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Synthetic Carbohydrate Vaccines: Synthesis and Immunogenicity of Tn Antigen Conjugates

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Abstract—A tumor-associated carbohydrate antigen, Tn antigen (GalNAcα1→*O*-Ser), was synthesized with a spacer arm, and assembled to dimeric and trimeric structures using *N*-*tert*-butyloxycarbonyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine as a key building block. The synthetic antigens were conjugated with OSA and their immunogenicity examined in mice. Mice immunized with dimeric or trimeric Tn antigen showed a stronger antibody (IgM) response to a Tn-glycoprotein (asialo-ovine submaxillary mucin) than mice immunized with monomeric Tn antigen. The dimeric and trimeric Tn antigens also induced measurable IgG responses. The dimeric Tn antigen was further coupled to a Starburst® dendrimer (5th generation) and to tripalmitoyl-*S*-glycerylcysteinyl-serine, a synthetic lipopeptide of the active moiety of a major lipoprotein of *Escherichia coli*. Unexpectedly, the Starburst® dendrimer conjugate did not stimulate any immune response specific to Tn antigen. On the other hand, immunization of mice with the lipopeptide conjugate produced not only a high IgM response but also significant IgG anti-Tn response without any carrier molecules or additional adjuvants. The production of IgG antibody is quite significant since carbohydrate antigens are in general known to produce only IgM antibody response. Being a totally synthetic, low-molecular weight, and carrier-free immunogen, the lipopeptide conjugate could be a prototype of synthetic carbohydrate vaccines.

Introduction

Cell-surface carbohydrates undergo dramatic changes as a consequence of malignant transformation.[1] The alteration is resulted from either incomplete glycosylation or neoglycosylation by tumor cells. Among the large number of known tumor-associated carbohydrate antigens (TACAs), Tn (GalNAcα1→*O*-Ser/Thr) and sialosyl Tn (NeuAcα2→6GalNAcα1→*O*-Ser/Thr) are the most specific to human cancers, and the most restricted in their expression on normal cells and tissues. These antigens represent the core structure of *O*-linked glycans exposed on mucin-type glycoproteins of tumor cells through blocked synthesis of carbohydrate chains. They are cryptic in normal cells because of further elongation to form complex oligosaccharides. Tn antigen was originally found as a very rare blood-group antigen, detected by naturally-occurring antibodies. Its occurrence (less than 1 out of 100,000 people) is often associated with hemolytic leukemia and leukopenia (Tn syndrome).[2] Springer *et al.* observed that many human cancers react with anti-Tn serum, i.e. A_1 serum exhaustively adsorbed with desialylated A_1 and O erythrocytes.[3] Tn structure was subsequently identified as α-GalNAc linked to Ser/Thr.[4] Although Tn was claimed to be a tumor-associated antigen, its expression and specificity were not established until the monoclonal antibody (mAb) approach was applied. Hirohashi *et al.*[5] established a mAb against human squamous cell lung carcinoma which showed strong reactivity with various other human cancers but also cross-reacted with blood group A. The epitope of this mAb was identified as Tn. Subsequent studies revealed that Tn is expressed in over 70 % of lung, colon, and stomach carcinomas.[6] Therefore, the carbohydrate antigens expressed on tumor cell surfaces have gained considerable interest as potential targets for diagnosis and immunotherapy of cancer, including cancer vaccines.[7]

The main idea of cancer vaccines is to induce a cancer-selective immunological attack in the tumor-bearing host, resulting in tumor rejection (active specific immunotherapy or ASI). ASI with whole tumor cells or cell fractions has so far been of limited success.[8] It is desirable to use chemically unambiguous antigens for ASI which are capable of generating specific immune responses. In recent years, cancer vaccines based on well-defined tumor-associated carbohydrate antigens have shown some promise in the suppression of tumor growth. These include purified ganglioside GM_2 coated on *Bacillus Calmette-Guérin*,[9] synthetic T antigen coupled to human serum albumin or keyhole limpet hemocyanin (KLH),[10] and purified asialo-ovine submaxillary mucin (A-OSM),[11] which contains a large amount of Tn antigen (> 96 % GalNAc by sugar analysis). However, most carbohydrate antigens elicit only humoral responses.[12] Since antigen-specific cellular immune responses are also important for successful ASI, an efficient presentation of synthetic carbohydrate antigens to the immune system needs to be designed such that these antigens will be recognized by B and T lymphocytes. Ideally, synthetic vaccines should elicit a strong immune response without the aid of macromolecular carriers or adjuvants, which then would eliminate irrelevant determinants and ambiguity in composition and structure.[13,14]

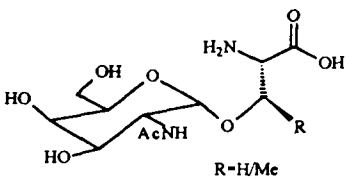
To develop totally synthetic carbohydrate vaccines, we have synthesized monomeric (Mono-Tn, **1**), dimeric (Di-Tn, **2**), and trimeric Tn (Tri-Tn, **3**) antigens with a spacer arm[15] (Fig. 1). These synthetic antigens have been subsequently conjugated with ovine serum albumin (OSA),[16] Starburst® dendrimer,[17] and tripalmitoyl-*S*-glycerylcysteinylserine (P_3CS), which is a highly potent B-cell and macrophage activator derived from the immunologically active *N*-terminal sequence of the principal lipoprotein of *Escherichia coli*[18] (Fig. 2). We report here the synthesis of these Tn antigens **1–3** and conjugates **4–8**, together with their immunogenicity in mice.[19] Our results indicate that Di-Tn–P_3CS conjugate **8** is a totally synthetic, low molecular weight, carrier-free immunogen that elicits immune responses against Tn-expressing glycoproteins. To our knowledge, this is the first example that a synthetic, small carbohydrate antigen can generate an immune response, especially IgG response, against a tumor-associated carbohydrate antigen without the use of a macromolecular carrier or an adjuvant.

Results and Discussion

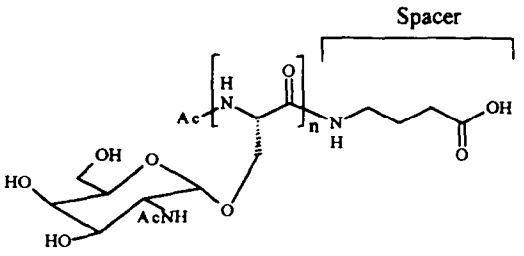
Synthesis of Mono-Tn (1), Di-Tn (2), and Tri-Tn (3) antigens

Synthesis of Tn, sialosyl Tn, and T antigens, and their clusters have been reported by several groups.[20] We have chosen the trichloroacetimidate method[21] for a practical large-scale (~50 mmol) synthesis of α-glycosyl amino acids (Scheme I). Glycosylation of the serine derivative **10** with glycosyl imidate **9**,[21] readily prepared from D-galactose through azidonitration, using trimethylsilyl trifluoromethanesulfonate as a promotor yielded a 5:1 mixture (72 % yield) of the α-[20g] (**12α**) and β-glycosides (**12β**). The α-glycoside was predominantly formed regardless of the anomeric configuration of **9** as previously reported.[22] These anomers were easily separated by silica-gel column chromatography. The azido groups in **12α** and **12β** were reduced by H_2S in pyridine–H_2O[23] and subsequently acetylated to give the protected Tn antigen **13α**[20g] (88 % yield) and its β-anomer **13β**[24] (86 % yield), respectively. Similar glycosylation of the threonine derivative **11** with **9** proceeded in the α-selectivity[20d] to give a 4:1 mixture (93 % yield) of **17α**[20g] and **17β**.[20d] Although these anomers have been reported to be separable in some degree by silica-gel column chromatography,[20d,g] in our hands these were so close together as to be inseparable. Separation of the anomers was, however, successful after reduction of the azido to the amino groups by H_2S in pyridine–H_2O (→**18α** and **18β**). Acetylation of **18α** and **18β** yielded the protected Tn antigen[20g] **19α** (54 % yield from **9**) and its β-anomer **19β** (12 % yield from **9**), respectively. Hydrogenolysis of **13α/13β** and **19α/19β** with 10 % Pd/C (→**14α/14β** and →**20α**[20g]**/20β**, respectively), followed by protection of the amino group by the acid-labile *tert*-butyloxycarbonyl (Boc) group using Boc_2O, provided the key glycosyl amino acids **15α/15β**[25] (95 %/97 % yields from **13α/13β**) and **21α/21β** (88 %/86 % yields from **19α/19β**), respectively, useful building blocks for the synthesis of Tn-glycopeptides.[15] Building block **15α** was used here for further study.

In order to preclude any steric interference with carrier molecules, a spacer was incorporated into the Tn antigen (Scheme II). Thus, 4-aminobutyric acid (**22**) and its methyl ester **23** were coupled to the *N*-hydroxysuccinimide (NHS) ester of **15α** (i.e. **16α**) to give **24** (89 % yield) and **25** (80 % yield), respectively. Sequential acidolysis with HCOOH, capping with Ac_2O, and saponification with 0.1 M NaOH in MeOH–H_2O furnished Mono-Tn (**1**) (71 % yield). The acid-labile glycosidic bond was resistant to the condition of acidolysis (HCOOH, rt, 1 h) due to the stabilizing effect of the *O*-acetyl groups in the GalNAc residue.[26] In addition, no products from β-elimination or racemization were detected during the saponification (0.1 M NaOH, rt, 15 min). It has been suggested that the epitopic structure of Tn glycoproteins might be a cluster of GalNAcα1→*O*-Ser/Thr residues.[27] The Di-Tn (**2**) and Tri-Tn (**3**) were, therefore, assembled by stepwise addition of **16α** to the N-terminal of the serine residue. The coupling sequence consisted of: (a) acidolysis with CF_3COOH (TFA) to unmask the amino group, (b) condensation with **16α** in the presence of *N*-ethyldiisopropylamine (DIEA) (for **3** repeat steps (a) and (b)), (c) transformation of the amino terminal *N*-Boc



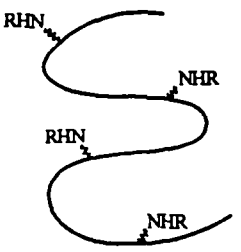
Tn Antigen



		n
Mono-Tn	1	1
Di-Tn	2	2
Tri-Tn	3	3

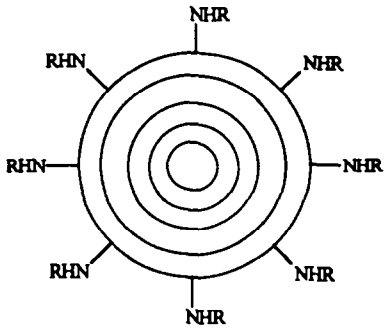
Synthetic Tn Antigens

Figure 1.



