1-*N*-Glycyl β-Oligosaccharide Derivatives as Stable Intermediates for the Formation of Glycoconjugate Probes†

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ABSTRACT: Incubation of reducing sugars in ammonium bicarbonate was found to be a simple procedure for the formation of β-D-glycosylamines of purified complex oligosaccharides in 70–80% yield. These provide valuable intermediates for the synthesis of a wide range of oligosaccharide probes and derivatives by acylation of the 1-amino function. The 1-amino function showed different rates of reactivity with different reagents. In general, interactions with large ring systems such as the fluorophores dansyl chloride and carboxyfluorescein gave 10–20% yields of products, which consisted of mixtures of both anomeric forms, whereas smaller acylating reagents gave near-quantitative yields of the desired β-D-derivatives. Steric effects may explain differences in reactivity. *N*-Chloroacetamido derivatives could be obtained in high yield with retention of the β-anomeric configuration. Subsequent ammonolysis of the chloroacetamido function afforded the corresponding *N*-glycyl β-derivatives. The linker thereby introduced retains the amino function, possesses the useful properties of fixed anomeric configuration, improved stability, and uniform reactivity with a variety of reagents, and is structurally analogous to an asparagine side chain. The potential therefore exists for the generation of oligosaccharide derivatives tailored for different applications.

Chemical derivatives of N-linked and O-linked oligosaccharides can provide valuable tools for analysis of the biological function of these important posttranslational modifications. They may also find applications in clinical research, in diagnostic medicine, and in the development of therapeutics. Approaches to the synthesis of saccharide–protein conjugates or "neoglycoproteins" have been extensively reviewed (Stowell & Lee, 1980; Aplin & Wriston, 1981; Ginsburg, 1987). In addition to neoglycoproteins, fluorescent [e.g., Hase et al. (1985)], biotinyl (Shao & Wold, 1987), and lipid-linked [e.g., Stoll et al. (1988)] oligosaccharide derivatives have proven useful in structural and biochemical analysis.

Stowell and Lee (1980) have defined the following prerequisite characteristics for glycon–aglycon linkages: (1) the modification should be specific, covalent, and stable to physiological conditions, (2) the conditions used for formation of the derivative should be chosen so as to avoid degradation of the carbohydrate, and (3) for in vivo applications, the linkage itself should not introduce bulky or highly interactive groups such as long-chain hydrocarbons, aromatic rings, or ionic groups, which may interfere nonspecifically with components of a biological system.

In general, the methods currently available do not conform to these specifications, since they may introduce nonbiological and potentially immunogenic groups linking the sugar to the required aglycon moiety. Such approaches may be chemically demanding, precluding their application to chemically sensitive biologically derived oligosaccharides available in limited quantities. However, these structures may be easily modified using reductive amination [e.g., Hase et al. (1985)]. In this approach reduction of a Schiff adduct between the aldehyde form of the sugar and a primary amine forms a stable covalent linkage between the two but leads to the fixation of the reducing terminal residue in an acyclic form. Formation of nonbiological determinants by ring-opening events may affect the biological activity or immunogenicity of the glycan moiety [e.g., Kamicker et al. (1977) and Yamamoto et al. (1982)]. These limitations have led us to investigate methods for chemical modification of oligosaccharides which do not require the introduction of complex, nonbiological linkages and which additionally do not affect the carbohydrate structure.

We have been examining the application of a method for the facile condensation of glycosylamines (Lihkosherstov et al., 1986; Kallin et al., 1989) to the synthesis of derivatives of oligosaccharides obtained from glycoproteins by chemical or enzymatic means. As a starting point for the formation of derivatives, glycosylamines have several desirable properties, in that they have a cyclic structure and they have a tendency to adopt the β-anomeric configuration, i.e., that which is found in the N-glycosidic linkage between proteins and carbohydrates. Replacement of the anomeric hydroxyl group of the reducing terminal residue of an oligosaccharide with a primary amino function introduces a reactive nucleophilic center which can be acylated to form a variety of novel glycoconjugates (Paul et al., 1979). We have therefore investigated the utility of glycosylamine derivatives of complex oligosaccharides as intermediates for the synthesis of novel glycoconjugates which should retain the maximum amount of "biological information" possible.

We report in this paper the application of glycosylamine chemistry to the synthesis of fluorescein and (dimethylamino)-naphthalenesulfonyl (dansyl) saccharides and demonstrate that direct modification of the 1-amino function of a glycosylamine is both inefficient and difficult to control in that a substantial fraction of the product adopts the "biologically incorrect" α-anomeric configuration. Subsequently, we have adopted the approach of converting the unstable glycosylamine to the stable 1-*N*-glycyl β-derivative. In addition to their improved stability, these intermediates are of fixed anomeric configuration and possess uniform reactivity toward a variety of reagents, suggesting that they may be invaluable in the generation of a broad spectrum of glycoconjugate probes.

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MATERIALS AND METHODS

Materials. N-Acetylglucosamine (GlcNAc)[1] (**1**) and N,N'-diacetylchitobiose (GlcNAcβ4GlcNAc) (**2**) were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). [1-^{14}C]-N-Acetylglucosamine was obtained from New England Nuclear (Du Pont U.K. Ltd., Stevenage, Herts, U.K.). D_2O (99.96 atom %) and hexadeuteriodimethyl sulfoxide (DMSO-d_6) (99.6 atom %) for NMR were purchased from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.). 5(6)-Carboxyfluorescein succinimido ester (FNHS) was obtained from Molecular Probes Inc. (Eugene, OR). (Dimethylamino)-naphthalenesulfonyl chloride (dansyl chloride) and chloroacetic anhydride were obtained from Fluka (Glossop, Derbyshire). All other reagents and solvents used were AR grade or the highest purity available.

The biantennary oligosaccharide, Galβ4GlcNAcβ2Manα6-(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβGlcNAc (**3**), and the core-fucosylated structure, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4(Fucα6)GlcNAc (**4**), were obtained from human serotransferrin and porcine thyroglobulin, respectively, using hydrazinolysis and purified using Bio-Gel P-4 gel filtration chromatography. The expected structures of the purified oligosaccharides were established using a combination of NMR and methylation analysis methods as previously described (Ashford et al., 1987).

Thin-layer chromatography was performed using Merck 10 × 20-cm glassbacked silica 60 F_{254} or 5- × 7.5-cm aluminum-backed silica 60 HPTLC plates obtained from BDH Ltd. (Poole, Dorset, U.K.). For TLC analysis of mono- and disaccharide glycosylamines, Merck silica 60 plates were developed using butan-1-ol/acetone/water (6:5:4 v/v: solvent A). Visualization of plates was performed under UV light (254 and 366 nm) following staining with *p*-aminobenzoic acid reagent (PABA), using ninhydrin and orcinol sprays. For oligosaccharides, silica 60 TLC plates were developed in acetonitrile/water systems (70:30 v/v) containing 0.05% diaminobutane (solvent B). Radioscanning of TLC plates was performed on the Berthold TLC linear analyzer using the manufacturer's software (IBM-PC-based TLC software, Berthold Ltd., St. Albans, Herts) run on an HP Vectra RS/20C computer.

