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Synthetic and Immunological Studies of 5'-N-phenylacetyl sTn to Develop Carbohydrate-Based Cancer Vaccines and to Explore the Impacts of Linkage between Carbohydrate Antigens and Carrier Proteins

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Abstract

5'-N-Phenylacetyl sTn (sTnNPhAc), an unnatural derivative of sTn antigen expressed by many tumors, and its α -linked protein conjugates were prepared and investigated to explore glycoconjugate cancer vaccines. sTnNPhAc α -KLH elicited a robust T cell-dependent immunity. The antiserum derived from sTnNPhAc α - or sTnNPhAc β -KLH-inoculated mice was similarly reactive to sTnNPhAc α and sTnNPhAc β but showed very little reactivity to sTn, NeuNPhAc α (2,3)GalNAc – a regioisomer of sTnNPhAc, isolated phenylacetyl group, and the linker employed to conjugate sTnNPhAc and carrier protein. It was concluded that the sTnNPhAc-elicited immunity was specific for the whole antigen rather than the phenylacetyl group or other partial structures of sTnNPhAc and that the reducing end configuration or linkage of sTnNPhAc did not affect its immunological identity. It was also concluded that a new linker designed to conjugate carbohydrates and proteins did not provoke any immune reaction and that the linker, as well as the associated new and convenient coupling strategy, can be safely used for the development of glycoconjugate vaccines.

Keywords

cancer; glycoconjugate; vaccine; carbohydrate; sTn antigen; linker

INTRODUCTION

Tumor-associated carbohydrate antigens (TACAs) are useful targets in the development of cancer vaccines (1–3). To form functional cancer vaccines, TACAs must be covalently conjugated to carrier proteins, because free carbohydrates only induce T cell-independent immune responses (4) which are not particularly useful in the treatment of cancer. Therefore, different linkers or spacers and coupling methods have been developed to facilitate the conjugation between carbohydrates and proteins (2,5). However, many linkers contain functionalities which are not compatible with the chemical synthesis of oligosaccharides, while late-stage installation of a linker to synthesized oligosaccharides can be difficult and usually needs multi-step transformations. To address these issues, our group developed a novel strategy for coupling synthetic oligosaccharides to proteins, which employs a simple linker as shown in Scheme 1 (6). The azido group utilized to attach the linker is a widely adopted protecting

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group in oligosaccharide synthesis, thus it can be installed as a protection of the oligosaccharide reducing end during the TACA synthesis. After oligosaccharides are accomplished, the azido group can be readily reduced to give a free amino group for introducing an unsaturated acyl linker, which in turn can be selectively oxidized to form an aldehyde functionality, enabling the reliable and effective coupling of synthetic TACAs to carrier proteins via reductive amination. This coupling strategy has been used to construct some hopeful conjugate vaccines (7,8).

Because all synthetic glycoconjugate vaccines contain unnatural linkers or spacers, it is natural to inquire how these linkers may affect the immunological properties of resultant vaccines. It has been reported that some linkers themselves may provoke a strong immune response (9, 10), which is not only unhelpful but rather detrimental, and that some linkers may even suppress the immune system and decrease its response to TACAs (9,11). Immunological studies of the synthetic glycoconjugates containing this new linker suggest that it is probably immunologically inert (7,8), which is a useful property and needs to be investigated in detail.

In nature, TACAs are directly α - or β -linked to membrane proteins or lipids on the cell surfaces, while most synthetic cancer vaccines have TACAs coupled to carrier proteins via unnatural linkers or anomeric linkages. Consequently, another question about these vaccines is whether this would affect the immunological identity of TACAs or whether the provoked immune system can recognize and target cancer cells that bear differently configured TACAs. For example, sialyl Tn (sTn, Figure 1) is a disaccharide TACA α -linked to the polypeptide backbone of mucins on cancer cells and is richly expressed by numerous tumors including breast, prostate, colorectal, ovarian, pancreatic, and gastric cancer (12–15). Due to its unique expression on cancer cells and its relevance to tumor metastasis (16), sTn has become an attractive target in cancer vaccine development (17–22). Recently, we have shown that sTn derivatives containing unnatural *N*-acyl sialic acids, for instance *N*-phenylacetyl sialic acid (NeuNPhAc), were much more immunogenic than the natural counterpart and that the sTn derivatives could elicit robust T cell-dependent immune responses (7). However, the glycoconjugates employed there, such as the keyhole limpet hemocyanin (KLH) conjugate of unnatural 5'-*N*-phenylacetyl sTn (sTnNPhAc) (1), had the sTn derivatives β -linked to carrier proteins. It is therefore natural to inquire whether the provoked immune system would interact with the α -linked antigens expressed on cancer cells following metabolic engineering (23, 24).

An even more interesting question about glycoconjugate vaccines is whether the elicited immune reactions are mainly against the whole carbohydrate antigens or they also direct at partial structures of the antigen, such as a segment of an oligosaccharide or the unnatural functionality of a conjugate vaccine consisting of an unnatural TACA derivative. An answer to this question should be helpful for understanding the functions and functional mechanisms of carbohydrate-based vaccines. It can also assist to address issues concerning the specificity of a novel strategy for cancer immunotherapy developed by our laboratory, where synthetic glycoconjugate vaccines consisting of unnatural TACA derivatives are combined with metabolic engineering of cancer cells for their expression of the unnatural TACA derivative (7,8,25–27).