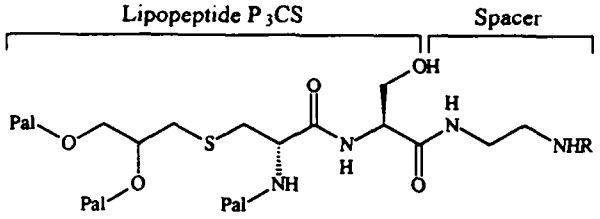
	R
4	Mono-Tn
5	Di-Tn
6	Tri-Tn

Conjugate with OSA
(Schematic)



R = Di-Tn

Conjugate with Starburst® dendrimer **7**
(Schematic)



Pal = $Me(CH_2)_{14}CO$—
R = Di-Tn

Conjugate with lipopeptide P_3CS **8**

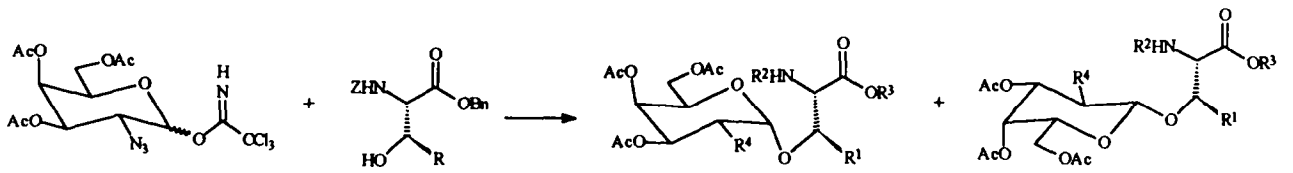
Figure 2. Synthetic Tn-antigen conjugates.

to *N*-Ac group, and (d) saponification with 0.1 M NaOH in MeOH–H_2O [for **2**: **24** → **26** → **28** → **2** (59 % yield in 3 steps); for **3**: **25** → **27** → **29** → **30** → **3** (48 % yield in 4 steps)]. Again, the glycosidic bond was stable to the acidolysis (TFA, rt, 10 min).

Synthesis of Di-Tn–lipopeptide P_3CS conjugate (8)

Jung *et al.*[28] have developed synthetic peptide vaccines with a length of approximately 15 amino acids by conjugating the peptide antigens with the lipopeptide P_3CS. Very recently, the lipoamino acid (P_3C-OH) **35** has been used to design a synthetic AIDS vaccine.[29] The lipoamino acid **35** was readily prepared as a diastereomeric mixture from cysteine in six steps according to the procedure of Wiesmüller *et al.*[30] Although we used the diastereomeric mixture of **35**, P_3CS with *R* configuration at the asymmetric carbon of the glyceryl unit is reported to be immunologically more active than the *S* isomer.[31]

A spacer was installed at the carboxyl group of the serine derivative **31** to generate amino functionality, which allows coupling of P_3CS to the carboxyl group of **2**, by attachment of the ethylenediamine derivative[32] **32** using the NHS ester method (Scheme III). The resulting **33** (89 % yield) was hydrogenolized with 10 % Pd/C to the amine **34** (quantitative) which was then joined to **35** by the 1-hydroxybenzotriazole (HOBt) method using 1,3-diisopropylcarbodiimide (DIC). Acidolysis of the product **36** (83 % yield) to the amine **37** (quantitative) with TFA, followed by coupling to **2** by the NHS ester method, completed the conjugation yielding **8** (57 % yield).



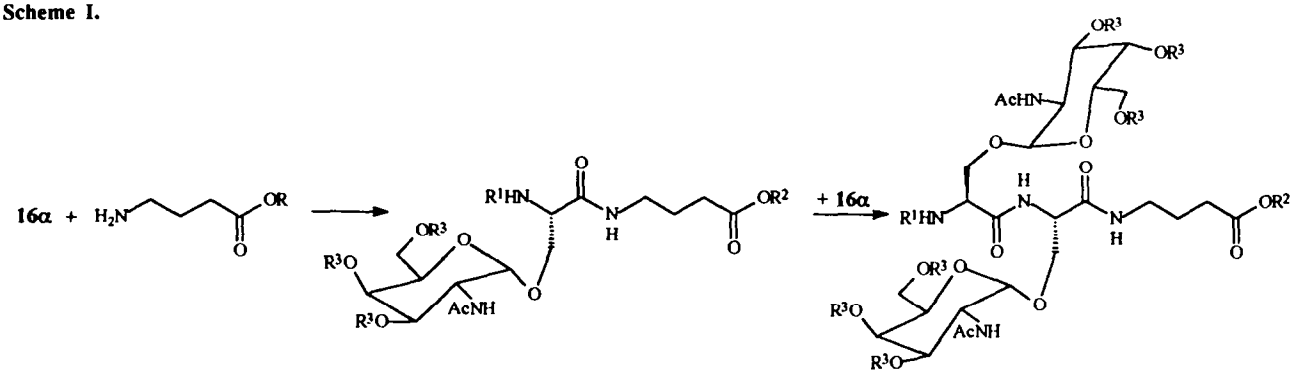
	R
10	H
11	Me

9 α β

	R^1	R^2	R^3	R^4
12α,β	H	Z	Bn	N_3
13α,β	H	Z	Bn	NHAc
14α,β	H	H	H	NHAc
15α,β	H	Boc	H	NHAc
16α	H	Boc	Su	NHAc
17α,β	Me	Z	Bn	N_3
18α,β	Me	Z	Bn	NH_2
19α,β	Me	Z	Bn	NHAc
20α,β	Me	H	H	NHAc
21α,β	Me	Boc	H	NHAc

Boc = tert-butyloxycarbonyl
Bn = benzyl
Su = succinimido

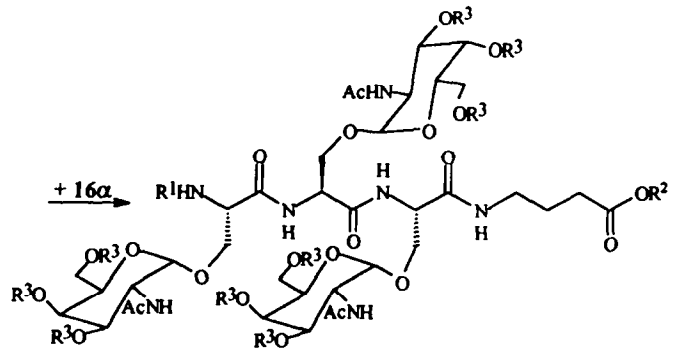
Scheme I.



	R
22	H
23	Me

	R^1	R^2	R^3
24	Boc	H	Ac
25	Boc	Me	Ac
1	Ac	H	H

	R^1	R^2	R^3
26	Boc	H	Ac
27	Boc	Me	Ac
28	Ac	H	Ac
2	Ac	H	H



	R^1	R^2	R^3
29	Boc	Me	Ac
30	Ac	Me	Ac
3	Ac	H	H

Scheme II.

Scheme IV summarizes the preparation of the serylserine–P_3CS conjugate **43**, which was needed as a control experiment. A spacer was introduced to the serine derivative **38** by the NHS ester method to give **39** (quantitative). Acidolysis with TFA (→ **40**, quantitative) was followed by coupling to **38** by the NHS ester method (→ **41**, 89 % yield). The transformation of the amino terminal *N*-Boc to *N*-Ac group and subsequent side-chain deprotection by hydrogenolysis with 10 % Pd/C yielded **42** (96 % yield). Conjugation of **42** with **37** was successful by the NHS ester method to furnish **43** (63 % yield).

Coupling of synthetic Tn antigens 1–3 to OSA: preparation of conjugates 4–6

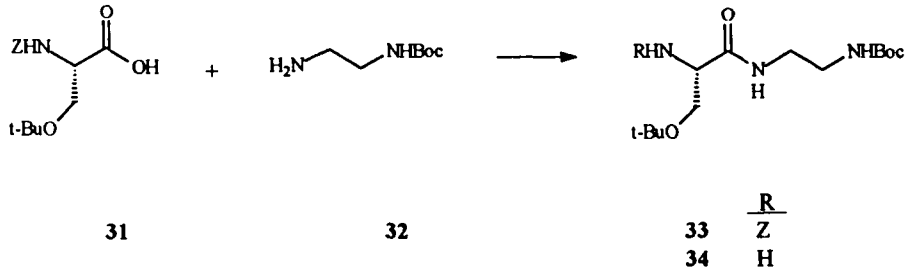
Tn antigen (**1**, **2**, or **3**) was incorporated to OSA utilizing the two-step coupling procedure. First, each antigen was converted to the NHS ester by treatment with NHS and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) in DMF, and then coupled to OSA by addition of the reaction mixture to the OSA preparation in phosphate buffer (PBS) at pH 7.4. The reaction progress was followed by monitoring the disappearance of the NHS ester on TLC. The unreacted Tn antigen which was the hydrolysis product of the NHS ester, NHS, *N*-(3-dimethylaminopropyl)-*N'*-ethylurea, and DMF were removed by exhaustive dialysis against PBS. The Tn activity of the conjugate was estimated by anti-Tn mAb (1E3) with A-OSM as the standard.[11] The conjugation typically afforded 250 μg of Tn antigen per 1 mg of OSA. We have not characterized these conjugates **4–6** fully, but the primary amino groups, including one *N*-terminal amino group and lysine ε-amino groups in OSA ($M_r \approx 66{,}000$, 66 lysine residues/mol),[33] are probably sites for coupling. These amino groups are known to be unprotonated at pH > 7.

Coupling of Di-Tn (2) to Starburst® dendrimer: preparation of conjugate 7

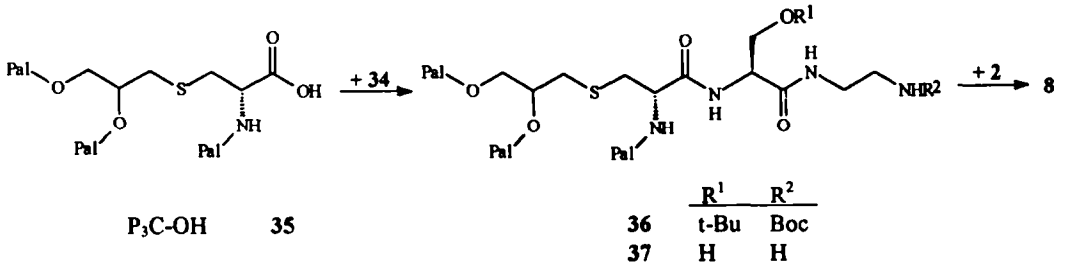
Starburst® dendrimers, constructed from methyl acrylate and ethylenediamine, are spherical polymers of well-controlled size, shape, molecular weight, and number of terminal amino groups depending on the 'generation' of the dendrimers. We used the 5th generation dendrimer (diameter: 40.1 Å; the number of terminal amino groups: 48.0) in this study. Application of the two-step coupling method described above to the dendrimer yielded the Di-Tn–Starburst® dendrimer conjugate (**7**). Complete incorporation of **2** was determined when the Kaiser test[34] was negative. The conjugate was purified by dialysis against PBS.