Samples for NMR analysis were prepared by lyophilization for 24–36 h prior to exchange with D_2O (3 × 500 μL, 99.96 atom % D_2O) with repeated rotoevaporation. For spectroscopy in DMSO, the lyophilization time was extended to 48 h, and a single addition of 500 μL of DMSO-d_6 was made. Fourier transform ^{1}H-NMR spectra were recorded on Bruker AM500 or 600 spectrometers controlled by an Aspect 3000 computer using the manufacturer's software. The probe temperature used (unless stated) was 300 K. Chemical shifts are reported relative to TMS = 0.0 ppm (D_2O) and DMSO (2.509 ppm). Acquisition parameters varied according to the nature of the experiment. Two-dimensional homonuclear correlation spectroscopy (COSY) was performed in the phase-sensitive mode using the time-proportional phase increment (TPPI) method of Marion and Wuthrich (1983). Spectra were multiplied by unshifted sine-bell functions and zero-filled in both dimensions prior to two-dimensional Fourier transformation. Partial assignments of the spectral resonances of derivatives were made by tracing connectivities using the COSY spectra, or by comparison with reference compounds. The COSY spectra generally provided unambiguous assignments of the H1, H2, and H3 protons for each monosaccharide.

Liquid scintillation counting was performed on a Beckmann LS-1701 scintillation counter. Standard quench curves were used to make quench corrections in dpm counting of fluorescent sugars using the H-number method (Beckman), implemented using the manufacturer's software. When corrected in this way, dilute samples containing fluorophore gave less than 1% variation from unquenched standards. For dpm counting, samples were dissolved in 100 μL of water and 10 mL of Beckman MP scintillant was added. For analysis of HPLC eluants, aliquots were transferred to plastic vials and 4 mL of scintillant was added. Samples scraped from TLC were transferred to glass scintillation vials. Water (200 μL) was then added, and the vials were vortexed. Scintillant (10 mL of Beckman MP) was then added, and the vials were stored in the dark overnight before counting.

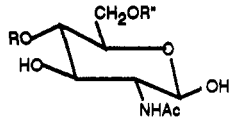
High-performance liquid chromatography (HPLC) was performed using a Waters HPLC system consisting of a Valco U6 manual injector and two Waters 510 pumps with an in-line degasser (Erma). Column eluants were monitored using a Waters 480 Lambda Max detector (UV–vis) and a Merck-Hitachi F1000 dual-monochromator fluorescence detector. The system was controlled by a DEC 380 computer running Waters HPLC software version 6.2. HPLC methods and gradient conditions are described in the figure legends.

Formation of Glycosylamines (**5–8**). Formation of the glycosylamines of N-acetylglucosamine (**1**), N,N'-diacetylchitobiose (**2**), and the oligosaccharides Gal-2 (**3**) and Gal-2F (**4**) (Figure 1) was performed according to a modification of the method of Lihkosherstov et al. (1986). Samples of oligosaccharide were rotoevaporated to dryness and resuspended in 200–500 μL of saturated ammonium bicarbonate made up in sterile water; additional solid ammonium bicarbonate was then added to maintain saturation of the solution during the incubation period. The tubes were sealed with Parafilm which was then punctured using a needle (to allow the escape of the ammonia and carbon dioxide evolved by decomposition of the salt) and then incubated at 30 °C for periods between 24 and 120 h.

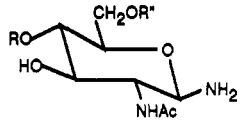
Aliquots of a solution of radiolabeled N-acetylglucosamine incubated in ammonium bicarbonate were withdrawn at 24-h time intervals, applied to a short column of Dowex AG50W-X12 (H^+), and eluted using distilled water. Unmodified sugar was eluted under these conditions and was quantitated by scintillation counting. The time course of formation of the glycosylamine was obtained from amount of radiolabel bound at each time. Time course measurements were also obtained using ^{1}H-NMR spectroscopy. Aliquots of oligosaccharide incubated in ammonium bicarbonate for various times were withdrawn, lyophilized, and exchanged into D_2O for analysis. The extent of reaction was measured by integration of the anomeric proton intensity of the product glycosylamine and the starting sugar (where possible) and comparison of these values with the integral of the acetamidomethyl singlet (3 times unit intensity). Accurate integrals were obtained by cutting out and weighing peaks.

Glycosylamine preparations were desalted by direct lyophilization of the reaction mixture. Samples of oligosaccharide which had been incubated in saturated ammonium bicarbonate (typically 100–200 μL) were diluted to 1 mL with distilled water and shell frozen. These were lyophilized at a chamber

[1] Abbreviations: COSY, two-dimensional *J*-correlated spectroscopy; dansyl, (dimethylamino)naphthalenesulfonyl; FITC, fluorescein isothiocyanate; FNHS, 5-carboxyfluorescein succinimido ester; Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; HPLC, high-performance liquid chromatography; Man, D-mannose; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 acid, sodium salt.

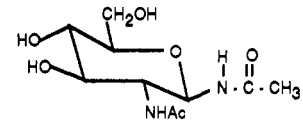


1 R=H R"=H
2 R=GlcNAc R"=H
3 R=Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ R"=H
4 R=Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ R"=Fuc



5 R=H R"=H
6 R=GlcNAc R"=H
7 R=Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ R"=H
8 R=Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ R"=Fuc

9



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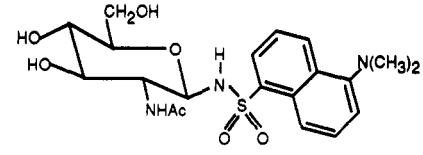

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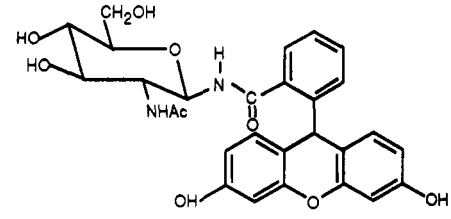

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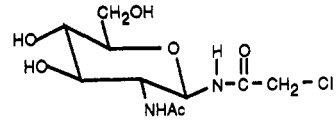

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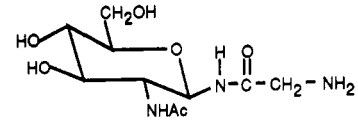

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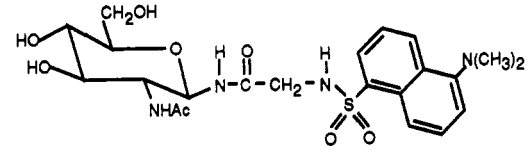

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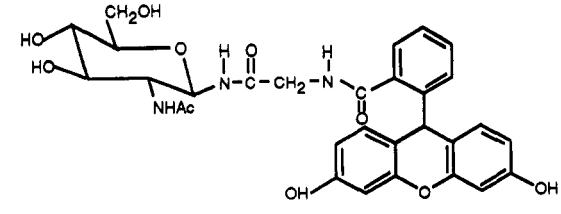

FIGURE 1: Compounds used in this study.

pressure of 8×10^{-2} atm with a condenser temperature of −45 °C. Sublimation of the salt could be accelerated by repeated addition of water at 6–8-h intervals. Lyophilized glycosylamine preparations were stored in a desiccator at −20 °C. Under these conditions, they were stable over at least a 1-year period.

