Chemical Glycobiology

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Chapter 10

Synthesis and Evaluation of Anticancer Vaccine Candidates, C-Glycoside Analogs of STn and PSA

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Neuraminic acids are biologically important and occupy the terminal position of the glycoconjugate glycans on the outside of cells. Sialyl-Tn (sTn) is found on tumor-associated glycoprotein antigens present on the surface of cancer cells, including those associated with carcinomas of the breast, prostate, pancreas, colon, ovary, lung, and stomach. The sTn antigen is well known as a prognostic indicator and has proven to be an effective target for cancer therapy. Conjugate vaccines of sTn-KLH show remarkable immunogenicity, resulting in the production of both IgM and IgG type antibodies. The sTn C-glycoside was designed and synthesized and its KLH-conjugate was evaluated for immunogenicity in mice. Neuraminic acid C-glycosides such as sTn and polysialic acid (PSA) might be useful in preparing immunogens for active immunization against neuraminic acid containing glycoconjugates in the design and preparation of anti-cancer vaccines.
Introduction

Ulosonic acids, or sialic acids (Figure 1), constitute a unique family of complex monosaccharides that are involved in many important biological functions (1–4). Their biological roles include facilitating cell-cell interactions (5), aggregation (6, 7) and development (8). Ulosonic acids are 8- and 9-carbon monosaccharides that contain an anomic carboxylate, a deoxyxylated methylene C-3 ring carbon, an hydroxylated 2- or 3-carbon side chain at C-6 and are differentially functionalized at C-5. Neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid) does not occur naturally, but many of its derivatives (Neu5Ac, KDN, KDO, Figure 1) are found widely distributed in animal tissues and in bacteria, especially in glycoproteins and gangliosides (2, 3). These sugars can be found in a wide variety of glycosidic linkages, mainly α(2,3) and α(2,6) to galactose or lactose but also as α(2,8) and α(2,9) in polysialic acids (2, 5). The most common sialic acid, N-acetylneuraminic acid (Neu5Ac), is a constituent of a large number of glycoconjugates (glycoproteins, glycopeptides, glycolipids, etc.) and is always found at the non-reducing termini of oligosaccharide chains. Neu5Ac represents an important biological receptor domain that interacts with enzymes, hormones, toxins, microbes, viruses and cells (9–11). For example, cell-cell recognition between circulating leukocytes in blood vessels and endothelial cells is believed to occur through the interaction between mammalian lectins (selectins) and Neu5Ac containing oligosaccharides ligands (10). Moreover, many pathogens employs Neu5Ac or other sialic acids to promote infection. Some viruses use hemagglutinin, a sialic acid binding lectin, others utilize neuraminidases, hydrolase-type enzymes that cleaves sialic acid glycosidic linkage, to gain entry into the cells they infect (12–15). Neisseria meningitides, a pathogenic bacteria, biosynthesizes an extracellular capsule composed of Neu5Ac homopolymers as camouflage to escape host immune response (13, 14).

![Chemical Structures]

Figure 1. The α-anomeric forms of some sialic acids, Neu5Ac (1), KDN (2) and KDO (3).

The linkage of neuraminic acid to glycoconjugates is among the most labile glycosidic linkages and can be cleaved in vitro under mildly acidic conditions.
Figure 2. O-glycosides vs C-glycosides stability.
Figure 3. Glycoprotein glycan catabolism
in nature. Thus, it can react with electrophilic type acceptors like ketones or aldehydes to generate the corresponding C-glycoside. Our laboratory has recently used this strategy to complete the synthesis of important C-glycosides targets (37, 43, 44).