To explore the problems mentioned above, we prepared glycoconjugate **3**, which has sTnNPhAc α -linked to KLH, and compared its immunological properties with that of the β -linked glycoconjugate **1**. The α -linked human serum albumin (HSA) conjugate of sTnNPhAc **4** was prepared and employed in assaying the immunological properties of **3**. Meanwhile, HSA conjugates **2**, **5**, **6**, and **7** containing sTnNPhAc β , natural sTn, the phenylacetylated linker, and NeuNPhAc α -(2,3)GalNAc α - a regioisomer of sTnNPhAc α , respectively, were also prepared

to check the reactivity of **1** and **3**-provoked antisera to sTn, β -linked sTnNPhAc, the linker, NeuNPhAc, the phenylacetyl functionality, and other partial structures of sTnNPhAc.

EXPERIMENTAL PROCEDURES

General Methods

NMR spectra were recorded on a 400 MHz spectrometer with chemical shifts reported in ppm (δ) in reference to SiMe₄ if not specified otherwise. Coupling constants (J) are reported in hertz (Hz). High resolution (HR) ESI MS was performed on a Micromass Autospec Ultima magnetic sector mass spectrometer or on Waters GCT time-of-flight mass spectrometers. MALDI TOFMS was obtained on a Bruker Ultraflex mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel GF₂₅₄ plates detected by charring with phosphomolybdic acid in EtOH or with 1% H₂SO₄/EtOH solution. Molecular sieves were dried under high vacuum at 170–180 °C for 6–10 h just before use. Glycoconjugates **1**, **2**, and **5** was prepared and reported previously (7), and glycoconjugate **7** was prepared for a different project and will be reported separately. Commercial anhydrous solvents and other reagents were used without further purification.

2-Azidoethyl 2-Acetamido-2-deoxy- α -D-galactopyranoside (**10**)

To a solution of *N*-acetyl-D- galactosamine (**8**, 1.2 g, 5.42 mmol) in 2-chloroethanol (15 mL) at 0 °C was added drop-wise acetyl chloride (0.47 g, 5.97 mmol), and the reaction mixture was heated at 70 °C for 4 h. The solution was concentrated, and the residue was co-evaporated with toluene and then purified by flash column chromatography (CH₂Cl₂/MeOH 10:1) to yield **9** as a white solid (0.66 g, 45%). ¹H NMR (D₂O, 400 MHz): δ 4.95 (d, J = 3.2 Hz, 1H, H-1), 4.14 (dd, J = 11.6 and 4.0 Hz, 1H, H-2), 4.02 (t, J = 6.8 Hz, 1H, H-4), 3.97–3.90 (m, 3H), 3.79–3.72 (m, 5H), 2.02 (s, 3H, NHAc); ¹³C NMR (D₂O, 100 MHz): δ 174.9, 97.5, 71.4, 68.7, 68.5, 67.8, 61.5, 50.1, 43.8, 22.2. The mixture of **9** (0.51 g, 1.80 mmol), NaN₃ (1.17 g, 18.0 mmol) and monobenzo-15-crown-5 (48 μ g, 0.18 mmol) in anhydrous DMF (5 mL) was stirred at 80 °C overnight. After cooling to rt, inorganic salts were removed by filtration and washed with CH₃CN. The filtrate and washings were combined and concentrated. The residue was purified by flash column chromatography (CH₂Cl₂/MeOH 10:1) to give **10** as a white solid (0.52 g, quantitative). Its spectroscopic data were consistent with that reported in the literature (28).

2-Azidoethyl 2-Acetamido-2-deoxy-3,4-O-(1-methylethylidene)- α -D-galactopyranoside (**11**)

To a solution of **10** (0.45 g, 1.55 mmol) in a mixture of DMF (4.5 mL) and 2,2-dimethoxypropane (9.0 mL) was added *p*-toluenesulfonic acid monohydrate (0.029 mg, 0.155 mmol) at 65 °C. After the solution was stirred at 65 °C for 5 h, it was cooled to rt, and Et₃N (0.5 mL) was added. The mixture was stirred for another 15 min, and it was concentrated and co-evaporated twice with toluene to remove traces of Et₃N. The residue was dissolved in MeOH/H₂O (10:1) (15 mL) and heated under refluxing for 30 min until TLC (CH₂Cl₂/MeOH 16:1) showed the complete disappearance of the reaction intermediate. The solution was then concentrated and co-evaporated with toluene. The residue was purified by flash column chromatography (CH₂Cl₂/MeOH 20:1) to give **11** as light yellow oil (0.47 g, 92%). *R*_f = 0.20 (CH₂Cl₂/MeOH 16:1). ¹H NMR (CDCl₃, 400 MHz): δ 5.62 (d, J = 10.0 Hz, 1H, NHAc), 4.87 (d, J = 3.2 Hz, 1H, H-1), 4.31 (dt, J = 9.2 and 3.6 Hz, 1H, H-2), 4.21 (dd, J = 4.8 & 2.4 Hz, 1H, H-4), 4.12–4.04 (m, 2H), 4.01–3.90 (m, 2H), 3.85 (dd, J = 11.6 & 4.0 Hz, 1H, H-3), 3.66–3.60 (m, 1H), 3.49–3.42 (dq, 1H), 3.36–3.29 (m, 1H, H-5), 2.03 (s, 3H, NHAc), 1.57, 1.34 (s, 2 $\times$ 3H, CH₃). ¹³C NMR (CDCl₃, 100 MHz): δ 170.5, 110.3, 98.3, 74.6, 73.6, 68.2, 67.8, 62.9, 50.6, 50.5, 28.1, 26.8, 23.6. HR ESI MS (*m/z*) calcd. for C₁₃H₂₂N₄NaO₆ (M + Na)⁺ 353.1437, found 353.1442.

