Synthesis of 1-*N*-Glycyl β-Oligosaccharide Derivatives. Reactivity of *Lens culinaris* Lectin with a Fluorescent Labeled Streptavidin Pseudoglycoprotein and Immobilized Neoglycolipid†

I. D. Manger,‡ S. Y. C. Wong, T. W. Rademacher, and R. A. Dwek*

Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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ABSTRACT: The lectin from *Lens culinaris* (lentil) has a binding specificity for glycopeptides bearing 6-O-linked fucose on the reducing terminus on complex-type N-linked oligosaccharides. Lentil lectin therefore provides an excellent example of a carbohydrate binding protein in which high-affinity interactions are dependent on the integrity of the oligosaccharide core structure. We report here the synthesis of the 1-*N*-glycyl β-derivative of Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4(Fucα6)-GlcNAc (Gal-2F) and its subsequent biotinylation and palmitoylation. The biotin derivative when bound to a streptavidin–fluorescein isothiocyanate (FITC) conjugate was able to bind to both concanavalin A (ConA) and lentil lectin affinity columns. In contrast, synthesis of the biotin derivative of the glycamine derivative of Gal-2F and subsequent binding to streptavidin–FITC afforded reactivity to a ConA affinity column but not to a lentil lectin affinity column. Lentil lectin also bound to plastic microtiter plates containing the adsorbed palmitoyl-1-*N*-glycyl β-derivative. No binding occurred when the homologous glycamine neoglycolipid was used. These results suggest the 1-*N*-glycyl β-derivative of oligosaccharides may have general utility as an intermediate in the synthesis of novel glycoconjugate probes.

Chemical derivatives of N-linked oligosaccharides can provide valuable tools for the analysis of their structure and function. A variety of methods for their formation are available [reviewed by Stowell and Lee (1981)], of which reductive amination is the most commonly employed. This procedure involves the formation of a covalent linkage by reduction of a Schiff adduct between the acyclic aldehyde form of the sugar and a primary amine and, therefore, leads to the destruction of the cyclic structure of the reducing terminal residue. This may affect the biological activity or immunogenicity of these structures.

In addition to the creation of nonbiological determinants by ring-opening events, the recognition of the chitobiosyl core or the α1–6- and α1–3-linked fucose residues commonly found attached to the reducing terminal residue may also be affected. At least two cases of high-affinity interactions between a lectin and an oligosaccharide have been proposed to depend on recognition of these residues. The lectin from common lentil (*Lens culinaris*) was initially shown to bind to α-mannose and α-glucose residues by agglutination assay (Makela, 1957). More precise definition of the specificity was provided by Kornfeld et al. (1981), who showed that lentil lectin has a high affinity for certain glycopeptides bearing 6-O-linked fucose on the reducing-terminal *N*-acetylglucosamine but not the analogous afucosylated structures. Yamamoto et al. (1982) confirmed that the lectin will bind to free oligosaccharides bearing these determinants, but they also observed that the integrity of the reducing-terminal *N*-acetylglucosamine residue affects the affinity of the interaction. Reduction of this residue to the alditol abolished binding to the immobilized lectin.

High-affinity interactions between glycopeptides containing N-linked oligosaccharides and the lectin from the common garden pea (*Pisum sativum*) are also partially dependent on the presence of α1–6-linked core fucose residue. However, in this case, the integrity of the linkage to peptide also appears to be important, since the immobilized lectin will not bind to free oligosaccharides, whether they are reduced or not (Yamamoto et al., 1982).

Fucose is not an effective competitive inhibitor for either of these two lectins, suggesting that this high-affinity interaction is not dependent upon the occupation of a subsite which simply binds fucose. Instead, it is possible that this core-dependent interaction involves a composite epitope with contributions from both the fucose and intact *N*-acetylglucosamine residues (in the case of pea lectin, the peptide also appears to contribute).

Lentil lectin therefore provides an excellent example of a carbohydrate binding protein in which high-affinity interactions are dependent on the integrity of the oligosaccharide core structure. That the high-affinity interaction between lectin and carbohydrate is abolished by reduction of the GlcNAc[1] residue to the alditol suggests that it may be difficult (if not impossible) to demonstrate binding to lentil lectin using carbohydrate probes generated by chemistry involving ring-opening events [e.g., reductive amination, such as in the neoglycolipid approach developed by Stoll et al. (1988)]. We have attempted to test this hypothesis and the effectiveness of glycosylamine derivatives as ligands in this interaction. In contrast to derivatives that can be obtained through reductive amination, glycosylamines retain a closed-ring structure and adopt the β-anomeric configuration observed in the N-glycosidic linkage between the carbohydrate and protein (Manger et al., 1992). Glycosylamines can therefore be chemically exploited to obtain a range of probes which should retain the

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‡ Present address: Department of Microbiology, St. Louis University Medical School, 1402 Grand Blvd., St. Louis, MO 63104.

[1] Abbreviations: ConA, concanavalin A; FITC, fluorescein isothiocyanate; Fuc, L-Fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HPLC, high-performance liquid chromatography; Man, D-mannose; NHS, *N*-hydroxysuccinimido ester; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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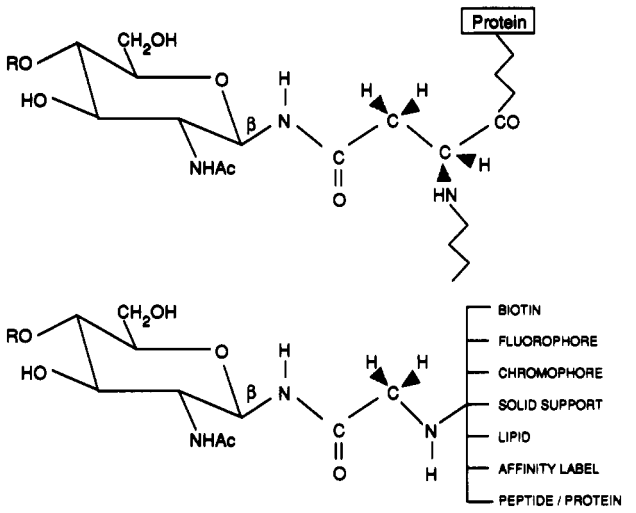


FIGURE 1: Structural similarity of the N-glycosidic linkage of oligosaccharides to peptide via an asparagine side chain (top) and the synthetic 1-*N*-glycyl β-oligosaccharide derivative to a probe (bottom).

