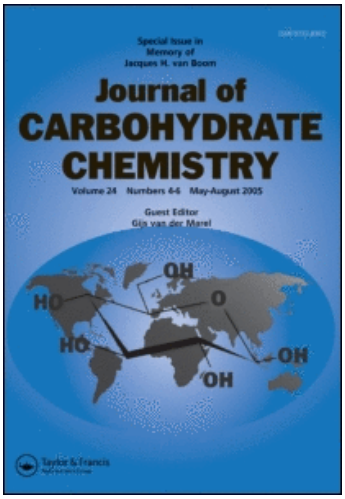

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IMMOBILIZATION OF REDUCING OLIGOSACCHARIDES TO MATRICES BY A GLYCOSYLAMIDE LINKAGE[1]

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ABSTRACT

A novel method is described for coupling of reducing oligosaccharides to matrices. A one pot, two-step reaction is used, which forms a glycosyl amide bond to the matrix. Unreacted oligosaccharide could easily be recovered. The new matrices were useful in affinity chromatography, e.g., for purification of lectins and antibodies.

INTRODUCTION

Molecular recognition is an important life process and occurs in a variety of phenomena such as fertilization, embryogenesis, cell migration, immune defense and microbial infections. Many of these interactions are known to be mediated by carbohydrate binding proteins (lectins) located on animal and plant cell membranes and bacterial pili.[2]

To study recognition phenomena between carbohydrates and lectins, a number of methods have been developed, some of them using saccharides immobilized on different matrices.[3] In general, two approaches have been used for preparation of immobilized saccharides.

The first approach is based on the total synthesis of an oligosaccharide starting from mono- or di- saccharides carrying a spacer with a functional group suitable for coupling to affinity matrices. There are a number of spacers available, the most common being the *p*-aminophenyl group.[4] Other examples include blood group A- and B-trisaccharides coupled to a *p*-aminophenylethyl group,[5] blood group Lewis a trisaccharide coupled to a 8-methoxycarbonyloctyl group,[6] bromoethyl glycosides,[7] and allyl-glycosides.[8]

A number of different methods have been used for coupling spacer glycosides to matrices, e. g., cyanogen bromide coupling,[9] isothiocyanate coupling[10] and acyl azide coupling.[11] The methods are essentially the same as preparing neo-glycoconjugates.

The advantage with a total synthesis approach is the complete control of structure allowing the preparation of modified structures, multivalent ligands, free choice of hydrophilic/hydrophobic spacers and functional group for the coupling. The main drawback is the time-consuming preparation of the oligosaccharide, which requires highly skilled carbohydrate chemists.

The second approach takes advantage of the large number of complex carbohydrates isolated from natural sources. These can be coupled to a functionalized or activated matrix, provided that methods are available to create a covalent linkage between the matrix and the saccharide.

One of the most used coupling methods for reducing sugars is reductive amination, either directly to a matrix[12] or by first reacting with a spacer[13,14] suitable for coupling as exemplified above. Both alternatives utilize the fact that reducing sugars exist partly in the aldehydo form and therefore can react with amino groups forming a Schiff base, which can be reduced to a stable amine with sodium cyanoborohydride. Other examples are coupling to epoxy substituted matrices[15] and divinylsulfone mediated coupling[16]. The methods used are often quite simple, but the reactions often use high pH, long reaction times and low coupling yields are obtained, requiring large quantities of sugars. The use of complex oligosaccharides is still uncommon although some reports have appeared.[17] Coupling of natural glycolipids to matrices has also been performed.[18]