Methyl (Ethyl 4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-5-phenylacetamido-D-glycer-D-galacto-non-2-ulopyranosid)onate (15)

A solution of **13** (4.0 g, 7.47 mmol) (**7,8**) and methanesulfonic acid (1.9 mL, 29.88 mmol) in anhydrous methanol (60 mL) was refluxed overnight. After the reaction was completed, the reaction mixture was neutralized with Et₃N to pH 8 and then concentrated under reduced pressure. The residue was dissolved again in methanol (60 mL) and Et₃N was added to adjust pH to 9. After the mixture was cooled to 0 °C, phenylacetic anhydride (3.8 g, 14.94 mmol) was added slowly and the mixture was stirred at rt for 1 h. The reaction mixture was concentrated under vacuum and the residue was purified by flash column chromatography (toluene/AcOEt/CH₃OH 5:5:1) to give **14** (2.47 g, 75%), which was dissolved in pyridine (12 mL) at 0 °C, followed by addition of DMAP (42 mg) and acetic anhydride (6 mL). After the mixture was stirred at 0 °C for 30 min and at rt for 5 h, it was diluted with ethyl acetate (100 mL), washed with 1 N HCl, brine, saturated NaHCO₃ aqueous solution and brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum. The residue was purified by flash column chromatography (AcOEt/hexanes 1:1) to give an anomeric mixture of **15** as a white solid (3.14 g, 92%). ¹H NMR (CDCl₃, 400 MHz): δ 7.37–7.22 (m, 5 H, aromatic H), 5.36 (t, *J* = 3.2 Hz, 1H), 5.25 (m, 1H), 5.17–5.04 (m, 2H), 4.76 (dd, *J* = 12.4 and 2.4 Hz, 1H), 4.24 (dd, *J* = 10.4 and 2.4 Hz, 1H), 4.15 (dd, *J* = 12.4 and 8.0 Hz, 1H), 4.05 (d, *J* = 10.4 Hz, 1H), 3.78 (s, 3H, COOCH₃), 3.44 (d, *J* = 2.4 Hz, 1H), 2.58–2.44 (m, 2H), 2.14 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.81 (s, 3H), 1.16 (t, *J* = 8.0 Hz, 3H). ¹³C NMR (CDCl₃, 100 MHz): δ 171.3, 170.9, 170.8, 170.7, 170.5, 168.6, 134.5, 129.7, 129.2, 127.6, 85.1, 72.7, 72.3, 69.0, 68.7, 62.6, 53.0, 49.7, 44.0, 37.5, 22.9, 21.2, 21.1, 21.0, 20.8, 14.3. HR ESI MS (*m/z*) calcd. for C₂₈H₃₇NNaO₁₂S (M + Na)⁺ 634.1934, found 634.1943.

2-Azidoethyl [Methyl 4,7,8,9-tetra-O-acetyl-3,5-dideoxy-5-phenylacetamido-D-glycero-α-D-galacto-2-nonulopyranosylonate]-(2→6)-O-2-acetamido-2-deoxy-α-D-galactopyranoside (16)

A mixture of **15** (0.32 g, 0.52 mmol), **11** (0.16 g, 0.48 mmol), and activated MS 3 Å (1.5 g) in anhydrous CH₃CN (5.0 mL) was stirred at rt for 2 h under an atmosphere of argon. After the mixture was cooled to –35 °C, NIS (0.44 g, 1.93 mmol) and TfOH (18 μL, 0.193 mmol) were added with stirring. The reaction was kept at –35 °C for 1 h and then quenched with the addition of Et₃N. The solid material was filtered off and washed with DCM. The combined filtrate and washings were washed with aqueous Na₂S₂O₃ and water, dried over anhydrous Na₂SO₄, and concentrated under vacuum. The residue was dissolved in 65% HOAc/H₂O (v/v, 5 mL) and heated at 65 °C for 1.5 h. The mixture was concentrated and the residue was purified by flash column chromatography (CH₂Cl₂/MeOH 30:1) to afford **16** as a white solid (212 mg, 52%), R_f = 0.35 (CH₂Cl₂/MeOH 15:1) and its β-anomer (64 mg, 15.7%), R_f = 0.40 (CH₂Cl₂/MeOH 15:1). **16**: ¹H NMR (CDCl₃, 400 MHz): δ 7.37–7.22 (m, 5 H, aromatic H), 5.98 (d, *J* = 8.4 Hz, 1H), 5.36–5.29 (m, 1H), 5.24 (dd, *J* = 8.4 and 2.0 Hz, 1H), 5.16 (d, *J* = 9.2 Hz, 1H), 4.89 (d, *J* = 3.2 Hz, 1H), 4.80–4.73 (m, 1H), 4.36–4.30 (m, 2 H), 4.09–3.99 (m, 3H), 3.98–3.92 (m, 2H), 3.89–3.82 (m, 2H), 3.78 (s, 3H, COOCH₃), 3.74 (dd, *J* = 10.4 and 3.2 Hz, 1H), 3.67–3.60 (m, 2H), 3.55–3.47 (m, 1H), 3.44 (d, *J* = 3.2 Hz, 2H), 3.37–3.30 (m, 1H), 2.61 (b, -OH), 2.54 (dd, *J* = 12.8 and 4.0 Hz, 1H, H-3'e), 2.15, 2.11, 2.05, 2.03 (4s, 4 × 3H, Ac), 1.90 (t, *J* = 12.0 Hz, 1H, H-3'a), 1.83 (s, 3H, Ac). ¹³C NMR (CDCl₃, 100 MHz): δ 172.6, 171.5, 171.1, 170.8, 170.5, 170.4, 168.3, 134.5, 129.7, 129.1, 127.6, 99.0, 98.0, 72.9, 70.6, 69.2, 69.1, 68.7, 68.4, 67.7, 67.5, 63.7, 62.7, 53.2, 50.8, 50.5, 49.5, 44.0, 37.8, 23.4, 21.3, 21.1, 21.0, 20.8. HR ESI MS (*m/z*) calcd. for C₃₆H₄₉N₅NaO₁₈ (M + Na)⁺ 862.2970, found 862.2983.