The product of the ammonium bicarbonate condensation method described above was compared with the products of the ammonia–methanol method described by Isbell and Frush (1958). Methanol (1 mL) was added to 10 mg of *N*-acetylglucosamine and dry ammonia gas passed through the solvent for 4 h to achieve saturation. The vial was sealed, and the mixture was stirred for 72 h at room temperature. Subsequently, the mixture was allowed to stand for 72 h at 4 °C. The residual methanol was then removed and the sample resuspended in D_2O prior to analysis by NMR. ^{1}H-NMR: (**5**) δ 4.14 (1 H, d, βH1), 3.88–3.42 (6 H, m, H2–H6′), 2.038 (3 H, s, CH_3); (**6**) δ 4.14 (1 H, d, 1-βH1), 4.58 (1 H, m, 2-H1), 3.88–3.40 (12 H, m, 1- and 2-H2–H6′); 2.03, 2.07 (6 H, 2 s, CH_3); (**7**) (anomeric region) δ 5.12 (1 H, d, 4H1), 4.92 (1 H, d, 4′H1), 4.65–4.55 (3 H, m, 2,5,5′H1); 4.50–4.45 (2 H, m, 6, 6′H1), 4.25, 4.20, and 4.10 (3 H, m, 3,4,4′H2), 4.14 (1 H, d, 1-βH1).

Synthesis of 1,2-Diacetamido-1,2-dideoxy-β-D-glucopyranose (**9**). Confirmation of the structure of **5** was obtained by trapping the 1-amino function by N-acetylation to form the 1,2-diacetamido derivative (**9**). Glycosylamine **5** [5 mg (22 μmol)] was dissolved in 200 μL of saturated sodium bicarbonate and cooled on ice. Acetic anhydride (10 μL) was added, and incubation was continued for 10 min at 0 °C. A second portion of anhydride was added and the sample warmed to room temperature. After 1 h, the sample was passed over a 0.5-mL column of Dowex AG50W-X12 (H^+) and eluted with water (5 column volumes). The eluant containing **9** was dried, redissolved in 1 mL of water, and counted to determine the recovery (greater than 95%). The sample was then lyophilized prior to analysis by NMR in D_2O and DMSO-d_6. ^{1}H-NMR: (D_2O) δ 5.05 (1 H, d, H1)m 3.89–3.45 (6 H, m, H2–H6′), 2.00 (3 H, s, CH_3); (DMSO) δ 8.05 (1 H, d, NH1), 7.78 (1 H, d, NH1), 4.79 (1 H, t, H1), 3.62–3.06 (6 H, m, H2–H6′), 1.85–1.80 (6 H, 2 s, CH_3); OH signals were not assigned.

Synthesis of Fluorescent Derivatives: *2-Acetamido-1,2-dideoxy-1-[(dimethylamino)naphthalenesulfonamido]-β-D-glucopyranose* (**10**) *and 2-Acetamido-1,2-dideoxy-1-fluoresceinamido-β-D-glucopyranoside Derivatives* (**11**). 2-Acetamido-1,2-dideoxy-1-(dimethylamino)naphthalenesulfonamido-β-D-glucopyranose (**10**) was obtained by treatment of **5** with (dimethylamino)naphthalenesulfonyl chloride (dansyl chloride) using conditions based on those of Gray (1972). **5** (10 μmol) was dissolved in 500 μL of 0.2 M sodium bicarbonate. Acetone (500 μL) containing 13.5 mg of dansyl chloride was then added (final concentration 50 mM) with stirring and the reaction allowed to continue at room temperature for 1 h. A brown solution containing some precipitated salt and some unreacted dansyl chloride was obtained. The mixture was dried and resuspended in 200 μL of 50% ethanol. The products were then separated using reverse-phase HPLC on a Spherisorb S50DS2SP column (8.0 × 300 mm), with UV (254 nm) and fluorescence detection (λ_{Exit} = 336 nm; λ_{Emiss} = 536 nm). The radioactive fractions were pooled, resuspended in water, and counted to obtain the yield. Following lyophilization, the purified derivative was analyzed by ^{1}H-NMR. ^{1}H-NMR: (**10**) δ 5.10 ($J_{1,2}$ = 5.25 Hz, 0.2 H, d, αH1), 4.50 ($J_{1,2}$ = 9.56 Hz, 0.8 H, d, βH1), 1.28 (0.6 H, s, α-methyl CH_3), 0.92 (2.4 H, s, β-methyl CH_3); other resonances were not assigned.

5 (10.2 μmol) was dissolved in 100 μL of 100 mM sodium carbonate–bicarbonate buffer, pH 8.5, and stirred at 0 °C. A 400-μL aliquot of 5(6)-carboxyfluorescein succinimido ester (FNHS) (32.5 mg/mL in DMF) was then added. Following

incubation at room temperature overnight, the reaction mixture was dried under vacuum, resuspended in 1 mL of water, acidified using glacial acetic acid, and extracted with ether. The ether fractions were washed with 1 mL of water and the water washings combined and rotoevaporated to dryness. The mixture obtained after ether extraction was purified by reverse-phase HPLC. Three peaks purified using this procedure were pooled, lyophilized, and analyzed using NMR. ^{1}H-NMR (**11**): (Fraction I) aromatic region, δ 8.2–6.5; 5.36, 5.26, 5.09 ($J_{1,2}$ ~ 9 Hz, βH1, 3 species); 4.02, 3.95, 3.82 (H2, 3 species); 3.92–3.58 (H3–H6′); 2.05 (methyl CH_3). (Fraction II) δ 5.29, 5.24, 5.15 ($J_{1,2}$ ~ 3.5 Hz, αH1, 3 species); other resonances were not assigned. Fraction III was not analyzed.

N-Chloroacetylation of Glycosylamines: 2-Acetamido-1-(chloroacetamido)-1,2-dideoxy-β-D-glucopyranose (**12**) *and Ammonolysis to 2-Acetamido-1,2-dideoxy-1-(N-glycinamido)-β-D-glucopyranose* (**13**). **5** (10 μmol) was resuspended in 100 μL of 1 M sodium bicarbonate followed by the addition of a 10-fold molar excess of chloroacetic anhydride. The pH was monitored using pH paper and additional base added if the pH dropped below 7.0. After 2 h at room temperature, a seond aliquot of base and anhydride was added. After a further 6 h, the mixture was desalted by passage over a mixed bed of Dowex AG50-X12(H^+) and AG3-X4A(OH^-) ion-exchange resins. The eluant was collected and evaporated to dryness. Recovery of radioactivity from the column following chloroacetylation was >95%. The mixture was analyzed using amino-phase HPLC as described by Mellis and Baenziger (1983) and by ^{1}H-NMR in D_2O. ^{1}H-NMR: δ 5.08 (1 H, d, βH1), 4.12 (2 H, m, CH_2), 3.88–3.49 (6 H, m, H2′–H6′), 2.01 (3 H, s, CH_3).

The mixture containing **12** (5 μmol) was stirred in saturated ammonium carbonate either at room temperature or at 50 °C in a sealed tube to prevent loss of ammonia by evaporation (Cheronis & Spitzmüeller, 1941). The reaction was monitored by amino-phase HPLC. The ammonium carbonate was then removed by evaporation and the putative 1-N-glycyl derivative purified using a short column of CM-Sepharose Fast Flow (Pharmacia), eluted first with water to elute the free sugar and then with 0.5 M ammonium carbonate. A portion of the purified N-glycyl derivative **13** was then N-acetylated and desalted as described above. The mixture was lyophilized before analysis by NMR in DMSO-d_6. ^{1}H-NMR: δ 7.84 (1 H, d, NH1), 7.88 (1 H, d, NH2), 8.09 (1 H, d, glycyl αNH).

