Efficient chemoenzymatic synthesis of biotinylated human serum albumin–sialoglycoside conjugates containing *O*-acetylated sialic acids†

Hai Yu,[a] Harshal A. Chokhawala,[a] Ajit Varki[b] and Xi Chen*[a]

Received 30th April 2007, Accepted 11th June 2007
First published as an Advance Article on the web 27th June 2007
DOI: 10.1039/b706507h

Sialyl Tn (STn) and sialyl lactoside derivatives containing *O*-acetylated sialic acid residues have been chemoenzymatically synthesized using a one-pot three-enzyme system and conjugated to biotinylated human serum albumin (HSA) using an adipic acid *para*-nitrophenyl ester coupling reagent. This approach provides an efficient and general protocol for preparing carbohydrate–protein conjugates containing base-sensitive groups.

Introduction

Carbohydrates presented on the surface of mammalian cells and microorganisms are believed to play important roles in various molecular recognition processes.[1] Many cell surface carbohydrates have terminal sialic acid residues[2] which are directly involved in a variety of biological and pathological processes, including bacterial and viral infection, regulation of the immune system,[1a,3] and cancer metastasis.[4]

Some sialic-acid containing carbohydrates are considered to be tumor-associated carbohydrate antigens (TACAs) which are overexpressed by certain types of cancers.[5] Sialyl–Tn antigen (STn, *O*-linked disaccharide Neu5Acα2,6GalNAc) is one of these TACAs. STn is expressed by 30% of breast carcinomas[6] and has been considered as an independent indicator for poor prognosis of cancer.[7] Synthetic STn conjugated to a protein carrier [keyhole limpet haemocyanin (KLH)] has been developed by Biomira, Inc. (Edmonton, Alberta, Canada) as a cancer vaccine (Theratope®) for breast cancer patients.[8] STn has also been used as one of seven epithelial cancer antigens and one of five prostate tumor-associated antigens in preparing unimolecular multivalent vaccines.[9] A variety of chemical methods have been developed for the synthesis of STn and other sialylated oligosaccharides.[10] More recently, enzymatic synthesis of STn oligosaccharides and glycopeptides has been achieved by using sialyltransferases, such as recombinant mouse ST6GalNAc-I,[11] chicken ST6GalNAc-I[12] and α2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST).[13] The only type of sialic acid residues in these compounds, however, is *N*-acetylneuraminic acid (Neu5Ac), the predominant form of naturally-occurring sialic acid. In nature, more than 50 structurally-different sialic acid forms have been found, including Neu5Ac, *N*-glycolylneuraminic acid (Neu5Gc), ketodeoxynonulosonic acid (KDN), and their methylated, sulfated, lactylated and singly/multiply acetylated derivatives.[2] Our groups have been interested in understanding the biological significance of naturally-occurring sialic acid modifications. One of the approaches to elucidate the function of sialic acid modifications is to synthesize sialosides with naturally-occurring modifications and couple them individually to an inert protein carrier for 96-well plate-based high-throughput screening of sialic acid binding proteins. A general coupling method would be necessary to produce sialoside–protein conjugates, including those containing base-sensitive groups (*e.g.* acetyl groups).

Among coupling reagents that have been used for producing carbohydrate–protein conjugates, succinimide esters are very convenient and effective for amide bond formation.[14] Their instability during chromatographic purification, however, limits their application. Maleimide-promoted conjugation, on the other hand, often results in disappointingly low yields as the result of a nonproductive disulfide dimerization of glycosides.[15] Homobifunctional squaric acid diesters (*e.g.* diethyl squarate or didecyl squarate) have been used commonly as efficient cross-linking reagents for preparing carbohydrate–protein conjugates.[16] The basic conditions used for coupling (usually an aqueous solution of pH 9), however, limit their application on compounds containing base-sensitive groups such as *O*-acetyl groups. Bis(*p*-nitrophenyl) esters are known cross-linking agents for compounds with primary amines.[17] Recently, one of these linkers, adipic acid *p*-nitrophenyl ester, has been used by the Bundle group as an efficient cross-linker for preparing neoglycoproteins.[18] The neutral conditions for the coupling reaction make this coupling reagent attractive. Here, we report that the adipic acid *p*-nitrophenyl ester can be used as a general and efficient coupling reagent for producing sialoside–protein conjugates containing base-sensitive groups. The method has been used for conjugating biotinylated human serum albumin (HSA) and chemoenzymatically-synthesized STn analogues containing Neu5Ac, Neu5Gc and their 9-*O*-acetylated derivatives. Conjugation of biotinylated human serum albumin (HSA) and chemoenzymatically-synthesized α2,6-linked sialyl lactosides containing Neu5Ac, Neu5Gc and their 9-*O*-acetylated derivatives has been similarly achieved. The retention of the *O*-acetyl group in the biotinylated HSA–sialoside conjugates throughout the coupling conditions is confirmed by sialidase hydrolysis of the conjugate followed by ESI-MS analysis.