Immunogenicity of Tn antigen conjugates 4–8

The conjugates **4–8** were examined for their ability to stimulate Tn antigen specific immune responses in mice. Sera from mice were measured for the presence of antibody titers against A-OSM[11] in an enzyme-linked immunosorbant assay (ELISA). Mice immunized with OSA conjugates **4–6** showed high-titer IgM response against Tn antigen (binding against A-OSM) (Fig. 3A). In addition, immunization with **5** and **6** induced

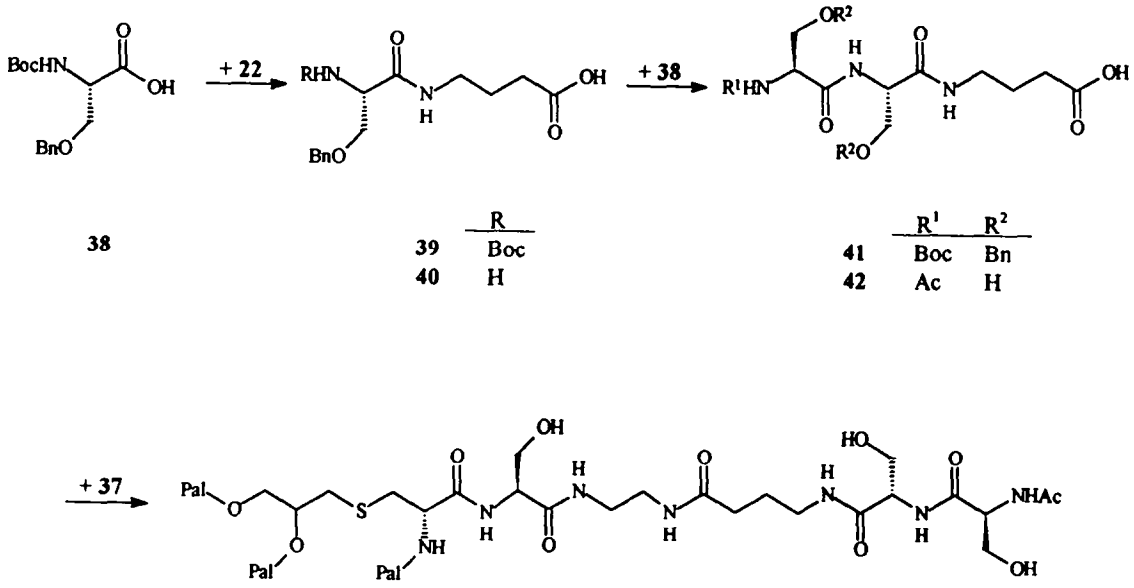


31 **32** **33** R = Z; **34** R = H



P_3C-OH **35** **36** R^1 = t-Bu, R^2 = Boc; **37** R^1 = H, R^2 = H

Scheme III.



38

	R
39	Boc
40	H

	R^1	R^2
41	Boc	Bn
42	Ac	H

43

Scheme IV.

measurable IgG anti-Tn antibody responses (Fig. 3B). This is significant since carbohydrate antigens are thought to stimulate B cells in the absence of any helper T cell enlistment and produce IgM antibody response.[12] As expected,[27] oligomers of Tn antigen **2** and **3** proved to be more effective in generating anti-Tn antibody response than monomeric Tn antigen (**1**).

It has been suggested that a high density of ganglioside GM_3 may play a role in mouse melanoma antigenicity.[35] However, no measurable levels of anti-Tn antibodies were detected in mice immunized with Di-Tn–Starburst® dendrimer conjugate **7**, in spite of the high density of Tn antigens on this conjugate (Fig. 3).

Interestingly, Di-Tn–P_3CS conjugate **8** generated not only high IgM antibody response (Fig. 4A) but also significant IgG anti-Tn response (Fig. 4B). Furthermore, these responses were much stronger than those with **5** and **6**. None of the control groups, immunized with **37**, **43**, and Intralipid®, showed any significant anti-Tn antibody responses. It is possible that the lipopeptide P_3CS is capable of enhancing the uptake of Tn antigen by the appropriate antigen presenting cells for increased immune response. Being a totally synthetic, low molecular weight, and carrier-free immunogen, this

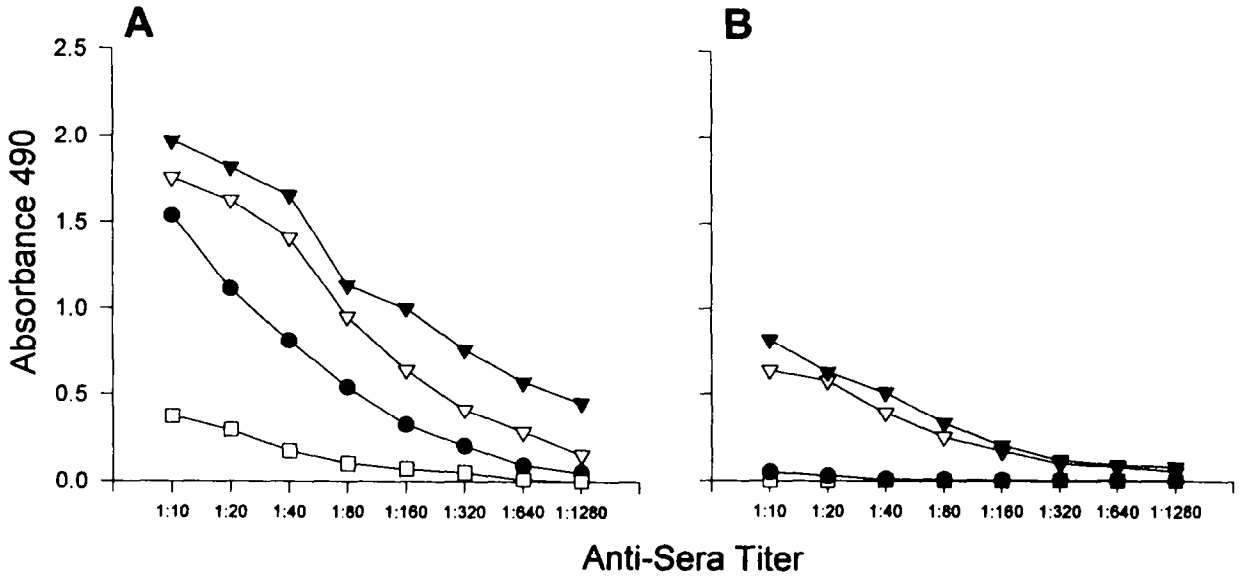


Figure 3. Serum anti-Tn IgM (A) and IgG (B) titers in mice immunized with either **4** (●), **5** (▽), **6** (▼), or **7** (□). All conjugates were suspended in Ribi® adjuvant at a concentration of 0.5 mg/mL according to the manufacturer's instructions. Mice were immunized and serum antibody titers were measured as described in Experimental Section.

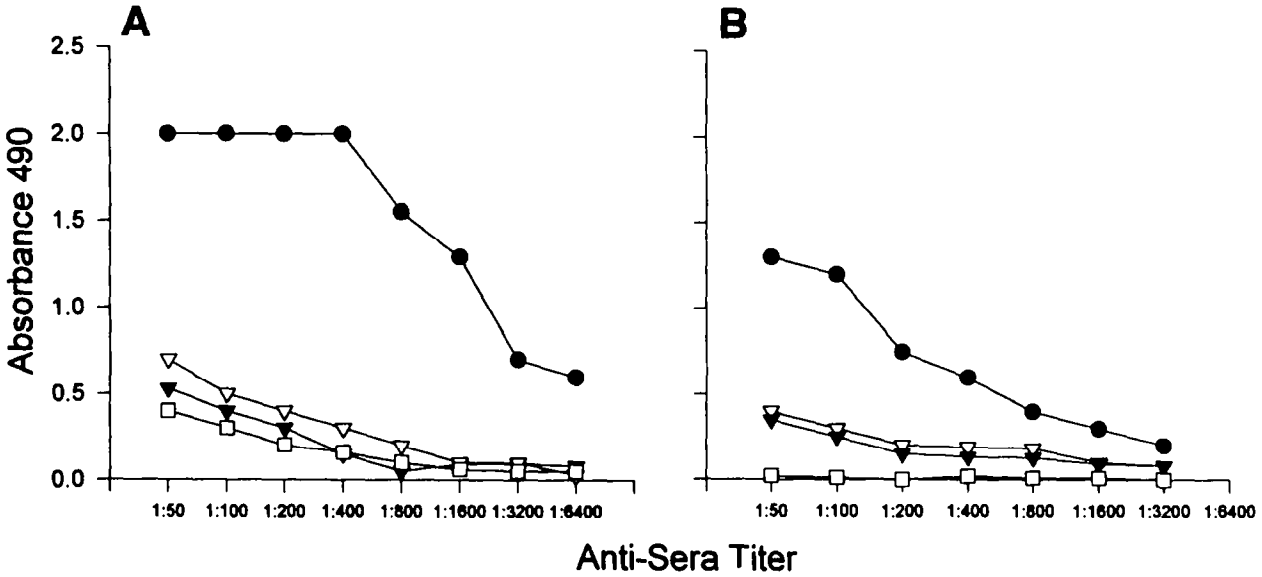


Figure 4. Serum anti-Tn IgM (A) and IgG (B) titers in mice immunized with either **8** (●), **37** (○), **43** (▼), or Intralipid® (▽). All conjugates were dissolved in 1:1 Intralipid®:PBS at a concentration of 0.5 mg/mL. Mice were immunized and serum antibody titers were measured as described in Experimental Section.

lipopeptide conjugate **8** could be a prototype of synthetic carbohydrate vaccines. Studies are underway to determine the effect of **8** directly on the stimulation of T cells.

Experimental Section

Synthesis

General. Melting points were measured with a Fisher–Johns melting point apparatus and are uncorrected. ^1H NMR spectra were measured at 309 °K on a Bruker WM-500 spectrometer with TMS in organic solvents and DSS in D_2O as internal standards. High-resolution mass spectral data (HRMS) were obtained on a JEOL JMS-HX 110 mass spectrometer in a FAB mode using *m*-nitrobenzyl alcohol as the matrix. Specific rotations were determined at 589 nm (Na line) at 23 °C with a Perkin–Elmer 241MC polarimeter. Flash column chromatogrphy (FCC) was performed as reported[36] using silica gel (mesh size 0.040–0.063 mm, EM Science Gibbstown, NJ). The protected amino acids **10**, **11**, **31**, and **38** were purchased from BACHEM Bioscience Inc. (Philadelphia, PA). Starburst® dendrimer (5th generation) was obtained from Polysciences, Inc. (Warrington, PA) and OSA from Sigma Chemical Co. (St. Louis, MO). Elemental analyses were provided by Galbraith Laboratories, Inc., Knoxville, TN.

