzkowitz, S. H.; Lloyd, K. O.; Livingston, P. O. *Cancer Immunol. Immunother.* **1995**, *41*, 185–192. (b) MacLean, G. D.; Reddish, M.; Koganty, R. R.; Wong, T.; Gandhi, S.; Smolenski, M.; Samuel, J.; Nabholtz, J. M.; Longenecker, B. M. *Cancer Immunol. Immunother.* **1993**, *36*, 215–222. (c) MacLean, G. D.; Reddish, M. A.; Bowen-Yacyshyn, M. B.; Poppema, S.; Longenecker, B. M. *Cancer Invest.* **1994**, *12*, 46–56. (d) MacLean, G. D.; Miles, D. W.; Rubens, R. D.; Reddish, M. A.; Longenecker, B. M. *J. Immunother.* **1996**, *19*, 309–316. (e) MacLean, G. D.; Reddish, M. A.; Koganty, R. R.; Longenecker, B. M. *J. Immunother.* **1996**, *19*, 59–68. (f) O'Boyle, K. P.; Markowitz, A. L.; Khorshidi, M.; Lalezari, P.; Longenecker, B. M.; Lloyd, K. O.; Welt, S.; Wright, K. E. *Hybridoma* **1996**, *15*, 401–408.
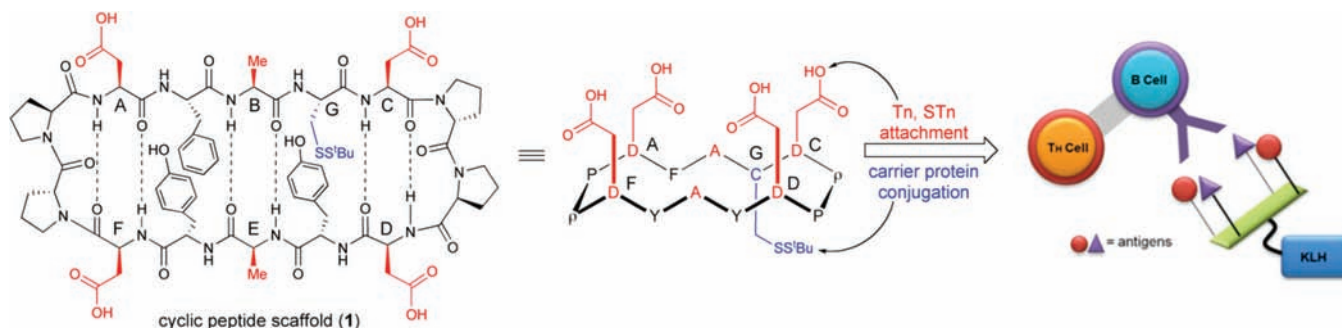
Figure 2. Cyclic peptide scaffold **1** and antibody response to multivalent-KLH conjugate. Tʜ Cell = T helper cell.
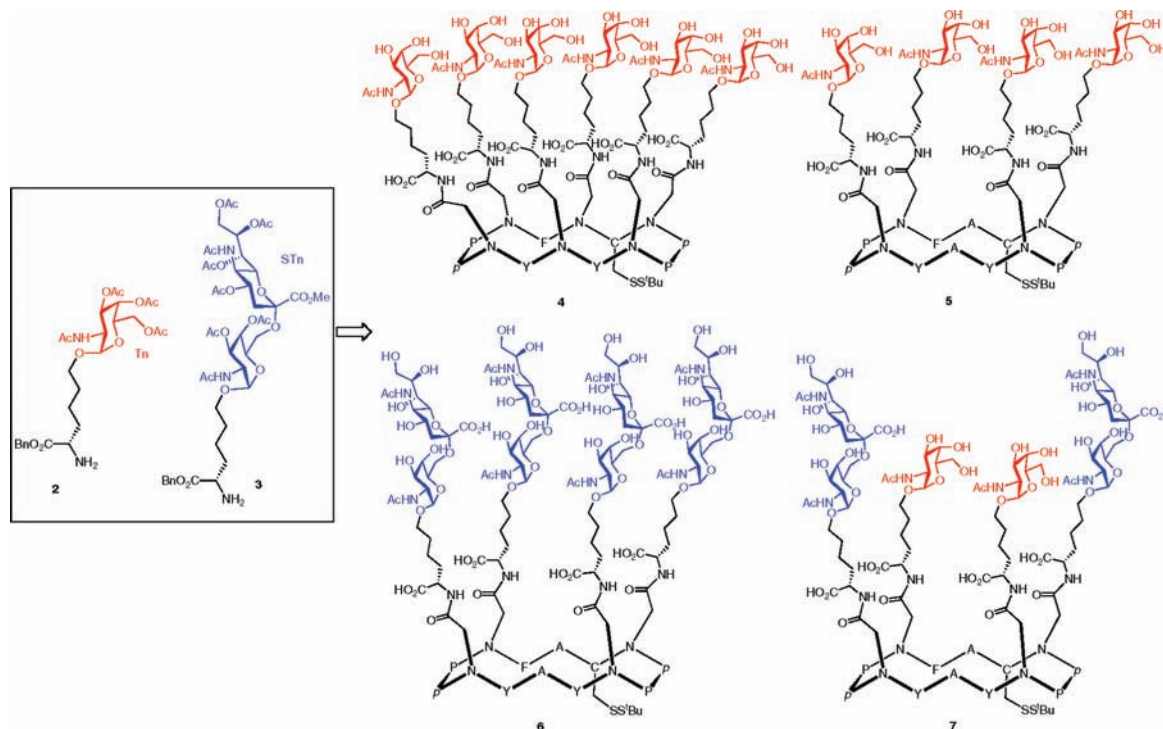
Figure 3. Protected glycosylamino acids and clustered antigens.