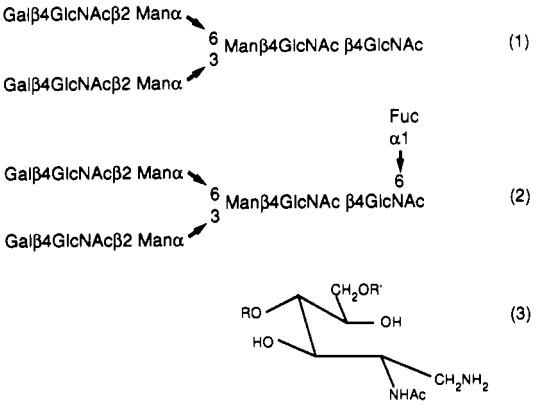


FIGURE 2: Biantennary complex-type oligosaccharides used in this study. Compound **1** is abbreviated as Gal-2 and compound **2** as Gal-2F. Compound **3** is the glycamine derivative of Gal-2F. Note the open-ring structure of the glycamine.

maximum amount of biological "information" possible (Figure 1).

MATERIALS AND METHODS

Human serotransferrin, porcine thyroglobulin, *L. culinaris* lectin–Sepharose 4B, and streptavidin–FITC conjugate were purchases from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Hydrazine and D_2O (99.96 atom %) were purchases from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.). Chloroacetic anhydride was obtained from Fluka (Glossop, Derbyshire). Bio-Gel P-4 (~400 mesh) and Dowex AG50W-X12 and AG3-X4A resins were purchased from Bio-Rad Laboratories Ltd. (Watford, Herts, U.K.). NHS-LC–biotin, NHS–biotin, and Selectispher-10 ConA high-performance lectin affinity chromatography (HPLAC) columns (5 cm × 5 cm) were obtained from Pierce and Warriner (Chester, Cheshire, U.K.). All other reagents and solvents used were AR grade or the highest purity available.

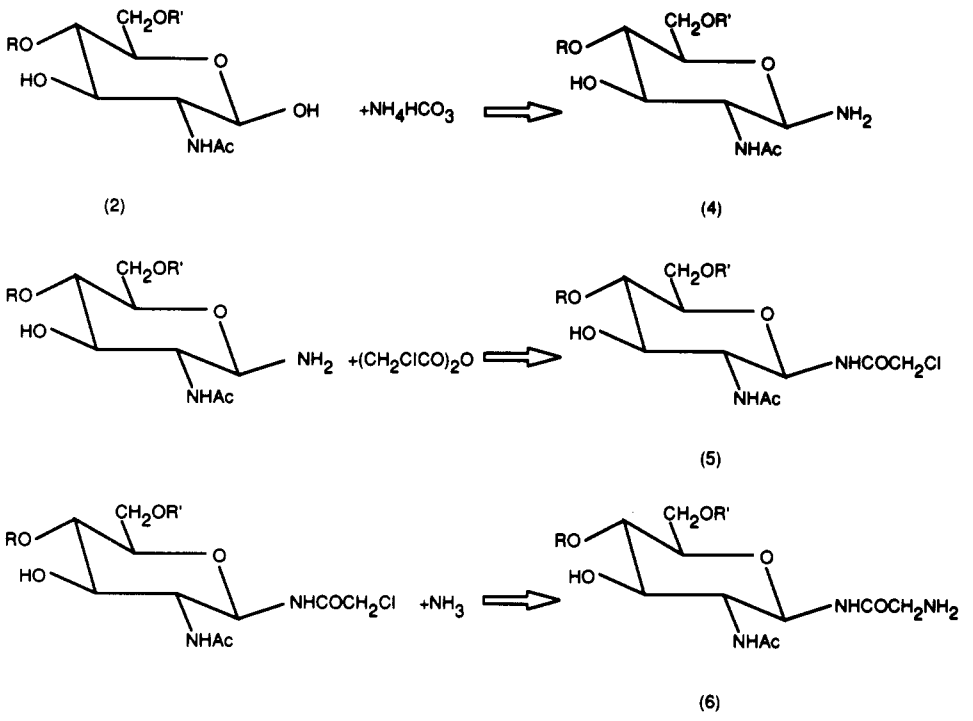
The biantennary oligosaccharide, Galβ4GlcNAcβ2Manα6-(Galβ4GlcNAcβ2Manα3)Manβ4GlcNAcβ4GlcNAc (Gal-2, compound **1**, Figure 2), and the core-fucosylated structure, Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcβ2Manα3)-Manβ4GlcNAcβ4(Fucα6)GlcNAc (Gal-2F, compound **2**, Figure 2), were obtained from human serotransferrin and porcine thyroglobulin, respectively, using hydrazinolysis and purified using Bio-Gel P-4 gel filtration chromatography. The expected structures of the purified oligosaccharides were confirmed using a combination of ^{1}H-NMR and methylation analysis methods as previously described (Ashford et al., 1987).