N-Benzyloxycarbonyl-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester (12α) and N-benzyloxycarbonyl-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl)-L-serine benzyl ester (12β). A 0.22 M solution of trimethylsilyl trifluoromethanesulfonate in dry CH_2Cl_2 (11.6 mL) was added dropwise to a mixture of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl trichloroacetimidate (**9**) (23 g, 48.4 mmol) and *N*-benzyloxycarbonyl-L-serine benzyl ester (**10**) (32 g, 97.2 mmol) in dry CH_2Cl_2 (1 L) vigorously stirred at −15 °C under dry N_2. After 30 min, the mixture was washed successively with ice-cold saturated aq. $NaHCO_3$ and H_2O. The organic-layer was dried (Na_2SO_4) and evaporated to give a 5:1 mixture of **12α** and **12β**. Purification by FCC (2:1, hexane:EtOAc) gave, in the first fraction, **12α** (17.7 g, 57 % based on **9**) as a colorless syrup: $[\alpha]_D$ +93 ° (*c* 3.0, $CHCl_3$) {lit.[20g] $[\alpha]_D$ +73.5 ° (*c* 1.55, $CHCl_3$)}.

The second fraction gave **12β** (4.7 g, 15 % based on **9**) as a colorless syrup: $[\alpha]_D$ −2 ° (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.02 (s, 3H), 2.04 (s, 3H), and 2.14 (s, 3H) (3 × OAc), 3.61 (dd, 1H, *J* = 11.0, 8.0 Hz, H-2), 3.72 (br t, 1H, *J* = 6 Hz, H-5), 3.95 (br d, 1H, *J* = 7 Hz) and 4.38 (br d, 1H, *J* = 7 Hz) (Ser β-CH_2), 4.70 (dd, 1H, *J* = 11.0, 6.5 Hz) and 4.09 (dd, 1H, *J* = 11.0, 4.0 Hz) (2 × H-6), 4.28 (d, 1H, *J* = 8.0 Hz, H-1), 4.60 (br m, 1H, Ser α-C*H*), 4.73 (dd, 1H, *J* = 11.0, 3.5 Hz, H-3), 5.14 (br s, 2 H, PhCH_2), 5.20 and 5.25 (AB-q, *J* = 12.0 Hz, PhCH_2), and 5.29 (d, 1H, *J* = 3.5 Hz, H-4); HRMS calcd for $C_{30}H_{35}N_4O_{12}$ $(M + H)^+$ 643.2251, found 643.2246.

The third fraction recovered **10** (11 g, 69 % recovery).

N-Benzyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester (13α). Hydrogen sulfide was bubbled into a solution of **12α** (5.54 g, 8.63 mmol) in 2:1 pyridine:H_2O (100 mL) at rt for 2 h. After removal of excess H_2S by a stream of N_2 followed by concentration to dryness, the residue was treated with Ac_2O–pyridine for 1 h. Evaporation and FCC (1:2, toluene:EtOAc) yielded **13α** (5.0 g, 88 %) as a colorless glass: $[\alpha]_D$ +83 ° (*c* 2.5 $CHCl_3$) {lit.[20g] $[\alpha]_D$ +74.5 ° (*c* 1.8, $CHCl_3$)}.

N-*Benzyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-galactopyranosyl)-L-serine benzyl ester (13β).* Reduction of **12β** with H_2S and subsequent acetylation as described above yielded **13β** (86 %) as colorless crystals: mp 156–157 °C; $[\alpha]_D$ −13 ° (*c* 1.0, $CHCl_3$) {lit.[24] mp 152 °C; $[\alpha]_D$ +12 ° (*c* 1, $CHCl_3$)}; ^1H NMR ($CDCl_3$) δ 1.99 (s, 3H), 2.02 (s, 3H), 2.09 (s, 3H), and 2.13 (s, 3H) (NAc and 3 × OAc), 3.82 (br t, 1H, *J* = 6.5 Hz, H-5), 3.88 (br t, 1H, *J* = 10.0 Hz, H-2), 3.92 (br d, 1H, *J* = 10.5 Hz) and 4.24 (dd, 1H, *J* = 10.5, 4.0 Hz) (Ser β-CH_2), 4.08 (dd, 1H, *J* = 11.0, 6.0 Hz) and 4.11 (dd, 1H, *J* = 11.0, 6.5 Hz) (2 × H-6), 4.54 (br s, 1H, Ser α-C*H*), 4.68 (d, 1H, *J* = 8.0 Hz, H-1), 5.12 (br s, 2H) and 5.20 (s, 2H) (2 × PhCH_2), 5.18 (dd, 1H, *J* = 11.5, 3.0 Hz, H-3), 5.32 (br d, 1H, *J* = 3.0 Hz, H-4).

O-*(2-Acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine (14α) and* O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-galactopyranosyl)-L-serine (14β).* These compounds were prepared from **13α** and **13β**, respectively, according to the reported procedure.[20g]

14α: colorless crystals (97 %); mp 174–175 °C; $[\alpha]_D$ +88 ° (*c* 0.1, MeOH) {lit.[20g] mp 177 °C; $[\alpha]_D$ +93 ° (*c* 0.5, MeOH)}.

14β: a colorless glass (98 %); $[\alpha]_D$ −31 ° (*c* 3.0, H_2O); ^1H NMR (D_2O) δ 2.00 (s, 3H), 2.01 (s, 3H), 2.08 (s, 3H), and 2.22 (s, 3H) (NAc and 3 × OAc), 4.00 (dd, 1H, *J* = 7.5, 3.5 Hz, Ser β-C*H*H), 4.76 (d, 1H, *J* = 8.5 Hz, H-1), 5.11 (dd, 1H, *J* = 11.0, 3.0 Hz, H-3), and 5.41 (br d, 1H, *J* = 3.0 Hz, H-4). Anal. calcd for $C_{17}H_{26}N_2O_{11}$: C, 47.00; H, 6.03; N, 6.45. Found: C, 47.17; H, 6.03; N, 6.39.

N-tert-*Butyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine (15α).* A solution of **14α** (2 g, 4.61 mmol) in MeOH (80 mL) containing DIEA (0.81 mL, 4.60 mmol) was treated with Boc_2O (1.10 mL, 4.64 mmol) at rt for 1 h. After treatment with IR-120 (H^+) resin, the mixture was concentrated to dryness. Purification by LH-20 column chromatography (acetone) yielded **15α** (2.41 g, 98 %) as a colorless foam: $[\alpha]_D$ +105 ° (*c* 1.5, acetone); ^1H NMR (CD_3OD) δ 1.47 (s, 9H, 3 × Boc-*Me*), 1.94 (s, 3H), 1.95 (s, 3H), and 2.13 (s, 3H) (NAc and 3 × OAc), 3.91 (dd, 1H, *J* = 10.5, 4.0 Hz) and 3.95 (dd, 1H, *J* = 10.5, 3.5 Hz) (Ser β-CH_2), 4.07 (dd, 1H, *J* = 11.0, 6.5 Hz) and 4.16 (dd, 1H, *J* = 11.0, 6.0 Hz) (2 × H-6), 4.29 (br t, 1H, *J* = 6 Hz, H-5), 4.16 (br s, 1H, Ser α-C*H*), 4.42 (dd, 1H, *J* = 11.5, 3.5 Hz, H-2), 4.91 (d, 1H, *J* = 3.5 Hz, H-1), 5.15 (dd, *J* = 11.5, 3.0 Hz, H-3), and 5.41 (br d, 1H, *J* = 3Hz); HRMS calcd for $C_{22}H_{35}N_2O_{13}$ ($M + H)^+$ 535.2139, found 535.2139. Anal. calcd for $C_{22}H_{34}N_2O_{13}$: C, 49.44; H, 6.41; N, 5.24. Found: C, 49.21; H, 6.43; N, 5.28.

N-tert-*Butyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-galactopyranosyl)-L-serine (15β).* Treatment of **14β** with Boc_2O as described above yielded **15β** (99 %) as colorless crystals: mp 157–158 °C; $[\alpha]_D$ −3 ° (*c* 1.0, acetone) {lit.[25] mp 157–159 °C; $[\alpha]_D$ +3 ° (*c* 1, $CHCl_3$)}.

N-*Benzyloxycarbonyl*-O-*(3,4,6-tri*-O-*acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl)-L-threonine benzyl ester*[20d,g] *(17α,β).* Glycosidation of **9** with **11** as described above gave a 4:1 mixture of **17α** and **17β** (93 % based on **9**): R_f 0.3, (3:2, hexane:EtOAc).

N-*Benzyloxycarbonyl*-O-*(3,4,6-tri*-O-*acetyl-2-amino-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (18α) and* N-*benzyloxycarbonyl*-O-*(3,4,6-tri*-O-*acetyl-2-amino-2-deoxy-β-D-galactopyranosyl)-L-threonine benzyl ester (18β).* A mixture of **17α,β** was treated with H_2S as described for the preparation of **13α**. Purification by FCC (5:1 toluene:EtOAc) yielded two fractions. The first fraction gave **18α** (57 % based on **9**) as a colorless syrup: $[\alpha]_D$ +47 ° (*c* 3.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.32 (d, 3H, *J* = 6.5 Hz, Thr-*Me*), 2.01 (s, 3H), 2.02 (s, 3H), and 2.10 (s, 3H) (3 × OAc), 3.05 (dd, 1H, *J* = 11.0, 3.5 Hz, H-2), 4.04 (br d, 2H, *J* = 6.5 Hz, 2 × H-6), 4.17 (br t, 1H, *J* = 6.5 Hz, H-5), 4.29 (m, 1H, Thr β-C*H*), 4.46 (br d, 1H, *J* = 8.5 Hz, Thr α-C*H*), 4.80 (dd, 1H, *J* = 11.0, 3.0 Hz, H-3), 4.85 (d, 1H, *J* = 3.5 Hz, H-1), 5.14 (s, 2H, PhCH_2), 5.15 and 5.23 (AB-q, 2H, *J* = 12.0 Hz, PhCH_2), 5.33 (br d, 1H, *J* = 3Hz, H-4); HRMS calcd for $C_{31}H_{39}N_2O_{12}$ ($M + H)^+$ 631.2503, found 631.2511.

The second fraction yielded **18β** (12 % based on **9**) as a colorless glass: $[\alpha]_D$ +4 ° (*c* 1.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.29 (d, 3H, *J* = 6.5 Hz, Thr-*Me*), 1.99 (s, 3H), 2.02 (s, 3H), and 2.07 (s, 3H) (3 × OAc), 3.05 (dd, 1H, *J* = 11.0, 8.0 Hz, H-2), 3.67 (br t, 1H, *J* = 7.0 Hz, H-5), 4.01 (dd, 1H, *J* = 11.0, 6.5 Hz) and 4.06 (dd, 1H, *J* = 11.0, 7.5 Hz) (2 × H-6), 4.16 (d, 1H, *J* = 8.0 Hz, H-1), 4.42 (br d, 1H, *J* = 9Hz, Thr α-C*H*), 4.47 (m, 1H, Thr β-C*H*), 4.65 (dd, 1H, *J* = 11.0, 3.0 Hz, H-3), 5.14 (s, 2H, PhCH_2), 5.15 and 5.21 (AB-q, 2H, *J* = 12.0 Hz, PhCH_2), 5.26 (br s, 1H, H-4); HRMS calcd for $C_{31}H_{39}N_2O_{12}$ ($M + H)^+$ 631.2503, found 631.2509.