**Synthesis of the C-Glycoside Analog of sTn**

Fully protected sTn C-glycoside analog 6 was prepared by C-glycosylation of the neuraminic acid sulfone donor 7 with the aldehyde acceptor 8 (Scheme 2). The sulfone donor 7 was easily prepared from neuraminic acid in four steps as previously described (42, 45). The critical intermediate, aldehyde acceptor 8, was prepared in 14 steps, from the commercially available diisopropylidene galactose derivative 9. The one carbon extension step was accomplished using a sequential iodination/cyanation early in the synthetic scheme because it is often a problematic step. Iodination of alcohol 9 gave the 6-iodo derivative 10 that was transformed to the corresponding cyano derivative 11 via a nucleophilic displacement with KCN in a modest yield (30%) probably due to unfavorable electronic and steric effects arising from the ring oxygen atom and the axially oriented oxygen at C-4 respectively. Reduction of the 6-cyano derivative 11 using Dibal-H afforded aldehyde 12. Quantitative reduction of aldehyde 12 with NaBH₄ in MeOH afforded the corresponding alcohol 13. De-isopropylideneation of 13 was then accomplished by treatment with amberlite IR-120 (H⁺) resin in water at 80 °C for 3 h and provided the 6-deoxy-D-galacto-heptopyranose 14. The one carbon extended galactal 15 was obtained in good yield from 14 using a one pot, three step procedure. First, peracetylation was accomplished using acetic anhydride and catalytic HBr/HOAc. Then, the
Scheme 1. Samarium mediated C-glycosylations.
anomeric acetate was converted to the corresponding bromide by treatment with excess HBr/HOAc. Finally, reductive elimination of the 1-bromo and 2-acetoxy groups using Zn/Cu afforded 15 (46). Azidonitration (47) of 15 with ceric ammonium nitrate (CAN) and sodium azide in dry acetonitrile afforded mainly the 2-azido-1-nitrate addition product having the desired galacto configuration as confirmed by $^1$H NMR spectroscopy. Treatment of the crude product with lithium bromide in dry acetonitrile under ionic conditions afforded 16 in moderate yield. Glycosylation of 16 with the N$^\beta$-benzoyl carbonyl protected OBn ester of 1-Serine 17 (48), in the presence of silver perchlorate afforded the glycopeptide 18 in good yield and stereoselectivity. Conversion of 18 by treatment with thioacetic acid in pyridine (49), afforded the desired glycopeptide product 19 in 92% yield. Selective enzymatic deacetylation of the C-7 primary acetyl group using an esterase from Rhodosporidium toruloides (50, 51) at pH 5 afforded 20 in quantitative yield. The site of the enzymatic deacetylation was unambiguously established as C-7 using $^1$H NMR spectra. The desired aldehyde acceptor 8 was then obtained via Swern oxidation (52).

The crucial C-glycosylation step was then accomplished using samarium chemistry developed in our laboratory (53). The neuraminic acid sulfone donor 7 was coupled to aldehyde acceptor 8 in the presence of freshly prepared SmI$_2$ to afford the fully protected sTn α-C-glycoside 6 as a diastereomeric mixture. Efforts to deoxygenate the bridge hydroxymethylene group by Barton deoxygenation failed. Chemical resolution was achieved by oxidizing the bridge hydroxyl group to ketone which was then stereoselectively reduced in the presence of Zn(BH$_4$)$_2$. After deacetylation, hydrogenolysis, amidation and saponification, 21 was afforded in > 90% de (37).

**Synthesis of a Double C-Glycoside Analog of sTn**

We then embarked in the synthesis of a double C-glycoside analog of sTn 31 (Schemes 3 and 4) (43). This target was designed to reduce the number of synthetic steps required to prepare 21, to facilitate its conjugation to KLH (Keyhole Limpet Hemocyanin) carrier protein, to further increase biological half-life and to enhance immunological response.

The α-C-glycoside derivative 27 was first synthesized in six steps from N-acetylglucosamine as described by Cipolla et al (Scheme 3) (54). The 6-hydroxyl group of compound 27 was then protected as the 6-TBDPS silyl ether followed by 3,4-isopropylidenation to afford compound 28. Based on our previous experience, we decided to double protect the acetamido group at C-2 position in order to avoid cyclic hemiaminal formation later in the scheme when the C-6 position will be oxidized to the corresponding aldehyde. Thus, 28 was treated
Scheme 2. Synthesis of the C-glycoside analog of sTh.
with p-chlorobenzoyl chloride in a mixture of pyridine and dichloromethane to give 29 in excellent yield. Chemoselective removal of the TBDPS protection at C-6 position using trihydrofluoride triethylamine afforded the corresponding alcohol 30 in quantitative yield (TBAF/acetone system proved to be inefficient for this transformation). We then decided to explore very mild conditions for the oxidation of C-6 hydroxyl group to the corresponding aldehyde in order to prevent C-5 epimerization. Hypervalent iodine reagents in ionic liquid have been previously reported as mild chemoselective and in some cases regioselective oxidative process (53). Thus, compound 30 was dissolved in 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF$_4$]) and treated with 1.5 equivalent of Dess-Martin periodinane for 3 hours. Extraction of the product from the ionic liquid was performed using diethyl ether and resulted in the isolation of a single product which was identified with HRMS and $^1$H NMR as the desired aldehyde 22. Because of its propensity for epimerization, the aldehyde was directly used in the C-glycosylation reaction.