[a]*Department of Chemistry, University of California-Davis, One Shields Avenue, Davis, CA, 95616, USA. E-mail: chen@chem.ucdavis.edu; Fax: +1 (1)530 752 8995; Tel: +1 (0)530 754 6037*
[b]*Departments of Medicine and Cellular & Molecular Medicine, University of California, San Diego, La Jolla, CA, USA*

† Electronic supplementary information (ESI) available: NMR spectra for the compounds **8**–**15**, **25**–**28**. See DOI: 10.1039/b706507h

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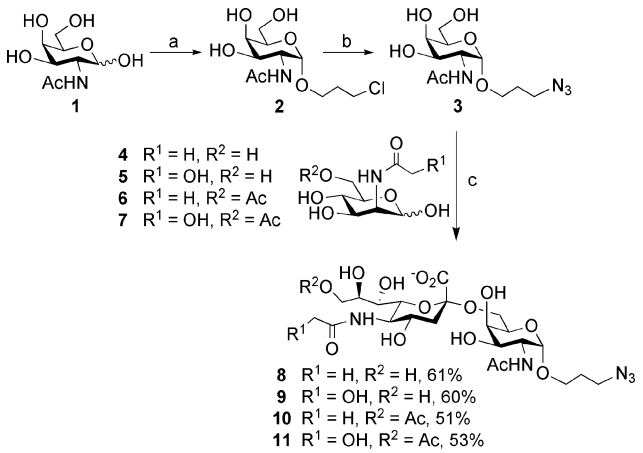
Results and discussion

Synthesis of sialyl–Tn (STn) derivatives

In order to prepare the sialyltransferase acceptor for chemoenzymatic synthesis of STn, 3-chloropropyl *N*-acetamido α-galactoside **2** was chemically synthesized by incubating *N*-acetylgalactosamine **1** with 3-chloropropanol at 70 °C in the presence of acetyl chloride. The chlorine group of **2** was displaced by an azido group by treating **2** with sodium azide in *N*,*N*-dimethylformamide and a catalytic amount of tetra-*n*-butylammonium iodide at 60 °C to form 3-azidopropyl-*N*-acetamido-α-galactoside **3** in quantitative yield (Scheme 1). Although **2** and **3** showed similar R_f values on TLC, they could be distinguished by ^{13}C NMR spectra. The chemical shift of the carbon linked to the azido group in **3** was 48.29 ppm, while that of the carbon linked to chlorine in **2** was 41.52 ppm.