syntheses in our lab and elsewhere.[13,14] In fact, we have recently employed this scaffold in the context of a separate program, directed toward the development of an HIV vaccine.[15]

The essential element of our cyclic peptide design is a pair of β-turn-inducing D-Pro-L-Pro sequences[16] at both ends of the macrocycle. Positions A−F (red, with side chain projecting "above" the plane of the scaffold) may contain handles for glycan attachment, whereas position G is a cysteine residue (blue, with side chain projecting from the "bottom" face of the scaffold), suitable for linkage to a carrier protein or biological marker. This scaffold is tunable in that differential placement of aspartate residues in positions A−F permits variation in the number and spacing of the glycan attachments (**1**, Figure 2).

In an effort to identify an optimal presentation of the carbohydrates on the cyclic peptide scaffold, we chose as our targets the four constructs shown in Figure 3. Structures

(10) (a) Kudryashov, V.; Glunz, P. W.; Williams, L. J.; Hintermann, S.; Danishefsky, S. J.; Lloyd, K. O. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3264–3269. (b) Slovin, S. F.; Ragupathi, G.; Musselli, C.; Olkiewicz, K.; Verbel, D.; Kuduk, S. D.; Schwarz, J. B.; Sames, D.; Danishefsky, S. J.; Livingston, P. O.; Scher, H. I. *J. Clin. Oncol.* **2003**, *21*, 4292–4298. (c) Zhang, S.; Walberg, L. A.; Ogata, S.; Itzkowitz, S. H.; Koganty, R. R.; Reddish, M.; Gandhi, S. S.; Longenecker, B. M.; Lloyd, K. O.; Livingston, P. O. *Cancer Res.* **1995**, *55*, 3364–3368.

(11) (a) Dumy, P.; Eggleston, I. M.; Cervigni, S.; Sila, U.; Sun, X.; Mutter, M. *Tetrahedron Lett.* **1995**, *36*, 1225–1258. (b) Dumy, P.; Renaudet, O. *Org. Lett.* **2003**, *5*, 243–246. (c) Singh, Y.; Dolphin, G. T.; Razkin, J.; Dumy, P. *ChemBioChem* **2006**, *7*, 1298–1314.

(12) (a) Jiang, L.; Moehle, K.; Dhanapal, B.; Obrecht, D.; Robinson, J. A. *Helv. Chim. Acta* **2000**, *83*, 3097–3112. (b) Favre, M.; Moehle, K.; Jiang, J.; Pfeiffer, B.; Robinson, J. A. *J. Am. Chem. Soc.* **1999**, *121*, 2679–2685, For a review, see: (c) Robinson, J. A. *Synlett* **2000**, 429–441.

(13) Grigalevicius, S.; Chierici, S.; Renaudet, O.; Lo-Man, R.; Deriaud, E.; Leclerc, C.; Dumy, P. *Bioconjugate Chem.* **2005**, *16*, 1149–1159.

(14) Wang, J.; Li, H.; Zou, G.; Wang, L. X. *Org. Biomol. Chem.* **2007**, *5*, 1529–1540.

(15) (a) Krauss, I. J.; Joyce, J. G.; Finnefrock, A. C.; Song, H. C.; Dudkin, V. Y.; Geng, X. J.; Warren, D.; Chastain, M.; Shiver, J. W.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2007**, *129*, 11042–11044. (b) Joyce, J. G.; Krauss, I. J.; Song, H. C.; Opalka, D. W.; Grimm, K. M.; Nahas, D. D.; Esser, M. T.; Hrin, R.; Feng, M.; Dudkin, V. Y.; Chastain, M.; Shiver, J. W.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 15684–15689.

(16) Bean, J. W.; Kopple, K. D.; Peishoff, C. E. *J. Am. Chem. Soc.* **1992**, *114*, 5328–5334.

