Michael Addition as the Key Step in the Syntheses of Sialyloligosaccharide–Protein Conjugates from *N*-Acryloylated Glycopyranosylamines

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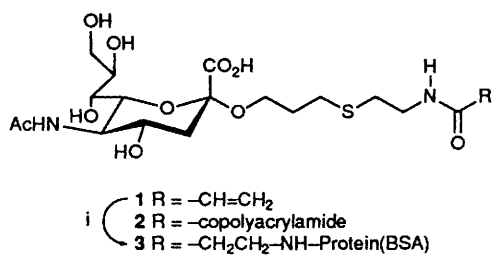
Sialic acid and sialyloligosaccharides having terminal *N*-acryloyl functionality in their aglycone portions were efficiently conjugated to the lysine groups of proteins through Michael additions under mild basic conditions; the same *N*-acryloyl precursors were also polymerized with acrylamide to form serologically useful antigens.

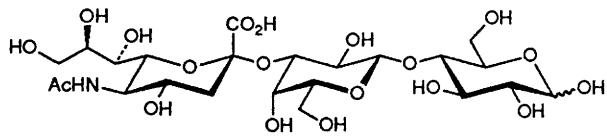
Sialyloligosaccharides are well known immunodominant components of cell wall glycolipids and glycoproteins.[1] They are of considerable interest because of their numerous biological functions. For example, they are known to participate in cellular recognition phenomena, to serve as receptors for influenza virus and to act as differentiation and as oncodevelopmental antigens.[2] It is not surprising therefore, that the syntheses of various sialyloligosaccharides have attracted intensive efforts.[3] However, stereospecific syntheses of α-linked sialyl glycosides are still in their infancy.

In our endeavours[4] to prepare multivalent forms of sialic acids (Neu5Ac, *N*-acetylneuraminic acid) and sialyloligosaccharides of definite anomeric configurations, efforts were concentrated on sialyloligosaccharide sequences directly obtained from abundant natural sources. In such studies, protein conjugates to be used as immunogens[5,6] and as inhibitors of hemagglutination of influenza A virus[6] were required. Protein conjugates, because of their recognized T-cell epitopes, constitute useful immunogens which can allow antibody class switching; a situation not encountered with pure glycolipids (gangliosides). Conversely, analogous polymer conjugates possessing only the carbohydrate portions in common with their protein counterparts represent advantageous screening antigens in enzyme linked immunosorbent assays (ELISA).[7] Hence, single sialyloligosaccharide derivatives amenable to both conjugation to proteins and to polymerization are worthy targets.

The structure of our model sialic acid conjugates are depicted in Scheme 1.[8] In the light of established reactivities of amines in Michael additions[9] (not previously applied to proteins), it was expected that an *N*-acryloyl precursor such as **1** would react as a Michael acceptor in protein additions. As

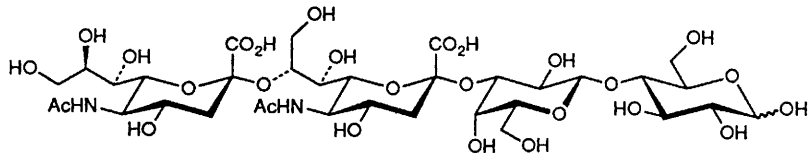


Scheme 1 *Reagents and conditions*: i, Borate buffer (0.4 mol dm^{-3}), pH 10, 37 °C, 48 h