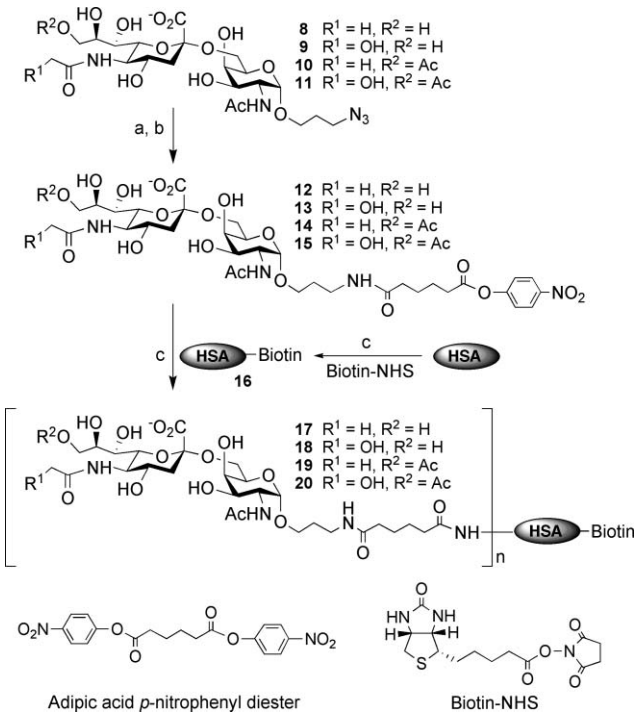


Scheme 1 Chemoenzymatic synthesis of STn antigen analogs using a one-pot three-enzyme system. (a) 3-Chloropropanol, acetyl chloride, 70 °C, 87%; (b) NaN_3, NaI, DMF, 60 °C, 95%; (c) **3** (20 mM), **4**, **5**, **6**, or **7** (30 mM), pyruvate (150 mM), CTP (30 mM), $MgCl_2$ (20 mM), a sialic acid aldolase, a CMP–sialic acid synthetase, Pd2,6ST, Tris-HCl (100 mM, pH 8.5 for **4** and **5**; pH 7.5 for **6** and **7**).

Chemoenzymatic sialylation of **3** was achieved using a highly efficient and convenient one-pot three-enzyme system established in our laboratory.[19,21] In this system—containing three recombinant bacterial enzymes including an *E. coli* K-12 aldolase,[20] an *N. meningitidis* CMP–sialic acid synthetase (NmCSS),[20] and a *Photobacterium damsela* α2,6-sialyltransferase (Pd2,6ST), *N*-acetylmannosamine (ManNAc), *N*-glycolylmannosamine (ManNGc) and their chemically synthesized 6-*O*-acetylated derivatives **4**–**7**[19b] were used as sialic acid precursors to obtain STn analogs containing Neu5Ac, Neu5Gc, 9-*O*-acetylated Neu5Ac, and 9-*O*-acetylated Neu5Gc **8**–**11** in yields varying from 51–61% (Scheme 1). The reactions were carried out at 37 °C in a Tris-HCl buffer (100 mM, pH 8.5 for substrates without *O*-acetyl groups; pH 7.5 for substrates with *O*-acetyl groups) containing Mg^{2+}, pyruvate, CTP, a sialic acid precursor selected from **4**–**7**, and **3** as a sialyltransferase acceptor. After the addition of appropriate amounts of aldolase (2.0 mg), NmCSS (0.8 mg) and Pd2,6ST (0.5 mg), the reaction was monitored by thin-layer chromatography (TLC) analysis (EtOAc : MeOH : H_2O : HOAc = 5 : 2 : 1 : 0.1, by volume) and stained with *p*-anisaldehyde sugar stain. Sialoside products were purified by Bio-Gel P-2 gel filtration chromatography and the structures of all sialylated products were characterized by ^1H and ^{13}C NMR as wells as high resolution mass spectrometry (HRMS).

Synthesis of biotinylated HSA–STn conjugates

As shown in Scheme 2, biotinylated HSA was obtained by incubating one equivalent of HSA with four equivalents of the *N*-hydroxy succinamide ester of biotin (biotin-NHS). The introduced biotin can be used to immobilize the final conjugates onto streptavidin-coated surface or to label the conjugates with streptavidin-linked molecules. In order to obtain sialoside and biotinylated HSA conjugates, half esters **12**–**15** were prepared by reducing the azido group in **8**–**11** to an amino group by catalytic hydrogenation (Pd/C in water) then reacting with an excess amount (5 equivalents) of adipic acid *p*-nitrophenyl diester[18a] in anhydrous DMF. The reaction was monitored by TLC (EtOAc : MeOH : H_2O : HOAc = 4 : 2 : 1 : 0.1, by volume) under UV light. The disappearance of the starting material and the appearance of a UV absorbent product with a larger R_f value indicated the completion of the reaction. Products **12**–**15** were purified using a short silica gel column to remove the unreacted adipic acid ester. The presence of the 9-*O*-acetyl group at the sialic acid residue in **14** and **15** was confirmed by proton NMR, indicating the stability of the *O*-acetyl group during hydrogenation and half ester formation.



Scheme 2 Conjugation of STn antigens to human serum albumin (HSA) using adipic acid *p*-nitrophenyl diester coupling reagent. (a) Pd/C, H_2, H_2O; (b) adipic acid *p*-nitrophenyl diester, DMF, rt, > 90%; (c) phosphate buffer (100 mM, pH 7.4). Average numbers of sialoside molecules incorporated were determined by MALDI-TOF.