Thin-layer chromatography was performed using Merck 10- × 20-cm glass-backed silica 60 F254 or 5- × 7.5-cm aluminium-backed silica 60 HPTLC plates obtained from BDH Ltd. (Poole, Dorset, U.K.). TLC plates were developed in acetonitrile/water (6:4 v/v) containing 0.05% diaminobutane (solvent A). Visualization of plates was performed under UV light (254 and 366 nm) and using ninhydrin and orcinol sprays. NMR analysis and high-performance liquid chromatography were performed as previously described (Manger et al., 1992).

Synthesis of 1-N-β-Glycosylamines (**4**). Formation of the 1-*N*-β-glycosylamines of the oligosaccharides Gal-2 (**1**) and Gal-2F (**2**) was performed according to the method described previously (Manger et al., 1992) and is illustrated schematically in Figure 3. Briefly, 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 periods between 24 and 120 h at 30 °C. The glycosylamine preparations were then desalted by direct lyophilization of the reaction mixture. Lyophilized glycosylamine preparations were stored in a desiccator at −20 °C.

Synthesis of the 1-N-Glycyl β-Derivative of Gal-2F (**6**). A 100-μg sample of the glycosylamine of Gal-2F (**4**) was converted to the 1-*N*-glycyl β-derivative (**6**) via the *N*-chloroacetamido derivative (**5**) using a combination of N-chloroacetylation and ammonolysis (Figure 3). A 100-μg sample of **4** was dissolved in 100 μL of 1 M sodium bicarbonate and cooled on ice. To this was added a crystal of solid chloroacetic anhydride (~500 μg), and the reaction mixture was allowed to warm to room temperature. The pH was monitored using pH paper and additional base added if the pH dropped below 7.0. Progress of the reaction was monitored using TLC (solvent A). After 2 h at room temperature, a second 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, evaporated to dryness, resuspended in 200 μL of water prior to analysis by ^{1}H-NMR. Ammonolysis of **5** was performed as described previously except that the removal of residual ammonium salts from the ammonolysis mixture was performed by paper chromatography on Whatman 3MMChr using butan-1-ol/ethanol/water (4:1:1 v/v) overnight. The 1-*N*-glycyl β-derivative **6** was eluted from the origin.

Synthesis of the Glycamine Derivative of Gal-2F (**3**). Another portion of the Gal-2F oligosaccharide was converted to the glycamine **3** according to the method of Honda et al. (1988). A 350-μL aliquot of methanol saturated with ammonium acetate was added to a tube containing 100 μg of dried oligosaccharide. The mixture was stirred for 30 min, and then 1 mg of sodium cyanoborohydride dissolved in 50 μL of methanol was added. After stirring for 40 h, the mixture was dried under vacuum and desalted by paper chromatography as described above. TLC analysis of the mixture in solvent A indicated the presence of a major orcinol- and ninhydrin-sensitive component with R_f ~0.1. Conversion to



R = Galβ4GlcNAcβ2Manα6(Galβ4GlcNAcManα3)Manβ4GlcNAcβ
R' = Fucα

FIGURE 3: Reaction scheme for the synthesis of the 1-*N*-glycyl β-derivatives. (Top) Glycosylamine formation; (middle) N-chloroacetylation; and (bottom) ammonolysis.

the glycamine was estimated as approximately 90% efficient by visual comparison with Gal-2F standards.

Synthesis of the N-Palmitoyl Derivative of the Gal-2F Oligosaccharide. A 50-μg sample of the purified oligosaccharide **2** was converted to the glycosylamine **4** and subsequently converted to the 1-*N*-glycyl β-derivative **6**.

The neoglycolipid derivative was synthesized by acylation of the glycyl amino function with *N*-(palmitoyloxy)succinimide (palmitic acid *N*-hydroxysuccinimido ester; Lapidot et al., 1967). Twenty-five micrograms of the 1-*N*-β-glycyl derivative **6** was dissolved in 10 μL of 0.1 M sodium bicarbonate, and to this was added 40 μL of methanol containing 1 mg of *N*-(palmitoyloxy)succinimide. Following stirring overnight, the mixture was acidified with glacial acetate acid and extracted with ether. The aqueous phase was then dried down, resuspended in water, and analyzed by TLC using solvent A. Visualization of the separation indicated the absence of the ninhydrin-sensitive component and the formation of a new orcinol-sensitive component which ran at the solvent front. In the TLC solvent system B 9:1 acetonitrile/water (v/v) containing 0.05% diaminobutane], this component has an R_f of 0.58 as compared to an R_f of 0.15 for the starting oligosaccharide. Since no residual glycyl derivative could be detected at the origin using ninhydrin or orcinol staining, the reaction was assumed to have proceeded to completion. The mixture was passaged over a C18 Bond-Elut cartridge eluted first with water and then with 80% acetonitrile. The acetonitrile fraction showed a single orcinol-sensitive component (R_f 0.58, solvent B). The purified glycolipid was stored at −20 °C.

A second glycolipid containing the Gal-2F structure was synthesized using the glycamine **3**. The *N*-palmitoylglycamine derivative (R_f 0.55, solvent B) was resuspended in water and stored at −20 °C. As for the 1-*N*-glycyl β-derivative, the N-palmitoylation step proceeded to completion.

TLC and Solid-Phase Lectin Binding Assay. Lentil lectin (100 μg) was labelled with ^{125}I using the Bio-Rad Enzymobead radioiodination system according to the manufacturer's instructions. The iodination mixture contained 10 nmol of Gal-2F to avoid potential modification of active site residues. The ^{125}I-labeled lentil lectin was purified by gel filtration on a Sephadex G-10 (PD-10) column equilibrated in phosphate-buffered saline (PBS), pH 7.2. Fractions eluting at the void were pooled and counted. The specific activity was 25Ci/mmol assuming 100% recovery of the lectin.