NeuSAc sulfone donor 7 was reacted with aldehyde 22 in the presence of 6 equivalents of SmI$_2$ in THF (Scheme 4) to afford the desired double C-glycoside product 32 in a modest 25% yield but with complete diastereoselectivity in favor of the S isomer, as demonstrated by molecular modeling and NOESY experiments. Complete deprotection was next accomplished in three steps. Deisopropylidenation using acetic acid at 80°C for 1 h, followed by deacetylation with NaOMe in MeOH and saponification of the methyl ester using 0.1M KOH afforded 33 in quantitative yield. After oxidative cleavage of the terminal olefin of 33, reductive amination was accomplished by treatment of the corresponding aldehyde with NaCNBH$_3$ in the presence of the KLH hapten to give target compound 31.

O-Linked conjugate vaccines of sTn-KLH have shown good immunogenicity, resulting in the production of both IgM and IgG type antibodies (56, 57). The KLH-conjugates of sTn C-glycosides were evaluated for immunogenicity in mice (Figure 5, Table 1). Female BALB/c mice, 5 weeks old, were immunized with O-linked sTn-KLH, C-linked sTn-KLH or double-C-linked sTn-KLH conjugate (1 or 10 μg) 3 times at two weeks interval. Seven days after the last immunization, mice were anesthetized with ether and blood samples were collected from abdominal vein. Antisera were separated by centrifugation. IgG and IgM antibody titers of the antisera against sTn antigen were assayed by ELISA. The titer was defined as highest dilution yielding an absorbance of greater than that of normal sera. Both KLH-conjugate of sTn C-glycosides lead to the production of IgM and IgG type antibodies. The C-glycosides were found to be more immunologically active than the natural O-linked sTn antigen (Table 1). Furthermore, the C-glycosides of sTn enhanced the production of the tightly binding IgG class of antibodies.
Synthesis of PSA C-disaccharide

Tumor related antigen often contains O-linked Neu5Ac-α(2,8)-Neu5Ac disaccharide units (58). The O-linkage being catabolically unstable, we have been interested in the synthesis of a C-linked Neu5Ac disaccharide (44). Indeed, vaccination with a catabolically stable sialic acid C-glycoside analog might enhance immunogenicity. Ultimately, the synthesis of C-linked polysialic acids (PSAs) would be of particular interest.

Polysialic acids are naturally occurring helical, linear homopolymers composed entirely of negatively charged sialic acid residues joined by α(2→8), α(2→9), or α(2→8)/α(2→9) alternating ketosidic linkages and are commonly found N-linked to a neural cell adhesion molecule (NCAM) (39). The precise function of PSA has not yet been established but a well-demonstrated property of PSAs is in cell-cell interaction and adhesion. It is postulated that alteration of PSA glycans in NCAM reduces cell adhesion and may be involved in invasive metastasis (60, 61). The unusual lability of PSA, their participation in developmental biology and their reappearance in various tumors make their C-glycosides analogs ideal targets for a wide array of experimental, biological and possibly therapeutic applications.

Retrosthetic analysis (Scheme 5) suggested that samarium mediated C-glycosylation of aldehyde acceptor 36 with Neu5Ac phenyl sulfone donor 7 would afford the nor-C-disaccharide target 35 (44). The success of this synthetic route relies on the design of aldehyde acceptor 36, which we envision could be synthesized using the previously reported sialic acid thiophenyl glycoside 39 (62).