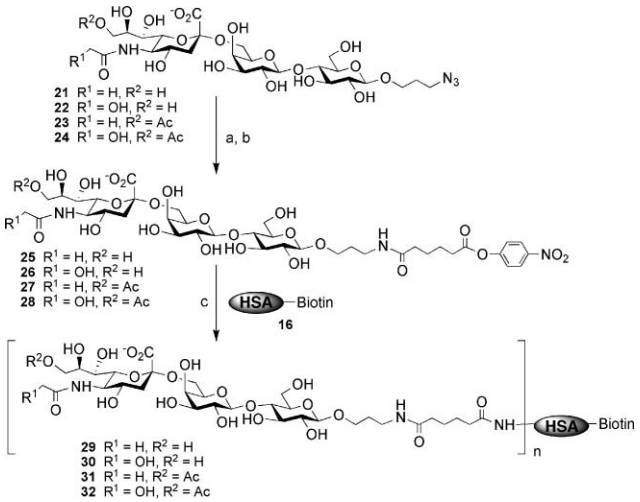
Finally, for coupling reactions, the purified sialoside half esters **12**–**15** (40 equivalents) were incubated individually with biotinylated HSA in phosphate buffer (100 mM, pH 7.4) for 24 h at room temperature before the produced conjugates were purified by HPLC using a gel filtration column. The purified conjugates were analyzed by MALDI-TOF mass spectroscopy to determine the numbers of the sialoside molecules that have been conjugated to the biotinylated HSA. The numbers obtained for the most populated conjugates are listed in Table 1. For example, MALDI-TOF data indicated a molecular ion of *m/z* 75752 for glycoconjugate **17**. By comparison to the *m/z* 67218 for biotinylated HSA **16**, one can estimate that **17** has an average of 12 sialoside molecules. The same estimation can be applied to other glycoconjugates **18**–**20**.

In order to study whether the *O*-acetyl group is preserved throughout the conjugation conditions, the conjugate **19**, containing Neu5,9Ac$_2$α2,6GalNAc, was treated with commercially-available *Arthrobacter ureafaciens* sialidase and *Clostridium perfingens* sialidase (type VI) (both sialidases can hydrolyze terminal 9-*O*-acetylated Neu5Ac residue) in ammonium acetate buffer at pH 5.0.[22] The sialidase reaction mixture was subjected to mass spectrometric analysis (ESI-MS). Only hydrolyzed Neu5,9Ac$_2$ was detected, and no signal was observed for Neu5Ac, indicating that the 9-*O*-acetyl group on Neu5,9Ac$_2$α2,6GalNAc was well preserved during the conjugation process.

Synthesis of biotinylated HSA–sialyl lactoside conjugates

In order to evaluate the generality of applying the adipic acid *p*-nitrophenyl diester in carbohydrate–protein conjugation, another set of sialoside–biotinylated HSA conjugates were prepared similarly. In this case, α2,6-linked sialyl lactosides **21**–**24** were synthesized by the one-pot three-enzyme system using 3-azidopropyl β-D-galactopyranose-(1→4)-β-D-glucopyranoside (LacβProN$_3$) as a sialyltransferase acceptor, as described previously.[19b] Following the same coupling protocol as described above for the production of biotinylated HSA–STn conjugates, biotinylated HSA–sialyllactose conjugates **29**–**32** were successfully obtained (Scheme 3). MALDI-TOF analysis gave the average numbers of sialosides incorporated into the conjugates (Table 1).

Comparing the results listed in Table 1, one can conclude that the degree of incorporation of a disaccharide STn sialoside to HSA (11–14) is similar to, but a little bit higher, than that for the conjugation of a trisaccharide sialyllactoside to HSA (10–12). Overall, 10 to 14 sialoside molecules were incorporated onto one molecule of HSA when 40-fold equivalents of activated STn or sialyl lactosides were used. These results are close to those reported for the coupling of oligosaccharides to bovine serum albumin (BSA) or tetanus toxoid (TT),[18b,c] although higher conjugation yields were achieved for coupling monosaccharides or a lactoside disaccharide to bovine serum albumin.[18a]



Scheme 3 Conjugation of sialyllactosides to human serum albumin (HSA) using adipic acid *p*-nitrophenyl diester coupling reagent. (a) H$_2$, Pd/C, H$_2$O; (b) adipic acid *p*-nitrophenyl diester, DMF, rt, > 90%; (c) phosphate buffer (100 mM, pH 7.4). Average numbers of sialoside molecules incorporated were determined by MALDI-TOF.