***N*-(2-*O*-{[(3,5-dideoxy-5-Phenylacetamido)-*D*-glycero- α -*D*-galacto-2-nonulosonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- α -*D*-galactopyranosyl}-ethyl) 4-Pentenamide (18)**

To a stirred solution of **16** (44.0 mg, 0.052 mmol) in MeOH (4 mL) was added a NaOMe solution in MeOH (0.1 N, 0.4 mL). After the mixture was stirred at rt overnight, it was neutralized with Amberlite 15 (H⁺) resin. The resin was filtered off and washed with MeOH. The filtrate and washings were combined and concentrated to a small volume (ca. 3 mL), to which was added an aqueous NaOH solution (0.5 N, 0.5 mL). After the solution was stirred at rt overnight, neutralized with 2 N HCl to pH 7. Then, the reaction mixture was concentrated in vacuum to give **17**, which was used directly in the next step without further purification. HR ESI MS (*m/z*) calcd. for C₂₇H₃₈N₅Na₂O₁₄ (M-H+2Na)⁺ 702.2211, found 702.2222. A solution of **17** in H₂O (4 mL) was stirred with 10% Pd/C under a H₂ atmosphere at rt overnight. After argon was introduced to remove H₂, MeOH (4 mL) and 4-pentenoic anhydride (18 μ L) were added sequentially. The mixture was stirred at rt overnight. After the catalyst was filtered off and washed with H₂O/MeOH, the filtrate and washings were combined and concentrated, the residue was first extracted with DCM to remove any non-polar materials and then purified on a Biogel P-2 column using distilled water as the eluent to give **18** after lyophilization as a white solid (28 mg, 75% from **16**). ¹H NMR (D₂O, 400 MHz): δ 7.41–7.30 (m, 5 H, Ar), 5.88–5.79 (m, 1H, CH=), 5.09 (s, 1H), 5.02 (t, *J* = 10.8 Hz, 1H, =CH), 4.81 (d, *J* = 3.2 Hz, 1H, H-1), 4.13 (dd, *J* = 11.2 and 3.2 Hz, 1H, H-2), 3.97–3.44 (m, 18H), 3.32 (d, *J* = 9.2 Hz, 1H), 3.30–3.26 (m, 1H), 2.71 (dd, *J* = 12.4 and 4.0 Hz, 1H, H-3'e), 2.38–2.28 (m, 4H), 2.01 (s, 3H, Ac), 1.65 (t, *J* = 12.4 Hz, 1H, H-3'a). ¹³C NMR (D₂O, 100 MHz): δ 176.5, 175.9, 174.7, 173.5, 137.2, 135.3, 129.4, 129.3, 127.6, 115.9, 100.5, 97.4, 72.8, 72.0, 69.8, 68.7, 68.6, 68.2, 68.0, 66.8, 63.9, 63.0, 52.2, 50.0, 42.9, 40.6, 39.2, 35.3, 29.6, 22.3. HR ESI MS (*m/z*) calcd. for C₃₂H₄₆N₃Na₂O₁₅ (M-H+2Na)⁺ 758.2724, found 758.2759.

***N*-(2-*O*-{[(3,5-dideoxy-5-Phenylacetamido)-*D*-glycero- α -*D*-galacto-2-nonulosonic acid]-(2 $\rightarrow$ 6)-*O*-2-acetamido-2-deoxy- α -*D*-galactopyranosyl}-ethyl) 4-Oxo-butanamide (19)**

To the stirred solution of **18** (25 mg, 0.035 mmol) in MeOH (5 mL) at –78 °C, ozone was bubbled until a blue color appeared and remained for 1 h. After introducing nitrogen to remove the remaining ozone, Me₂S (0.5 mL) was added at –78 °C. The resultant solution was allowed to warm to rt over a period of 1 h and stand for another 1 h before it was condensed in vacuum. The crude product was purified by a Biogel P-2 column using distilled water as the eluent to give **19** after lyophilization as a white solid. ¹H NMR (D₂O, 400 MHz): δ 7.40–7.30 (m, 5 H, Ar), 5.42–5.35 (m, 1H, hydrated CHO), 4.82 (d, *J* = 3.2 Hz, 1H, H-1), 4.15–4.09 (m, 1H), 3.98–3.46 (m, 19H), 3.36–3.28 (m, 2H), 2.73–2.54 (m, 2H), 2.47–2.32 (m, 2H), 2.01 (s, 3H, Ac), 1.96–1.84 (m, 1H), 1.68–1.60 (m, 1H). ¹³C NMR (D₂O, 100 MHz): δ 178.5, 175.9, 174.7, 173.7, 135.3, 129.3, 129.2, 127.6, 100.5, 97.4, 72.8, 72.1, 69.9, 69.8, 68.7, 68.6, 68.3, 67.9, 66.6, 64.0, 63.0, 52.2, 50.0, 42.9, 40.6, 39.2, 32.3, 29.0, 27.4, 22.2. HR ESI MS (*m/z*) calcd. for C₃₁H₄₄N₃Na₂O₁₆ (M-H+2Na)⁺ 760.2517, found 760.2552.