We first considered the use of t-butyldiphenylsilyl (TBDPS) protection in order to differentiate the C-9 primary hydroxyl group. Unfortunately, after successful isopropylidene protection of 7,8-hydroxyl groups we encountered decomposition problems during the deprotection step of the 9-OTBDPS group using TBAF. We then considered another strategy involving a selective ring opening of 8,9-p-methoxybenzylidene ring (63). First, regioselective protection of 8,9-diol gave 40 in 94% yield, which was then regioselectively opened using aluminium (III) chloride and BH₃·NMe₃ to afford the 9-p-methoxybenzyl (PMB) ether 41 in 60% yield (Scheme 6). Once the differentiation of the C-9 position was accomplished, we focused our attention on the protecting groups at 4,7,8 positions. Modeling suggested that allyl protection should allow efficient C-glycosylation with the bulky nucleophile derivative of 7. The ease of introduction, electron-donating properties, orthogonality, and ease of removal made OAll an ideal protecting group for synthesis of desired aldehyde acceptor 36 (64). Perallylation of 41 under standard conditions resulted in unexpected
Scheme 4. Synthesis of the double C-glycoside analog of sTn.
Figure 5. KLH conjugates of sTn O- and C-glycosides.
Scheme 5. Retrosynthetic scheme for the synthesis of PSA C-disaccharide 35.
Scheme 6 Synthesis of C-Neu5Ac disaccharide. a) p-MeOPhCH(OMe)$_2$, CSA, MeCN, 94%; b) BH$_3$NMe$_3$, AlCl$_3$, MS 4A 60%; c) AlBr, BaO/Ba(OH)$_2$,8H$_2$O, DMF, 75%; d)CH$_2$(CH$_3$)COOCCH$_3$, TsOH, 63 °C, 80%; e) CAN, 74%; f) Dess-Martin reagent, 70%; g) 7. 5 equiv 0.1N SmI$_2$, THF, 35%; h) Ac$_2$O, pyr.; i) 2-(2-hexylthioethoxy)ethoxyethanol. THFMe, MS 4A.
Table I. Immunological Evaluation of O- and C-Glycosides of Tn and sTn

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<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>O,O-sTn-KLH</td>
<td>1860 ± 1278</td>
<td>480 ± 981</td>
</tr>
<tr>
<td>O,C-sTn-KLH</td>
<td>11520 ± 2862</td>
<td>2560 ± 2147</td>
</tr>
<tr>
<td>C,C-sTn-KLH</td>
<td>7360 ± 4005</td>
<td>2330 ± 1198</td>
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Female BALB/c mice were immunized three times with 1 μg/mouse (O,C) or 10 μg/mouse (C,C and O,O) of KLH conjugates. Seven days after the third immunization, IgG and IgM titers were measured by ELISA. Data are expressed as the mean ± SD of 5 mice.

lactamization. Alternately, a mixture of barium oxide and barium hydroxide gave the desired, albeit transesterified, allylated compound 42 in 75% yield (65). Then, the N-acetyl group was double protected in order to avoid the formation of an hemiaminal ring later in the scheme (during oxidation of the 9-hydroxyl to the corresponding aldehyde). N-acetylation of 42 afforded 43 in 80% yield (66). Deprotection of the PMB was accomplished using cerium ammonium nitrate and gave the 9-hydroxyl derivative 44, which was further oxidized to the corresponding aldehyde acceptor 45 in 70% yield (67). C-Glycosylation of the aldehyde 45 with Neu5Ac sulfone donor 7 in the presence of freshly prepared SmI₂ (68) gave the desired nor-α(2,8)-C-neuraminic acid disaccharide 46 in 35% yield, as a diastereomeric mixture (R/S 1:1) at the bridge hydroxymethylene group. The two diastereomers were separated by flash chromatography and the R isomer was acetylated at the bridge hydroxyl group to give 47. Hapten conjugation was then accomplished by O-glycosylation of 2-[2-(2-benzylthioethoxy)ethoxy]ethanol using methyl triflate as promoter.

**Conclusion and Perspectives**

Samarium mediated C-glycosylation has proven to be an effective method for the synthesis of α-C-glycosides of Neu5Ac. We have successfully used this strategy to synthesize α-C-glycosides analogs of sTn antigen and polysialic acids. These catabolically stable, carbohydrate based, potential antinecancer vaccines shown remarkable immunogenicity, resulting in the production of both IgM and IgG type antibodies. We have shown that the synthesis of polysialic acid type C-disaccharide can be accomplished via the same procedure. Although
very promising, the yield of the C-glycosylation need to be improved to apply this reaction to the polymerization reactions that would allow us to prepare C-linked polysialic acid derivatives (Figure 6).

References