Scheme 1. Synthesis of Cyclic Peptide Scaffolds
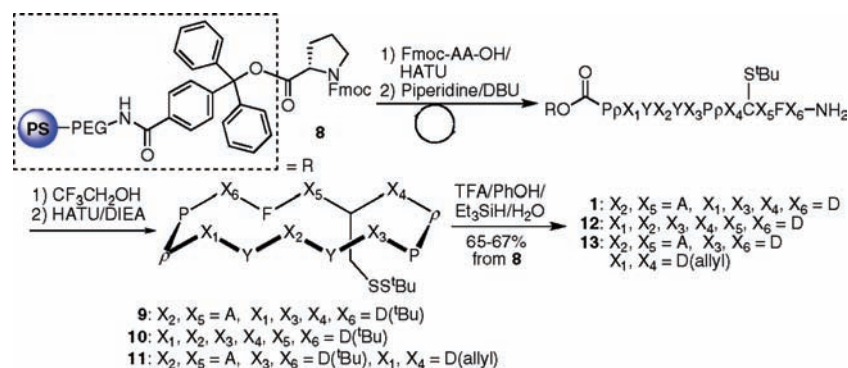
4 and **5** incorporate six and four replicate copies of the Tn antigen, respectively, and construct **6** presents four copies of the STn disaccharide. We also sought to prepare a multiantigenic construct, **7**, incorporating both the Tn and STn antigens.

Protected O-linked glycosylamino acids **2** and **3** (Figure 3) were prepared from the L-hydroxynorleucine benzyl ester, according to our previously established protocol.[17] These Tn and STn "cassettes," which we originally employed in earlier approaches to the synthesis of clustered antigens, serve as useful building blocks for glycal assembly. In this system, the N-termini of both the Tn and STn cassettes serve as handles for coupling to the peptide scaffold, and the remaining carboxyl function may provide a handle for further elaboration (i.e., as T-helper or additional B-epitope attachments).

Cyclic peptides **9**, **10**, and **11** (containing 4 or 6 aspartate residues) were prepared in parallel through automated solid-phase synthesis from the prolinated trityl resin **8** (Scheme 1). Cleavage from the resin, macrocyclization,[18] and deprotection of the *tert*-butyl esters of the aspartate and tyrosine residues furnished **1**, **12**, and **13** in good overall yields.

With the components of the target structures in hand, we directed our efforts toward the covalent attachment of the carbohydrate antigens to the scaffolds. The Lansbury aspartylation reaction[19] is often employed in glycopeptide synthesis. However, the standard Lansbury aspartylation protocol employs a glycosidic amine as the coupling partner of activated aspartic acid, rather than the primary amino acid nitrogen, which would be required by our strategy. In considering the application of this protocol to our own system, there was concern that altered nucleophilicity of the amine might favor the emergence of nonproductive pathways. For example, the competing, relatively facile cyclization of the peptide itself might lead to the formation of aspartimide.[20] Indeed, this side reaction was found to be a problem in the coupling of peptide **12** with glycosylamino acid **2**. Under standard conditions (HOAt, HATU, DIEA/DMSO), formation of the undesired aspartimide was predominant, with little

indication of the requisite hexavalent product, **14**. Upon close investigation, it was found that the HOAt, which is generally used as an activation additive, along with HATU, plays a significant role in promoting undesired aspartimide formation. Fortunately, activation of the aspartic acids with HATU alone in the presence of DIEA in DMSO effectively minimized aspartimide formation, and consequently allowed high yielding conversion to the desired hexavalent product, **14** (Scheme 2).

Having identified workable coupling conditions, we were able to assemble the tetravalent constructs **15** and **16** in a similar fashion. These less congested glycopeptides exhibit different spatial arrangements, yet they still express the epitopes in highly clustered fashions. Following global deprotection, the highly clustered antigens **4**, **5**, and **6** were in hand.

We next turned to the synthesis of the unimolecular multiantigenic construct, **7**,[21] wherein both the Tn and STn carbohydrate antigens are displayed on the peptidic backbone. This type of multivalent construct is intended to reflect the degree of carbohydrate heterogeneity associated with most cancers.[22] There is significant variation in the types of carbohydrates which are overexpressed on the tumor cell surface, even within a particular cancer type. By combining clusters of both the Tn and STn antigens within a single cyclic peptide scaffold, we would hope to induce a more effective immune response, in which the antibodies raised would target a greater proportion of transformed tumor cells. It is not unlikely that careful strategic considerations in the peptide scaffold design could well influence the effectiveness of a multiantigenic construct.

(17) Keding, S. J.; Endo, A.; Danishefsky, S. J. *Tetrahedron* **2003**, *59*, 7023–7031.

(18) The macrocyclization was essentially instantaneous at room temperature, indicating that cross-strand hydrogen bonding may preorganize the acyclic peptide for cyclization. Moreover, downfield ^{1}H NMR chemical shifts of selected **NH** resonances, slow D_2O exchange times, and the temperature profile of these chemical shifts provided preliminary evidence of the desired β-sheet character of the peptide scaffold.

(19) Cohen-Anisfeld, S. T.; Lansbury, P. T. *J. Am. Chem. Soc.* **1993**, *115*, 10531–10537.

(20) (a) Bodanszky, M.; Natarajan, S. *J. Org. Chem.* **1975**, *40*, 2495–2499. (b) Bodanszky, M.; Kwei, J. Z. *Int. J. Pept. Protein Res.* **1978**, *12*, 69–74. (c) Tam, J. P.; Riemen, M. W.; Merrifield, R. B. *Pept. Res.* **1988**, *1*, 6–18. (d) Mergler, M.; Dick, F.; Sax, B.; Weiler, P.; Vorherr, T. *J. Pept. Sci.* **2003**, *9*, 36–46. (e) Lauer, J. L.; Fields, C. G.; Fields, G. B. *Lett. Pept. Sci.* **1994**, *1*, 197–205.