Procedure for the Coupling between 19 and KLH or HSA

A solution of **19** (10 mg), KLH or HSA (10 mg), and NaBH₃CN (10 mg) in 0.1 M NaHCO₃ (0.3 mL, pH 7.5–8.0) was allowed to stand at rt in the dark for 4 days with occasional shaking. The reaction mixture was then purified by a Biogel A 0.5 column using 0.1 M phosphate buffered saline (PBS) (*I* = 0.1, pH = 7.8) as the eluent. The fractions containing the glycoconjugates, positively characterized by both the bicinchoninic acid (BCA) assay for proteins and the Svennerholm method for sialic acid, were combined and dialyzed against distilled water for 2 d. It was then lyophilized to give the expected glycoconjugates **3** and **4** (ca. 10 mg) as white powders.

2-Pent-4-enamidoethyl 2-Phenylacetate (21)

To a solution of *N*-(2-hydroxyethyl)-4-pentenamide (**20**, 0.3 g, 2.1 mmol) in pyridine (5 mL) were added phenylacetic anhydride (1.06 g, 4.2 mmol) and DMAP (25 mg, 0.21 mmol) at 0°C. After stirring at rt overnight, the mixture was extracted with AcOEt, and the extract was washed with saturated NaHCO₃ aqueous solution and brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum. The residue was purified by flash column chromatography (AcOEt/hexanes 1:2) to give **21** as oil (0.5 g, 92%). ¹H NMR (CDCl₃, 400 MHz): δ 7.35–7.26 (m, 5 H, Ar), 5.81–5.73 (m, 1H, CH=), 5.62 (brs, 1 H, NH), 5.06–4.97 (m, 2H, =CH₂), 4.16 (t, *J* = 6.0 Hz, 2H), 3.63 (s, 2H, PhCH₂), 3.47 (dd, *J* = 10.4 and 5.6 Hz, 2H), 2.29 (dd, *J* = 14.8 and 5.6 Hz, 2H), 2.16 (t, *J* = 7.2 Hz, 2H). ¹³C NMR (CDCl₃, 100 MHz): δ 172.5, 171.8, 137.1, 134.1, 129.4, 128.9, 127.5, 115.8, 63.8, 41.5, 38.8, 35.8, 29.6. HR GCEI MS (*m/z*) calcd. for C₁₅H₁₉NO₃ (M)⁺ 261.1365, found 261.1372.

4-Oxo-4-[2-(2-phenylacetoxylethylamino)]butanoic Acid (22)

To the solution of **21** (0.1 g, 0.383 mmol) in DMF (2 mL) was added OsO₄ (1 mg, 3.83 μmol) at rt. After it was stirred for 5 min, oxone (0.94 g, 1.53 mmol) was added in one portion and the mixture was stirred at rt for 3 h. Na₂SO₃ was added to reduce the remaining Os(VIII). The reaction mixture was extracted with AcOEt, and the extract was washed with 1N HCl and brine, dried over Na₂SO₄, and concentrated vacuum. The residue was purified by flash column chromatography (AcOEt) to give **22** as a white solid (0.07 g, 65%). ¹H NMR (CDCl₃, 400 MHz): δ 7.35–7.26 (m, 5 H, Ar), 5.98 (brs, 1 H, NH), 4.16 (t, *J* = 5.6 Hz, 2H), 3.63 (s, 2H, PhCH₂), 3.48 (q, *J* = 5.6 Hz, 2H), 2.64 (t, *J* = 5.6 Hz, 2H), 2.38 (t, *J* = 6.4 Hz, 2H). ¹³C NMR (CDCl₃, 100 MHz): δ 176.6, 172.8, 172.0, 134.0, 129.5, 128.9, 127.5, 63.5, 41.4, 39.0, 30.8, 29.7. HR GCEI MS (*m/z*) calcd. for C₁₄H₁₇NO₅ (M)⁺ 279.1107, found 279.1106

Synthesis of Conjugate 6

To the stirred solution of **22** (10 mg, 0.036 mmol), *N*-hydroxysuccinimide (9.0 mg, 0.078 mmol), and DMAP (catalytic amount) in anhydrous CH₂Cl₂ (2 mL) was added DIC (9.9 mg, 0.078 mmol) at 0°C. After the mixture was stirred at 0°C for 2 h, it was concentrated under vacuum, and the residue was dissolved in anhydrous DMF (0.5 mL) and cooled to 0°C. Then, a 0.1 mL solution of HSA (10 mg) in 50 mM carbonate buffer (pH 9.1) was added drop-wise. After the mixture was stirred at 0°C overnight, it was diluted with water and washed with AcOEt. The aqueous layer was dialyzed against H₂O and then lyophilized to give **6** as a white powder (9 mg).

