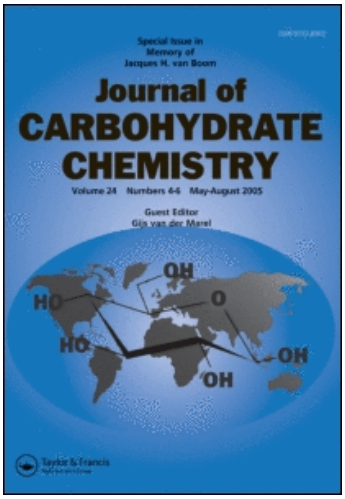

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Derivatization Procedures for Reducing Oligosaccharides, Part 3: Preparation of Oligosaccharide Glycosylamines, and Their Conversion Into Glycosaccharide - Acrylamide Copolymers

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DERIVATIZATION PROCEDURES FOR REDUCING OLIGOSACCHARIDES, PART * 3: PREPARATION OF OLIGOSACCHARIDE GLYCOSYLAMINES, AND THEIR CONVERSION INTO OLIGOSACCHARIDE-ACRYLAMIDE COPOLYMERS

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ABSTRACT

Reducing oligosaccharides were converted in good yields into the corresponding primary glycosylamines by treatment with aqueous ammonium bicarbonate. The glycosylamines were then acryloylated and the obtained oligosaccharide *N*-acryloyl glycosylamines were copolymerized with acrylamide. High molecular weight, linear copolymers were obtained, which were useful as antigens in immunoassays.

INTRODUCTION

Carbohydrate structures, exposed on the surface of cells or occuring in soluble form in body fluids, are important in many

* Part one and two is reference 1 and 25, respectively

biological recognition processes. To investigate such processes, low molecular weight oligosaccharides are sometimes not satisfactory. Therefore, a great deal of effort has been devoted to the development of techniques[1-4] for attaching oligosaccharides to larger molecules such as proteins, to give high molecular weight, multivalent conjugates. The obtained "neoglycoproteins" can be used as immunizing antigens to produce carbohydrate-directed antibodies, or as antigens in immunoassays to detect such antibodies.

However, for some applications, it is not ideal to use protein conjugates. For example, in immunoassays using carbohydrate antigens, the presence of protein epitopes is sometimes undesireable. An alternative to proteins in these cases are water-soluble, weakly immunoreactive polymers of the polyacrylamide type. Several reports have recently appeared describing the preparation of oligosaccharide-acrylamide copolymers, both linear[5-14] and crosslinked.[15-17] The general strategy for preparation of these conjugates has been to attach an olefinic group to a carbohydrate, and then copolymerize this derivative with acrylamide. The olefinic group has been introduced into the carbohydrate molecule either as an allyl glycoside at an early stage in a synthetic scheme[5-7,9-12,15], by acryloylation of an amino function of a mono- or oligosaccharide derivative,[8,11,13,14,17] or by other methods.[16]

To date, few reports have appeared [14,18] on the attachment of an olefinic group onto a *reducing* oligosaccharide. Reducing oligosaccharides of great complexity and structural variety can be isolated from natural sources such as milk,[19] urine,[20] and faeces,[20] and also from chemical or enzymatic hydrolyzates of glycoproteins,[21] glycolipids,[22,23] or lipopolysaccharides.[24] We have previously reported a technique for attachment of oligosaccharides from natural sources to proteins.[1,25] We now report a method for the attachment of an olefinic group to the anomeric position of reducing oligosaccharides. The oligosaccharides were first converted into the corresponding β-glycosylamines,[26] which were then *N*-acryloylated. The formed *N*-acryloyl glycosylamines were readily copolymerized with acrylamide to form high molecular weight, linear polymers. These polymers were useful as coating antigens in ELISA assays.