N-*Benzyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (19α) and* N-*benzyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β-D-galactopyranosyl)-L-threonine benzyl ester (19β).* Conventional acetylation of **18α** and **18β** with Ac_2O–pyridine gave **19α** and **19β**, respectively.

19α: a colorless foam (95 %); $[\alpha]_D$ +63 ° (*c* 1.0, $CHCl_3$) {lit.[20g] $[\alpha]_D$ +65 ° (*c* 1.1, $CHCl_3$)}.

19β: colorless crystals (97 %); mp 155–156°C; $[\alpha]_D$ −12 ° (*c* 3.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.21 (d, 3H, *J* = 6.0 Hz, Thr-*Me*), 1.91 (s, 3H), 1.99 (s, 6H), and 2.10 (s, 3H) (NAc and 3 × OAc), 3.70 (br t, 1H, *J* = 6.5 Hz, H-5), 3.74 (br q, 1H, *J* = 8 Hz, H-2), 4.00 (dd, 1H, *J* = 11.0, 6.0 Hz) and 4.06 (dd, 1H, *J* = 11.0, 7.0 Hz) (2 × H-6), 4.38 (dd, 1H, *J* = 6.0, 2.5 Hz, Thr β-C*H*), 4.42 (dd, 1H, *J* = 6.5, 2.5 Hz, Thr α-C*H*), 4.68 (d, 1H, *J* = 8.5 Hz, H-1), 5.12 and 5.15 (AB-q, 2H, *J* = 12.5 Hz, PhCH_2),

5.17 and 5.21 (AB-q, 2H, J = 12.5 Hz, PhCH_2), 5.24 (dd, 1H, J = 11.0, 3.5 Hz, H-3), 5.28 (br d, 1H, J = 3.5 Hz, H-4); HRMS calcd for $C_{33}H_{41}N_2O_{13}$ $(M + H)^+$ 673.2608, found 673.2610. Anal. calcd for $C_{33}H_{40}N_2O_{13}$: C, 58.92; H, 5.99; N, 4.16. Found: C, 59.01; H, 5.98; N, 4.07.

O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine (20α) and O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-L-threonine (20β). Hydrogenolysis of **19α** and **19β**, as described for the preparation of **14α** and **14β**, yielded **20α** and **20β**, respectively.

20α: colorless crystals (91 %); mp 167–169 °C; $[\alpha]_D$ +61 ° (*c* 1.0, H_2O) {lit.[20g] mp 170 °C; $[\alpha]_D$ +62.3° (*c* 1.03, H_2O)}.

20β: a colorless glass (87 %); $[\alpha]_D$ –40 ° (*c* 1.5, EtOH); ^1H NMR (D_2O) δ 1.29 (d, 3H, J = 6.5 Hz, Thr-*Me*), 1.98 (s, 3H), 1.99 (s, 3H), 2.07 (s, 3H), and 2.19 (s, 3H) (NAc and 3 × OAc), 3.58 (d, 1H, J = 6.5 Hz, Thr α-C*H*), 4.09 (dd, 1H, J = 11.0, 8.5 Hz, H-2), 4.12 (br t, 1H, J = 6Hz, H-5), 4.21 (br d, 2H, J = 6Hz, 2 × H-6), 4.25 (qd, 1H, J = 6.5, 6.5 Hz, Thr β-C*H*), 4.73 (d, 1H, J = 8.5 Hz, H-1), 5.08 (dd, 1H, J = 11.0, 3.0 Hz, H-3), 5.39 (br d, 1H, J = 3Hz, H-4); HRMS calcd for $C_{18}H_{29}N_2O_{11}$ $(M + H)^+$ 449.1771, found 449.1777.

N-tert-Butyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine (21α) and N-tert-butyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-L-threonine (21β). Treatment of **20α** and **20β** with Boc_2O, as described for the preparation of **15α** and **15β**, gave **21α** and **21β**, respectively.

21α: a colorless glass (97 %); $[\alpha]_D$ +91 ° (*c* 2.0, acetone); ^1H NMR (CD_3OD) δ 1.31 (d, 3H, J = 6.5 Hz, Thr-*Me*), 1.48 (s, 9H, 3 × Boc-*Me*), 1.94 (s, 3H), 1.96 (s, 3H), 2.03 (s, 3H), and 2.13 (s, 3H) (NAc and 3 × OAc), 4.10 (dd, 1H, J = 11.5, 7.0 Hz) and 4.12 (dd, 1H, J = 11.5, 6.0 Hz) (2 × H-6), 4.22 (br s, 1H, Thr α-C*H*), 4.32 (br t, 1H, J = 6.5 Hz, H-5), 4.40 (dd, 1H, J = 11.5, 4.0 Hz, H-2), 4.40 (m, 1H, Thr β-C*H*), 4.95 (d, 1H, J = 4.0 Hz, H-1), 5.10 (dd, 1H, J = 11.5, 3.0 Hz, H-3), and 5.39 (br d, 1H, J = 2.5 Hz, H-4); HRMS calcd for $C_{23}H_{36}N_2O_{13}Na$ $(M + Na)^+$ 571.2115, found 571.2116.

21β: a colorless glass (99 %); $[\alpha]_D$ –4 ° (*c* 1.0, acetone); ^1H NMR ($CDCl_3$) δ 1.23 (d, 3H, J = 6.5 Hz, Thr-*Me*), 1.46 (s, 9H, 3 × Boc-*Me*), 1.97 (s, 3H), 2.01 (s, 3H), 2.06 (s, 3H), and 2.15 (s, 3H) (NAc and 3 × OAc), 3.89 (br t, 1H, J = 6Hz, H-5), 3.99 (br m, 1H, H-2), 4.11 (dd, 1H, J = 11.5, 6.0 Hz), and 4.21 (dd, 1H, J = 11.5, 6.0 Hz) (2 × H-6), 4.36 (br m, 1H, Thr α-C*H*), 4.41 (qd, 1H, J = 6.5, 2.5 Hz, Thr β-C*H*), 4.75 (br d, 1H, J = 8 Hz, H-1), 5.22 (br d, 1H, J = 9Hz, H-3), 5.35 (br d, 1H, J = 3Hz, H-4); HRMS calcd for $C_{23}H_{36}N_2O_{13}Na$ $(M + Na)^+$ 571.2115, found 571.2102.

N-[N-tert-Butyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (24). A mixture of **15α** (500 mg, 0.94 mmol), NHS (160 mg, 1.39 mmol), and EDC (268 mg, 1.40 mmol) in dry CH_2Cl_2 (25 mL) was stirred at rt for 40 min. The mixture was washed with precooled H_2O (3 × 30 mL), dried (Na_2SO_4), and concentrated to give *N-(tert*-butyloxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-de-oxy-α-D-galactopyranosyl)-L-serine succinimido ester (**16α**) as a colorless syrup (> 95 % purity based on the ^1H NMR spectroscopy): ^1H NMR ($CDCl_3$) δ 1.48 (s, 9H, 3 × Boc-*Me*), 1.96 (s, 3H), 1.99 (s, 3H), 2.06 (s, 3H), and 2.16 (s, 3H) (NAc and 3 × OAc), 2.88 (br s, 4 H, 2 × Su-CH_2), 3.99–4.10 (m, 3H) and 4.14 (dd, 1H, J = 11.0, 6.0 Hz) (2 × H-6, Ser β-CH_2), 4.20 (br dd, 1H, J = 6.5, 6.5 Hz, H-5), 4.69 (br ddd, 1H, J = 10.5, 10.5, 3.5 Hz, H-2), 4.92 (d, 1H, J = 3.5 Hz, H-1), 4.94 (br m, 1H, Ser α-C*H*), 5.09 (dd, 1H, J = 11.0, 3.0 Hz, H-3), and 5.41 (br d, 1H, J = 3.0 Hz, H-4).

After dissolution of **16α** in dry DMF (20 mL), the mixture was cooled in an ice bath and a precooled solution of 4-aminobutanoic acid (**22**) (109 mg, 1.0 mmol) in H_2O (4 mL) containing DIEA (190 μL, 1.09 mmol) was added dropwise. The mixture was stirred at rt for 30 min, treated with IR-120 (H^+) resin, and concentrated to dryness. Purification by LH-20 column chromatography (acetone) yielded **24** (515 mg, 89 % based on **15α**) as a colorless syrup: $[\alpha]_D$ +82 ° (*c* 2.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.48 (s, 9H, 3 × Boc-*Me*), 1.88 (br quintet, 2H, J = 6.0 Hz, NCH$_2$CH_2CH$_2$CO), 1.98 (s, 3H), 2.01 (s, 3H), 2.05 (s, 3H), and 2.16 (s, 3H) (NAc and 3 × OAc), 2.42 (br t, 2H, J = 6.0 Hz, NCH$_2$CH$_2$CH_2CO), 3.23 (br m, 1H) and 3.57 (br m, 1H) (NCH_2CH$_2$CH$_2$CO), 3.80 (dd, 1H, J = 10.0, 5.0 Hz) and 3.94 (br s, 1H) (Ser β-CH_2), 4.05–4.18 (m, 3H, 2 × H-6, H-5), 4.29 (br s, 1H, Ser α-C*H*), 4.60 (ddd, 1H, J = 11.0, 11.0, 3.5 Hz, H-2), 4.93 (d, 1H, J = 3.5 Hz, H-1), 5.18 (dd, 1H, J = 11.0, 3.0 Hz, H-3), and 5.36 (br d, 1H, J = 3.0 Hz, H-4); HRMS calcd for $C_{26}H_{42}N_3O_{14}$ $(M + H)^+$ 620.2667, found 620.2659. Anal. calcd for $C_{26}H_{41}N_3O_{14}$: C, 50.40; H, 6.67; N, 6.78. Found: C, 50.65; H, 6.65; N, 6.69.

Methyl N-[N-tert-butyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoate (25). Reaction of **16α** with methyl 4-aminobutanoate (**23**) as described above yielded **25** (80 % based on **15α**) as a colorless syrup: $[\alpha]_D$ +49 ° (*c* 2.0, $CHCl_3$); HRMS calcd for $C_{27}H_{44}N_3O_{14}$ $(M + H)^+$ 634.2823, found 634.2818. The ^1H NMR spectrum ($CDCl_3$) was almost identical with that of **24**, except for the presence of a signal at δ 3.69 (s, 3H, COO*Me*).