(21) (a) Ragupathi, G.; Koide, F.; Livingston, P. O.; Cho, Y. S.; Endo, A.; Wan, Q.; Spassova, M. K.; Keding, S. J.; Allen, J.; Ouerfelli, O.; Wilson, R. M.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 2715–2725. (b) Allen, J. R.; Harris, C. R.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 1890–1897. (c) Ragupathi, G.; Coltart, D. M.; Williams, L. J.; Koide, F.; Kagan, E.; Allen, J.; Harris, C.; Glunz, P. W.; Livingston, P. O.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13699–13704. Keding, S. J.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11937–11942.

(22) (a) Zhang, S. L.; Cordon-Cardo, C.; Zhang, H. S.; Reuter, V. E.; Adluri, S.; Hamilton, W. B.; Lloyd, K. O.; Livingston, P. O. *Int. J. Cancer* **1997**, *73*, 42–49. (b) Zhang, S. L.; Zhang, H. S.; Cordon-Cardo, C.; Reuter, V. E.; Singhal, A. K.; Lloyd, K. O.; Livingston, P. O. *Int. J. Cancer* **1997**, *73*, 50–56.

Scheme 2. Syntheses of Clustered Glycopeptides
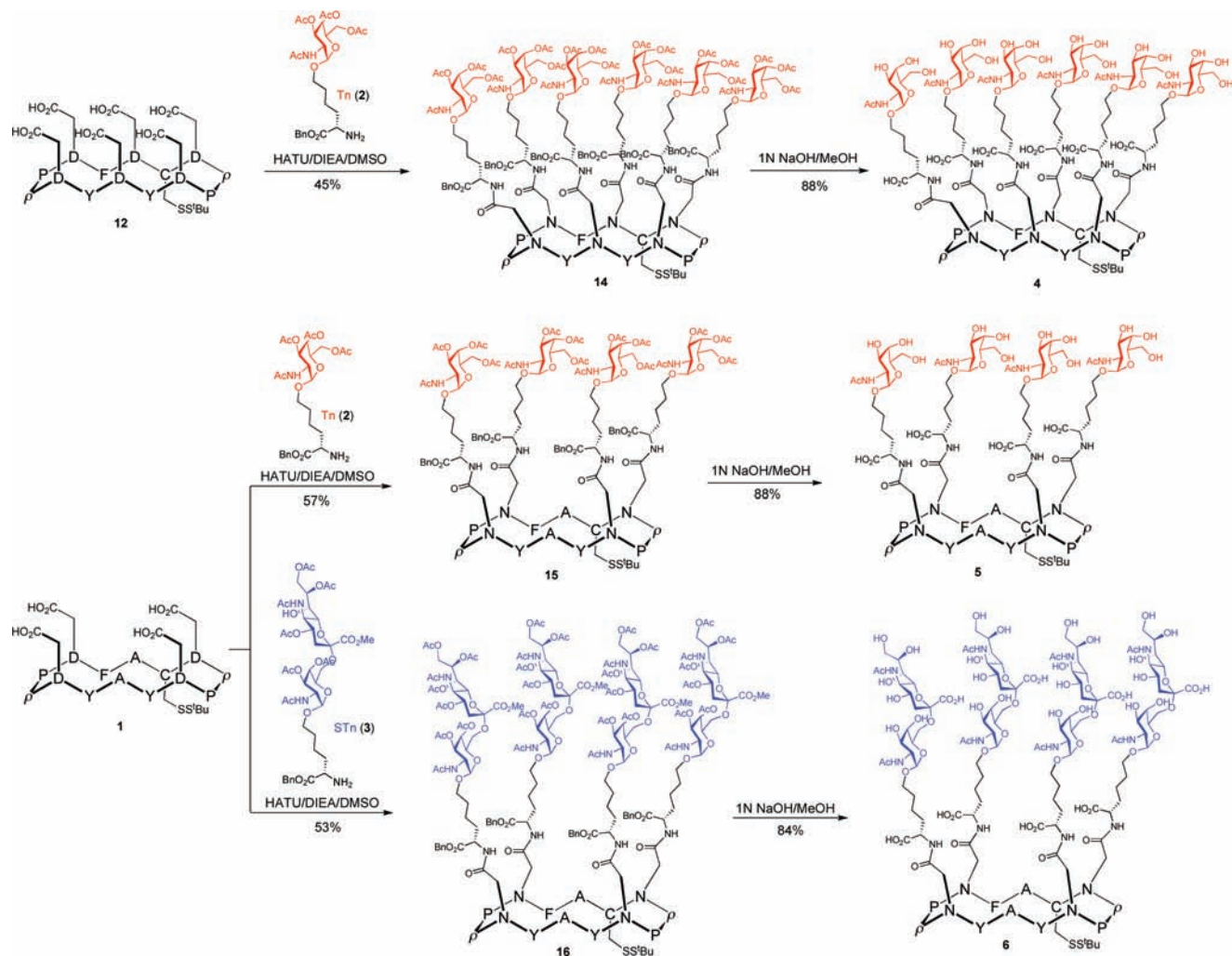
Initial efforts to reach cyclic peptide **17** were unsuccessful, due to complications arising from aspartimide formation at position 8, leading to the undesired product, **17a** (Scheme 3). Unlike the bulky Asp-β-*tert*-butyl esters, the less hindered Asp-β-allyl ester is susceptible to undesired aspartimide formation, arising from intramolecular nucleophilic attack by the amide nitrogen at the aspartyl C-terminus.[23] However, aspartimide formation of the Asp-β-allyl ester at position 5 was impeded by the presence of proline in position 6. Thus, we initiated a design decision to transpose the -β-allyl and -β-*tert*-butyl protecting groups of positions 8 and 12. Gratifyingly, no evidence of aspartimide formation was found in the synthesis of cyclic peptide **18**.