Neoglycolipid derivatives were separated on Si60 plates using solvent B and using 60:35:5 chloroform/methanol/water (v/v) (solvent C). In the latter system, the derivatives ran at the solvent front. Lectin binding to these neoglycolipid derivatives was performed using a microtiter plate method (Karlsson & Stromberg, 1987).

The two neoglycolipid derivatives were each diluted in 50% methanol to give an approximately 10 μg/mL solution. From this, 5-fold serial dilutions in 50% methanol were made, and 50 μL of each dilution was added (in triplicate) to wells in a 96-well microtiter plate. The solvent was then allowed to evaporate overnight at room temperature. The plates were blocked by the addition of 100 μL of 3% BSA in PBS, pH 7.4, and incubated at room temperature for 2 h. The plates were then rinsed three times with PBS. Lectin binding was assayed by the addition of 50 μL per well of 10 mM Tris-buffered saline (TBS), pH 8.0/3% BSA (TBS-BSA) containing ^{125}I-labeled lentil lectin (1×10^7 cpm/mL). The plate was incubated for 2 h at room temperature and then washed five times in TBS-BSA. The wells were cut out and counted in an LKB Minigamma counter.

Synthesis of Biotinylated Derivatives of the Gal-2F Oligosaccharide (**7**–**10**). Biotin and LC–biotin derivatives of the 1-*N*-glycyl β-derivative and glycamine derivative of the Gal-2F oligosaccharide were synthesized by N-acylation of

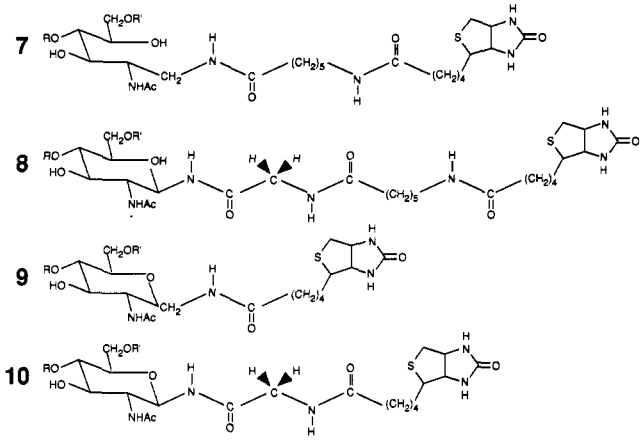


FIGURE 4: Various biotin derivatives used in this study. Note difference in total length between glycamine (**7** and **9**) and 1-*N*-glycyl β-derivatives despite an equivalent length of biotin spacer.

the amino function with the appropriate NHS ester (see Figure 4 for structures).

(*A*) *Biotin Conjugates* (*Structures* **9** *and* **10**). The 1-*N*-glycyl-β- (**6**) and glycamine (**3**) oligosaccharide derivatives (28 nmol) were dissolved in 50 μL of 0.1 M sodium bicarbonate buffer and cooled on ice with stirring. One hundred microliters of a 1 μg/μL solution of biotin–NHS in dimethylformamide was then added. The mixture was allowed to warm to room temperature and the reaction continued for 4 h. A further 50 μL of base and 100 μL of biotin solution were added, and incubation was continued for a further 4 h. The progress of the reaction was monitored by TLC (solvent A). As the reaction was allowed to proceed, the intensity of the orcinol-sensitive components close to the origin decreased. However, the biotinylated and free sugars were imperfectly resolved, giving an orcinol-sensitive smear between R_f 0.70 and 0.80. When no more orcinol-sensitive material could be observed at the origin, the mixture was dried, resuspended in water, and passed over a short tandem column of Dowex AG50W-X12 and Dowex AG3-X4A eluted with water to remove sodium ions and excess biotin. The column eluate was dried, resuspended in 100 μL of water, and stored at −20 °C.

(*B*) *NHS-LC–Biotin* (*Structures* **7** *and* **8**). The reaction of the 1-*N*-glycyl-β- (**6**) and glycamine (**3**) oligosaccharide derivatives with an LC–biotin derivative was carried out in a similar fashion except that NHS-LC–biotin was added directly to the vial containing 50 μg of each Gal-2F derivative dissolved in 100 μL of 0.1 M sodium bicarbonate. The reaction was monitored by TLC; the free sugar and its biotin conjugate were again only partially resolved by solvent A. Each derivative was desalted, dried, resuspended, and stored at −20 °C as described above.

Formation of Streptavidin–Gal-2F Pseudoglycoproteins and Lectin Affinity Chromatography. Biotinyl oligosaccharides (see Figure 4 for structures **7**–**10**) synthesized according to the above procedures were complexed with a streptavidin–FITC (StrF) conjugate to form fluorescent pseudoglycoproteins (ΨGP) (Shao & Wold, 1987). Each of the Gal-2F biotin conjugates was dried and resuspended in 100 μL of a 10 mg/mL solution of streptavidin–FITC conjugate in PBS, pH 7.4, containing 0.05% sodium azide, which was then gently agitated overnight at room temperature. The mixture was then dialyzed exhaustively against 50 mM sodium acetate/0.5 M sodium chloride/1 mM $CaCl_2$/1 mM $MgCl_2$ buffer, pH 5.1 (ConA start buffer), to remove free fluorophore.

StrF–Gal-2F pseudoglycoproteins were purified from free StrF using ConA high-performance lectin affinity chromatography (HPLAC). Fluorescence of the column eluant was detected using a Merck Hitachi F1000 detector (excitation 488 nm; emission 530 nm).

The column was equilibrated in ConA start buffer at a flow rate of 1 mL/min. Samples of ΨGPs were injected, and the column was washed for 5 min to elute the free StrF. The retained glycoconjugate was eluted with 10 mM methyl α-glucoside in the start buffer. Fractions containing fluorescent pseudoglycoprotein eluted using this buffer were then pooled, concentrated to approximately 200 μL using Centricon 10 concentrators, and dialyzed exhaustively against 10 mM Tris-buffered saline, pH 8.0, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (LL buffer).