N-[N-Acetyl-O-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (Mono-Tn, 1). Acidolysis of **24** (70 mg, 0.11 mmol) with HCOOH (3 mL) at rt for 1 h, followed by lyophilization and vacuum drying over NaOH pellets, gave a pale yellow solid. This was dissolved in MeOH (5 mL) and the pH was adjusted to 8.0 with Et_3N. The mixture was then treated with Ac_2O (25 μL, 24 mmol) at rt for 1 h and concentrated. The residue was dissolved in 2:1

MeOH:H_2O (10 mL) containing 1 M aq. NaOH (1 mL). After 15 min, neutralization with IR-120 (H^+) was followed by column chromatography on P-2 (H_2O). Lyophilization yielded **1** (35 mg, 71 %) as a colorless amorphous solid: $[\alpha]_D$ +100 ° (*c* 0.6, MeOH); ^1H NMR (D_2O) δ 1.72 (quintet, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 1.99 (s, 3H) and 2.02 (s, 3H) (2 × NAc), 2.24 (t, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.21 (m, 2H, $NCH_2CH_2CH_2CO$), 3.93 (br d, 1H, *J* = 3.0 Hz, H-4), 4.10 (dd, 1H, *J* = 11.0, 3.5 Hz, H-2), 4.48 (br t, 1H, *J* = 5.5 Hz, Ser α-C*H*), and 4.85 (d, 1H, *J* = 3.5 Hz, H-1); HRMS calcd for $C_{17}H_{30}N_3O_{10}$ $(M + H)^+$ 436.1931, found 436.1935.

N-*[*N-tert-*Butyloxycarbonyl-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (26).* Acidolysis of **24** (500 mg, 0.81 mmol) with TFA (5 mL) at rt for 10 min, followed by lyophilization and vacuum drying over NaOH pellets, yielded a pale yellow foam (>99 % purity on TLC: R_f 0.4, 2:1:1 BuOH:MeOH:H_2O). This was dissolved in dry DMF (15 mL) containing DIEA (285 μL, 1.64 mmol), and the pH of the mixture was adjusted to 8.0 with an additional amount of DIEA. A solution of **16**α, prepared from **15**α (500 mg, 0.94 mmol) as described above, in dry DMF (10 mL) was added dropwise. After 2 h, the mixture was treated with IR-120 (H^+) resin and concentrated to dryness. Purification by LH-20 column chromatography (acetone) yielded **26** (585 mg, 70 % based on **24**) as a colorless syrup: $[\alpha]_D$ +93 ° (*c* 2.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.47 (s, 9H, 3 × Boc-*Me*), 1.93 (br m, 2H, $NCH_2CH_2CH_2CO$), 1.97 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 2.06 (s, 3H), 2.15 (s, 3H), and 2.17 (s, 3H) (2 × NAc and 6 × OAc), 2.50 (m, 2H, $NCH_2CH_2CH_2CO$), 3.22 (ddd, 1H, *J* = 14.0, 5.0, 5.0 Hz) and 3.60 (ddd, 1H, *J* = 14.0, 6.5, 6.5 Hz) ($NCH_2CH_2CH_2CO$), 3.77 (dd, 1H, *J* = 10.0, 6.0 Hz), 3.81 (dd, 1H, *J* = 10.0, 6.0 Hz), and 3.89 (m, 2H) (2 × Ser β-CH_2), 4.03–4.15 (m, 4H, 4 × H-6), 4.17 (m, 2H, 2 × H-5), 4.40 (br s, 1H) and 4.52 (m, 1H) (2 × Ser α-C*H*), 4.56 (ddd, 1H, *J* = 11.5, 8.0, 3.5 Hz) and 4.63 (ddd, 1H, *J* = 11.5, 10.0, 3.5 Hz) (2 × H-2), 4.96 (d, 1H, *J* = 3.5 Hz) and 5.00 (d, 1H, *J* = 3.5 Hz) (2 × H-1), 5.14 (dd, 1H, *J* = 11.5, 3.0 Hz) and 5.21 (dd, 1H, *J* = 11.5, 3.5 Hz) (2 × H-3), and 5.36 (br s, 2H, 2 × H-4); HRMS calcd for $C_{43}H_{66}N_5O_{24}$ $(M + H)^+$ 1036.4097, found 1036.4072. Anal. calcd for $C_{43}H_{65}N_5O_{24}$: C, 49.85; H, 6.32; N, 6.76. Found: C, 50.17; H, 6.30; N, 6.65.

Methyl N-*[*N-tert-*butyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoate (27).* Acidolysis of **25** with TFA and subsequent coupling reaction with **16**α as described above yielded **27** (73 % based on **25**) as a colorless syrup: $[\alpha]_D$ +90 ° (*c* 0.4, $CHCl_3$). Anal. calcd for $C_{44}H_{67}N_5O_{24}$: C, 50.33; H, 6.43; N, 6.67. Found: C, 50.51; H, 6.40; N, 6.69. The ^1H NMR spectrum ($CDCl_3$) was almost identical with that of **26**, except for the presence of a signal at δ 3.68 (s, 3H, COO*Me*).

N-*[*N-*Acetyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (28).* Acidolysis of **26** with TFA as described above (>95 % purity on TLC: R_f 0.4, 2:1:1 BuOH:MeOH:H_2O), followed by acetylation with Ac_2O–pyridine, yielded, after purification by LH-20 column chromatography (acetone), **28** (92 %) as a colorless glass: $[\alpha]_D$ +78 ° (*c* 1.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.92 (br quintet, 2H, *J* = 6.0 Hz, $NCH_2CH_2CH_2CO$), 1.98 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.04 (s, 3H), 2.07 (s, 3H), 2.11 (s, 3H), 2.15 (s, 3H), and 2.16 (s, 3H) (3 × NAc and 6 × OAc), 2.43 (br t, 1H, *J* = 6.0 Hz) and 2.45 (br t, 1H, *J* = 6.0 Hz) ($NCH_2CH_2CH_2CO$), 3.19 (ddd, 1H, *J* = 18.5, 5.5, 5.5 Hz) and 3.60 (ddd, 1H, *J* = 18.5, 6.0, 5.5 Hz) ($NCH_2CH_2CH_2CO$), 3.79 (dd, 1H, *J* = 10.0, 5.0 Hz) and 3.85–3.94 (m, 3H) (2 × Ser β-CH_2), 4.02–4.16 (m, 4 H, 4 × H-6), 4.20 (br t, 1H, *J* = 6.5 Hz) and 4.21 (br t, 1H, *J* = 6.0 Hz) (2 × H-5), 4.52–4.64 (m, 3H, 2 × H-2, Ser α-C*H*), 4.75 (br ddd, 1H, *J* = 7.5, 5.0, 5.0 Hz, α-C*H*), 4.97 (br d, 2H, *J* = 2.0 Hz, 2 × H-1), 5.13 (dd, 1H, *J* = 11.5, 3.0 Hz) and 5.16 (dd, 1H, *J* = 11.5, 3.0 Hz) (2 × H-3), 5.35 (br s, 2H, 2 × H-4), and 6.51 (d, 1H, *J* = 9.0 Hz); HRMS calcd for $C_{40}H_{60}N_5O_{23}$ $(M + H)^+$ 978.3679, found 978.3698. Anal. calcd for $C_{40}H_{59}N_5O_{23}$: C, 49.13; H, 6.08; N, 7.16. Found: C, 49.41; H, 6.09; N, 7.08.

N-*[*N-*Acetyl*-O-*(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (Di-Tn, 2).* A solution of **28** (300 mg, 0.31 mmol) in 2:1 MeOH:H_2O (20 mL) was treated with 1 M aq. NaOH (2 mL) at rt for 15 min. The mixture was then neutralized with IR-120 (H^+) and purified by P-2 column chromatography (H_2O). Lyophilization from H_2O yielded **2** (202 mg, 91 %) as a colorless amorphous solid: $[\alpha]_D$ +130 ° (*c* 0.8, DMF); ^1H NMR (D_2O) δ 1.75 (quintet, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 1.99 (s, 3H), 2.00 (s, 3H), and 2.02 (s, 3H) (3 × NAc), 2.35 (t, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.17 (dt, 1H, *J* = 13.5, 7.0 Hz) and 3.24 (dt, 1H, *J* = 13.5, 7.0 Hz) ($NCH_2CH_2CH_2CO$), 3.91 (br d, 1H, *J* = 4.0 Hz) and 3.93 (br d, 1H, *J* = 4.0 Hz) (2 × H-4), 4.11 (dd, 1H, *J* = 7.0, 3.5 Hz) and 4.13 (dd, 1H, *J* = 7.0, 3.5 Hz) (2 × H-2), 4.57 (t, 1H, *J* = 5.5 Hz) and 4.60 (t, 1H, *J* = 5.5 Hz) (2 × Ser α-C*H*), 4.85 (d, 1H, *J* = 3.5 Hz) and 4.86 (d, 1H, *J* = 3.5 Hz) (2 × H-1); HRMS calcd for $C_{28}H_{48}N_5O_{17}$ 726.3045, found 726.3012.

Methyl N-*[*N-tert-*butyloxycarbonyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoate (29).* Acidolysis of **27** with TFA followed by coupling to **16**α, as described for the preparation of **26**, yielded **29** (70 % based on **27**) as a colorless syrup: $[\alpha]_D$

+81 ° (*c* 2,0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.44 (br s, 9H, 3 × Boc-*Me*), 1.86 (br m, 2H, $NCH_2CH_2CH_2CO$), 1.96 (s, 3H), 1.97 (s, 3H), 1.98 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.03 (s, 3H), 2.04 (s, 6H), 2.06 (s, 3H), 2.15 (s, 9H) (3 × NAc and 9 × OAc), 2.44 (br t, 2H, *J* = 6.5 Hz, $NCH_2CH_2CH_2CO$), 3.23–3.37 (br m, 2H, $NCH_2CH_2CH_2CO$), 3.68 (s, 3H, COO*Me*), 4.96 (d, 1H, *J* = 3.5 Hz), 4.98 (d, 1H, *J* = 3.5 Hz), and 4.99 (d, 1H, *J* = 3.5 Hz) (3 × H-1), 5.11 (dd, 1H, *J* = 11.0, 3.0 Hz), 5.13 (dd, 1H, *J* = 11.0, 3.0 Hz), and 5.18 (dd, 1H, *J* = 11.5, 3.0 Hz) (3 × H-3), and 5.38 (br s, 3H, 3 × H-4); HRMS calcd for $C_{61}H_{92}N_7O_{34}$ $(M + H)^+$ 1466.5680, found 1466.5718.

Methyl N-*[*N*-acetyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoate (30).* Acidolysis of **29** with TFA followed by acetylation as described above yielded **30** (99 %) as a colorless glass: $[\alpha]_D$ +99 ° (*c* 2.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.86 (br quintet, 2H, *J* = 7 Hz, $NCH_2CH_2CH_2CO$), 1.97 (s, 3H), 1.98 (s, 6H), 1.99 (s, 3H), 2.00 (s, 3H), 2.03 (s, 3H), 2.04 (s, 6H), 2.05 (s, 3H), 2.06 (s, 3H), 2.15 (s, 6H), and 2.16 (s, 3H) (4 × NAc and 9 × OAc), 2.43 (br t, 2H, *J* = 7 Hz, $NCH_2CH_2CH_2CO$), 3.23–3.36 (br m, 2H, $NCH_2CH_2CH_2CO$), 3.67 (s, 3H, COO*Me*), 4.95 (d, 1H, *J* = 3.5 Hz), 4.98 (d, 1H, *J* = 3.0 Hz), and 4.95 (d, 1H, *J* = 3.0 Hz) (3 × H-1), 5.11 (dd, 1H, *J* = 3.0, 11.0 Hz), 5.14 (dd, 1H, *J* = 11.0, 3.0 Hz), and 5.16 (dd, 1H, *J* = 3.0, 11.0 Hz) (3 × H-3), and 5.37 (m, 3H, 3 × H-4); HRMS calcd for $C_{58}H_{86}N_7O_{33}$ $(M + H)^+$ 1408.5270, found 1408.5340. Anal. calcd for $C_{58}H_{85}N_7O_{33}$: C, 49.47; H, 6.08; N, 6.96. Found: C, 49.73; H, 6.07; N, 7.13.