RESULTS AND DISCUSSION

Pure oligosaccharides, isolated from human milk,[19] urine,[20] or other sources were treated with aqueous ammonium bicarbonate,

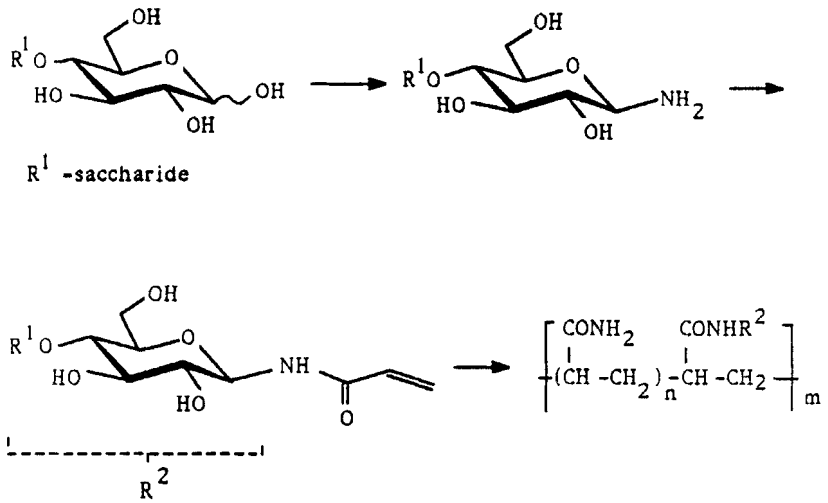


Fig.1: Conversion of reducing sugars to glycosylamines, N-acryloyl glycosylamines and polyacrylamide copolymers.

essentially as previously described,[25] to give the corresponding glycosylamines (Fig.1). The yields were as reported in Table 1. In all cases where oligosaccharides terminating with 4-linked glucose were used, β-pyranosidic glycosylamines were the main product. Less than 5 % of the bis-β-glycosylamine[27,28] was detected (NMR, H-1 at δ 4.32). No α-anomeric product signals were detected in any of the NMR spectra.

For a closer inspection of the reaction path leading to the glycosylamines, the reaction between lactose and ammonium bicarbonate was performed in D_2O and monitored by ^1H NMR spectroscopy. It was shown (Fig.2), that after 24 h at room temperature, about 50 % of the lactose had been converted. After 5 days, more than 95 % of the starting material had disappeared. However, only minor amounts of the glycosylamine **1** (H-1 at δ 4.12, H-2 at δ 3.20) were detected. Instead, signals from another major product (H-1 at δ 4.70, H-2 at δ 3.38) were present. This product was assumed to be the *N*-glycosylcarbamate **2**. Signals from another, unidentified minor product (H-1 at δ 6.29, H-2 at δ 4.71) were also present. That **2** was indeed present in the reaction mixture was

Table 1: Conversion of reducing oligosaccharides to the corresponding glycosylamines

Oligosaccharide	Glycosylamine, yield (%)
Lactose	82
Lacto-N-tetraose[a]	82
Lacto-N-fucopentaose I[b]	81
Lacto-N-fucopentaose II[c]	78
Lacto-N-difucohexaose I[d]	88
2'-Fucosyllactose	83
3'-Siallylactose	74
A-tetrasaccharide[e]	88
Cellobiose	72

a. β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

b. α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)D-Glc

c. β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

d. α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

e. α-D-GalNAc*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc

indicated by a FAB-MS spectrum of the mixture, where peaks corresponding to the acid of **2** (m/z 386) and **2** (m/z 403) were detected. After processing of the reaction mixture by concentration, adsorption to a cation exchange column, elution with methanolic ammonia and concentration, the ^1H NMR spectrum of a D_2O solution showed the presence of pure **1** (H-1 at δ 4.12), the yield was 82 %. Therefore, any N-glycosylcarbamate(**2**) present in the reaction mixture must have decomposed into the glycosylamine (**1**) during processing, an expected reaction for this type of derivative.[29] The importance of **2** as an intermediate was indicated by the fact, that reaction of lactose with concentrated aqueous solutions of ammonium acetate, ammonium formiate, or ammonium chloride gave less than 10 % conversion (TLC and NMR evidence) after 5 days at room temperature.