Lentil lectin affinity chromatography was performed according to the method of Yamamoto et al. (1982). Aliquots of the ConA-purified ΨGPs were applied to a 3.5-cm × 6-mm column of lentil lectin–Sepharose 4B equilibrated in LL buffer. The column was then washed using the starting buffer and eluted with the same buffer containing 500 mM methyl α-mannoside. One-milliliter fractions were collected; 100-μL aliquots of each fraction were removed and diluted to 1 mL in the starting buffer, and fluorescence was measured in a Perkin-Elmer LS-5 spectrophotometer.

RESULTS

Formation and Characterization of Glycosylamines. Formation of the glycosylamines 1 and 2 was performed as described in Materials and Methods. Analysis of the reaction mixtures following 96-h incubation in ammonium bicarbonate using thin-layer chromatography (solvent A) indicated the presence of two orcinol-sensitive components. The first was a minor components with an R_f of ~0.4 that comigrated with the starting oligosaccharide. The second component barely moved off the origin (R_f ~0.05) and was ninhydrin-sensitive. TLC of the Gal-2F derivative in the same system indicated a similar decrease in orcinol staining intensity of the starting material (R_f ~0.35) and formation of a new component with R_f ~0.05. No other orcinol-sensitive components could be observed in this system (data not shown).

^{1}H-NMR analysis of the two glycosylamine preparations (Gal-2 and Gal-2F) was performed following lyophilization. An expansion of the anomeric region of the 1-D ^{1}H-NMR spectrum of the glycosylamine derivative of the Gal-2 and Gal-2F oligosaccharides is shown in Figures 5 and 6 (bottom panels) and can be compared with the 1-D spectrum of the starting oligosaccharides, respectively (top panels). Changes consistent with the conversion of the reducing-terminal residue to the amine were found. First, there is the expected reduction in intensity of the H1 resonances of GlcNAc-1 associated with the two anomeric forms of the starting oligosaccharide at 4.70 (β) and 5.19 ppm (α) and the appearance of the H1 resonance of the glycosylamine which resonates at 4.14 ppm ($J_{1,2}$ ~ 9.6 Hz) between the H2 protons of the Man-4 (4.19 ppm) and 4′ residues (4.10 ppm). In the Gal-2F glycosylamine, the fucose H5 multiplet falls into the region between the Man-4 and 4′ H2 resonances and overlaps with the βH1 resonance (Figure 6).

These observations confirm both the closed-ring structure of the reducing-terminal residue in the derivatives and their anomeric configuration (β). Integration of the βH1 resonance indicates that, after 96 h, about 80% of the oligosaccharides

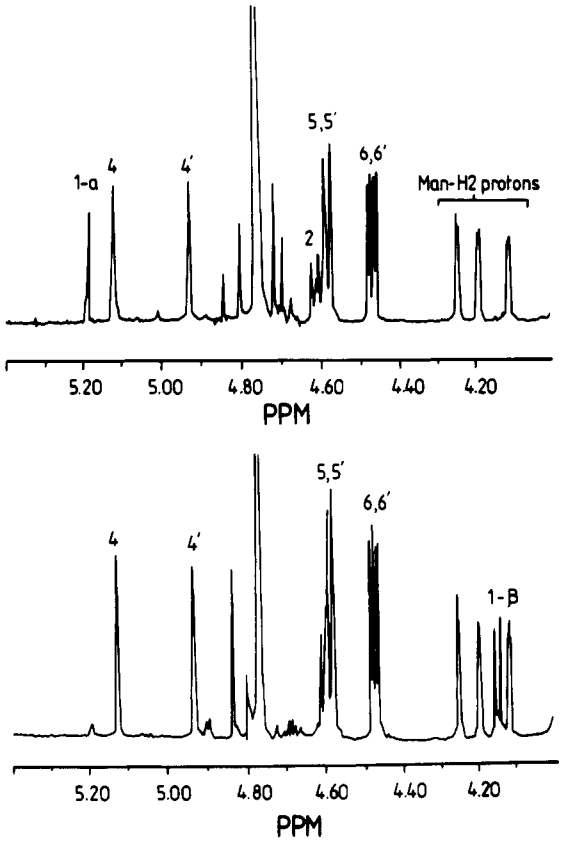


FIGURE 5: Comparison of the anomeric region of the 1-D spectrum of the Gal-2 oligosaccharide (top panel) and its glycosylamine derivative (lower panel), with assignments based on those of Vliegenthart et al. (1983). The βH1 of GlcNAc-1 of the glycosylamine falls between the Man4 and 4' H2 resonances ($J_{1,2}$ = 9.6 Hz). The integral intensity of H1 indicates the formation of this derivative in approximately 70% yield.

were converted to this form. The H1, H2, and H3 protons of the β-mannose residue in the core trisaccharide were also sensitive to the anomericity of the reducing terminus; the shift between the two anomers induces changes of the order of 0.005–0.01 ppm (Vliegenthart et al., 1983), and this was used to confirm the transition from an equilibrium mixture of the two anomers to the β-anomeric glycosylamine product. Signals associated with the monosaccharides beyond Man-3 were not affected.