N-[N-Acetyl-O-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl-O-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl-O-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic acid (Tri-Tn, 3). Saponification of **30** with 2:1 MeOH:H_2O containing 12 % 1M aq. NaOH, as described for the preparation of **2**, yielded **3** (94 %) as a colorless amorphous solid: $[\alpha]_D$ +140 ° (*c* 0.2, H_2O); ^1H NMR (D_2O) δ 1.71 (quintet, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.00 (br s, 9H) and 2.02 (s, 3H) (4 × NAc), 2.21 (t, 2H, *J* = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.14 (dt, 1H, *J* = 13.5, 7.0 Hz) and 3.22 (dt, 1H, *J* = 13.5, 7.0 Hz) ($NCH_2CH_2CH_2CO$), 3.89–3.95 (m, 3H, 3 × H-4), 4.09–4.16 (m, 3H, 3 × H-2), 4.54 (t, 1H, *J* = 5.5 Hz) and 4.59–4.67 (m, 2H) (3 × Ser α-C*H*), and 4.86 (m, 3H, 3 × H-1); HRMS calcd for $C_{39}H_{65}N_7O_{24}Na$ $(M + Na)^+$ 1038.3980, found 1038.3989.

N-(N-Benzyloxycarbonyl-O-tert-butyl-L-seryl)-N'-tert-butyloxycarbonyl-1,2-diaminoethane (33). A solution of *N*-benzyloxycarbonyl-*O-tert*-butyl-L-serine (**31**) (1.0 g, 3.39 mmol), HOBt (458 mg, 3.39 mmol), and DIC (531 μL, 3.39 mmol) in dry DMF (20 mL) was stirred at rt for 30 min. Subsequently, a solution of *N-tert*-butyloxycarbonyl-1,2-diaminoethane[32] (**32**) (542 mg, 3.39 mmol) in dry DMF (10 mL) was added dropwise and the mixture was stirred for an additional 1 h. The mixture was treated with IR-120 (H^+) resin and concentrated. FCC (1:1, toluene:EtOAc), followed by crystallization from EtOAc, gave **33** (1.32 g, 89 %) as colorless crystals: mp 115–116 °C; $[\alpha]_D$ +7.0 ° (*c* 4.0, MeOH); ^1H NMR ($CDCl_3$) δ 1.18 (s, 9H, 3 × *t*-Bu-*Me*), 1.43 (s, 9H, 3 × Boc-*Me*), 3.24 (br s, 2H) and 3.37 (br s, 2H) (NCH_2CH_2N), 3.38 (m, 1H) and 3.82 (br m, 1H) (β-CH_2), 4.20 (br m, 1H, α-C*H*), and 5.13 (s, 2H, $PhCH_2$); HRMS calcd for $C_{22}H_{35}N_3O_6Na$ $(M + Na)^+$ 460.2423, found 460.2417. Anal. calcd for $C_{22}H_{35}N_3O_6$: C, 60.39; H, 8.06; N, 9.60. Found: C, 60.61; H, 8.07; N, 9.52.

N-(O-tert-Butyl-N-tert-butyloxycarbonyl-L-seryl)-1,2-diaminoethane (34). A mixture of **33** (250 mg, 0.57 mmol) and 10 % Pd/C (25 mg) in MeOH (15 mL) was stirred under H_2 (1 atm) at rt for 2 h. After filtration, the filtrate was concentrated to give **34** (173 mg, quantitative) as a colorless syrup: $[\alpha]_D$ –5.0 ° (*c* 3.5, MeOH); ^1H NMR (CD_3OD) δ 1.18 (s, 9H, 3 × *t*-Bu-*Me*), 1.43 (s, 9H, 3 × Boc-*Me*), 3.17 (t, 2H, *J* = 6.0 Hz, NCH_2CH_2NHBoc), 3.29 (br t, 2H, *J* = 6.0 Hz, NCH_2CH_2NHBoc), 3.37 (t, 1H, *J* = 5.5 Hz, α-C*H*), 3.50 (dd, 1H, *J* = 8.0, 5.5 Hz) and 3.52 (dd, 1H, *J* = 8.0, 5.5 Hz) (β-CH_2); HRMS calcd for $C_{14}H_{21}N_2O_4$ $(M + H)^+$ 281.1501, found 281.1532.

N-[N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,2S)-propyl]-L-cysteyl-O-tert-butyl-L-seryl]-N'-(tert-butyloxycarbonyl)-1,2-diaminoethane (36). DIC (70 μL, 0.44 mmol) was added dropwise to a stirring solution of *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-L-cysteine[30] (**35**) (400 mg, 0.44 mmol) and HOBt (66 mg, 0.48 mmol) in dry CH_2Cl_2 (10 mL) under dry N_2 in an ice-bath, and the mixture was stirred at rt for 30 min. A solution of **34** (135 mg, 0.45 mmol) in dry CH_2Cl_2 (5 mL) was added and the mixture was stirred at rt for 1.5 h. Concentration, followed by FCC (1:1, $CHCl_3$:EtOAc), yielded **36** (436 mg, 83 %) as a colorless solid: ^1H NMR ($CDCl_3$) δ 0.88 (t, 9H, *J* = 6.5 Hz, 3 × Pal-*Me*), 1.18 (br s, 9H, 3 × *t*-Bu-*Me*), 1.23–1.34 (m) and 1.62 (m) (39 × Pal-CH_2), 1.43 (br s, 9H, 3 × Boc-*Me*), 2.32 (m, 6H, 3 × Pal-CH_2CO), 2.74 (br dd, 1H, *J* = 13, 5.5 Hz) and 2.78 (br dd, 1H, *J* = 13.5 Hz) (SCH_2CHOCO), 2.96 (dd, 0.5 H, *J* = 13.0, 7.0 Hz), 2.97 (dd, 0.5 H, *J* = 13.0, 5.5 Hz), 3.04 (dd, 0.5 H, *J* = 13.0, 6.0 Hz), and 3.06 (dd, 0.5 H, *J* = 13.0, 6.0 Hz) (Cys β-CH_2), 3.21–3.45 (m, 4 H, NCH_2CH_2N), 3.84 (m, 1H) and 3.89 (m, 1H) (Ser β-CH_2), 4.14 (br dd, 1H, *J* = 12, 6 Hz) and 4.33 (br dd, 1H, *J* = 12, 5 Hz) (CH_2OCO), 4.37–4.42 (m, 1H), 4.46 (br q, 0.5 H, *J* = 6 Hz), and 4.51 (br q, 0.5 H, *J* = 6 Hz) (Ser α-C*H* and Cys α-C*H*), 5.19 (m, 1H, C*H*OCO); HRMS calcd for $C_{68}H_{130}N_4O_{10}SNa$ $(M + Na)^+$ 1217.9408, found 1217.9437. Anal. calcd for $C_{68}H_{130}N_4O_{10}S$: C, 68.30; H, 10.96; N, 4.69; S, 2.68. Found: C, 68.54; H, 10.93; N, 4.67; S, 2.76.

N-[N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,2S)-propyl]-L-cysteyl-L-seryl]-1,2-diaminoethane (37). Acidolysis of **36** (200 mg, 0.17 mmol) with TFA (5 mL) at rt for 1 h, followed by evaporation, left a colorless solid. Lyophilization from *t*-BuOH–AcOH yielded the AcOH

salt of **37** (185 mg, quantitative) as a colorless amorphous solid: ^{1}H NMR (3:1 $CDCl_3$:CD_3OD) δ 0.89 (t, 9H, J = 6.5 Hz, 3 × Pal-*Me*), 1.28 (m) and 1.62 (m) (39 × Pal-CH_2), 1.89 (s, 3H, *Ac*OH), 2.29 (br dd, 3H, J = 8, 8 Hz) and 2.34 (br dd, 3H, J = 8, 8 Hz) (3 × Pal-CH_2CO), 2.76 (dd, 1H, J = 14.0, 6.5 Hz), 2.79 (dd, 0.5 H, J = 14.0, 6.0 Hz), and 2.82 (dd, 0.5 H, J = 14.0, 6.0 Hz) (SCH_2CHOCO), 2.86–3.09 (m, 4 H, Cys β-CH_2 and CH_2NH_2), 3.45 (m, 2H, CONHCH_2), 3.77 (dd, 1H, J = 11.5, 4.5 Hz) and 3.96 (br dd, 1H, J = 11.5, 4.5 Hz) (Ser β-CH_2), 4.17 (m, 1H) and 4.36 (m, 1H) (CH_2OCO), 4.38 (m, 1H) and 4.48 (m, 1H) (Ser α-C*H* and Cys α-C*H*), 5.18 (m, 1H, C*H*OCO); HRMS calcd for $C_{59}H_{115}N_4O_8S$ $(M + H)^+$ 1039.7778, found 1039.7843.