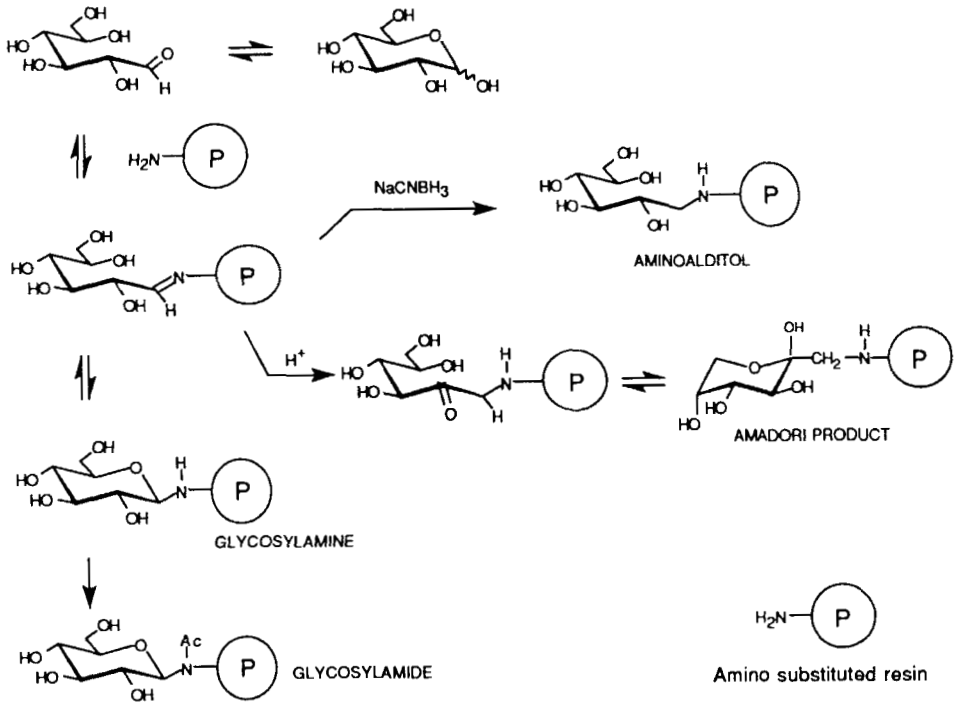

FIG 1. Schematic Overview of Amine Reactions with Reducing Saccharides.

We now report a novel method of immobilizing reducing oligosaccharides to a matrix. The method utilizes the fact that a glycosylamine bond can be formed between a reducing oligosaccharide and a matrix carrying an amino group and that this labile linkage can be stabilized by acetylation to form a glycosylamide bond. The methodology is simple and can be performed as a one pot reaction. The yield is almost quantitative when considering the possibility of recovering unreacted sugar.

RESULTS AND DISCUSSION

It is well known that reducing sugars react with ammonia or amines through a series of equilibria to yield glycosylamines in high yields.[19] However, these compounds are unstable in water below a certain pH value. Several possibilities exist to stabilize the C - N linkage as shown for glucose in FIG. 1.

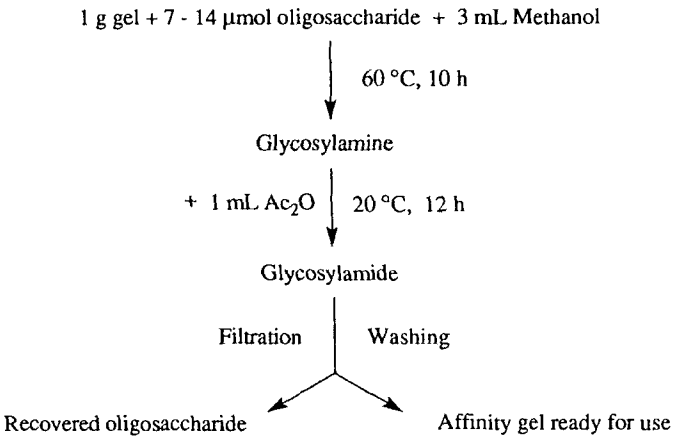

FIG. 2 Procedure for obtaining glycosylamide substituted matrices.

The most common stabilizing reaction is reductive amination of the intermediate imine with sodium cyanoborohydride, which creates a stable secondary amine. A second possibility to obtain a stable linkage is to perform the reaction between the reducing sugar and the amine under more harsh and acidic conditions. Then the intermediate imine undergoes an Amadori rearrangement, forming a secondary amine linkage. A third possibility is to stabilize the initially formed glycosylamine by treatment with an acid anhydride or acid chloride to yield a stable glycosylamide linkage.[20] We have investigated this latter possibility. Oligosaccharides were reacted with matrices containing amino groups, and the matrix was then *N*-acetylated with acetic anhydride to give a stable product well suited for affinity chromatography. The glycosylamide linkage obtained resembles the naturally occuring linkage found in N-linked glycoproteins. The treatment with acetic anhydride also effects acetylation of the remaining amino groups and a neutral matrix is obtained with no ion exchange properties.

We used a synthetic, non-carbohydrate matrix, Fractogel TSK HW 65 (F) which is substituted with a short hydrophilic, amino-containing spacer arm ($-OCH_2CHOHCH_2NH_2$). The degree of substitution was approximately 150 μmol amino groups/g wet matrix.

The procedure used is outlined in FIG. 2.