Following selective *tert*-butyl deprotection of **18** under the conditions described in Scheme 1, intermediate **13** was in hand. We then undertook the sequential attachment of the Tn and STn carbohydrate antigens. Eventually, coupling of Tn **2** with peptide **13** proceeded efficiently to produce the Tn glycopeptide construct, as shown in Scheme 4. The liberation of additional reaction sites by palladium-mediated allyl deprotection[24] resulted in a high yield of divalent

product **20**. Addition of STn **3** to activated **20** and subsequent global deacetylation completed the synthesis of tetravalent antigen **7**.

An intriguing question arose concerning the clustered antigen design with regard to the potential flexibility of linkers that attach the glycans to the cyclic peptide scaffold. Cross-linking elements between carbohydrates would rigidify the construct and prevent undesired spreading of glycan units. This should better mimic a tightly clustered antigen and enhance the cluster-recognizing antibody response. Ring-closing metathesis (RCM) has proven to be a powerful tool for the formation of peptide macrocycles in numerous settings.[25] In particular, fascinating work of Kiessling[26] on the synthesis of multivalent glycoconjugates has further encouraged us to explore the ruthenium-catalyzed metathesis for the incorporation of cross-linker in our system, which would lead to highly clustered cross-linked constructs. Our

(23) Mergler, M.; Dick, F.; Sax, B.; Weiler, P.; Vorherr, T. *Pept. Sci.* **2003**, *9*, 36–46.

(24) Kunz, H.; Waldmann, H. *Angew. Chem., Int. Ed.* **1984**, *23*, 71–72.

(25) (a) Blackwell, H. E.; Sadowsky, Jack., D.; Howard, R. J.; Sampson, J. N.; Chao, J. A.; Steinmetz, W. E.; O'Leary, D. J.; Grubbs, R. H. *J. Org. Chem.* **2001**, *66*, 5291–5302. (b) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606–9614. (c) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **1998**, *37*, 3281–3284. (d) Schmiedeberg, N.; Kessler, H. *Org. Lett.* **2002**, *4*, 59–62. (e) Kazmaier, U.; Maier, S. *Org. Lett.* **1999**, *1*, 1763–1766. (f) Jarvo, E. R.; Copeland, G. T.; Papaioannou, N.; Bonitatebus, P. J., Jr.; Miller, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 11638–11643.