Analysis of the Carbohydrate Loading Levels of Glycoconjugates 3 and 4 (8)

The solution of an exactly weighed glycoconjugate (0.5 mg) in distilled water (1.0 mL) was mixed with the resorcinol reagent (2.0 mL). The mixture was heated in a boiling water for 30 min. It was cooled to rt, and to this mixture was added an extraction solution (1-butanol acetate and 1-butanol, 85:15 v/v, 3.0 mL). The mixture was shaken vigorously before it was allowed to stand still for ca. 10 min to allow the organic layer to separate well from the inorganic layer. The organic layer was transferred to a 1.0-cm cuvette, and its absorbance at 580 nm was determined by an UV-Vis spectrometer, using a blank organic solution as the control. The sialic acid content of the glycoconjugate was determined against a calibration curve created with the standard NeuNPhAc solution analyzed under the same conditions. The carbohydrate loading of each glycoconjugate was calculated according to the following equation.

$$\text{sTnNPhAc loading (w/w \%)} = \frac{\text{sTnNPhAc content (mg) in sample}}{\text{weight of the sample (mg)}} \times 100\%$$

Immunization of Mouse

A total of 0.1 mL of an emulsion of sTnNPhAc α -KLH **3** containing 2 μ g of sTnNPhAc and Titermax Gold adjuvant (Sigma) was intramuscularly injected to each of 5 female C57BL/6 mice at the age of 6–8 weeks (Jackson Laboratories, Bar Harbor, ME) on day 0, day 14, day 21 and day 28, respectively. The mice were bled prior to the initial immunization on day -1 and after immunization on day 27 and day 35. Blood samples of 5 mice collected on each date were pooled and then clotted to obtain serum, which was stored at -80°C before use.

ELISA

ELISA plates were incubated with a 100 μ L solution of sTnNPhAc α -HSA (**4**, 2 μ g/mL), or other HSA conjugates including **2**, **5**, **6**, and **7**, in the coating buffer (0.1 M bicarbonate, pH 9.6) at 4°C overnight, which was followed by washing 3 times with PBS buffer containing 0.05% Tween-20. Pooled antisera diluted 1:300 to 1:72900 in serial half-log dilutions in PBS buffer containing 0.02% sodium azide (100 μ L/well) were added in the coated ELISA plates, and the plates were incubated at 4°C overnight. The plates were washed again with PBS buffer and then incubated with alkaline phosphatase linked goat anti-mouse kappa, IgM, or IgG2a antibody (1:1000 dilution) or with alkaline phosphatase linked anti-mouse IgG1 or IgG3 antibody (1:2000 dilution) for 1 h at rt. Finally, the plates were washed with PBS buffer and developed with 100 μ L of PNPP solution (1.67 mg/mL in PNPP buffer) for 30 min at rt for colorimetric readout using a plate reader at 405 nm wavelength. For titer analysis, the OD values were plotted against dilution numbers, and a best-fit line was obtained. The equation of this line was used to calculate the dilution number at which an OD value of 0.5 was achieved, and this dilution number gives the antibody titer.

RESULTS AND DISCUSSION

The synthesis of sTnNPhAc, as well as its protein conjugates **3** and **4**, is shown in Scheme 2. First, a new and short route was developed to prepare glycosyl acceptor **11** from *N*-acetylgalactosamine **8**. Treating **8** with 2-chloroethanol in the presence of a catalytic amount of acetyl chloride produced 2-chloroethyl glycoside **9** (**28**), of which the anomeric α -configuration was affirmed by ^1H NMR ($J_{1,2} = 3.2$ Hz). The chlorine atom in **9** was then substituted by an azido group after reacting with sodium azide at 80°C in the presence of monobenzo-15-crown-5 to afford **10** in a quantitative yield. The reaction progress was monitored by ^{13}C NMR since on TLC the R_f values of **9** and **10** were similar, but their ^{13}C NMR spectra were very different. The chemical shift of the carbon linked to the azido group in **10** was 50.6, while the same carbon in **9** appeared at δ 43.8. Finally, the 3,4-hydroxyl groups in **10** were selectively protected as acetone under acidic conditions to give a high yield (92%) of **11**.

For the synthesis of sTn antigen and related derivatives, the most difficult step is probably the sialylation reaction. In our previous synthesis of sTn derivatives (**7**), Boons method (**29**) was used to obtain good yields and stereoselectivity for α -sialosides. In the present work, we planned to directly use *N*-phenylacetylthiosialoside **15** as a glycosyl donor with the hope of saving a few synthetic steps. Thus, after *N*-acetyl sialic acid (NeuNAc, **12**) was transformed into **13** by an established procedure (**7**), its *N*-acetyl group was replaced with a phenylacetyl group in two steps including deacetylation under acidic conditions to remove both *O*- and *N*-acetyl groups and then selective phenylacetylation of the free amino group. Thereafter, the hydroxyl groups were protected again by acetyl groups to give **15** as a mixture of α and β anomers. Since both α and β anomers could act as glycosyl donors, the mixture was directly applied to the subsequent sialylation reaction.

The sialylation of **11** by **15** was performed in CH₃CN using *N*-iodosuccinamide (NIS) and triflic acid (TfOH) as promoters to give a clean reaction (judged by TLC). ¹H NMR of the product showed that it was a mixture of α - and β -anomers (3.3:1.0). The anomers were nearly inseparable by column chromatography. Fortunately, after the isopropylidene group was removed, the resultant diol products were readily separated to afford the desired α -disaccharide **16** (52%), as well as the β anomer (16%). The α -configuration of sialic acid in **16** was assigned on the basis of the unique downfield shift of the NMR signal of H-3'e (δ 2.54) compared to that of β -linked sialic acids (δ 2.42), which have been observed in all sialosides. Moreover, the strong correlation between C-1' and H-3'a, but not between C-1' and H-3'e, in the HMBC spectrum of **16** suggests that the dihedral angle between C-1' and H-3'a is close to 180°, which further proves the α -configuration of the sialyl residue in **16**. Therefore, the sialylation reaction using **15** as a sialyl donor gave an excellent yield and good stereoselectivity. Finally, all protecting groups in **16** were removed after treatments first with a methanolic solution of NaOMe and then with a methanolic/aqueous solution of NaOH to afford **17**, which was directly used in the next step for conjugation to proteins.

