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DIRECT ACCESS TO NEOGLYCOPROTEINS AND GLYCOPOLYMERS FROM SINGLE PRECURSORS. SYNTHESIS OF T-ANTIGEN AND N-ACETYL-LACTOSAMINE-β-D-(1→6)-α-D-GalNAc CONJUGATES

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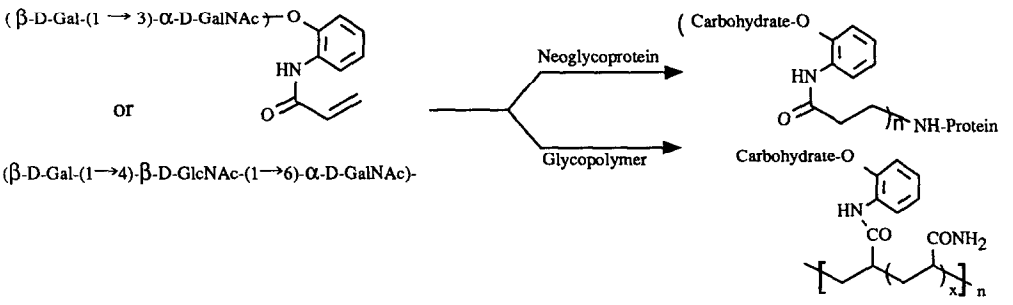
Abstract: Michael addition and acrylamide copolymerization of single N-acryloylated carbohydrate precursors of the Thomsen Friedenreich antigen (T-antigen) and an ABH type 2 human blood group trisaccharide determinant afforded both neoglycoprotein and glycopolymer conjugates suitable for immunochemical studies.

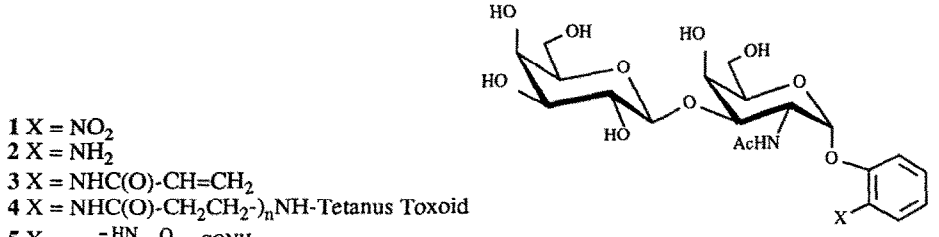
Some carbohydrate sequences[1] and the cryptic Thomsen-Friedenreich (T) antigen in particular (β-D-Gal-(1→3)-α-D-GalNAc-O-serine/threonine)[2] have been well established as tumor-associated markers. Advances in mapping these carbohydrate determinants have been made through the syntheses of well defined conjugates. Although there has been numerous syntheses of the T-antigen containing a wide variety of aglycons,[3] there has been no systematic studies on the involvement of the aglyconic regions (Serine/Threonine) in the antibody combining sites. It is possible that the epitope density, more than the exact nature of the peptide sequences anchoring the T-antigen (and others) might be responsible for the high antigenic expression. Therefore, efficient access to immunogenic neoglycoproteins from which antibodies can be raised, coupled to the synthesis of antigenic glycopolymers which could be used for the screening of anti-carbohydrate antibodies are still valuable goals.

We have recently designed a general strategy which allows direct access to both neoglycoprotein and glycopolymer[4] conjugates.[5] The strategy is based on the synthesis of single N-acryloylated carbohydrate precursors having dual reactivities.[6]

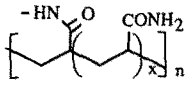


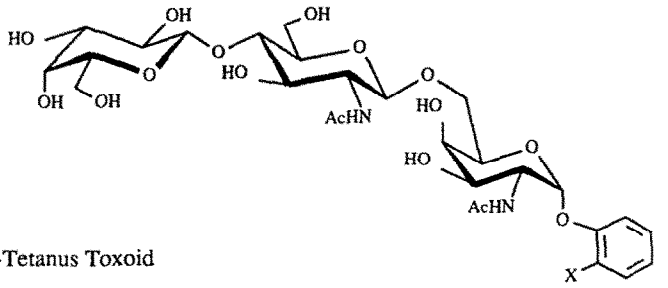
The conjugated double bond of the N-acryloyl residues serves as Michael acceptor for the nucleophilic additions of protein units (either from ϵ-lysine-NH_2 or cysteine-SH groups) and at the same time can serve as comonomers in polymerization with acrylic type monomers. Thus, access to immunogenic neoglycoproteins is seconded by access to antigenic glycopolymers which are used in immunoassays, the carbohydrate residues being the only part in common to both conjugates. The above strategy has been substantiated herein with the T-antigen (β-D-Gal-(1→3)-α-D-GalNAc) and a trisaccharide (β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-α-D-GalNAc) constituting an ABH type 2 glycoprotein determinant of human blood group activities.[7]