Scheme 3. Cyclic Peptide Scaffold Design for Multi-Antigen Attachments
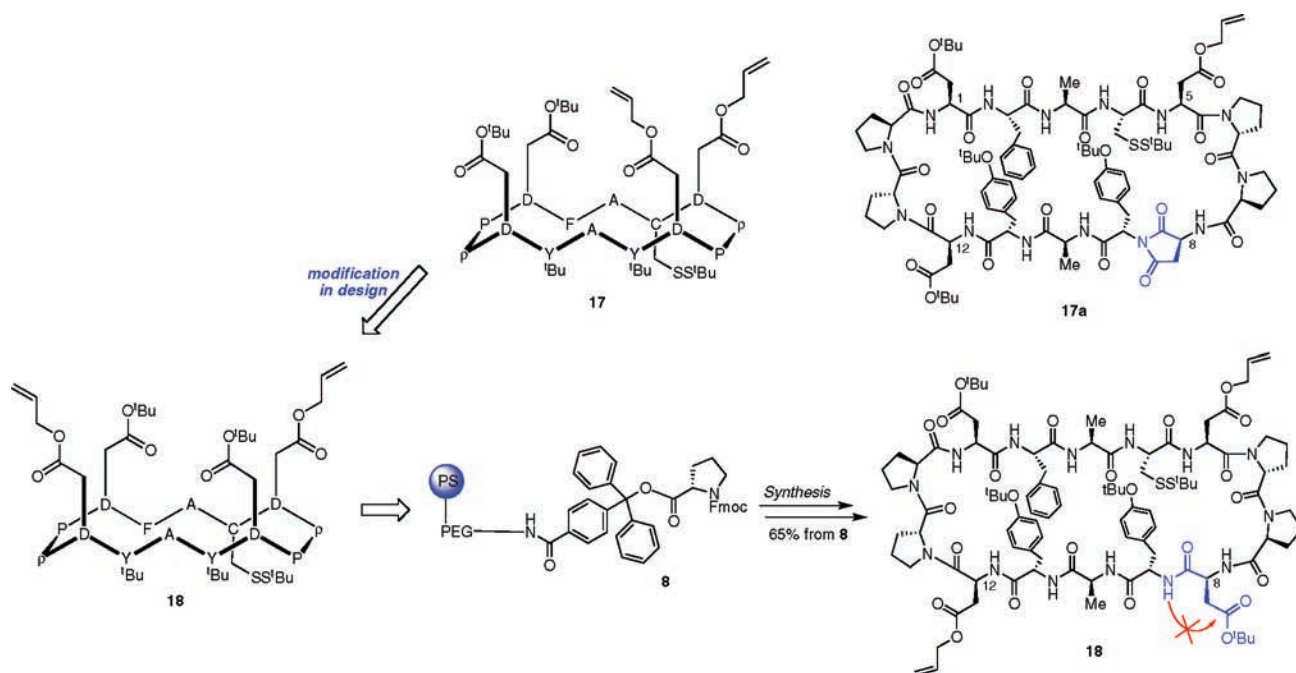
Scheme 4. Synthesis of Unimolecular Multiantigenic Glycopeptide **7**
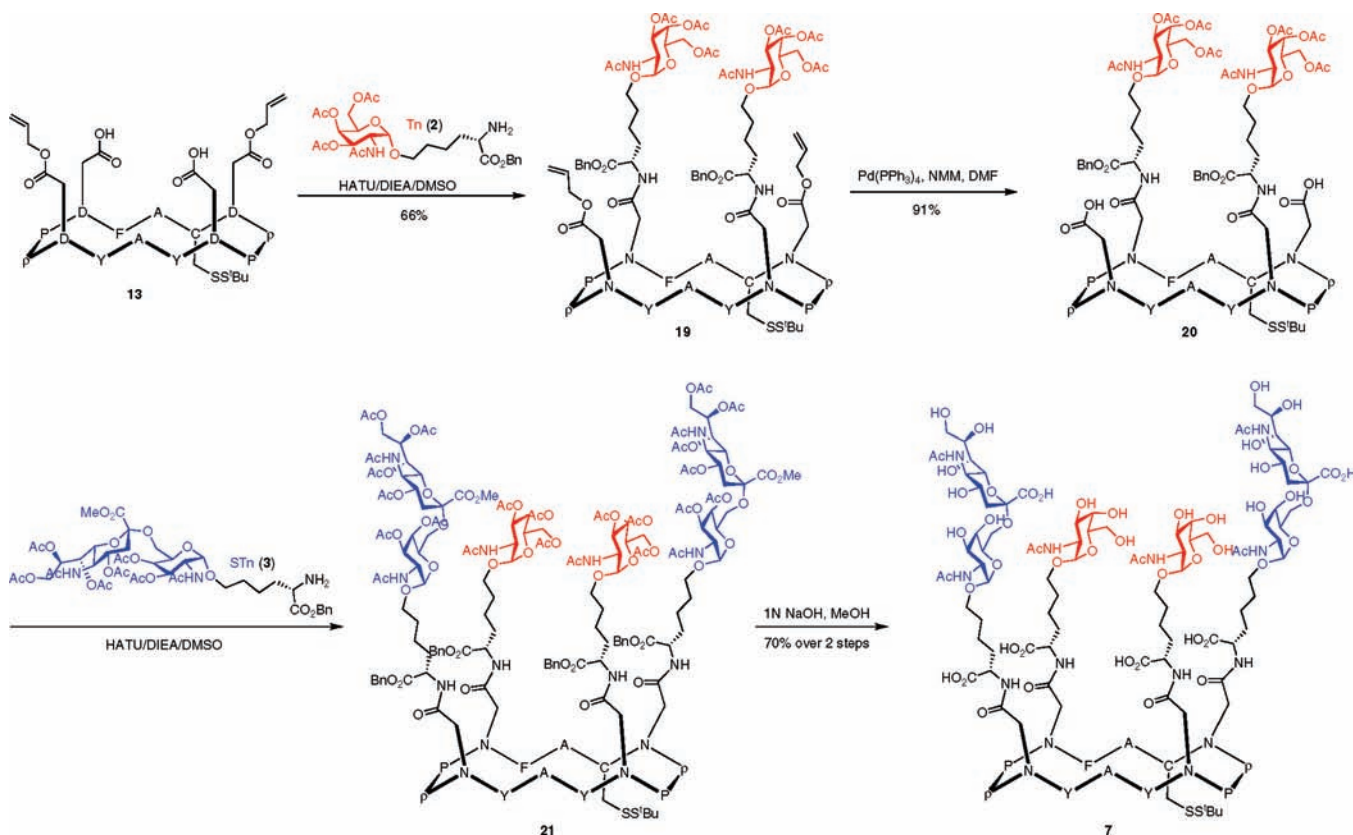
initial attempted RCM of di-Tn precursor **23** employing Grubbs catalyst (**24**) proceeded to generate spacer-linked construct **27** in modest yield (Scheme 5). Variation of the reaction conditions, including higher catalyst loading, was unable to induce complete conversion of **23**. Additionally, mass spectra of isolated RCM products indicated the existence of inseparable contaminants in which a methylene group was deleted,[27a] presumably due to the ruthenium complex-