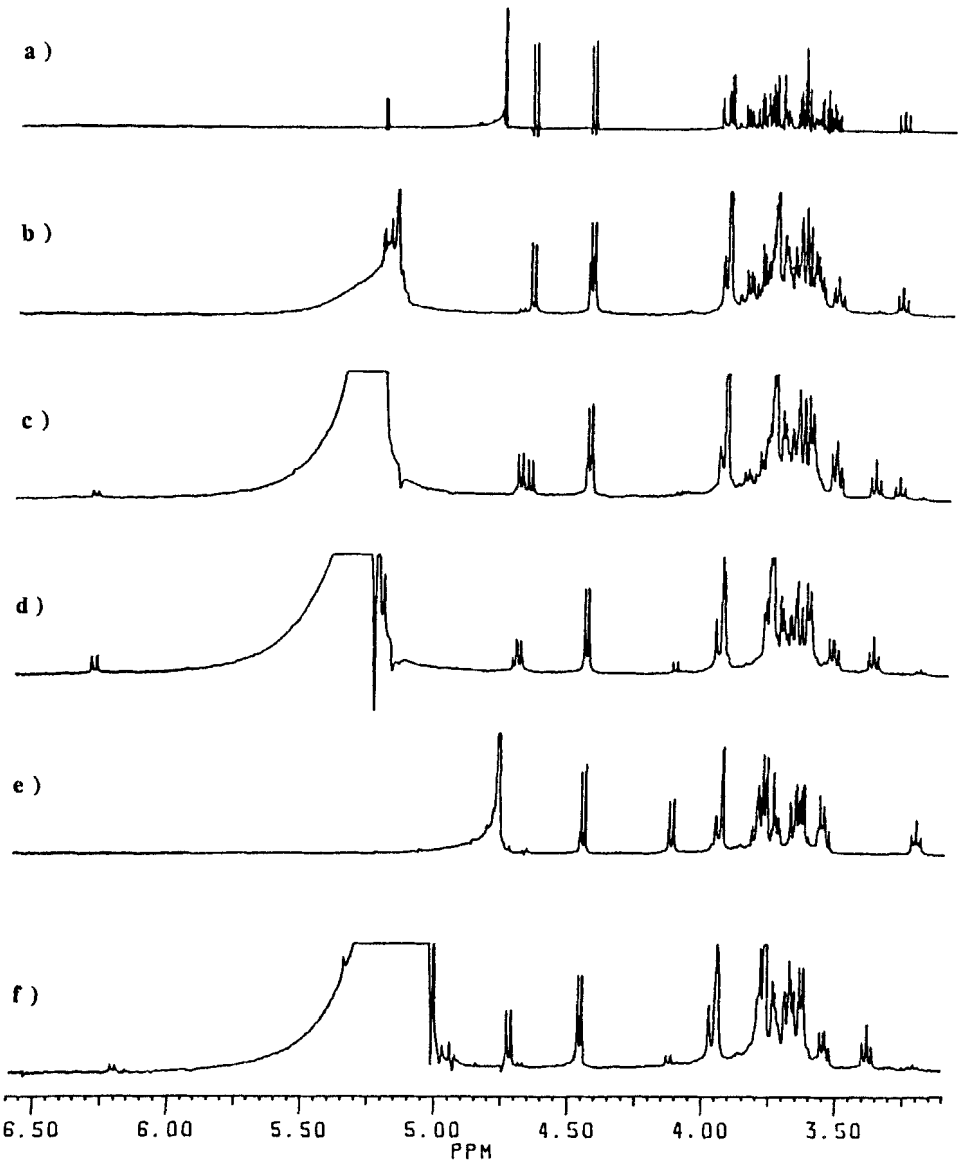
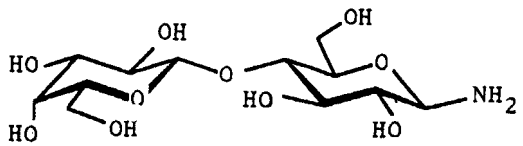


Fig. 2 : ^{1}H-nmr spectra in D_2O at 300 K of: **a**) lactose, **b-d**) the reaction mixture of lactose and ammonium bicarbonate after 4 h reaction (**b**), 23 h reaction (**c**) and 6 d reaction (**d**), **e**) lactose glycosylamine **1** (obtained after processing of the reaction mixture by ion exchange chromatography), **f**) pure **1** 4 h after dissolving in saturated ammonium bicarbonate in D_2O.