The protein conjugates of **17** were prepared by an established procedure (6). First, the azido group in **17** was reduced to form a primary amine, which was regioselectively acylated for the introduction of a pentenoyl group. The product **18** was purified with a Biogel P2 column using distilled water as the eluent, and its identity and purity were confirmed by NMR and HR MS. Next, the C=C double bond in **18** was selectively oxidized by ozone in methanol to afford aldehyde **19** which was purified with a Biogel P2 column. The ¹H NMR spectra of **19** showed the hydrated aldehyde proton signals (δ 5.3–5.5) with the disappearance of signals of protons linked to C=C double bond. Finally, **19** was coupled to carrier proteins, KLH and HSA, by reductive amination carried out in a 0.1 N NaHCO₃ buffer solution in the presence of NaBH₃CN. Aldehyde **19** was used in excess, and unreacted **19** was readily separated from the conjugate products on a Biogel A 0.5 column. Column fractions containing the desired glycoconjugates, which gave positive results in both sialic acid assays and protein assays, were combined and dialyzed against water. Lyophilization of the resultant aqueous solution afforded **3** and **4** as white fluffy solids. Glycoconjugate **7** used as a coating antigen in this study was prepared from the 2,3-linked disaccharide by a similar procedure.

The antigen loading levels of glycoconjugates **3**, **4**, and **7** were analyzed by measurements of their sialic acid contents as reported in the literature (8). It was found that they contained 4.0–5.3% (w/w) of sugars, respectively. These loading levels were similar to those obtained previously (7). The KLH conjugate **3** was used as a vaccine for immunization of animals, and the HSA conjugates **4** and **7** were employed as the coating antigen in immunological assays.

HSA conjugate **6**, which contains the linker and the phenylacetyl group as an ester, was prepared according to Scheme 3. Acylation of **20** by phenylacetic anhydride followed by oxidative cleavage of the C=C double bond in the resultant **21** using OsO₄ and oxone (30) afforded carboxylic acid **22**, which was then converted into an active ester **23** for conjugation with HSA. Attempt to purify **23** by silica gel column chromatography resulted in decomposition on the column, therefore it was directly applied to the conjugation step without purification. Because **6** did not contain sialic acid, its hapten loading level was determined by MALDI TOFMS. The molecular weight of **6** was found to be 80,186. Based on the molecular weights of HSA (66,592) and **22** (262), we estimated that in average each HSA molecule was modified by 51 copies of the phenylacetylated linker in **6**.

The immunological property of **3** as a vaccine was examined in C57BL/6 mouse, and the animal immunizations and the enzyme-linked immunosorbent assays (ELISA) to evaluate antibody responses were performed according to the protocols described for the β -linked conjugate **1** (7). Thus, 0.1 mL of an emulsion of **3** (containing 2 μ g of carbohydrate antigen) and Titermax

Gold adjuvant were injected to each of a group of 5 mice intramuscularly on day 0, 14, 21 and 28, respectively. These mice were bled three times on day -1, day 27, and day 35, respectively, prior to and after immunization. Blood samples of all 5 mice collected on each date were pooled for the preparation of antisera, and each serum would give a mean antibody concentration. The titers of total antibody and individual antibody isotypes including IgM, IgG1, IgG2a, and IgG3 of the antisera were assessed by ELISA. The antibody titer is the serum dilution number at which the optical density (OD) of an ELISA experiment reaches 0.5. Consequently, the ELISA OD values of each serum were plotted against serum dilution numbers, and the best-fit line was obtained for the calculation of antibody titer. sTnNPhAc α -HSA **4** was used as the capture antigen to coat plates in ELISA for the detection of sTnNPhAc-reactive antibodies, and the plate-bound antibodies were analyzed by various anti-antibodies (7). The use of HSA conjugate as the capture antigen in ELISA allowed us to eliminate any interference potentially caused by KLH and detect antibodies specific for sTnNPhAc or the linker. Similarly, HSA conjugates of other antigens were employed to detect antibodies reactive to the specific antigen.

Figure 2 shows the observed total antibody and IgM, IgG1, IgG2a, and IgG3 antibody titers of the obtained sera. Day -1 serum did not show any binding to **4**, while day 27 and day 35 antisera both displayed high total antibody titers, with the latter higher than the former, suggesting a progressive immune response to **3** produced in animals. Detailed analysis of the antibody isotypes revealed that **3** provoked mainly IgG1 antibody, which indicates a strong T cell-dependent immune response. As usual, IgM antibody as the first line of antibody response to foreign antigens was also observed. These results were very similar to those derived from the β -linked conjugate vaccine **1** (7). It is apparent that the unnatural sTn derivative sTnNPhAc, whether it is α - or β -linked to a carrier protein, could form functional vaccines that elicited strong T cell-dependent immunity useful for the treatment of cancer.