Conclusions

In summary, we have demonstrated that the one-pot three-enzyme system is very efficient in synthesizing STn analogs containing different forms of sialic acid. In addition, we have also proven that the adipic acid *p*-nitrophenyl ester is a very effective and general coupling reagent to produce carbohydrate–protein conjugates, including those with base-sensitive groups. The conjugates can now be used to study the significance of 9-*O*-acetyl groups in the binding properties of sialoside to various lectins and antibodies that recognize sialic acids.

Experimental

General methods

Chemicals were purchased and used without further purification. ^{1}H NMR (300, 400, or 600 MHz) and ^{13}C NMR (75 or 100 MHz) spectra were recorded on a Varian Mercury-300, a Varian Inova-400, or a Varian Inova-600 spectrometer. Low and high resolution electrospray ionization (ESI) mass spectra were obtained at the Mass Spectrometry Facility in the Ohio State University. Silica gel 60 Å (40–63 μm, Sorbent technologies) was used for flash chromatography. Analytical thin-layer chromatography was performed on silica gel plates 60 GF$_{254}$ (Sorbent technologies) using anisaldehyde stain for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA). *Arthrobacter*

Table 1 The average numbers of sialosides that have been conjugated to biotinylated HSA determined by MALDI-TOF analysis

Conjugate	**17**	**18**	**19**	**20**	**29**	**30**	**31**	**32**	**16** (Biotin-HSA)
Peak *m/z* in MALDI-TOF spectrum	75 752	76 826	77 853	75 971	76 827	77 079	75 938	75 871	67 218
Average number of sialosides per conjugate	12	13	14	11	11	12	10	10	0

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ureafaciens (type VI) sialidase was purchased from MP Biomedicals. *Clostridium perfingens* sialidase (type VI) was from Sigma.

Starting materials

Sodium pyruvate and ManNAc **4** were bought from Sigma. GalNAc derivative **3**, ManNGc **5**, 9-OAc-ManNAc **6**, 9-OAc-ManNGc **7**, and sialosides **21**–**24** were synthesized as described previously.[19,20]

3-Azidopropyl *O*-[sodium (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate]-(2→6)-*O*-2-acetamido-2-deoxy-α-D-galactopyranoside (8, Neu5Acα2,6GalNAcαProN$_3$)

Yield, 61%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 4.70 (d, 1H, J = 3.6 Hz), 3.97 (dd, 1H, J = 3.6 and 10.8 Hz), 3.87 (dd, 1H, J = 4.2 and 7.8 Hz), 3.83 (d, 1H, J = 3.0 Hz), 3.76–3.61 (m, 5H), 3.55–3.26 (m, 9H), 2.56 (dd, 1H, J = 4.8 and 12.0 Hz, H-3$_{eq}$″), 1.87, 1.86 (2s, 2CH$_3$), 1.74 (m, 2H), 1.51 (t, 1H, J = 12.0 Hz, H-3$_{ax}$″); ^{13}C NMR (75 MHz, D_2O) δ 175.16, 174.68, 173.57, 100.49, 97.20, 72.69, 71.91, 69.74, 69.62, 68.66, 68.38, 67.61, 65.28, 63.97, 62.75, 51.99, 50.07, 48.29, 40.40, 28.06, 22.18, 22.08. HRMS (ESI) m/z calcd for $C_{22}H_{36}N_5O_{14}Na_2$ (M+Na) 640.2054, found 640.2070.