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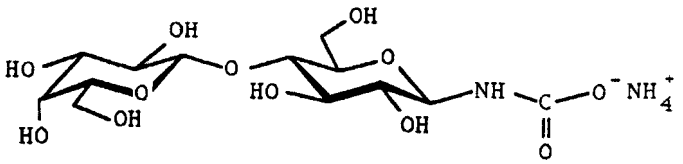


2

The influence of pH on the stability of glycosylamine solutions was also investigated. Lactose glycosylamine (1) was dissolved in phosphate buffers of different pH and the solutions were monitored polarimetrically. Solutions in the pH range 8.0-10.0 were found to undergo little change during several days at room temperature. This was also confirmed by the observation, that the ^1H NMR spectrum of 1 in D_2O (10 mg/mL, pH 7.7) did not change during this period. Lowering the pH increased the rate of decomposition of 1. At pH 5, for example, 1 was completely converted to lactose in less than 1 hour. However, at low pH (0.5 M aqueous HCl) 1 was again stable. These results are in good agreement with those reported for similar glycosylamines.[30] However, we also noted a lower stability of glycosylamines in borate buffers. At pH 10.0 in 0.1 M borate buffer, lactose glycosylamine was converted to lactose in less than 5 min (optical rotation and TLC evidence). The reason for this needs to be investigated.

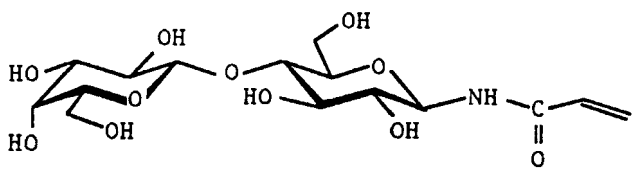
On basis of the above stability investigations, it was concluded that *N*-acylation of the glycosylamines in hydroxylic solvents should be possible, provided that the acylation reaction is fast and selective for amino groups, and that the pH during the reaction can be kept above 8. Indeed, several acylating agents were successfully used, and this communication reports on the results with acryloyl chloride. Treatment of the glycosylamines in table 2 with acryloyl chloride in aqueous methanol using sodium carbonate as buffer gave satisfactory yields of the corresponding *N*-acryloyl glycosylamines (Fig.1, Table 2). The *N*-acryloyl glycosylamines were, as predicted,[30] much more stable towards hydrolysis than the glycosylamines. However, the

Table 2: Acryloylation of glycosylamines

Glycosylamine	N-acryloylglycosylamine (%)
Lactose	88
Lacto-N-tetraose	74
Lacto-N-fucopentaose I	53*
Lacto-N-fucopentaose II	61*
2′-Fucosyllactose	92
A-tetrasaccharide	65*

* Yield not optimized

presence of the acryloyl group introduced a marked tendency to self-polymerization,[13,14] therefore addition of small amounts of a radical inhibitor to the solutions of these compounds was necessary during some operations.



3

Radical copolymerization of the oligosaccharide *N*-acryloyl glycosylamines with acrylamide in aqueous solution using ammonium persulfate/tetramethylethylenediamine (TEMED) as the initiator system[5] gave linear polymers (Fig.1, Table 3). The carbohydrate contents of the polymers were determined by integration of appropriate signals in the ^{1}H NMR spectra, and also independently by the anthrone-sulfuric acid colorimetric method.[31] The yield (weight of polymer bound carbohydrate divided by weight

Table 3: Synthesis of copolymers

oligo-saccharide	yield of copolymer*	charged ratio**	polymer ratio**	$[\alpha]_D$
Lactose	54	1:3	1: 4.6	+ 7
Lacto-N-tetraose	82	1:4	1: 5	- 1
Lacto-N-fucopentaose I	59	1:4	1: 6	- 11
A-tetrasaccharid	48	1:6	1:14	+ 18
2'-Fucosyllactos	65	1:6	1: 9	- 30

*Yield calculated from starting *N*-acryloylglycosylamine

**Ratio = ratio glycosylamide/ $CHCH_2$-unit

of carbohydrate in the reaction mixture) of carbohydrate incorporated into the polymer varied from 48 to 82 %. The molar ratio oligosaccharide groups/$CH\text{-}CH_2$ groups in the polymer agreed well with the corresponding ratio in the pre-polymerization mixture (Table 3). This is in contrast to what is the case[6,9,12] when allyl glycosides are copolymerized with acrylamide. Here lower yields are obtained and much lower oligosaccharide/$CH\text{-}CH_2$ ratios are found in the polymer than in the reaction mixture, since the reactivity of allyl glycosides in radical reactions is lower than that of acrylamide. Obviously, *N*-acryloyl sugar derivatives have a higher reactivity[11] in this respect.