Comparison of the Efficiency of Formation of Fluorophore Conjugates of the Glycosylamine **5** *and 1-N-Glycyl Derivative* **13**. The efficiencies of N-acylation of the glycosylamine and N-glycyl derivatives of N-acetylglucosamine were compared by preparing their N-dansyl and fluorescein derivatives as described above. **5** and **13** (5 μmol of each) were treated as described above for the synthesis of **10** and **11**. The reaction products **10**, **11**, **14**, and **15** were then separated using C18 reverse-phase HPLC. Fractions containing the starting material and the fluorescent conjugates were then pooled, dried, and counted to obtain the overall yields based on starting sugar.

RESULTS

Formation and Characterization of Glycosylamines. Formation of the glycosylamines **5**–**8** was performed as described in Materials and Methods. The time course of the formation of radiolabeled **5** showed that the reaction reached a maximum at approximately 85% modification after 96–120 h. The kinetics of formation measured using the resin-binding method were in close agreement with determinations by NMR (Figure 2). TLC analysis of this reaction mixture (solvent A) showed a major ninhydrin-sensitive component with an R_f value of 0.41. TLC analysis of the mixture of **7** (solvent B) and orcinol staining revealed a major component which was also ninhydrin sensitive (**7**, R_f ~0.05) and a small amount of the starting oligosaccharide **3** with R_f ~0.4 (data not shown).

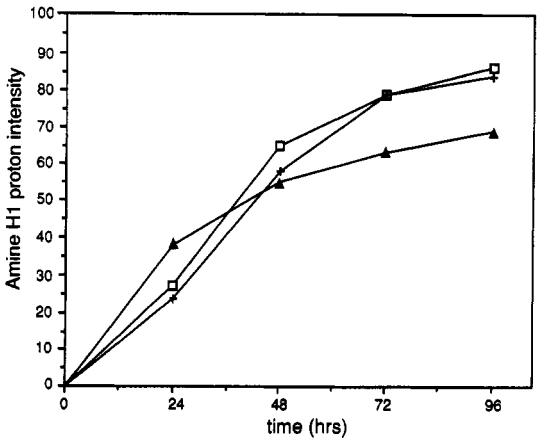

FIGURE 2: Time course of formation of glycosylamine derivatives **5** (GlcNAc, □), **6** (*N,N′*-diacetylchitobiose, +), and **7** (biantennary Gal-2, ▲) obtained by measurement of the βH1 proton (δ = 4.14 ppm) intensity for the derivative and comparison with a unit intensity integral. Yields of oligosaccharide glycosylamines were generally 70–80%.

Glycosylamines have previously been isolated from ammonium bicarbonate solutions by binding to cation-exchange resins and recovery by elution with ammonia (Lihkosherstov et al., 1986; Kallin et al., 1989). However, we have been unable to recover glycosylamines in good yields using either Amberlyst-15 or Dowex AG50-X12(H^+) resins. The latter observation was used as the basis of the binding assay described above. Complete removal of the salt from the sugar is essential, since ammonia would inhibit subsequent N-acylation reactions. In practice, direct lyophilization of the reaction mixture proved to be the most simple and nonselective method for desalting.

^{1}H-NMR analysis of **5** gave a spectrum (Figure 3) in accordance with the expected structure, with simplification of the H2–H6′ resonance envelope reflecting the transition from an anomeric mixture of **1** to a product which exists predominantly as one anomer. The resonances of the anomeric protons of the starting monosaccharide at 5.19 ppm (α) and 4.70 ppm (β) were reduced in intensity, and a single anomeric resonance was observed at 4.14 ppm ($J_{1,2}$ = 9.78 Hz; βH1 proton of the product glycosylamine). The H2 proton is also shifted upfield in the glycosylamine (3.61 ppm) compared to the starting sugar (3.66 ppm), and the other ring protons (H3–H6′) show slight upfield shifts (~0.01 ppm) from the values for the free monosaccharide.

The β-anomeric form of **5** and the starting monosaccharide are the major components of the product mixture, but the anomeric region of the spectrum contained a number of other minor resonances. A low-intensity doublet resonance could be identified at 4.74 ppm ($J_{1,2}$ ~ 9.3 Hz). Kallin et al. (1989) have assigned this resonance to the H1 proton of the carbamate salt of the glycosylamine, which they suggested to be the final product of the reaction of ammonium bicarbonate with the sugar. They showed that the carbamate was converted to the free amine on exposure to an acidic cation-exchange resin. This resonance was less than 5% of unit intensity, suggesting that if it is a major intermediate in the formation of the glycosylamine, lyophilization is sufficient to cause its decomposition into carbon dioxide and the free amine. The latter can therefore be obtained without recourse to exposure of the

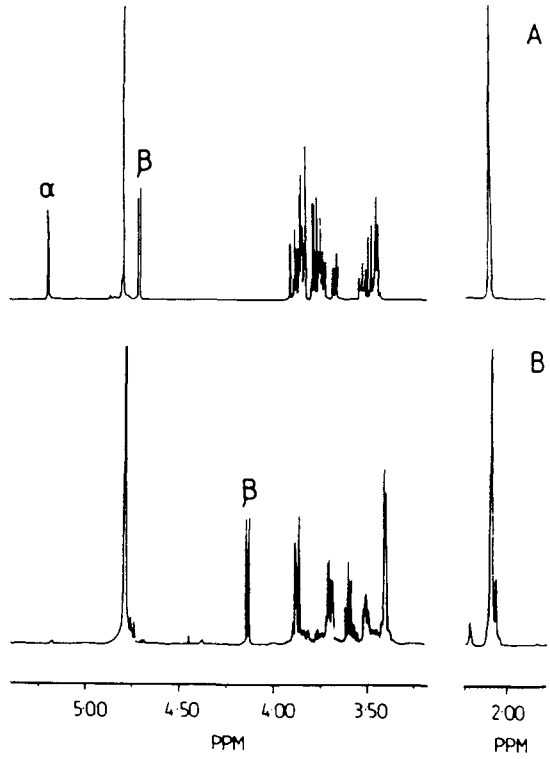

FIGURE 3: 1-D NMR spectrum of the glycosylamine derivative of *N*-acetylglucosamine (**5**) obtained after 96-h incubation in ammonium bicarbonate (lower panel). Comparison with the starting sugar (**1**) (upper panel) indicates a decrease in intensity of the anomeric protons of the free sugar (5.19 ppm-α and 4.70 ppm-β) and the formation of the β-glycosylamine (H1 = 4.14 ppm) and the carbamate salt (4.74 ppm). Significant changes are also apparent in the H2–H6′ signal envelope (3.3–3.8 ppm.), consistent with the formation of a product which exists essentially in a single (β) anomeric configuration.