3-Azidopropyl *O*-[sodium (5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate]-(2→6)-*O*-2-acetamido-2-deoxy-α-D-galactopyranoside (9, Neu5Gcα2,6GalNAcαProN$_3$)

Yield, 60%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 4.71 (d, 1H, J = 3.6 Hz), 3.97 (dd, 1H, J = 3.6 and 11.4 Hz), 3.95 (s, 2H), 3.87 (dd, 1H, J = 3.6 and 7.8 Hz), 3.83 (d, 1H, J = 3.6 Hz), 3.77–3.58 (m, 8H), 3.53–3.26 (m, 8H), 2.57 (dd, 1H, J = 4.8 and 12.6 Hz, H-3$_{eq}$″), 1.87 (s, CH$_3$), 1.74 (m, 2H), 1.53 (t, 1H, J = 12.0 Hz, H-3$_{ax}$″); ^{13}C NMR (75 MHz, D_2O) δ 175.97, 174.71, 173.60, 100.56, 97.25, 72.47, 72.00, 69.68, 68.71, 68.37, 68.16, 67.68, 65.35, 64.03, 62.77, 61.16, 51.73, 50.12, 48.35, 40.49, 28.10, 22.13. HRMS (ESI) m/z calcd for $C_{22}H_{36}N_5Na_2O_{15}$ (M+Na) 656.2003, found 656.2015.

3-Azidopropyl *O*-[sodium (9-*O*-acetyl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate]-(2→6)-*O*-2-acetamido-2-deoxy-α-D-galactopyranoside (10, Neu5,9Ac$_2$α2,6GalNAcαProN$_3$)

Yield, 51%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 4.74 (d, 1H, J = 3.6 Hz), 4.28 (dd, 1H, J = 2.4 and 11.4 Hz), 4.06 (dd, 1H, J = 6.6 and 12.0 Hz), 4.00 (dd, 1H, J = 3.6 and 10.8 Hz), 3.97–3.47 (m, 5H), 3.40–3.29 (m, 6H), 2.59 (dd, 1H, J = 4.8 and 12.6 Hz, H-3$_{eq}$″), 1.99, 1.90, 1.89 (3s, 3CH$_3$), 1.77 (m, 2H), 1.54 (t, 1H, J = 12.0 Hz, H-3$_{ax}$″); ^{13}C NMR (75 MHz, D_2O) δ 175.13, 174.69, 174.53, 173.56, 100.52, 97.21, 72.50, 69.66, 69.39, 68.67, 68.40, 67.63, 65.82, 65.31, 64.00, 51.96, 50.08, 48.30, 40.42, 28.07, 22.19, 22.07, 20.40. HRMS (ESI) m/z calcd for $C_{24}H_{38}N_5Na_2O_{15}$ (M+Na) 682.2160, found 682.2174.

3-Azidopropyl *O*-[sodium (9-*O*-acetyl-5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate]-(2→6)-*O*-2-acetamido-2-deoxy-α-D-galactopyranoside monosodium salt (11, Neu9Ac5Gcα2,6GalNAcαProN$_3$)

Yield, 53%; white foam. ^{1}H NMR (400 MHz, D_2O) δ 4.70 (d, 1H, J = 4.0 Hz), 3.97 (d, 1H, J = 9.6 Hz), 4.04–3.97 (m, 2H), 3.95 (s, 2H), 3.93–3.26 (m, 14H), 2.56 (dd, 1H, J = 4.4 and 12.4 Hz, H-3$_{eq}$″), 1.95, 1.86 (2s, 2CH$_3$), 1.73 (m, 2H), 1.52 (t, 1H, J = 12.0 Hz, H-3$_{ax}$″); ^{13}C NMR (75 MHz, D_2O) δ 175.87, 174.67, 173.52, 173.57, 100.51, 97.20, 72.21, 69.68, 69.44, 68.65, 68.20, 68.27, 67.61, 65.77, 65.30, 64.04, 61.10, 51.63, 50.07, 48.28, 40.46, 28.08, 22.07, 20.40. HRMS (ESI) m/z calcd for $C_{24}H_{38}N_5O_{16}$ (M-Na) 652.2313, found 652.2333.