mediated isomerization[27] of the terminal olefin of **23** (Figure 4, top) prior to ring closure. Although a significant increase in yield was observed when the Hoveyda-Grubbs II catalyst (**25**)[28] was employed, terminal olefin isomerization could not be suppressed. Gratifyingly, no isomerization of **23** occurred with the use of Hoveyda–Grubbs I catalyst (**26**),[29] which afforded the desired RCM product **27** in good yield (Scheme 5 and Figure 4 (bottom)). The product olefins, a mixture of
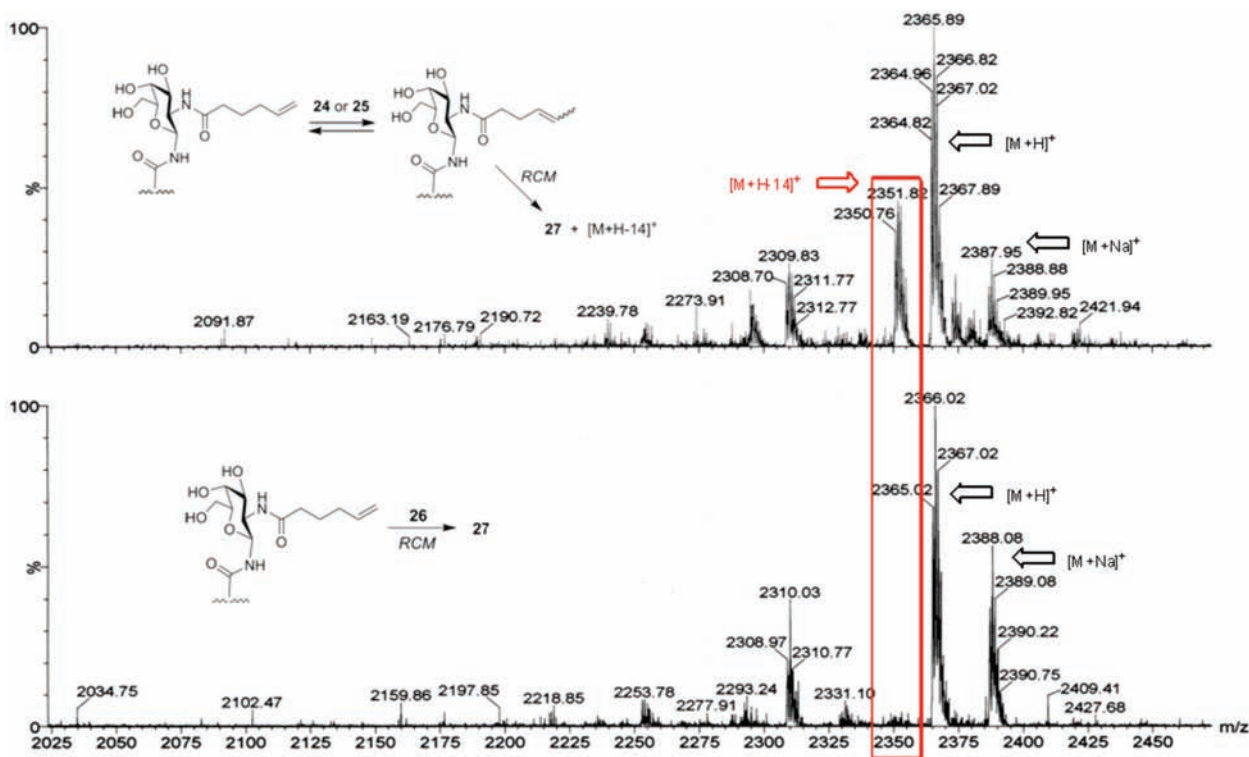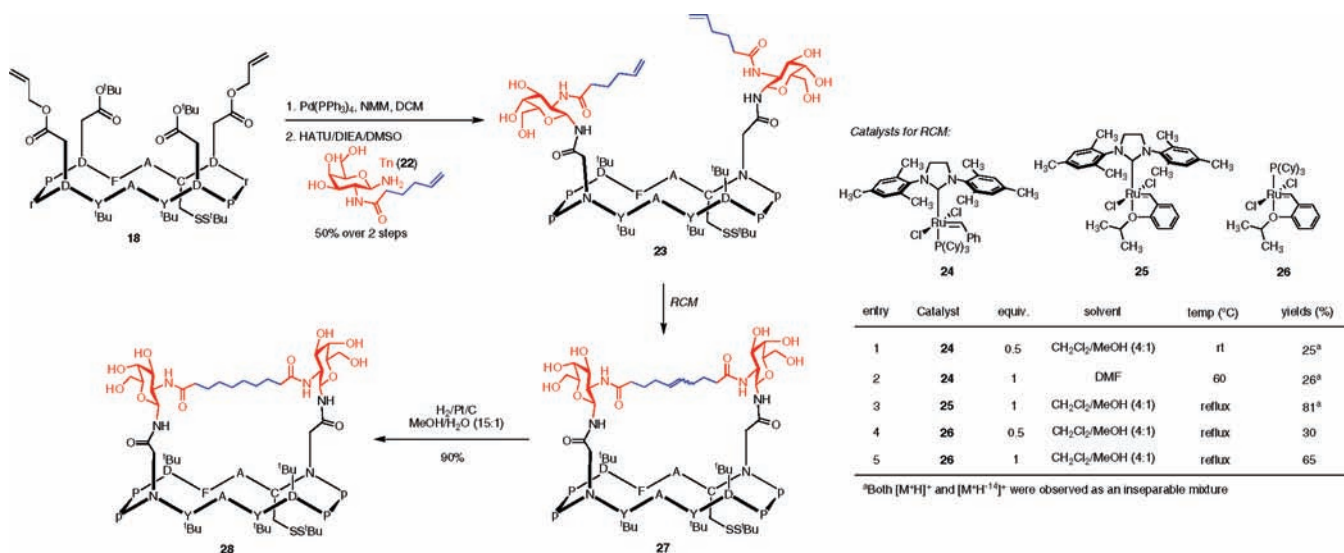
Figure 4. Low-resolution mass spectra of RCM products.