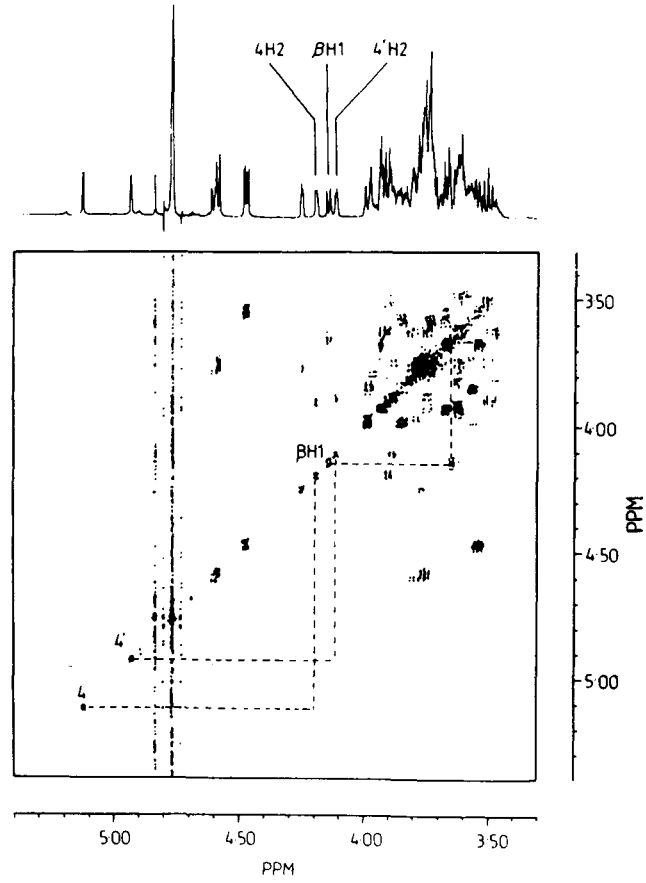

FIGURE 4: 2-D COSY spectrum of the glycosylamine derivative of *N*-acetylglycosamine (**5**) showing the connectivity network from the βH1 proton at 4.14 ppm used in assignment. Shifts are relative to TMS = 0.0 ppm, 300 K.

mixture to an acidic environment, lessening the twin problems of dimerization and hydrolysis of the glycosylamine associated with this treatment.

The α-anomer of the glycosylamine could not be identified unambiguously. A potential candidate for the H1 resonance of this structure was apparent at 4.79 ppm ($J_{1,2}$ = 5.12 Hz), in close proximity to the solvent. Analysis of this component in the 2-D COSY spectrum (Figure 4) indicated that it shows a cross peak with a signal at 3.92 ppm, but couplings beyond this putative H2 signal could not be identified. Kallin et al. (1989) reported that no α-anomeric structures could be identified in their glycosylamine preparations. Two further very low-intensity signals (~0.5%) at 5.11 and 5.02 ppm could be identified in some spectra of this derivative, corresponding to the α- and β-H1 signals for *N*-acetylmannosamine, suggesting that epimerization at C2 occurs to a slight extent in this system. Neither the α-anomeric or C2-epimeric products were reported by Lihkosherstov et al. (1986); however, these authors relied on the selective crystallization of the product, presumably from a mixture of components.

The primary amino function of the glycosylamine could not be observed directly by NMR in DMSO-d_6 because of exchange broadening, but the identity of the products of the ammonium bicarbonate reaction was confirmed by trapping the amino function by N-acetylation (Risley & Van Etten, 1985; Lihkosherstov et al., 1986). N-Acetylation of **5** afforded the expected β-1,2-diacetamido derivative **9** in 76% yield. ^{1}H-NMR in D_2O revealed no firm evidence of the equivalent α-anomeric structure in either the 1-D or 2-D spectra, although a candidate αH1 doublet was observed at 5.70 ppm ($J_{1,2}$ = 5.2 Hz). This signal was of approximately 0.5–1% integral intensity, and its coupling network could not be traced. Analysis of **9** in DMSO-d_6 revealed the expected NH signals with chemical shifts and coupling constants in accordance with previous determinations for acetamido sugars in this solvent (Bush et al., 1980).

The 1-D spectrum of the glycosylamine derivative of *N*,*N*′-diacetylchitobiose (**6**) indicated that resonance associated with the reducing-terminal residue undergo similar shifts to those observed for *N*-acetylglycosamine, with the GlcNAc-1 H1 resonance shifting upfield to 4.14 ppm. The H1 proton of GlcNAc-2 (nonreducing) is sensitive to the anomericity of GlcNAc-1, and since the starting material exists as a mixture of the two anomers, this proton gives rise to two superimposed doublets (4.594 ppm-α and 4.585 ppm-β; Boyd et al., 1985). Formation of the β-anomeric glycosylamine results in the partial collapse of this multiplet into a doublet at 4.58 ppm. Further assignments of this derivative were not made.

NMR spectra of the glycosylamine derivative of the Gal-2 oligosaccharide (**7**) are shown in Figure 5. Comparison with the 1-D spectrum of the starting oligosaccharide **3** [partially assigned by Vliegenthart et al. (1983)] indicates changes associated with the conversion of the reducing-terminal residue to the amine. The H1 resonance of GlcNAc-1(NH_2) falls between the H2 protons of the Man-4 (4.19 ppm) and the 4′ residue (4.10 ppm) at 4.14 ppm ($J_{1,2}$~9.6Hz). Integration of this resonance indicates that, after 96 h, about 75% of the sugar is converted to the amine, in good agreement with the kinetics obtained from the cation-exchange resin binding assay with the radiolabeled monosaccharide. Similar kinetics were also obtained in NMR analyses of the formation of **5** and **6** (see Figure 2), suggesting that the time course of the reaction