To screen the ability of the gels to bind lectins, each gel was tested with lectins of known specificity. In some cases, carbohydrate recognizing

monoclonal antibodies were used. The results obtained are summarized and included in TABLE 1.

As shown in TABLE 1, the immobilization reaction gave in most cases a high yield of coupling. Unreacted and unchanged oligosaccharide can easily be recovered after freeze drying of the supernatant. This makes the total yield near quantitative, which is important since many of the oligosaccharides are only avaliable in small quantities at high cost.

In most cases where a lower yield was obtained (**10, 11, 19, 26**) the reducing residue was a 2-acetamido-2-deoxy sugar. The reason for the inferior yield here is unknown. In the case of **16** (LNT) the low yield could possibly be attributed to the low solubility of LNT in methanol, since the fucosylated analogs of LNT (**20, 21, 24**), which are methanol soluble, gave a normal yield.

The degree of oligosaccharide substitution was estimated using two different methods. In the first method, the gel was hydrolyzed to liberate the immobilized sugar and the monosaccharide residues were quantified with GLC as alditol acetates. In the second method, the supernatants were collected, freeze dried and weighed. Most recovered oligosaccharides were analyzed by HPLC and in most cases found to be essentially unaffected by the treatment except in the case of sialylated structures where by-products were seen. These were not further characterized. The two analytical methods showed a good correlation, although the sugar analysis gave a slightly lower value.

The substituted gels (**7, 11, 12, 13**) showed no detectable loss of coupled carbohydrate or ability to bind lectins after being subjected to the following conditions:

24 h at pH 2.5 and 4 °C.

24 h at pH 10.0 and 4 °C.

24 h in 6 M guandinium hydrochloride-Tris buffer pH 7.5 at 4 °C.

Autoclaving at 120 °C for 20 minutes at pH 7.5.

Due to the difficulty of analyzing the matrix directly to demonstrate the type of linkage between the sugar and the matrix, a model reaction, using the same derivatization conditions as described above, was performed between the monosaccharide glucose and ethanolamine (mimicking the spacer moiety on the matrix). This reaction produced, according to NMR and FAB-MS data, the glycosylamide *N*-acetyl-*N*-(2′-hydroxyethyl)-β-D-glucopyranosyl-amine. Because of slow rotation around the C-N bond,[21] the NMR spectra at room temperature showed the presence of two rotamers. Warming produced

TABLE 1. Result of Oligosaccharide Coupling to Fractogel and Biological Testing of the Corresponding Affinity Matrices.

Trivial name or abbreviation	Structure	Binding properties[a]	Yield (%)[b]
Disaccharides			
1 Melibiose	Galα1-6Gal	A (+++)	
2 Lactosamine	Galβ1-4GlcNAc	B (+), F(+), H (++)	36
3 Maltose	Glcα1-4Glc	A (+++)	32
4 Isomaltose	Glcα1-6Glc	A (++)	48
5 Gentiobiose	Glcβ1-6Glc	A (++), E(+), I (++)	50
6 Chitobiose	GlcNAcβ1-4GlcNAc	H (+++)	43
Trisaccharides			
7 2′-Fucosyllactose	Fucα1-2Galβ1-4Glc	C (++), F (++)	82
8 Globotriose	Galα1-4Galβ1-4Glc	A (++), G(+++)	80
9 A-Trisaccharide	GalNAcα1-3Gal 2 Fucα1	G (++), K(+)	20
10 Man2GlcNAc	Manα1-3Manβ1-4GlcNAc	A (++)	30
11 Chitotriose	GlcNAcβ1-4GlcNAcβ1-4GlcNAc	H (+++)	9
12 3′Sialyllactose	Neu5Acα2-3Galβ1-4Glc	H (+++)	73
13 6′Sialyllactose	Neu5Acα2-6Galβ1-4Glc	H (++)	78
14 Maltotriose	Glcα1-4Glcα1-4Glc	A (++)	84
15 Isomaltotriose	Glcα1-6Glcα1-6Glc	A (++)	88
Tetrasaccharides			
16 LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	B (++), F (++)	8
17 A-Tetrasaccharide	GalNAcα1-3Galβ1-4Glc 2 Fucα1	K (+++)	83
18 Globotetraose	GalNAcβ1-3Galα1-4Galβ1-4Glc	A (++)	82
19 Chitotetraose	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc	H (+++)	6
Pentasaccharides			
20 LNF I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	L (+++)	90
21 LNF II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	M (+++)	77