The average molecular weights of the polymers (usually in the range of 100 - 500 kDa) were determined from the gel filtration elution volume using dextran standards for calibration (Fig.3). The molecular weights so determined agreed well with those determined by ultrafiltration through filters with different pore sizes.

In order to find optimal conditions for obtaining a high molecular weight polymer, several sets of experiments were performed with the *N*-acryloyl derivative of lactose. It was found that, as expected,[32-34] the molecular weight increased with

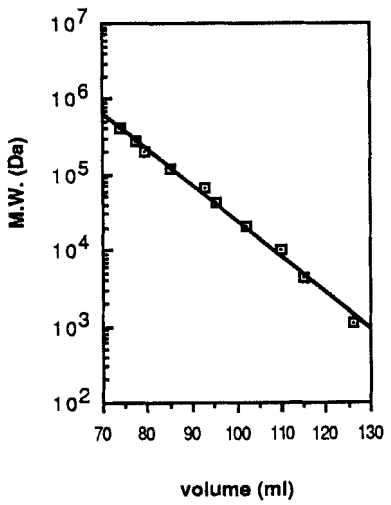


Fig.3: Calibration curve for ten different dextran standards on Fractogel HW 55(F) (85 cm x 2 cm).

decreasing concentration of ammonium persulfate and increasing concentration of monomers. However, too high a concentration of monomer resulted in an insoluble product. The reaction temperature is expected[32-33] to affect polymer molecular weight, but the effect was found to be moderate when varying between 0 °C and 20 °C. We also found little effect of pH (5 and 9) of the reaction mixture on the molecular weight distribution of the polymer, and there was no detectable change when the acrylamide to sugar monomer ratio was changed from 10:1 to 2:1. Factors that were found to be important for a good result were the purity of the monomers and an oxygen-free reaction mixture[7]. Factors that were not investigated but are known[32-34] to affect polymer molecular weight are initiator type and presence of chain transfer agents such as salts or alcohols.

The obtained copolymers exhibited strong specific binding to antibodies against the carbohydrate portion when used as coating antigens in ELISA assays. Thus, as reported before,[6,11] carbohydrate-acrylamide copolymers are alternatives to glycolipid or glycoprotein antigens in immunological assays. Other biological properties of carbohydrate-acrylamide copolymers, such as the ability to inhibit or promote various biological processes are being investigated in this laboratory.

EXPERIMENTAL

General Procedures. Degassed distilled water was used. All reactions except the preparation of glycosylamines were performed under nitrogen. Concentrations were performed at < 30 °C (bath). Optical rotations were recorded at 21 °C with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded at 27 °C in D_2O with a Bruker AM 500 instrument, using acetone methyl signals (δ_H 2.225 and δ_C 23.2) as internal standards. The FAB-MS spectra were recorded with a VG ZAB-SE mass spectrometer. The primary beam consisted of xenon atoms with a maximum energy of 8 keV. The samples were dissolved in thioglycerol and the positive ions were extracted and accelerated over a potential of 10 kV. Thin layer chromatography was performed on silica gel 60 F_{254} (Merck, Darmstadt, FRG) using 4:3:3:2 ethyl acetate : acetic acid : methanol : water as eluant. The spots were visualized by charring with 5 % sulfuric acid. Acrylamide (enzyme grade, Eastman Kodak C:o, Rochester, NY, USA) was used without further purification. Bond-Elut C-18 and SCX cartridges and Sepralyte C-18 silica gel were from Analytichem International (Harbor City, USA). Bio-Gel P2 (Bio-Rad, Richmond, USA) and Fractogel TSK HW-55(F) (Merck, Darmstadt, FRG) columns were packed and eluted with water. Dextran standards were from Pharmacosmos (Viby, Denmark). Ultrafiltration equipment (Omega cells) were from Filtron AB (Bjärred, Sweden). The tetrahydrofuran (Riedel-de Haën, FRG) used contained 250 mg/L of 2,6-di-*tert*-butyl-4-methylphenol as stabilizing agent.