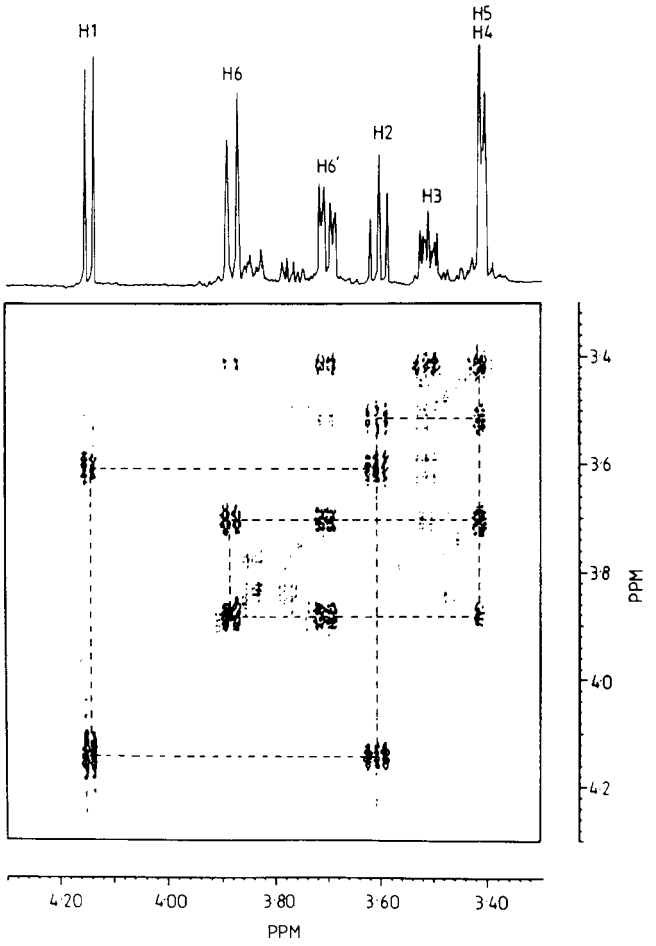

FIGURE 5: 1-D and 2-D NMR spectrum of the oligosaccharide glycosylamine derivative **7** obtained after 96-h incubation in ammonium bicarbonate, showing the H1–H2 connectivities of the anomeric proton (δ = 4.14 ppm) of GlcNAc-1 and those of the Man-4 (H1 = 5.12 ppm) and 4′ (H1 = 4.92 ppm). Comparison with the starting sugar **3** indicates a decrease in intensity of the anomeric protons (5.19 ppm-α and 4.70 ppm-β) and the formation of the glycosylamine (H1 = 4.14 ppm) and the carbamate salt (4.74 ppm). Changes are also apparent in the H2–H6′ signal envelope for GlcNAc-1 and the subterminal GlcNAc, indicating that **7** exists essentially in a single (β) anomeric configuration. In the fucosylated derivative **8**, the H1 proton of GlcNAc-1 overlaps with the fucose H5 but can be resolved upon titration of the amine (Manger et al., unpublished data). Assignments based on Vliegenthart et al. (1983).

is independent of the type of sugar. The ^{1}H-NMR spectra of oligosaccharide glycosylamine derivatives are described in the following paper (Manger et al., 1992).

Direct condensation of **1** with free ammonia in methanol (Isbell & Frush, 1958) afforded the desired glycosylamine **5** in approximately 15% yield. ^{1}H-NMR analysis also indicated that while approximately 55% of the sugar remained as free *N*-acetylglucosamine, an additional 20% of the monosaccharide was converted to the bisglycosylamine [δ_{H1} = 4.32 ppm: d, $J_{1,2} \sim 9.28$ Hz] during the treatment. A number of additional anomeric signals apparent in this mixture at δ = 4.82, 4.52, and 4.10 ppm were not assigned. Clearly the ammonium bicarbonate method offers improved yields and minimizes potential side reactions.

Synthesis of 2-Acetamido-1,2-dideoxy-1-[(dimethylamino)naphthalenesulfonamido]-β-D-glucopyranoside (**10**). N-Dansylation of **5** was performed as described in Materials and Methods. Reverse-phase HPLC analysis of the reaction products revealed two radioactive peaks. The major radioactive peak eluted in the column breakthrough (Figure 6, lower panel) and consisted of a mixture of free *N*-acetylglucosamine and the residual glycosylamine. The *N*-dansyl monosaccharide derivative (**10**) appeared as a second radioactive peak (with associated UV absorption) eluting at 27.0 min. A large peak of dansylsulfonic acid (Dans-OH) derived from hydrolysis of the reagent eluted at 15–17 min (upper panel). Typical yields of the derivative obtained using this method were 10–15% based on starting sugar.

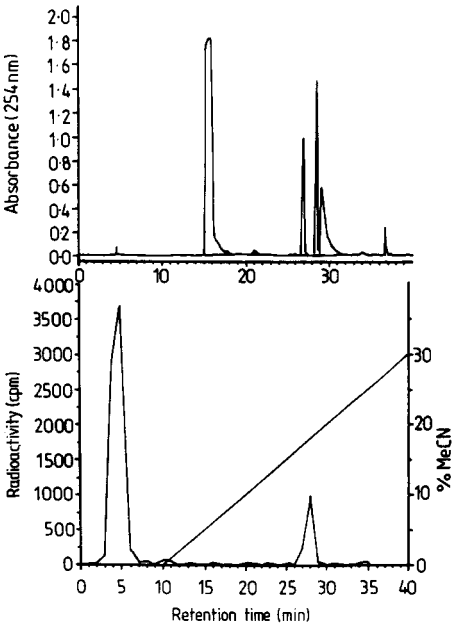

FIGURE 6: Separation of dansylation products by reverse-phase HPLC. Aliquots of the reaction mixture were applied to a Spherisorb S50DS2SP column equilibrated in water containing 0.1% TFA. The flow rate was 2.65 mL/min. After a 5-min wash at the start conditions, products were eluted using a gradient of acetonitrile (containing 0.1% TFA) from 0% to 30% at 1%/min. One-minute fractions were collected. Detection was by UV (254 nm) (upper panel) and by scintillation counting aliquots of the eluate (lower panel).

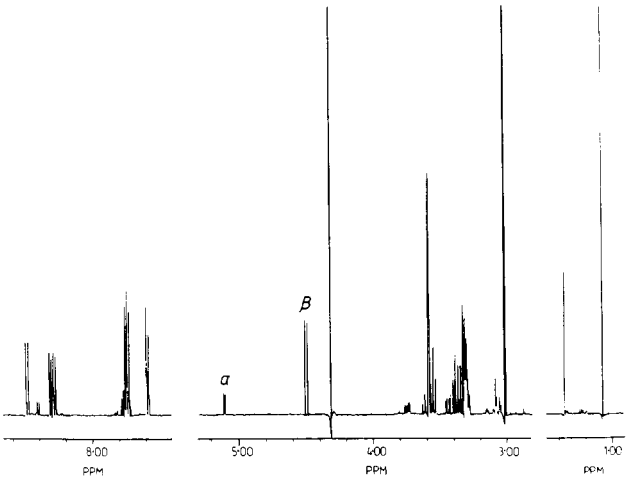

FIGURE 7: 1-D ^{1}H-NMR spectrum of *N*-dansyl derivative of *N*-acetylglucosamine (**10**) at 330 K, pH 4.5.

Analysis of the 1-D ^{1}H-NMR spectra (Figure 7) of the putative *N*-dansyl derivative **10** indicated the presence of both α- and β-anomeric forms which cochromatograph on reverse-phase HPLC. The anomeric region contained two doublet resonances at 4.48 ppm (approximately 80% of unit intensity) and 5.10 ppm (approximately 20% of unit intensity). Examination of the coupling constants suggests that the major signal ($J_{1,2}$ = 9.56 Hz) corresponds to a β-anomer structure, while the minor component ($J_{1,2}$ = 5.25 Hz) appears to adopt

an α-anomeric configuration. Coupling constants of this magnitude may characterize α-anomeric glycosylamine derivatives, since a similar $J_{1,2}$ value was obtained for the putative α-anomeric structure identified in the 1,2-diacetamido derivative. The coupling constant of the β-anomer of the dansyl derivative ($J_{1,2}$ = 9.56 Hz) compares with that of the β-D-glycosylamine ($J_{1/2}$ = 9.78 Hz) and is similar in magnitude to other β-linked derivatives. The acetamidomethyl singlet was also split into two components with a 4:1 ratio of intensities.

Synthesis of 2-Acetamido-1,2-dideoxy-1-fluoresceinamido-β-D-glucopyranoside Derivatives (**11**). The 5(6)-carboxyfluorescein conjugate **11** was prepared using the FNHS reagent as described in Materials and Methods. TLC analysis of the reaction mixture (solvent A) indicated that **11** ran as a band with R_f 0.85–0.90 and was not resolved from some residual fluorescent material remaining after ether extraction. TLC analysis of the glucosamine derivative obtained under the same conditions indicated the formation of the fluorescent derivative (R_f ~0.90) in approximately 85% yield (data not shown).