The T-antigen and the D-lactosamine-trisaccharide were prepared as their ortho nitrophenyl glycosides **1** and **6** respectively.[8] Reduction of the nitro group with ammonium formate and 5% Pd on charcoal in methanol containing few drops of formic acid at reflux for few minutes (<10 min) afforded quantitative yields of the unstable ortho-aniline derivatives **2** and **7** after filtration and washing from celite. Excess ammonium formate was removed after repeated evaporation from methanol and freeze drying.

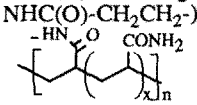


1 X = NO_2
2 X = NH_2
3 X = NHC(O)-CH=CH_2
4 X = NHC(O)-CH_2CH_2-)$_n$NH-Tetanus Toxoid
5 X =





6 X = NO_2
7 X = NH_2
8 X = NHC(O)-CH=CH_2
9 X = NHC(O)-CH_2CH_2-)$_n$NH-Tetanus Toxoid
10 X =



The ortho-aniline derivatives **2** and **7** were immediately transformed into their corresponding N-acrylamide derivatives **3** and **8** using previously described procedures.[5-6] Thus, cooled (0°C) methanolic solutions of **2** and **7** were individually treated with anionic resin (OH^-) to which was added dropwise a slight excess of acryloyl chloride in chloroform. After a few minutes, the conversions were completed. The resin was filtered, washed with methanol and the filtrates were evaporated to dryness. Compound **3** was slightly contaminated with acrylamide originating from acryloylation of residual ammonium formate (^{1}H-NMR) and was further purified by size exclusion chromatography on Sephadex LH-20 column using methanol/water as eluant (9/1 by vol)(95% overall yield). Compound **8** was also obtained in almost quantitative yield without

the need for chromatography.[9]

The homologous neoglycoproteins **4** and **9** were then prepared by Michael addition. It is noteworthy to emphasize that the Michael additions not only occurred through thiol group but also occurred through the ϵ-amino groups of the lysine residues without substantial protein degradations as judged by SDS electrophoresis. Thus, treatment of the N-acryloylated derivatives **3** and **8** in 0.2 M carbonate buffer at pH 10.0 for 3 days at room temperature with tetanus toxoid followed by centrifugation and exhaustive dialysis afforded the neoglycoproteins **4** and **9** in 82 and 97% yield respectively (weight basis).[10]

Similarly, glycopolymers **5** and **10** were obtained by radical initiated copolymerization of **3** or **8** with acrylamide in de-oxygenated water using ammonium persulfate ($(NH_4)_2 S_2 O_8$) and N, N, N', N'-tetramethylethylenediamine (TMEDA) at room temperature overnight as previously described.[11] The glycopolymers **5** and **10** were purified and isolated by dialysis and freeze drying. ^{1}H-NMR spectroscopy was used to determine the carbohydrate contents of the glycopolymers. The T-antigen containing copolyacrylamide **5** showed a carbohydrate to acrylamide ratio of 1 to 23 (molar basis; 21% by weight) while the trisaccharide glycopolymer **10** had a hapten to acrylamide ratio of 1:40 (18% by weight).

The antigenicity of the T-antigen neoglycoprotein **4** was first demonstrated by agar gel diffusion and by enzyme linked lectin assays (ELLA) using unlabeled and horseradishperoxidase labeled peanut lectin (100 µl/well of serial two fold dilution from 1 mg/mL). Figure 1 shows a typical binding assay using **4** as coating antigen in microtiter plates (1 µg/well). The enzyme activity in the wells was measured by using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as enzyme substrate and recording the optical density at 410 nm on a Dynatech MR 600 spectrophotometer. The immunogenicities of the protein conjugates are being evaluated in rabbits and the results will be presented in due course.