N-*{*N-*Palmitoyl*-S-*[2,3-bis(palmitoyloxy)-(2R,2S)-propyl]*-L-*cysteyl*-L-*seryl]*-2-*aminoethyl*-*{*N^4-*[*N-*acetyl*-O-*(2-acetamido*-2-*deoxy*-α-D-*galactopyranosyl)*-L-*seryl*-O-*(2-acetamido*-2-*deoxy*-α-D-*galactopyranosyl)*-L-*seryl]]*-4-*aminobutanamide (8)*. A solution of **2** (250 mg, 0.34 mmol), NHS (40 mg, 0.35 mmol), and EDC (66 mg, 0.34 mmol) in dry DMF (5 mL) was stirred at rt for 4 h. At this time TLC indicated the presence of one major product (R_f 0.3, 1:1 $CHCl_3$:MeOH). The reaction mixture was added dropwise to a milky solution of **37** (350 mg, 0.32 mmol as the AcOH salt) in dry DMF (15 mL) containing DIEA (60 μL, 0.34 mmol), and the mixture was stirred at rt for 1 h. After concentration, the residue was washed three times with H_2O by decantation. FCC (65:25:4 $CHCl_3$:MeOH: H_2O) yielded **8** (343 mg, 57 % based on **2**) as a colorless amorphous solid: ^{1}H NMR (65:25:4 $CDCl_3$:CD_3OD:D_2O) δ 0.89 (t, 9H, J = 6.5 Hz, 3 × Pal-*Me*), 1.28 (m) and 1.62 (m) (39 × Pal- C*H*$_2$), 1.77 (br quintet, 2H, J = 7 Hz, $NCH_2CH_2CH_2CO$), 2.02 (s, 3H), 2.03 (s, 3H), and 2.04 (s, 3H) (3 × NAc), 2.20 (br t, 2H, J = 7 Hz, $NCH_2CH_2CH_2CO$), 2.28 (br t, 2H, J = 7.5 Hz), 2.32 (br t, 2H, J = 7.5 Hz), and 2.34 (br t, 2H, J = 7.5 Hz) (3 × Pal-CH_2CO), 2.77 (m, 2H, SCH_2CHOCO), 2.83–2.94 (m, 1H) and 3.00–3.08 (m, 1H) (Cys β-CH_2), 3.20 (br t, 2H, J = 7 Hz, $NCH_2CH_2CH_2CO$), 3.24–3.41 (m, 4H, NCH_2CH_2N), 3.87 (br d, 1H, J = 3 Hz) and 3.90 (br d, 1H, J = 3 Hz) (2 × H-4), 4.82 (br d, 1H, J = 4 Hz) and 4.83 (br d, 1H, J = 4 Hz) (2 × H-1), 5.18 (m, 1H, C*H*OCO); HRMS calcd for $C_{87}H_{160}N_9O_{24}S$ 1747.1256, found 1747.1176.

N-*(*N-tert-*Butyloxycarbonyl*-O-*benzyl*-L-*seryl)*-4-*aminobutanoic acid (39)*. A mixture of *N-tert*-butyloxycarbonyl-*O*-benzyl-L-serine (**38**) (1 g, 3.39 mmol), NHS (390 mg, 3.39 mmol), and EDC (649 mg, 3.39 mmol) in dry CH_2Cl_2 (20 mL) was stirred at rt for 30 min. The mixture was washed with ice-cold water (3 × 20 mL), dried (Na_2SO_4), and concentrated to dryness. The residue was dissolved in DMF (10 mL) and a precooled solution of **22** (350 mg, 3.39 mmol) in H_2O (5 mL) containing Et_3N (472 μL, 3.39 mmol) was added dropwise. After 1 h, the mixture was treated with IR-120 (H^+) and concentrated to dryness. The residue was dissolved in CH_2Cl_2 (20 mL), washed with H_2O (4 × 20 mL), and dried (Na_2SO_4). Evaporation yielded **39** (1.28 g, quantitative) as a colorless syrup: $[\alpha]_D$ +15 ° (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H, 3 × Boc-*Me*), 1.84 (quintet, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.35 (t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.34 (m, 2H, $NCH_2CH_2CH_2CO$), 3.59 (dd, 1H, J = 9.0, 6.0 Hz) and 3.91 (dd, 1H, J = 9.5, 4.0 Hz) (Ser β-CH_2), 4.50 and 4.55 (AB-q, 2H, J = 12 Hz, PhCH_2); HRMS calcd for $C_{19}H_{29}N_2O_6$ $(M + H)^+$ 381.2025, found 381.2024.

N-*(*O-*Benzyl*-L-*seryl)*-4-*aminobutanoic acid (40)*. Acidolysis of **39** with TFA as described for the preparation of **26** yielded **40** (quantitative) as a TFA salt: ^{1}H NMR (D_2O) δ 1.73 (quintet, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.31 (t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.16 (dt, 1H, J = 13.0, 7.0 Hz) and 3.27 (dt, 1H, J = 13.0, 7.0 Hz) ($NCH_2CH_2CH_2CO$), 3.84 (dd, 1H, J = 11.0, 5.0 Hz) and 3.87 (dd, 1H, J = 11.0 4.0 Hz) (Ser β-CH_2), 4.16 (br t, 1H, J = 4.5 Hz, Ser α-C*H*), 4.52 and 4.64 (AB-q, 2H, J = 12.0 Hz, PhCH_2); HRMS calcd for $C_{14}H_{21}N_2O_4$ $(M + H)^+$ 281.1501, found 281.1504.

N-*(*N-tert-*Butyloxycarbonyl*-O-*benzyl*-L-*seryl*-O-*benzyl*-L-*seryl)*-4-*aminobutanoic acid (41)*. Coupling of **38** to **40** as described for the preparation of **39**, followed by FCC (2:1:0.01 $CHCl_3$:acetone:AcOH), gave **41** (89 %) as a colorless syrup: $[\alpha]_D$ +6 ° (*c* 0.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H, 3 × Boc-*Me*), 1.79 (quintet, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$), 2.33 (br s, 2H, $NCH_2CH_2CH_2CO$), 3.21 (dt, 1H, J = 19, 6.5 Hz) and 3.29 (dt, 1H, J = 19, 6.5 Hz) ($NCH_2CH_2CH_2CO$), 3.58 (dd, 1H, J = 9.5, 4.5 Hz) and 3.88 (br dd, 1H, J = 9, 4 Hz) (Ser β-CH_2), 3.70 (dd, 1H, J = 9.5, 5.5 Hz) and 4.08 (br d, 1H, J = 9.5 Hz) (Ser β-CH_2), 4.30 (m, 1H) and 4.55 (m, 1H) (2 × Ser α-C*H*); HRMS calcd for $C_{29}H_{39}N_3O_8Na$ $(M + Na)^+$ 580.2625, found 580.2629.

N-*(*N-*Acetyl*-L-*seryl*-L-*seryl)*-4-*aminobutanoic acid (42)*. Acidolysis of **41** (620 mg, 1.11 mmol) with TFA (5 mL) at rt for 20 min, followed by lyophilization, gave a pale yellow glass (R_f 0.6, 2:1:1 *n*-BuOH:MeOH:H_2O). The residue was dissolved in DMF (20 mL) containing Et_3N (0.5 mL) and treated with Ac_2O (105 μL, 1.11 mmol) at rt for 1 h. After concentration, the residue was dissolved in 5:1 EtOH:H_2O (20 mL) and stirred in the presence of 10 % Pd/C (50 mg) under H_2 (1 atm) for 2 h. After filtration, the filtrate was concentrated to give **42** (342 mg, 96 %) as a colorless glass: $[\alpha]_D$ −37 ° (*c* 1.5, H_2O); ^{1}H NMR (D_2O) δ 1.72 (quintet, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.00 (s, 3H, Ac), 2.32 (t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.18 (dt, 1H, J = 13.5, 7.0 Hz) and 3.20 (dt, 1H, J = 13.5, 7.0 Hz) ($NCH_2CH_2CH_2CO$), 3.74–3.84 (m, 4 H, 2 × Ser β-CH_2), and 4.33 (t, 1H, J = 5.0 Hz) and 4.39 (t, 1H, J = 5.0 Hz) (2 × Ser α-C*H*); HRMS calcd for $C_{12}H_{22}N_3O_7$ $(M + H)^+$ 320.1451, found 320.1452.

N-*{*N-*Palmitoyl*-S-*[2,3-bis(palmitoyloxy)-(2R,2S)-propyl]*-L-*cysteyl*-L-*seryl]*-2-*aminoethyl*-*(*N^4-L-*seryl*-L-*seryl)*-4-

aminobutanamide (43). Conjugation of **42** with **37** was carried out as described for the preparation of **8**. The succinimido ester of **42** showed R_f 0.7 on TLC (1:1 $CHCl_3$:MeOH). Purification by FCC (6:1, $CHCl_3$:MeOH) yielded **43** (63 % based on **42**) as a colorless solid: ^1H NMR (3:1, $CDCl_3$:CD_3OD) δ 0.89 (t, 9H, J = 6.5 Hz, 3 × Pal-*Me*), 1.27 (m) and 1.63 (m) (39 × Pal-CH_2), 1.81 (br quintet, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$), 2.05 (s, 3H, NAc), 2.18 (m, 2H, $NCH_2CH_2CH_2CO$), 2.27 (br t, 2H, J = 7.5 Hz), 2.32 (br t, 2H, J = 7.5 Hz), and 2.35 (br t, 2H, J = 7.5 Hz) (3 × Pal-CH_2CO), 2.77 (m, 2H, CH_2OCO), 2.89 (m, 1H) and 3.02 (m, 1H) (Cys β-CH_2), 3.17–3.48 (m, 6H, NCH_2CH_2N and $NCH_2CH_2CH_2CO$), 5.18 (m, 1H, *CH*OCO); HRMS calcd for $C_{71}H_{133}N_7O_{14}SNa$ $(M + Na)^+$ 1362.9493, found 1362.9543.

Coupling of synthetic Tn antigens 1–3 to OSA and Starburst® dendrimer: preparation of conjugates 4–7. Typical procedure. The synthetic Tn antigen (**1**, **2**, or **3**; 5 molar excess to the lysine residues) was treated with NHS and EDC (both used in an equimolar amount to the Tn antigen) in DMF at rt for 4 h. The NHS esters of **1**, **2**, and **3** showed R_f 0.5, 0.3, and 0.1, respectively, on TLC (1:1, $CHCl_3$:MeOH). The reaction mixture was added dropwise to the OSA preparation (4 mg/mL in 0.1 M PBS, pH 7.4) or the Starburst® dendrimer preparation (5th generation, 1.2×10^{17} particles/mL in 0.1 M PBS, pH 7.4) with gentle stirring in an ice-bath. The reaction progress was followed by monitoring the disappearance of the NHS ester on TLC (1:1, $CHCl_3$:MeOH). For the coupling to the Starburst® dendrimer, the progress was also monitored by the Kaiser test. After stirring at rt for 6–8 h, the mixture was exhaustively dialyzed against PBS at 4 °C. The Tn activity of the conjugate was determined by anti-Tn mAb (1E3) with A-OSM (expressing > 96 % Tn antigen) as standard according to the previously outlined procedure.[11] The conjugation typically afforded 250 μg of Tn antigen per 1 mg of OSA.

Immunology

Mice. Six- to 8-week-old female CAF mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Antigen preparation and immunizations. Before immunization, the conjugates **4–7** were suspended in Ribi adjuvant composed of monophosphoryl lipid A and trehalose dimycolate (RibiImmuno Chem, Hamilton, MT) at a concentration of 0.5 mg Tn activity/mL, according to the manufacturer's instruction. The conjugate **8** and the control antigens **37** and **43** were dissolved in 50 % Intralipid® composed of soybean oil, egg yolk phospholipids, and glycerin (KabiVitrum, Inc., Clayton, NC) in PBS at a concentration of 0.5 mg antigen/mL. A group of six mice was immunized twice (one week apart) with 100 μg antigen subcutaneously at the base of the tail and at the neck.

Serum antibody titers. Blood was drawn retro-orbitally from mice 7 days after the second immunization. Sera were titered against A-OSM, prepared from OSM as described previously,[11] in ELISA as reported.[7] Anti-Tn IgG and IgM titers were detected by horseradish peroxidase-conjugated goat anti-mouse μ- and γ-chain specific antibodies.

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