α-D-Neup5Ac-(2→3)-β-D-Galp-(1→4)-β-D-Glc-OH

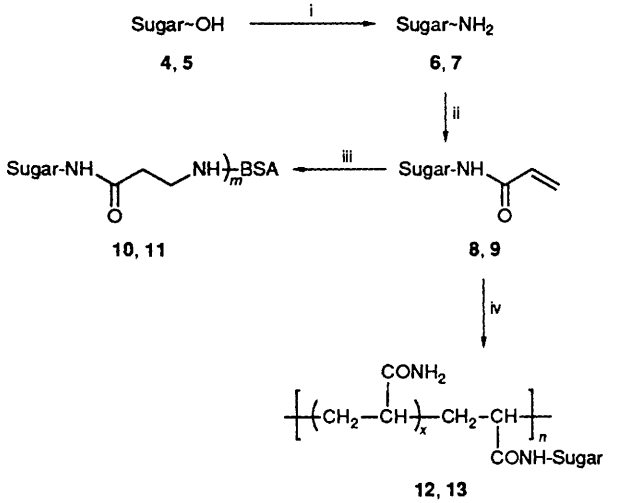
4



α-D-Neup5Ac-(2→8)-α-D-Neup5Ac-(2→3)-β-D-Galp-(1→4)-β-D-Glc-OH

5

Scheme 2



Scheme 3 *Reagents and conditions*: i, Sat. NH_4HCO_3, 37 °C, 3 days, lyophilized; ii, CH_2=CHCOCl, Na_2CO_3, MeOH–H_2O (1:1 v/v), 48–52% overall; iii, 0.1 mol dm^{-3} carbonate buffer, pH 10.0, 37 °C, 48 h, dialysis; iv, CH_2=$CHCONH_2$, H_2O, $(NH_4)_2S_2O_8$, TMEDA, 25 °C, 16 h

expected, compound **1** could be covalently linked to bovine serum albumin (BSA) in aqueous buffers at pH greater than 8–8.5 through a Michael reaction. Amino acid analysis of the resulting conjugates **3** revealed that the lysine residues had been modified (BSA contains only one free cysteine group) and that it contained up to 16 sialic acid residues as determined by both amino acid analysis and by the resorcinol[10] and colorimetric assays (±2 residues). The reactions were shown to be dependent on the nature of the buffers used. For example, no appreciable incorporation occurred in phosphate buffers or in sodium hydroxide solutions (pH 9–10), while the reactions were efficient in both borate and carbonate buffers. No extensive protein degradation was observed after three days at 37 °C with pH up to 10.5 as shown by SDS-PAGE electrophoresis.

In order to expand the generality of the Michael reactions to immunologically important sialyloligosaccharides **4** and **5** (Scheme 2), the syntheses of *N*-acryloylated glycosylamines **8** and **9** were undertaken. The general strategy for the syntheses of both proteins **10** and **11** and polymer conjugates **12** and **13** derived from single precursors is depicted in Scheme 3. Thus, sialyloligosaccharides **4** and **5** isolated by a simplified[11] procedure from bovine colostrum were derivatized to their corresponding *N*-acryloyl glycosylamines **8** and **9** by a slight modification of the literature procedure.[12] The glycosylamines **6** and **7** derived from the reducing sugars **4** and **5** by reaction with saturated aqueous ammonium hydrogen carbonate solutions (3 days; 37 °C) were not isolated but directly *N*-acryloylated (acryloyl chloride, Na_2CO_3, MeOH–H_2O) after exhaustive lyophilization to remove the excess of ammonium salts. The products **8** and **9** were purified by gel permeation chromatography on Sephadex G-10 columns using water as eluent. Final purifications, when required, were performed by reverse phase HPLC using an Altex O.D.S. column (10 mmol dm^{-3} Et_3N–HOAc buffer, pH 4.8). Last traces of triethylamine were removed by cation exchange resin (H^+ form). The *N*-acryloyl derivatives were stored as their sodium salts after neutralization with NaOH (0.1 mol dm^{-3}) and freeze-drying.