Preparative C18 reverse-phase HPLC fractionation of **11** yielded three radioactive fractions (Figure 8a,b). NMR analysis indicated that each peak contained a complex mixture of components. Only fractions I and II gave spectra in which monosaccharide resonances could be identified, while fraction III appeared to contain a large quantity of free fluorophore. In the spectrum of fraction I, three doublet anomeric resonances were observed at 5.35, 5.24, and 5.09 ppm ($J_{1,2}$ = 9.66, 9.82, and 9.78 Hz, respectively) and therefore appeared to consist of conjugates in which the monosaccharide adopted the β-anomeric configuration. By contrast, three anomeric doublets at 5.29, 5.24, and 5.15 ppm ($J_{1,2}$ = 3.5, 3.46, and 3.42 Hz, respectively) were observed in the spectrum of fraction II. No anomeric resonances with larger coupling constants could be identified, suggesting that this fraction consisted solely of α-anomeric fluorophore derivatives.

The FNHS reagent therefore appeared to yield three species in each of the two anomeric configurations, which in contrast to the dansyl derivatives could be completely separated by HPLC. Quantitation of fractions I and II by scintillation counting suggested that the ratio is approximately 2.5:1 β- to α-anomer, presenting additional evidence that the formation of products in which the α-anomer is a significant component may be a general property of the interaction of glycosylamines with aromatic ring systems (such as fluorophores) in aqueous media in which mutarotation of the glycosylamine can occur. Similar results were obtained in attempts to modify glycosylamines with fluorescein isothiocyanate (data not shown).

Synthesis of 2-Acetamido-1,2-dideoxy-1-(N-glycinamido)-β-D-glucopyranose (**13**). N-Chloroacetylation of a sample of **5** afforded the expected 1-(chloroacetamido) derivative **12** in an overall yield of ~75%. NMR analysis of the reaction mixture indicated the presence of a doublet resonance at 5.08 ppm ($J_{1,2}$ = 9.77 Hz) corresponding to the βH1 proton of **12**. An additional doublet resonance of approximately 0.5% intensity was be observed at 5.77 ppm ($J_{1,2}$ ~ 5 Hz). This may be the H1 resonance of a putative α-anomeric structure. Further assignments of this component were not possible owing to its very low intensity. **12** was therefore found to be almost exclusively in the β-anomeric configuration. Owing to the resonance stabilization of the amide bond, this anomer ratio is retained throughout subsequent manipulations.

Ammonolysis of the chloroacetamido function in saturated ammonium carbonate was used to convert **12** to the 1-*N*-glycyl β-derivative **13**. The reaction was found to be extremely slow at room temperature but gave rise to a single product,

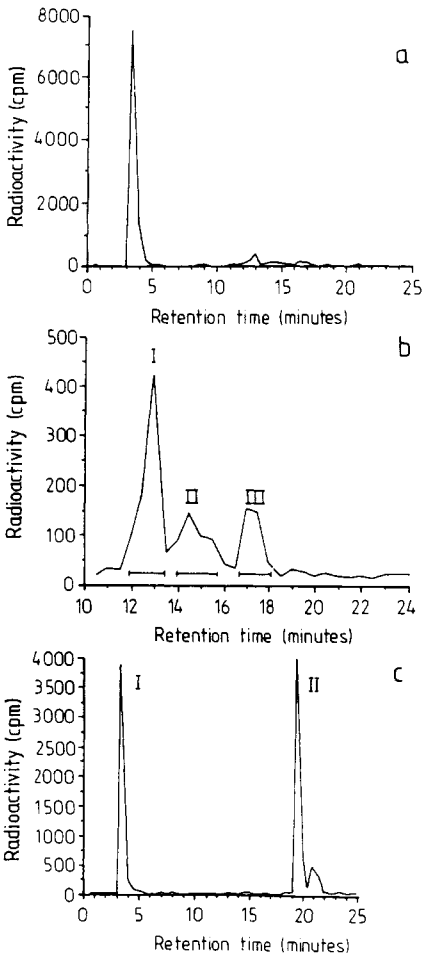

FIGURE 8: Reverse-phase HPLC separation of FNHS-GlcNAc-NH_2 reaction products. The ether-extracted mixture was applied to an S50DS2SP column (8 × 250 mm) equilibrated in 70% water/30% MeCN. The flow rate was 2.0 mL/min. After a 5-min wash under these conditions, the column was eluted using a linear gradient of MeCN at 1%/min to 50%. Fractions (0.5 min) were collected. Aliquots (50 μL) were withdrawn and counted to obtain the profile in (a). Expansion of the radioactivity elution profile between 10 and 25 min shows the three fluorophore fractions (b) which were subsequently analyzed by NMR. (c) Reverse-phase HPLC of 1-(*N*-glycylamino)-β-*N*-acetylglucosamine–fluorescein conjugate (**15**) (compare with panel a). An aliquot of the reaction mixture following ether extraction was applied to the column. (I) Free *N*-acetylglucosamine and glycyl derivative (18% of total radioactivity). (II) Fluorescein conjugate; the heterogeneity in this peak may reflect different fluorescein isomers in the FNHS prep. These were also identified in the conjugate prepared by direct acylation of the glycosylamine with FNHS.

suggesting that the use of a significant excess of ammonia did not promote the formation of higher amines. The reaction rate was improved considerably when the mixture was subjected to ammonolysis at 50 °C (Cheronis & Spitzmüeller, 1941). The reaction was essentially completed after only 8 h. ^{1}H-NMR analysis confirmed the structure of 1-*N*-glycyl β-derivative.

For comparison of the β-glycosylamine and the 1-*N*-glycyl β-derivatives as acceptors in the formation of probes, samples of each were converted to their dansyl and carboxyfluorescein derivatives and purified as previously described (compare panels a and c of Figure 8). The addition of a short linker arm using the chloroacetylation–ammonolysis technique enabled the formation of the *N*-dansyl monosaccharide **14** in approximately 70% yield from **1** and the carboxyfluorescein conjugate **15** in about 55% yield from **1**. For comparison, direct acylation of the amine with these fluorophores yielded

approximately 10% of **10** and **11**, which consists of mixtures of both anomers. These results indicate that *N*-glycyl β-derivatives have improved properties over the free β-glycosylamine in terms of their reactivity toward complex aromatic fluorophores. In addition, the β-anomeric configuration is fixed prior to formation of the conjugate, and the problem of the creation of products with the "incorrect" anomeric configuration (i.e., α) is therefore substantially eliminated.

DISCUSSION

In order to examine the potential of glycosylamines as the starting point for the generation of glycoconjugate probes, we have investigated the synthesis of fluorescent derivatives. Such derivatives have many potential applications, e.g., the use of the increased sensitivity offered by fluorescence over other detection methods (such as radiolabeling or UV spectroscopy) in the separation, isolation, and structural analysis of oligosaccharides. For these applications, pyridylamino derivatives of oligosaccharides generated by reductive amination have proved useful and found widespread application in a number of analytical applications. They offer improved detection sensitivity over conventional tritium-radiolabeling techniques and can be separated by reverse or bonded aminophase HPLC (Hase et al., 1979, 1984; Tomiya et al., 1988; Oku et al., 1990). Both pyridylamino oligosaccharides (Morita et al., 1988) and the fluorescent lactose derivative 2-aminopyridylaminoethyl 4-lactoside (Sato et al., 1988) have been used for the purpose of assaying glycosyltransferase activities.