Preparation of Glycosylamines: Solid ammonium bicarbonate was added until saturation to a solution of oligosaccharide (50 mg) in water (2.5 mL). The mixture was stirred in an open vessel at room temperature for 3 - 7 days. Ammonium bicarbonate was added at intervals, saturation was assured by always keeping a portion of solid salt present in the mixture. When TLC indicated no more conversion, the mixture was diluted with water (5 mL) and concentrated to half the original volume. The residue was diluted to 20 mL with water and concentrated to 5 mL. This process was repeated once, then the residue was diluted to 10 mL and lyophilized. The crude product was purified by dissolving in water (1 mL) and passing the solution through a cation exchange resin (Bond-Elut SCX, H^+-form, 0.5 g cartridge). After washing the

resin with water, the glycosylamine was eluted with 2 M ammonia in 1:1 methanol-water (2.5 mL). The eluate was concentrated to 1 mL and then lyophilized.

4-*O*-(β-D-Galactopyranosyl)-β-D-glucopyranosylamine (1). Treatment of lactose (50 mg) as described above gave 1 (41 mg, 82 %), $[\alpha]_D$ +37° (*c* 1.0, water), lit.[35] $[\alpha]_D$ +38.5 (water).

NMR data: ^{13}C, δ 61.1 (C-6), 61.9 (C-6′), 69.4 (C-4′), 71.8 (C-2′), 73.4 (C-3′), 74.8 (C-2), 76.0 (C-3), 76.2 (C-5′), 76.5 (C-5), 79.5 (C-4), 85.7 (C-1), 103.7 (C-1′); ^1H, δ 3.20 (dd, $J_{1,2}$ 8.7, $J_{2,3}$ 9.4 Hz, H-2), 3.54 (dd, $J_{1',2'}$ 7.8, $J_{2',3'}$ 9.9 Hz, H-2′), 3.55 (ddd, $J_{4,5}$ 9.6, $J_{5,6a}$ 5.0, $J_{5,6b}$ 2.3 Hz, H-5), 3.62 (dd, $J_{2,3}$ 9.4, $J_{3,4}$ 8.7 Hz, H-3), 3.64 (dd, $J_{3,4}$ 8.7, $J_{4,5}$ 9.6 Hz, H-4), 3.66 (dd, $J_{2',3'}$ 9.9, $J_{3',4'}$ 3.4 Hz, H-3′), 3.72 (ddd, $J_{4',5'}$ 1.1, $J_{5',6'a}$ 3.8, $J_{5',6'b}$ 8.1 Hz, H-5′), 3.75 (dd, $J_{5',6'a}$ 3.8, $J_{6'a,6'b}$ 11.6 Hz, H-6′a), 3.78 (dd, $J_{5,6a}$ 5.0, $J_{6a,6b}$ 12.1 Hz, H-6a), 3.79 (dd, $J_{5',6'b}$ 8.1, $J_{6'a,6'b}$ 11.6 Hz, H-6′b), 3.92 (dd, $J_{3',4'}$ 3.4, $J_{4',5'}$ 1.1 Hz, H-4′), 3.94 (dd, $J_{5,6b}$ 2.3, $J_{6a,6b}$ 12.1 Hz, H-6b), 4.11 (d, $J_{1,2}$ 8.7 Hz, H-1), 4.45 (d, $J_{1',2'}$ 7.8 Hz, H-1′).

Anal. Calcd for $C_{12}H_{23}NO_{10} \times H_2O$: C, 40.1; H, 7.0; N, 3.9. Found: C, 40.3; H, 6.8; N, 3.8. A FAB-MS spectrum showed an M+1 ion at m/z 342.