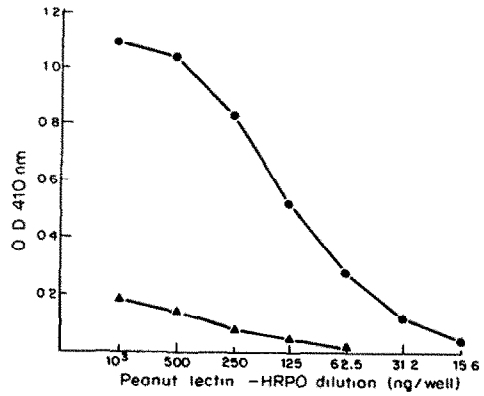


Fig. 1 Enzyme Linked Lectin Assay (ELLA) of neoglycoprotein **4** as coating antigen at 1 µg (●) and 0.1 µg (▲) per well using horseradish peroxidase labeled peanut lectin.

Acknowledgments

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4. We have recently proposed the term "glycopolymer" in replacement to "pseudopolysaccharide" in analogy to "glycoprotein" and "glycolipid". The name glycopolymer should thus be applicable to both soluble (antigenic) and insoluble (affinity media) polymers to which carbohydrates are covalently attached.
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9. All materials gave satisfactory spectroscopic analyses. Compounds **1-3** had R_F of 0.32, 0.23, 0.33 respectively in acetonitrile/acetone/10% acetic acid (5/3/1 by vol). Compounds **6-8** had R_F of 0.15, 0.09, and 0.12 respectively in the same solvent. TLC plates were run on pre-coated silica gel plates 60-F254 (E. Merck). ^{1}H-NMR data, δ ppm (D_2O), ref. with HOD at 4.83 ppm, **3**: 7.15-7.44 (m, 4H, aryl), 6.57 (dd, 1H, J_{cis}=10.2, J_{trans} = 17.0 Hz, C(O)CH=), 6.40 (dd, 1H, J_{gem} = 1.2 Hz, C=CH_2 trans), 5.96 (dd, 1H, C=CH_2 cis), 5.71 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.53 (dd, 1H, $J_{2,3}$ = 11.1 Hz, H-2), 4.43 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1'), 4.30 (d, 1H, J = 2.4 Hz, H-4 or H-4'), 4.03-4.11 (m, 2H), 3.92 (dd, 1H, $J_{3,4}$ = 3.0, $J_{4,5} \leq$ 1 Hz, H-4 or H-4'), 3.77-3.51 (m, 7H), 2.05 (s, 3H, NHAc). **8** (D_2O) ref. to acetone (2.216 ppm): 7.16-7.47 (m, 4H, aryl), 6.55 (dd, 1H, J_{cis} = 10.1, J_{trans} = 16.8 Hz, C(O)CH=), 6.37 (dd, 1H, $J_{gem} \leq$ 1 Hz, =CH trans), 5.94 (dd, 1H, =CH cis), 5.69 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.47 (d, 1H, $J_{1,2}$ = 7.4 Hz, H-1'), 4.43 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1"), 3.5-4.3 (m, 18H), 2.04, 1.84 (2s, 2 x 3H, NHAc).
10. **Protein conjugation-(4, 9):** To the N-acryloylated carbohydrate precursors (2.0 mg, 4 μmol, **3**; 2.9 mg, 4 μmol, **8**) was added purified tetanus toxoid (5.1 mg, 33 nmol) in 0.2 M sodium carbonate buffer pH 10.0 (1 mL). The suspensions were homogenized by mixing on a Vortex mixer. The Michael additions were allowed to proceed for three days at room temperature after which time insoluble particles were centrifuged at 14,000 g in a Fisher Microcentrifuge Model 235 B for 5 min. The homogeneous reaction mixtures were then dialysed against distilled water (4 x 1L) and lyophilized to provide **4** (5.8 mg) and **9** (7.9 mg).
11. **Glycopolymer conjugates-(5, 10):** To the N-acryloylated carbohydrate precursors (5.0 mg, 9.5 μmol, **3**; 3.4 mg, 4.65 μmol. **8**) were added acrylamide (10.4 mg, 146 μmol and 6.5 mg, 91.5 μmol respectively) dissolved in distilled deoxygenated water (200 μL). TMEDA (2 μL) followed by 5 μL of ammonium persulfate solution (50 mg mL^{-1}) were then added and the reaction mixtures were stirred overnight at room temperature. Completion of polymerization were evaluated by the disappearance of the starting glycoside monomers by TLC (acetonitrile, acetone, 10% acetic acid, 5:3:1). More TEMED may be required if there is some unreacted starting material. The reaction mixtures were diluted with warm water (2 mL) and dialysed against distilled water to provide **5** (3.0 mg) and **10** (9.9 mg) after freeze-drying ^{1}H-NMR (D_2O) showed **5** and **10** to contain carbohydrate to acrylamide ratios of 1:23 and 1:40 respectively.