The application of fluorescent neoglycoproteins bearing mono- and disaccharides to identify cell surface carbohydrate binding components by fluorescence microscopy and flow cytometry has been studied by a number of workers, notably Gabius et al. (1987) and Monsigny et al. (1988). In an alternative approach, Yednock et al. (1987a,b) employed fluorescent beads coated with a phosphomonoester–mannan fragment rich in mannose and mannose 6-phosphate to identify a lymphocyte homing receptor related, or identical, to the 90K glycoprotein bearing the MEL-14 antigen. While the application of fluorescent derivatives of free oligosaccharides to studies of this type has not so far been investigated, it is possible that targeted drug delivery studies would benefit from the availability of derivatives of more complex oligosaccharides bearing probes that could be interrogated by flow cytometry.

The methodology discussed in this paper enables glycosylamine derivatives of oligosaccharides to be obtained in high yield and is clearly superior to previously available methods involving either direct condensation (Isbell & Frush, 1958) or multistep synthesis via the protected glycosyl azide (Nakabayashi et al., 1988). The improved efficiency of the ammonium bicarbonate method over condensation in ammonia–methanol has been suggested to be a consequence of the sequestration of the amine as the carbamate or its salt (Kallin et al., 1989). Reduction of the concentration of the free glycosylamine would (1) shift the position of the equilibrium away from formation of the acyclic immonium ion suggested to be intermediate in both hydrolysis and dimerization and (2) decrease the availability of the free amine as the nucleophile in dimerization to form the bis structure. The carbamate salt appears to decompose during the subsequent workup, either by exposure to cation-exchange resins or simply through lyophilization. The latter procedure may hold advantages over attempts to purify the amine using ion-exchange resins, since exposure to mild acidic conditions promotes both hydrolysis and formation of the dimer. Provided subsequent manipulations of the amine are performed with adequate buffering, these undesirable side reactions are insignificant. We have shown that this procedure can be equally well applied to the formation of glycosylamines of purified complex oligosaccharides such as **3** and **4**, thereby providing valuable intermediates for the synthesis of a wide range of oligosaccharide probes and derivatives by acylation of the 1-amino function.

The low reactivity of the 1-amino function with fluorophores was found to correlate with the formation of products which consisted of mixtures of both anomeric forms. In a previous report of the synthesis of the *N*-dansyl monosaccharide **10** (Risley et al., 1988), a similar yield was obtained (approximately 13% following purification). However, these authors reported that less than 5% of the product adopted the α-configuration. In our hands, the α-anomer was approximately 20% of the product. The discrepancies suggest that the anomeric configuration is difficult to control, such that N-dansylation of glycosylamines which exist exclusively as the β-anomer leads to the formation of a mixture of products consisting of both anomeric forms.

A possible explanation for the observed ratio of the two anomeric forms of the fluorescent monosaccharide derivatives is that these represent an equilibrium established between the two anomers by mutarotation. However, mutarotation is strongly dependent upon the availability of electrons from the nitrogen atom and cannot occur in N-acylated derivatives in which there is resonance stabilization of the amide bond. The observed incidence of the α-anomer is considerably greater than that for equilibrium mixtures of other glycosylamines with bulky substituents [reviewed in Paulsen and Pflüghaupt (1980)], suggesting that the observed ratio of the two forms is more likely to arise through some other mechanism.

Alternatively, the α-form of the glycosylamine may react significantly more rapidly with the fluorophore. The glycosylamine would undergo rapid mutarotation under the mild basic conditions employed, and as a result the anomer ratio in the final product does not reflect that of the starting compound. The observed differences in the rates of reaction and the anomeric ratio of products in the reactions with less hindered electrophiles such as acetic anhydride suggest that this rate dependence may result from steric effects. While steric arguments would suggest that the favored interaction of the two reacting species would occur with the amino function in the equatorial position, i.e., the β-anomer, steric control of the rates of breakdown of the intermediate generated from each anomer might also account for the observed ratio. For practical purposes, the slow reaction with aromatic fluorophores and establishment of product mixtures to which the "biologically incorrect" anomer contributes significantly represent a significant problem in establishing a general methodology for the use of glycosylamines to obtain glycoconjugate probes.

In addition to steric effects on the rate and outcome of the reaction, electronic effects may contribute to the significant differences in reactivity between amino functions at C2 and C1. The pK_a values for the two amino groups (C1 = 5.2, C2 = 7.7) suggest that the 1-amino function is a comparatively poor nucleophile. However, it reacts rapidly and quantitatively with acetic anhydride. Although the product of this reaction (the 1,2-diacetamido derivative) cannot be further modified, this observation suggested that the more useful *N*-(haloacetamido) derivatives could also be obtained in high yields and with retention of the anomeric configuration of the starting glycosylamine. Since steric effects appeared to play an

important role in determining the outcome of the reaction, a linker introduced via the haloacetamido function was used to move the site of N-acylation away from the anomeric center. Ammonolysis was found to efficiently replace the halogen atom and yields the 1-*N*-glycyl β-derivative. Fluorescent glycoconjugates were obtained in much higher yields via the 1-*N*-glycyl β-sugar than by direct acylation. This methodology also eliminates the problem of the synthesis of products with the "incorrect" anomeric configuration. The linker itself is structrally analogous to the N-glycosidic linkage between the carbohydrate and the asparagine side chain of glycoproteins. It should therefore be stable under physiological conditions. An additional advantage is that *N*-glycyl β-derivatives are more stable than the glycosylamine, both for storage purposes and in handling during coupling. Haloacetamido derivatives themselves can also be used to alkylate thiols (Thomas, 1977).

N-(Haloacetamido) and *N*-glycyl derivatives of monosaccharides have been synthesized by other workers (Paul et al., 1980; Cowley et al., 1971). In both of these reports, the derivatives were obtained through the protected glycosylamines, and in the case of the *N*-glycyl derivative by direct coupling of the glycosylamine to the *N*-benzyloxycarbonyl)-protected amino acid, followed by deprotection of the amino function. In neither case were the properties of these as intermediates in the formation of carbohydrate derivatives recognized or exploited. The method described here offers superior yields to those previously reported, primarily because the glycosylamine can be obtained in a simple one-step condensation. We have also shown that larger oligosaccharides can be converted to these synthetically useful intermediates in acceptable yields.

The potential therefore exists for the generation of a myriad of different oligosaccharide derivatives tailored for different applications.

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Registry No. **1**, 14131-68-1; **2**, 35991-83-4; **3**, 71496-52-1; **4**, 78392-81-1; **5**, 4229-38-3; **6**, 102039-77-0; **7**, 133677-64-2; **8**, 143216-73-3; **9**, 6205-72-7; **10**, 133677-70-0; **11**, 143216-74-4; **12**, 73982-39-5; **13**, 6255-72-7; **14**, 143216-75-5; **15**, 143216-76-6; FNHS, 117548-22-8; dansyl chloride, 605-65-2.