We also investigated the reactivity of the antiserum obtained with sTnNPhAc α -KLH **3** to natural sTn, sTnNPhAc β , NeuNPhAc α (2,3)GalNAc α , the linker between the antigen and protein, and isolated phenylacetyl functionality by means of ELISA, and the results are depicted in Figure 3. In these studies, ELISA plates were first coated with sTnNPhAc α -HSA **4**, sTn-HSA **5**, sTnNPhAc β -HSA **2**, NeuNPhAc α (2,3)GalNAc α -HSA **7**, and PhAc/linker-HSA **6**, respectively, and then treated with the day 35 antiserum described above. It was demonstrated that the antiserum showed very strong binding to **2**, with a titer only slightly lower than that of **4**, indicating that the sTnNPhAc α -elicited antibodies could hardly differentiate the reducing end anomeric configurations of the capture antigens in ELISA. On the other hand, the antiserum produced a very low antibody titer with **7**-coated plate and did not exhibit major cross-reactivity with **5**- and **6**-coated plates. The significantly lower reactivity of the antiserum to **7** than to **4** suggest that the majority of antibodies raised by the glycoconjugate vaccine were specific to the whole antigen, sTnNPhAc, rather than to its partial structures such as NeuNPhAc and the phenylacetyl group or to the linker, as glycoconjugates **4** and **7** are only different in terms of their inner glycosidic linkage, α (2,6)- vs α (2,3)-linkage, while they showed very different reactivity to the antiserum. The extremely low reactivity of the antiserum to sTn-HSA **5**, which differs from **4** only in the acyl group linked to the sialic acid 5-*N*-position, further proves that the **3**-induced immune response was specific to sTnNPhAc. Meanwhile, because **5** contains the same linker, its low reactivity to the antiserum further proves that no significant level of antibodies against the linker was induced. Moreover, the low reactivity of the antiserum to **6** also suggests that the antiserum did not contain a significant level of antibodies against the linker or the phenylacetyl group in a slightly different form, namely as an ester.

We have also examined the reactivity of the antiserum obtained with sTnNPhAc β -KLH **1** (7) to natural sTn, α -linked sTnNPhAc, the linker, and the isolated phenylacetyl functionality. For ELISA, again, the plates were first coated with sTn-HSA **5**, sTnNPhAc α -HSA **4**, sTnNPhAc β -HSA **2**, and PhAc/linker-HSA **6**, respectively, and then treated with the anti-

sTnNPhAc β serum. As shown in Figure 4, the anti-sTnNPhAc β serum had very strong binding to both **4**- and **2**-coated plates, but only a very low antibody titer was observed with **6**-coated plate and no cross-reactivity was detected with **5**-coated plate. These results further support the conclusions derived from the experiments described above.

In summary, a new and highly efficient method was developed for the synthesis of sTnNPhAc antigen and its α -linked protein conjugates. Immunological studies of sTnNPhAc α -KLH in mice have shown that it could provoke a strong and robust T cell-dependent immune response, and the results were very similar to those previously obtained with sTnNPhAc β -KLH (7). Moreover, the antisera derived from sTnNPhAc α - and sTnNPhAc β -KLH-inoculated mice showed very little difference in reacting with sTnNPhAc α - and sTnNPhAc β -coated plates in ELISA experiments, while the antisera did not show significant binding to natural sTn, NeuNPhAc α (2,3)GalNAc – an α (2,3)-linked analog of sTnNPhAc, or to the free phenylacetyl functionality and the linker. These results, together with the results from our previous studies (7,8), led us to conclude that sTnNPhAc can indeed elicit a specific immune response and the elicited immune response recognize the whole antigen instead of merely the unnatural functionality or other partial structures of sTnNPhAc. More interestingly, it seems that the reducing end configuration of sTnNPhAc or its unnatural linkage to conjugate proteins had very little or no influence on its immunological identity or on the recognition of sTnNPhAc by the provoked antibodies. These results have proved the designing principles of glycoconjugate vaccines that have carbohydrate antigens coupled to carrier proteins via artificial linkages, which is very important for the understanding of the functions of carbohydrate-based vaccines and for future design of novel glycoconjugate vaccines. They can also help to explain why an unnatural GM3 analog-provoked antibody was specifically cytotoxic to cancer cells that were metabolically engineered to express the same GM3 analog (26), but not to normal cells, though normal cells could also use the engineering precursor to express unnatural sialic acids or even a low level of the unnatural GM3 analog. The results have further demonstrated that the new linker does not have any obvious influence on the immunological properties of the resultant glycoconjugates and that the linker does not provoke any immune response. Therefore, this linker and the associated coupling strategy can be safely employed in the development of glycoconjugate cancer vaccines. The conclusions of this work should be of general significance in the field of conjugate vaccine research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of sTn antigen and the conjugates used in this research

Figure 2. Total antibody titers of day -1, day 27, and day 35 sera and IgM, IgG1, IgG2a, and IgG3 antibody titers of day 35 serum determine by ELISA with **4** as the coating antigen. All antibody titers were obtained with pooled sera of five replicate animals.

Figure 3.

Titers of total antibody reactive to sTnNPhAc α , sTn, sTnNPhAc β , NeuNPhAc α 2,3GalNAc, and PhAc/linker, respectively, in the pooled day 35 serum derived from five replicate mice inoculated with sTnNPhAc α -KLH **3**. For ELISA, after the plates were coated with **4**, **5**, **2**, **7**, and **6**, respectively, they were treated with the antiserum, and the antibodies bound to the plates were then analyzed.

Figure 4.

Titers of total antibody reactive to sTn, sTnNPhAc α , sTnNPhAc β , and PhAc/linker, respectively, in the pooled serum derived from five replicate mice inoculated with sTnNPhAc β -KLH **1**. For ELISA, after the plates were coated with **5**, **4**, **2**, and **6**, respectively, they were treated with the antiserum, and the antibodies bound to the plates were then analyzed.

Scheme 1.

Scheme 2.

Scheme 3.