22	LST a	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	D (+),H(++), J(++)	88
		Neu5Acα2 6		
22	LST b	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	H (++)	72
24	LND I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	L (+++)	83
	Oligosaccharides			
		Neu5Acα2 6		
25	Disialyl LNT	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	H (+++)	74
		Galβ1-4GlcNAcβ1-2Manα1-6		
26	GM1 Octasaccharide	Manβ1-4GlcNAc		<5
		Galβ1-4GlcNAcβ1-2Manα1-3		
27	Sialyl Lewis[a]	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1	N (+++)	80

a. The following lectins and monoclonal antibodies with claimed specificity were used for testing affinity properties:

Lectins

	Taxonomic names	**Common Name**	**Sugar specificity**
A.	Canavalia ensiformis	Con A, Jack bean	α-D-Man, α-D-Glc, α-D-Gal
B.	Erythrina cristagalli		β-D-Gal(1-4)GlcNAc
C.	Ulex europaeus	Ulex, gorse	α-L-Fuc/ $(GlcNAc)_2$
D.	Limulus polyphemus	Horseshoe crab	Neu5Ac(2-6) /GlcNAc
E.	Pisum sativum	Garden pea,	α-D-Man
F.	Arachis hypogaea	Peanut	β-D-Gal(1-3)-D-GalNAc
G.	Glycine max	Soybean	D-GalNAc
H.	Triticum vulgaris	Wheat germ	D-$(GlcNAc)_2$ / NeuNAc
I.	Lens culinaris	Lentil	α-D-Man
J.	Sambuccus nigra	Elderberry	Neu5Ac(α2-6)Gal/GalNAc

Monoclonal antibodies:

Cell line:		**Known Specificity:**
K	A003 (40/5G7)	GalNAcα1-3Galβ1-3(4)Glc 2 4(3) Fucα1 (Fucα1)
L.	510/1G3	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 (Fucα1)

(continued)

TABLE 1. Continued

M.	Le^a01	(318/2B3)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 (Fucα1)
N.	α-S-LeA	923/9C2 1C8	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1

The ability to bind the lectin (100 μg /250 μl gel) was estimated as (+) if 10%or less was bound, (++) if 10-85% was bound and (+++) if >85% was bound to the gel. The ability to bind the monoclonal antibodies were estimated likewise.

b. The yield was calculated as the incorporated sugar on the gel according to sugar analysis and recovered oligosaccharide. The recovered oligosaccharide is not included in the yield.

spectra with selective line broadening, indicating faster rotamer conversion. No Amadori rearrangment products were observed in this model reaction.

Two of the affinity gels obtained have been extensively used for other affinity chromatography purifications.

Globotriose gel (**8**) was used to purify Shiga toxin directly from a crude cell free mixture of *Shigella dysenteriae*.[22] A 1224-fold purification of the toxin was achieved in one step with a recovery of 87%. Globotetraose (**18**), which also contains the Galα1-4Gal sequence, also retained the toxin whereas a number of other glycosylated gels did not.

A-Tetrasaccharide gel (**17**) has been used for removing blood group Anti-A IgG and IgM antibodies from human blood group O-plasma[23] and was found to be as effective as other matrices produced by other methods.

In summary, a new method has been developed for immobilizing oligosacharides to matrices. The procedure is simple and gives high yields of affinity gels, which were shown to adsorb lectins and carbohydrate-binding antibodies efficiently.

EXPERIMENTAL

General Methods: Oligosaccharides were obtained from BioCarb Chemicals, Sweden. Fractogel TSK HW 65 (F) was obtained from Merck,

Germany. Derivatization to an amino form was performed by treatment with epichlorohydrine and subsequent opening of the epoxide with ammonia as described.[24] Lectins were purchased from Sigma, USA. Monoclonal antibodies were obtained from MonoCarb AB, Lund, Sweden. Elderberry extract was obtained according to known method.[25] Methanol and acetic anhydride were of analytical grade. 1H and 13C NMR-spectra were recorded with a JNM-GX-270 instrument. FAB-MS spectrum was recorded with a NERMAG R10-10 instrument. GLC was performed on a Hewlett Packard 5890 instrument fitted with a flame ionization detector. HPLC chromatography was performed on a Nucleosil C18 column with RI-detection.