Formation and Analyses of 1-N-Glycyl β-Derivatives of the Gal-2F Oligosaccharide. A sample of the glycosylamine derivative of the Gal-2F oligosaccharide was converted to its 1-*N*-glycyl β-derivative using N-chloroacetylation/ammonolysis as described in Materials and Methods. ^{1}H-NMR analysis of the intermediate *N*-chloroacetamido derivative of Gal-2F is shown in Figure 7. Chemical shift assignments were derived from the 2-D COSY spectrum of the *N*-chloracetamido derivatives of GlcNAc-NH_2 (data not shown). The CH_2 protons of the chloromethyl function could not be identified owing to overlap from the fucose H5 multiplet (4.15–4.10 ppm). The yield estimated from integration of the H1 resonance was 56% of the total oligosaccharide. Following ammonolysis, the oligosaccharide derivative was desalted using paper chromatography. TLC (solvent A) of the resulting mixture indicated the presence of two orcinol-sensitive components of approximately equal intensity with R_f values 0.05 and 0.73. The latter component comigrated with the starting sugar. The component with R_f 0.05 gave a positive ninhydrin test (data not shown).

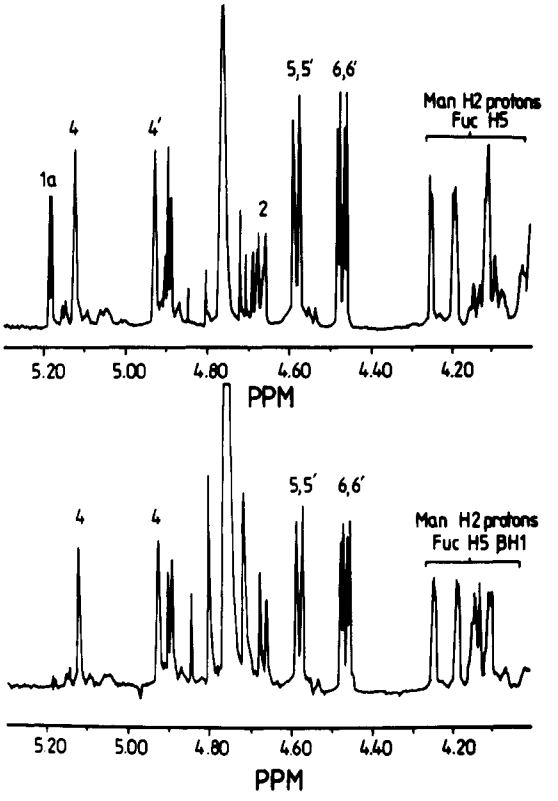


FIGURE 6: Expansion of the anomeric region of the 1-D spectrum of the Gal-2F oligosaccharide (top panel) and its glycosylamine derivative. The H1 proton of GlcNAc-1 in the derivative overlaps with the fucose H5 [assignments based on Vliegenthart et al. (1983)].

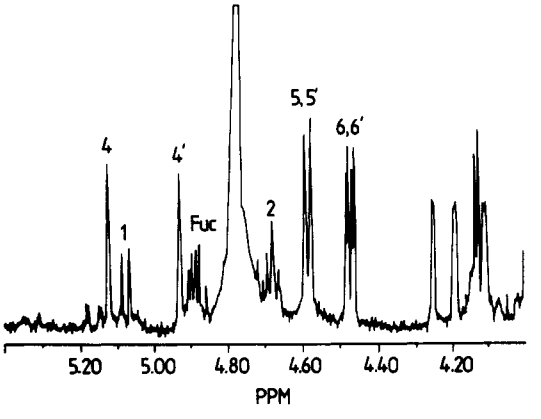


FIGURE 7: 1-D ^{1}H-NMR spectrum of the *N*-chloroacetamido derivative of the Gal-2F oligosaccharide. The H1 proton reports the amide function at C1 (δ = 5.07 ppm). The integral intensity of this proton indicates that the yield of the desired derivative is approximately 56%.

L. culinaris Lectin Binding Studies. Figure 8 shows the ability of ^{125}I-labeled *L. culinaris* lectin to bind to immobilized glycolipids using the microtiter plate method. There was no binding of the lectin to plates containing the immobilized glycamine neoglycolipid. In contrast, the lectin was found to bind to plates containing the 1-*N*-glycyl β-neoglycolipid.

Conversion to the biotinyl derivative was performed by N-acylation of the glycamine derivative or 1-*N*-glycyl β-derivative with the appropriate *N*-hydroxysuccinimido ester as described in Materials and Methods.

The pseudoglycoproteins consisting of biotinylated oligosaccharide and fluorescein-labeled streptavidin were purified using ConA affinity chromatography (Figure 9). Unbound fluorescent protein (tetrameric streptavidin with no sites occupied by biotinyl oligosaccharide) was eluted in the void,

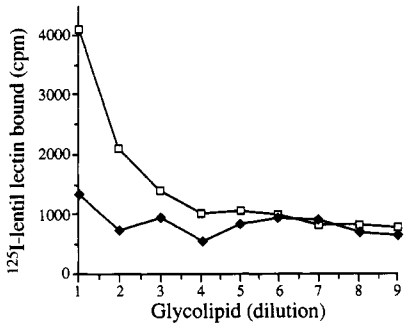


FIGURE 8: Binding of ^{125}I-labeled *L. culinaris* lectin to immobilized *N*-palmitoyl-Gal-2F derivatives. The neoglycolipid derivatives were diluted in 50% methanol (starting concentration 10 μg/mL), and 50 μL of each dilution was added to microtiter plate wells. The solvent was allowed to evaporate overnight at room temperature. The plates were then blocked with BSA. The ability of lentil lectin to bind to the plates was assessed by the addition of ^{125}I-labeled lentil lectin for 2-h at room temperature. Each point is the mean of three blank substracted determinations. Fivefold serial dilutions (1–9) of the glycyl derivative (open squares) and the glycamine derivative are shown (solid diamonds).