General procedure for enzymatic sialylation of 3 in one-pot three-enzyme system

ManNAc **4**, ManNGc **5**, or their 6-*O*-acetylated derivatives **6** or **7** (50–100 mg, 1.5 equiv.), GalNAcαProN$_3$ **3** (1.0 equiv.), sodium pyruvate (7.5 equiv.), CTP (1.5 equiv.) were dissolved in 5 mL Tris-HCl buffer (100 mM, pH = 8.5 for **4** and **5**, pH = 7.5 for **6** and **7**). After the addition of *E. coli* K-12 sialic acid aldolase (2.0 mg), *N. meningitidis* CMP-sialic acid synthetase (NmCSS) (0.8 mg), and Pd2,6ST (0.5 mg), the total volume of the reaction mixture was brought to 10 mL by adding water. The reaction solution was incubated at 37 °C with agitation at 140 rpm for 2–4 hours. The reaction was monitored by TLC analysis (EtOAc : MeOH : H_2O : HOAc = 5 : 2 : 1 : 0.1, by volume) and stained with *p*-anisaldehyde sugar stain. When no further product was detected, 10 mL of 95% EtOH was added to the reaction. The precipitates were removed by centrifugation. The supernatant was concentrated by rotary evaporation and purification by a Bio-Gel P-2 gel filtration column to give STn products.

Protocol for preparing biotinylated HSA–sialoside conjugates using adipic acid *p*-nitrophenyl diester coupling reagent

A sialoside selected from **8**–**11** or **21**–**24** (20–30 mg, 0.03–0.05 mmol) was dissolved in water (2 mL) and the mixture was stirred under hydrogen atmosphere in the presence of 10% palladium on charcoal for overnight to afford the reduced product. Adipic acid *p*-nitrophenyl ester (5 mol equiv.) in anhydrous DMF was added. The reaction was monitored by TLC (EtOAc : MeOH : H_2O : HOAc = 4 : 2 : 1 : 0.1, by volume) under UV light. The disappearance of the starting material and the appearance of a UV absorbent product with a larger R_f value indicated the completion of the reaction. After stirring for overnight, the reaction mixture was concentrated and purified by flash chromatography to afford the sialoside half ester selected from **12**–**15** or **25**–**28**. For the conjugation reaction, a sialoside half ester (~ 40 mol equiv.) was added to a solution containing biotinylated human serum albumin (2.0 mg mL^{-1}) in phosphate buffer (pH 7.4, 100 mM). The mixture was stirred gently at room temperature for 24 h. The resulting solution was concentrated using a centrifugal filter (Centricon Plus-20) and purified by high performance liquid chromatography (HPLC) or fast-performance liquid chromatography (FPLC) using a gel filtration column (Superdex 75 10/300 GL column) to afford biotinylated HSA–sialoside conjugate. The obtained HSA–sialoside conjugates were charactered by MALDI-TOF.

Compound 12 (*p*-nitrophenyl ester of 8)

^{1}H NMR (600 MHz, D_2O) δ 8.16 (d, 2H, J = 9.0 Hz), 7.20 (d, 2H, J = 9.0 Hz), 4.70 (d, 1H, J = 3.6 Hz), 3.97–3.09 (m, 21H),

2.56–2.53 (m, 3H), 2.14 (m, 2H), 1.85 (s, 3H, NHC(O)CH_3), 1.84 ((s, 3H, NHC(O)CH_3), 1.65 (m, 2H), 1.54–1.46 (m, 3H).

Compound 13 (*p*-nitrophenyl ester of 9)

^{1}H NMR (600 MHz, D_2O) δ 8.16 (d, 2H, $J = 9.0$ Hz), 7.21 (d, 2H, $J = 9.0$ Hz), 4.71 (d, 1H, $J = 3.6$ Hz), 3.97 (dd, 1H, $J = 3.6$ and 11.4 Hz), 3.95 (s, 2H, CH_2OH), 3.85–3.10 (m, 22H), 2.58–2.55 (m, 3H), 2.15 (m, 2H), 1.86 (s, NHC(O)CH_3), 1.66 (m, 2H), 1.56–1.45 (m, 3H).

Compound 14 (*p*-nitrophenyl ester of 10)

^{1}H NMR (600 MHz, D_2O) δ 8.18 (d, 2H, $J = 9.0$ Hz), 7.22 (d, 2H, $J = 8.4$ Hz), 4.74 (d, 1H, $J = 3.6$ Hz), 4.22 (dd, 1H, $J = 1.8$ and 11.4 Hz), 4.03–3.11 (m, 17H), 2.58–2.53 (m, 3H), 2.16 (m, 2H), 1.95 (s, 3H, OC(O)CH_3), 1.87 (s, 3H, NHC(O)CH_3)., 1.86 (s, 3H, NHC(O)CH_3), 1.67 (m, 2H), 1.57–1.48 (m, 3H).