Standard Procedure For Preparing Glycosylamidated Affinity Gels: Amino Fractogel (10 g suction filtered) was thoroughly washed with methanol several times on a sintered glass funnel to remove water. Oligosaccharides was added to a suspension of gel in 30 mL methanol in a plastic covered bottle with a screwcap. Typically, 7-14 μmol/g gel (except entry **1** and **3**, where 75 μmol/g was used) was added. The closed bottle was shaken gently in a water bath for 10 h at 60 °C. After cooling to below room temperature, 10 mL of cold acetic anhydride was added and the suspension was left at room temperature for 12 h. The gel was filtered off and washed with 10 mL of methanol. The gel was thereafter washed and suspended in 50 mL of water, swirled for 10 min and filtered. The procedure was repeated four times. The collected methanol and water washings were combined and immediately frozen.

Determination of the Degree of Substitution by GLC: [26] The glycosylated gel was filtered off on a sintered glass funnel. A 25 mg sample of the gel was hydrolyzed in 20 mL of 4 M trifluoroacetic acid at 100 °C for 4 hours together with an internal standard (Perseitol). The supernatant was concentrated, the residue taken up in water, and 5 mg of sodium borohydride was added. After 3 hours, excess sodium borohydride was destroyed by adding acetic acid and the solution was concentrated to dryness. The dry residue was heated for 4 hours with 5 mL of a mixture containing equal amounts of acetic acid and pyridine. Ethanol (5 mL) was added and the sample was concentrated to dryness. The alditol acetates were extracted with chloroform after addition of water.

Gravimetric Estimation of the Degree of Substitution: The reaction and washing solvents were collected as described above and freeze dryed. The

residue was dissolved in water and filtered through a 0.45 μ filter to remove fines from the gel. The resulting clear solution was once more freeze dryed. By weighing the residue, the amount of immobilized oligosaccharide could be calculated.

HPLC-Analysis of Recovered Oligosaccharides: Recovered material was analyzed by injecting on a C18 column and eluting with appropriate water/methanol mixtures. The peaks were detected by RI and compared to an authentic sample. In most cases the recovered oligosaccharides were essentially unaffected by the treatment. In certain cases a number of byproducts were found. By comparision with the original sample it could be concluded that these by-products in most cases were present at the outset and thus only impurities which had been enriched by the treatment.

Biological Testing of the Affinity Matrices: 250 μL gel was packed in a column and equilibrated with 0.05 M Tris HCl pH 7.5, 1 mM NaCl, 1 mM $MnCl_2$, and 1mM $CaCl_2$. 100 μg Lectin was applied and the the column was washed with the application buffer. The bound material was eluted with 6 M urea, 0.05 M glycin pH 2.5. The absorbance at 280 nm was followed and the amount of bound lectin was calculated from the difference between the area for the bound and unbound material. A blank run was always performed prior to the application of the lectin. Monoclonal antibody affinity testing was performed by the same method.

Model reaction: Preparation of *N*-acetyl-*N*-(2′-hydroxyethyl)-β-D-gluco-pyranosylamine. Ethanolamine (170 mg, 2.7 mmol) and D-glucose (250 mg, 1.4 mmol) was stirred with methanol (25 mL) at 50 °C for 16 h, then cooled to room temperature. Acetic anhydride (10 mL) was added, and after 16 h at room temperature, the mixture was concentrated. The residue was purified by column chromatography on silica gel (50 g), using 15:3:3:2 ethyl acetate-acetic acid-methanol-water as eluant. The main fraction (260 mg, 71 %) was chromatographically homogeneous in several TLC systems, and gave positive and negative ion FAB-MS spectra indicating a molecular weight of 265. The NMR spectrum in D_2O at room temperature showed presence of two compounds with similar structure, in the ratio 1:2. This was interpreted as arising from two (slowly on the NMR timescale) exchanging rotamers around the C-N bond, a known phenomenon.[21] Heating the sample to 85 °C gave selective line broadening, especially of the H-1 signals, indicating faster rotamer conversion (intermediate exchange). NMR data: ^1H, δ 5.40 ($J_{1,2}$ 9.2 Hz, H-1a), 4.98 ($J_{1,2}$ 8.0 Hz, H-1b), 2.23, 1.90 (two s, methyl); ^{13}C, δ 87.7, 83.7 (2

C-1), 79.0, 78.8, 77.6, 77.1, 70.9, 70.2, 70.0 (ring C), 61.6, 61.3, 60.4 (C-6 and $CH_2\underline{C}H_2O$), 47.06, 43.9 ($\underline{C}H_2CH_2O$), 22.6, 22.2 (methyl C).

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