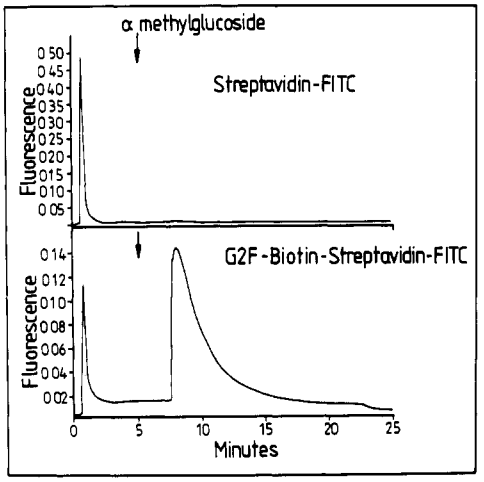


FIGURE 9: ConA HPLAC affinity purification of Gal-2F pseudoglycoprotein. Detection was by fluorescence (excitation 488 nm; emission 530 nm). The top panel shows the elution of a control streptavidin–FITC conjugate. The bottom panel shows the elution of a Gal-2F pseudoglycoprotein. The arrow indicates the start of elution buffer containing 10 mM methyl α-glucoside.

and the fluorescent pseudoglycoproteins were eluted with 10 mM methyl α-glucoside. No fluorescent material eluted with 0.5 M methyl α-mannoside. Each of the four pseudoglycoproteins appeared to be bound equally effectively to the immobilized lectin. They all possess the branched trimannosyl determinant important for high-affinity binding (Brewer & Bhattacharyya, 1986), and this carbohydrate determinant appeared to be equally accessible for each type of pseudoglycoprotein irrespective of the configuration of GlcNAc-1 of the oligosaccharide core or the length of spacer between biotin and the carbohydrate probe. ConA-positive fluorescent pseudoglycoproteins were analyzed for their ability to bind to lentil lectin Sepharose 4B. The elution profiles of each of the four Gal-2F–StrF pseudoglycoproteins from this column are shown in Figure 10. They clearly show that only the biotin–LC–*N*-glycyl-β-ΨGP (panel C) shows binding to lentil lectin (35% of the total fluorescence) and can be specifically eluted by methyl α-mannoside (fractions 7–12). The biotin–*N*-glycyl-β-pseudoglycoprotein (panel A) and the two glycamine derivatives (panels B and D) did not have any significant interaction with the immobilized lectin.

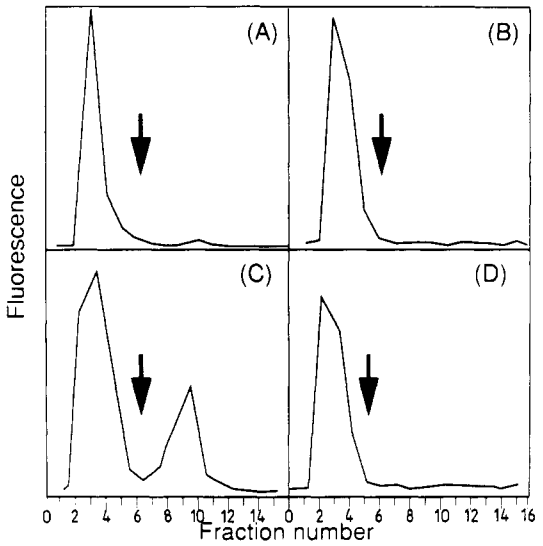


FIGURE 10: *L. culinaris* lectin affinity chromatography of the Gal-2F pseudoglycoprotein (ΨGP). The arrow indicates the point where elution with 500 mM methyl α-mannoside commenced. Panels: (A) biotin–glycyl–ΨGP; (B) biotin–glycamine ΨGP; (C) LC–biotin–glycyl-ΨGP; (D) LC–biotin glycamine ΨGP. Aliquots of the ConA-purified ΨGPs were applied to a 3.5-cm × 6-mm column of lentil lectin–Sepharose 4B. The column was washed using 5 column volumes of starting buffer and then eluted with the same buffer containing 500 mM methyl α-mannoside.

DISCUSSION

The anomericity of the products and the overall efficiency of N-acylation reactions of glycosylamines are strongly dependent on the nature of the electrophile. In particular, steric effects appear to be important, since we have previously shown that acylation with aromatic fluorophores such as dansyl chloride gives low yields of anomeric mixtures of products (Manger et al., 1992). For this reason, we adopted the use of a simple spacer function to move the site of reaction away from the anomeric center (Figure 1). The approach we adopted was based on the observation that N-acetylation of the 1-amino function gives a good yield of the desired β-1,2-diacetamido derivative. Although this derivative cannot be further modified, N-haloacetylation can provide intermediates with useful properties. For example, replacement of the halogen atom using ammonia yields the 1-*N*-glycyl β-glycosylamine derivative. Further, we have previously reported that 1-*N*-glycyl β-derivatives have improved properties over the free glycosylamine in terms of their reactivity toward complex aromatic fluorophore probes. The addition of a short spacer arm using the combination of chloroacetylation–ammonolysis enables the formation of conjugates in much higher yields than in the direct acylation method and substantially eliminates the problem of the creation of products with the "incorrect" anomeric configuration (Manger et al., 1992).

In order to determine the biological properties of neoglycoconjugates synthesized by this novel method, we investigated the reactivity of *L. culinaris* lectin with Gal-2F oligosaccharide and its various derivatives using two different binding assays.

The binding of ^{125}I-labeled lentil lectin to immobilized Gal-2F glycyl and glycamine neoglycolipid derivatives was studied using the microtiter plate method of Karlsson and Stromberg (1987). This method involves the immobilization of Gal-2F neoglycolipids by the hydrophobic interactions between the plastic plate and the C_{16} lipid portion of the neoglycolipids. The results are shown in Figure 8. The assay indicates that

the lectin will bind effectively to the glycyl lipid but not to the glycamine-derived structure.