Compound 15 (*p*-nitrophenyl ester of 11)

^{1}H NMR (600 MHz, D_2O) δ 8.19 (d, 2H, $J = 8.4$ Hz), 7.23 (d, 2H, $J = 9.6$ Hz), 4.70 (d, 1H, $J = 4.0$ Hz), 4.24–3.13 (m, 21H), 3.97 (s, 2H, CH_2OH), 2.56 (m, 3H), 2.17 (m, 2H), 1.96 (s, 3H, OC(O)CH_3), 1.87 (s, 3H, NHC(O)CH_3), 1.67 (m, 2H), 1.60–1.50 (m, 5H).

Compound 25 (*p*-nitrophenyl ester of 21)

^{1}H NMR (600 MHz, D_2O) δ 8.18 (d, 2H, $J = 9.6$ Hz), 7.22 (d, 2H, $J = 9.0$ Hz), 4.28 (d, 1H, $J = 7.8$ Hz), 4.23 (d, 1H, $J = 8.4$ Hz), 3.82–3.12 (m, 27H), 2.58–2.52 (m, 3H), 2.14 (m, 2H), 1.86 (m, 2H), 1.85 (s, 3H), 1.68–1.55 (m, 5H).

Compound 26 (*p*-nitrophenyl ester of 22)

^{1}H NMR (600 MHz, D_2O) δ 8.00 (d, 2H, $J = 8.4$ Hz), 6.78 (d, 2H, $J = 7.4$ Hz), 4.30 (d, 1H, $J = 7.8$ Hz), 4.24 (d, 1H, $J = 7.8$ Hz), 3.94 (s, 2H, CH_2OH), 3.81–3.01 (m, 27H), 2.55 (dd, 1H, $J = 5.4$ and 12.6 Hz, H-$3_{eq}''$), 2.10–2.06 (m, 4H), 2.02 (m, 2H), 1.74 (m, 2H), 1.58 (t, 1H, $J = 12.6$ Hz, H-$3_{ax}''$), 1.44–1.37 (m, 4H).

Compound 27 (*p*-nitrophenyl ester of 23)

^{1}H NMR (400 MHz, D_2O) δ 8.19 (d, 2H, $J = 9.2$ Hz), 7.22 (d, 2H, $J = 8.8$ Hz), 4.28 (d, 1H, $J = 7.6$ Hz), 4.07–3.11 (m, 28H), 2.57–2.50 (m, 3H), 2.15 (m, 2H), 1.95 (s, 3H, OC(O)CH_3), 1.86 (s, 3H, NHC(O)CH_3), 1.74 (m, 2H), 1.67–1.52 (m, 5H).

Compound 28 (*p*-nitrophenyl ester of 24)

^{1}H NMR (600 MHz, D_2O) δ 8.18 (d, 2H, $J = 9.0$ Hz), 7.22 (d, 2H, $J = 9.0$ Hz), 4.27 (d, 1H, $J = 7.8$ Hz), 4.24–4.22 (m, 2H), 4.02 (dd, 1H, $J = 6.0$ and 12.0 Hz), 3.95 (s, 2H, CH_2OH), 3.93 (m, 1H), 3.85–3.10 (m, 24H), 2.56 (m, 3H), 2.15 (m, 2H), 1.96 (s, 3H, OC(O)CH_3), 1.94 (m, 2H), 1.67–1.55 (m, 5H).

Sialidase treatment of biotinylated HSA–sialoside conjugate 19 and MS analysis

To confirm the presence of *O*-acetylated sialosdies in HSA conjugates, conjugate **19** (1.6 mg mL^{-1}) was incubated in a final volume of 15 µL with *Arthrobacter ureafaciens* sialidase (0.75 mU) and *Clostridium perfingens* sialidase (type VI) (15 mU) for 1 h at 37 °C in ammonium acetate buffer (pH 5.0, 100 mM). Mass spectrometric analysis (ESI-MS) of the reaction solution was carried out immediate and showed the mass ion of Neu5,9Ac_2 at 350.20 a.m.u (M-H). No signal for de-*O*-acetylated Neu5Ac was observed in the MS spectrum.

Acknowledgements

This work was supported by NIH R01GM076360 and the start-up funds to X. C. from the Regents of the University of California.

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