***N*-Acryloylation of Glycosylamines:** Sodium carbonate (100 mg) and methanol (1.0 mL) was added to a solution of the glycosylamine (0.14 mmol) in water (1.0 mL). The mixture was stirred at 0 °C while acryloyl chloride (60 μL, 0.74 mmol) in tetrahydrofuran (0.5 mL) was added during 5 min. After 10 min, the solution was diluted with water (3 mL) and concentrated to 2 mL. The solution was again diluted with water (2 mL), 200 μL of 0.5 % 2,6-di-*tert*-butyl-4-methylphenol in tetrahydrofuran (inhibitor solution) was added, and the solution was concentrated to 1-2 mL. This solution was applied onto a C-18 silica gel column (2.0 x 5.0 cm), packed in water. Elution with water gave salts, unreacted glycosylamine, and reducing sugar in the first fractions, and the desired product in the later fractions. In some cases, elution of the product was preferably speeded up by adding methanol to the eluant. The fractions containing product were pooled, mixed with a few drops of inhibitor solution, and concentrated to 2 mL. This solution was purified by gel filtration on a Bio-Gel P2 column. Appropriate fractions were pooled and lyophilized.

***N*-Acryloyl-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosylamine (3).** Treatment of 1 (50 mg) with acryloyl

chloride (60 mL) as described above gave **3** (51 mg, 88 %), $[\alpha]_D$ -7° (*c* 0.5, water). NMR data: ^{13}C, δ 60.7 (C-6), 61.9 (C-6′), 69.4 (C-4′), 71.8 (C-2′), 72.3 (C-2), 73.3 (C-3′), 75.9 (C-3), 76.2 (C-5′), 77.3 (C-5), 78.6 (C-4), 80.1 (C-1), 103.7 (C-1′), 130.20, 130.25 ($CH=CH_2$), 170.2 (C=O); ^1H, δ 3.49 (dd, $J_{1,2}$ 9.2, $J_{2,3}$ 9.2 Hz, H-2), 3.56 (dd, $J_{1',2'}$ 7.8, $J_{2',3'}$ 9.9 Hz, H-2′), 3.67 (dd, $J_{2',3'}$ 9.9, $J_{3',4'}$ 3.4 Hz, H-3′), 3.70 (m, H-5), 3.72 (m, H-3), 3.73 (m, H-4), 3.75 (m, H-5′), 3.77 (dd, $J_{5',6'b}$ 3.8, $J_{6'a,6'b}$ 11.6 Hz, H-6′b), 3.80 (dd, $J_{5',6'a}$ 8.2, $J_{6'a,6'b}$ 11.6 Hz, H-6′a), 3.82 (dd, $J_{5,6b}$ 4.4, $J_{6a,6b}$ 12.3 Hz, H-6b), 3.93 (dd, $J_{3',4'}$ 3.4, $J_{4',5'}$ 1.6 Hz, H-4′), 3.94 (dd, $J_{5,6a}$ 2.1, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.46 (d, $J_{1',2'}$ 7.8 Hz, H-1′), 5.08 (d, $J_{1,2}$ 9.2 Hz, H-1), 5.87 (dd, J 3.7 and 7.9 Hz, $CH=CH_2$), 6.31 (m, $CH=CH_2$).

Anal. Calcd for $C_{15}H_{25}NO_{11}$: C, 45.6; H, 6.4; N, 3.5. Found: C, 41.1; H,6.1; N, 3.6. A FAB-MS spectrum showed an M+1 ion at m/z 396.

Copolymerization of *N*-Acryloylglycosylamines with Acrylamide: A solution of the *N*-acryloylglycosylamine (52 μmol) and acrylamide (210 μmol, 15 mg) in distilled water (400 μL) was deaerated by flushing with nitrogen for 20 min. The solution was then stirred at 0 °C and *N,N,N′,N′*-tetramethylethylenediamine (2 μL) and ammonium persulfate (1 mg) were added. The mixture was slowly stirred at 0 °C for 2 h, and then at room temperature overnight. The viscous solution was diluted with water (1 mL) and purified by gel filtration on Fractogel HW 55(F). Fractions containing polymer were pooled and lyophilized.

Copolymer of *N*-Acryloyl-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosylamine and Acrylamide: Treatment of **3** (20 mg) with acrylamide (7.2 mg, 2 eq) as described above gave copolymer (18 mg, 54 % calculated from **3**), $[\alpha]_D$ +7° (*c* 0.1, water). Analysis of the material by ^1H NMR spectroscopy (D_2O, 50 °C) showed presence of approximately 1 lactose unit per 4.6 $CHCH_2$ units (theoretical value: 1/3). The molecular weight distribution of the copolymer, as determined by gel filtration, was 50 - 1000 kDa, centered around 300 kDa.

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