Scheme 5. Synthesis of Cross-Linked Glycopeptide **28**

entry	Catalyst	equiv.	solvent	temp (°C)	yields (%)
1	24	0.5	CH$_2$Cl$_2$/MeOH (4:1)	rt	25[a]
2	24	1	DMF	60	26[a]
3	25	1	CH$_2$Cl$_2$/MeOH (4:1)	reflux	81[a]
4	26	0.5	CH$_2$Cl$_2$/MeOH (4:1)	reflux	30
5	26	1	CH$_2$Cl$_2$/MeOH (4:1)	reflux	65

[a] Both [M+H]$^+$ and [M+H-14]$^+$ were observed as an inseparable mixture

E- and *Z*-isomers, were then hydrogenated to furnish **28**. The protected aspartyl residues of **28** may serve as additional handles for further modification of the construct; i.e. attach-ment of multiple different antigens, to appropriately reflect the heterogeneity of target cancers.

Conclusions

In summary, we have described the design and synthesis of multivalent anticancer vaccine constructs. The individual carbohydrate-based antigens were prepared in well-defined and highly clustered modes by convergent synthesis. In addition, the successful introduction of a cross-linker provides a wider scope of future modifications in epitope design either through spacer length variations or additional glycan attachments on the scaffolds. Upon conjugation to KLH carrier protein, the anticipated higher molecular ratio of Tn or STn versus KLH

(26) (a) Yang, Z.-Q.; Puffer, E. B.; Pontrello, J. K.; Kiessling, L. L. *Carbohydr. Res.* **2002**, *337*, 1605–1613. (b) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2002**, *124*, 14922–14933. (c) Strong, L. E.; Kiessling, L. L. *J. Am. Chem. Soc.* **1999**, *121*, 6193–6196. (d) Kanai, M.; Mortell, K. H.; Kiessling, L. L. *J. Am. Chem. Soc.* **1997**, *119*, 9931–9932. (e) Schuster, M. C.; Mortell, K. H.; Hegeman, A. D.; Kiessling, L. L. *J. Mol. Catal. A: Chem.* **1997**, *116*, 209–216. (f) Mortell, K. H.; Weatherman, R. V.; Kiessling, L. L. *J. Am. Chem. Soc.* **1996**, *118*, 2297–2298. (g) Mortell, K. H.; Gingras, M.; Kiessling, L. L. *J. Am. Chem. Soc.* **1994**, *116*, 12053–12054.

could offer an additional benefit in terms of immunogenicity. We expect that these highly clustered anticancer constructs will help in gaining further insight into the way in which carbohydrate clusters are recognized by the immune system. Conjugation to KLH carrier protein and immunological investigations will be reported in due course.

(27) (a) Wan, Q.; Cho, Y. S.; Lambert, T. H.; Danishefsky, S. J. *J. Carbohydr. Chem.* **2005**, *24*, 425–440. (b) Hu, Y.-J.; Dominique, R.; Das, S. K.; Roy, R. *Can. J. Chem.* **2000**, *78*, 838–845. (c) Wipf, P.; Rector, S. R.; Takahashi, H. *J. Am. Chem. Soc.* **2002**, *124*, 14848–14849. (d) Cadot, C.; Dalko, P. I.; Cossy, J. *Tetrahedron Lett.* **2002**, *43*, 1839–1841.

(28) Garber, S. B.; Kingsbury, J. S.; Gray, B. L.; Hoveyda, A. H. *J. Am. Chem. Soc.* **2000**, *122*, 8168–8179.

(29) Kingsbury, J. S.; Harrity, J. P. A.; Bonitatebus, P. J., Jr.; Hoveyda, A. H. *J. Am. Chem. Soc.* **1999**, *121*, 791–799.