Compounds **8** and **9** were obtained in 48–52% overall yields from their reducing precursors **4** and **5**. The anomeric configurations of all the glycopyranosylamine moieties were in the β-form as determined by the observed signals in the ^1H NMR spectra (300 MHz, D_2O) of **8** and **9** (doublets, δ 5.10, $J_{1,2}$ 9.2 Hz, H-1 Glc).†

Like their monosaccharide counterpart **1**, these *N*-acryloylated derivatives **8** and **9** were also found to give Michael

† Satisfactory spectral data have been obtained for each compound isolated. **8**: FAB MS for $C_{26}H_{42}O_{19}N_2$: (M − 1) *m/z* 685; ^1H NMR (D_2O) δ: 6.326 (ABX, 1H, H_c), 6.307 (ABX, 1H, H_a), 5.872 (ABX, 1H, H_b), 5.096 (d, 1H, $J_{1,2}$ 9.2 Hz, βGlc-H1), 4.545 (d, 1H, $J_{1,2}$ 7.9 Hz, Gal-H1), 4.116 (dd, 1H, $J_{2,3}$ 9.8, $J_{3,4}$ 3.1 Hz, Gal-H3), 3.489 (t, 1H, $J_{2,3}$ = $J_{1,2}$ = 9.0 Hz, βGlc-H2), 2.761 (dd, 1H, $J_{3a,3e}$ 12.4, $J_{3e,4}$ 4.6 Hz, NeuAc-H3eq), 2.030 (s, 3H, N*Ac*) and 1.800 (t, 1H, $J_{3a,3e}$ = $J_{4a,4}$ = 12.0 Hz, NeuAc-H3ax); **9**: FAB MS for $C_{37}H_{59}O_{27}N_3$: (M − 1) *m/z* 977; ^1H NMR (D_2O) δ: 6.329 (ABX, 1H, H_c), 6.310 (ABX, 1H, H_a), 5.873 (ABX, 1H, H_b), 5.092 (d, 1H, $J_{1,2}$ 9.3 Hz, βGlc-H1), 4.549 (d, 1H, $J_{1,2}$ 8.0 Hz, Gal-H1), 4.105 (dd, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 3.1 Hz, Gal-H3), 3.491 (t, 1H, $J_{2,3}$ = $J_{1,2}$ = 8.9 Hz, βGlc-H2), 2.790 (dd, 1H, $J_{3a,3e}$ 12.4, $J_{3e,4}$ 4.6 Hz, NeuAc'-H3eq), 2.678 (dd, 1H, $J_{3a,3e}$ 12.4, $J_{3e,4}$ 4.6 Hz, NeuAc-H3eq), 2.030 (s, 3H, NeuAc'-N*Ac*), 2.067 (s, 3H, NeuAc-N*Ac*), 1.763 (t, 1H, $J_{3a,3e}$ = $J_{3a,4}$ = 12.0 Hz, NeuAc'-H3ax) and 1.744 (t, 1H, $J_{3a,3e}$ = $J_{3a,4}$ = 12.0 Hz, NeuAc-H3ax).

additions efficiently with protein (BSA and tetanus toxoid for **8**). When solutions (0.1 mol dm^{-3} Na_2CO_3 buffer, pH 10.0) of the proteins and the Michael acceptors **8** and **9** were allowed to react for two days at 37 °C and then processed by dialysis and lyophilization, the protein conjugates **10** and **11** were obtained as white powders. The sialic acid contents of the conjugates were determined by the resorcinol method which showed 15–19 Neu5Ac residues per BSA molecule (for **8**, 48 Neu5Ac residues were coupled to tetanus toxoid).

Water-soluble copolyacrylamide polymers **12** and **13** were also prepared using conditions already described for the synthesis of **3** from **1** [acrylamide, $(NH_4)_2S_2O_8$, *N,N,N',N'*-tetramethylethylenediamine (TMEDA); 16 h; 25 °C].[8] The polymers have sialic acid contents of ≈14% (w/w)[10] and showed broad molecular weight distribution by size extrusion chromatography on Sepharose CL-4B ($M \approx 100\,000$). The antigenicities of some of the protein and polymer conjugates were demonstrated by double immunodiffusion, quantitative precipitation and by ELISA using the lectin from *Triticum vulgaris* (wheat germ agglutinin)[13] as model in binding assays. Work is in progress to evaluate the immunogenicities of the protein conjugates.

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