The Gal-2F oligosaccharides were modified at the reducing terminus with biotin by the N-acylation of the *N*-glycyl-β- (closed ring) and glycamine (open ring) derivatives. In order to allow for possible steric effects on accessibility to the 6-*O*-linked core fucose residue, we also tested the analogous biotin–LC derivatives in which the valerate side chain of the biotin moiety is further extended with an aminocaproate linker. The biotinyl oligosaccharide derivatives were then complexed to fluorescein-labeled streptavidin, yielding a panel of fluorescent "pseudoglycoproteins" (Shao & Wold, 1987; Shao et al., 1987) with closed- and open-chain configurations at the oligosaccharide core.

The requirement of an intact core structure for binding of Gal-2F to lentil lectin is immediately apparent from comparison of the LC–glycyl profiles in Figure 10 (C and D), since only in the case of the long-chain (LC) glycyl derivative (panel C) is significant binding observed. Accessibility to the core fucose residue also appears to be an important factor in determining the efficiency of binding. Comparison of the results for the biotin and LC–biotin–*N*-glycyl β-pseudoglycoproteins (panels A and C) shows clearly that the nonextended biotin derivatives (panel A) are not appreciably bound. A pseudoglycoprotein obtained by complexing the LC–biotin–*N*-glycyl β-derivative of the Gal-2 oligosaccharide which lacks the core fucose residue was not bound by the lentil lectin column, confirming previous observations on the lectin (data not shown). Yamamoto et al. (1982) have shown that the interaction between lentil lectin and its ligand can be abolished by fixing the reducing-terminal residue in a ring-open configuration. The origins of this effect are unclear. It may result from destruction of a composite epitope involving the fucose and elements of the ring structure of the reducing-terminal residue; in this respect the configuration of the anomeric centre of the oligosaccharide may be important (Yamamoto et al., 1982). Alternatively, the flexibility of this segment of the molecule may be increased by reduction and this may effect the orientation of the reducing terminus with respect to the lectin subsite with which it normally interacts. The effects of oligosaccharide mobility on the affinity of oligosaccharide–protein interactions have been examined by Carver (1988).

Steric effects on accessibility to the biotin binding site have been noted in other applications (Green et al., 1971) and were particularly important in determining the efficiency of enzymatic processing of the pseudoglycoprotein derivatives constructed by Shao et al. (1987). The difference in the length of the extended and nonextended biotin derivatives is approximately 9 Å, which is the reported depth below the protein surface of the binding site for biotin in the avidin monomer (Green et al., 1971).

Not all of the ConA-positive Gal-2F pseudoglycoprotein bound to the lentil lectin affinity resin. This is consistent with the finding of Yamamoto et al. (1982) which showed that a considerable proportion of the nonreduced Gal-2F oligosaccharide did not bind to a lentil lectin–Sepharose 4B column. It was suggested that the nonbinding fraction contained a mixture of the open-chain and α-D-anomeric forms of the oligosaccharide. However, this is clearly not an adequate explanation for our results since our NMR analyses indicated that the biotinylated Gal-2F oligosaccharide exists almost exclusively in the β-configuration. It is also unlikely that the unbound fraction represents FITC–streptavidin dissociated from the biotinylated glycans, although Shao et al. (1987) reported that biotinylated glycans have at least 10-fold reduced affinity for avidin/streptavidin.

Possible explanations for nonbinding Gal-2F pseudoglycoprotein are that the chromatographic conditions for binding glycopeptide efficiently on immobilized lentil lectin are not optimal for binding free nonreduced oligosaccharide or pseudoglycoprotein and/or that our preparation contained a mixture of ConA-positive pseudoglycoproteins with differing affinity for immobilized lentil lectin. The former possibility has not been thoroughly investigated, but we observed that the binding of radiolabeled nonreduced Gal-2F oligosaccharide to lentil lectin affinity column is affected by flow rate and column length (unpublished results). The latter possibility could be due to the fact that streptavidin contains four biotin sites per tetramer and our preparation would contain a mixture of species with one to four biotinylated glycans per streptavidin molecule since excess streptavidin was used. It is possible that multivalent binding of Gal-2F oligosaccharide to lentil lectin is important in achieving binding constants sufficiently high to ensure tight binding on the solid-phase affinity support. Consistent with this possibility is our recent finding that 80% of the total Gal-2F pseudoglycoprotein formed in the presence of excess biotinylated oligosaccharides to ensure full occupancy of streptavidin binding sites bound to immobilized lentil lectin. The bound pseudoglycoprotein was not eluted even with 0.5 M methyl α-mannoside. In contrast 100% of the total Gal-2 pseudoglycoprotein applied was recovered in the wash fractions (data not shown). The ability of glycamine pseudoglycoproteins with full occupancy of streptavidin binding sites to bind to immobilized lentil lectin has not been investigated. However, it is clear from our results (Figure 10) that, under the same chromatographic conditions, the glycamine derivatives would have much lower affinity for lentil lectin than the 1-*N*-glycyl β derivatives.

In conclusion, we have investigated a simple condensation method for the formation of β-D-glycosylamines of purified complex oligosaccharides. These provide valuable intermediates for the synthesis of a wide range of oligosaccharide derivatives by acylation of the 1-amino function. We have reported here the synthesis of the 1-*N*-β-glycyl derivative of a fucosylated biantennary oligosaccharide and its subsequent biotinylation. An investigation of one application of these carbohydrate probes confirms that the "information" inherent in the structure of the core of the fucosylated biantennary oligosaccharide is important for its interaction with *L. culinaris* lectin, suggesting that, despite its popularity, reductive amination of oligosaccharides, as a technique for formation of glycoconjugates, should be used with caution. The chemical simplicity of the procedure outlined here suggests that this represents both a viable alternative to the use of reductive amination and an opportunity to obtain a wide range of oligosaccharide derivatives.

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