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Saccharide Display on Microtiter Plates

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Summary

New insight into the importance of carbohydrates in biological systems underscores the need for rapid synthetic and screening procedures for them. Development of an organic synthesis-compatible linker that would attach saccharides to microtiter plates was therefore undertaken to facilitate research in glycobiology. Galactosyllipids containing small, hydrophobic groups at the anomeric position were screened for noncovalent binding to microtiter plates. When the lipid component was a saturated hydrocarbon between 13 and 15 carbons in length, the monosaccharide showed complete retention after aqueous washing and could be utilized in biological assays. This alkyl chain was also successfully employed with more complex oligosaccharides in biological assays. In light of these findings, this method of attachment of oligosaccharides to microtiter plates should be highly efficacious to high-throughput synthesis and analyses of carbohydrates in biological assays.

Introduction

Carbohydrates, with a diversity that far surpasses that of amino or nucleic acids, are the most abundant family of natural products. Adhesion, recognition, protein stability, transport, and folding are all greatly influenced by saccharides. Their structural variability also makes them ideal for cellular storage and stabilization [1, 2]. Certain oligosaccharides and glycoconjugates, such as members of the aminoglycoside family, exhibit important medicinal properties as well [3]. Despite the biological importance of carbohydrates, however, their function is still largely unknown as compared to the information on proteins and nucleic acids.

Two major obstacles face scientists working on these still-unanswered questions: inherent synthetic difficulties in creating diverse carbohydrate libraries and a dearth of techniques available for their high-throughput analysis. Whereas these issues are well resolved for both proteins and nucleic acids [4, 5], only recently have great strides been made for oligosaccharides. Polymer-supported synthesis and one-pot reactions are finally placing the synthesis of large carbohydrate libraries within reach [6–11]. Coupling one-pot strategies with solid-phase screening would therefore produce diverse libraries with direct evaluation.

Previously, researchers have applied solid-phase combinatorial strategies to the synthesis and screening of carbohydrate libraries against biotin-conjugated lectins [12]. On-bead screening protocols have also helped to identify other saccharide-lectin interactions by allowing the observation of labeled lectins [10]. Structural elucidation of library members was acheived by attachment of specific chemical tags at each combinatorial step and subsequent analysis of these tags on desired compounds after screening. This method, however, may prove impractical for larger and more complex libraries. Another method utilizing carbohydrate microarrays on glass slides was able to display dextrans effectively to anti-carbohydrate antibodies, but the carbohydrate's molecular weight significantly influenced immobilization and limited its application to smaller, synthetic oligosaccharides [13].

An alternative to these approaches would be immobilization on multiwell plate surfaces. Previously, this referred to specific lectins immobilized to microtiter plate surfaces and screened against a variety of carbohydrates in solution [14]. This technique, though, is hindered by its application to only one lectin at a time. Immobilization of the carbohydrate would allow for a variety of biological molecules in an ELISA-type fashion and could also reveal biologically important carbohydrate structural motifs.

Currently, several methods are available for immobilization of small molecules onto microtiter plates. Most commonly employ covalent linkage, which can be cleaved upon photolytic or chemical treatment. Noncovalent attachment for the most part has only been applied to large molecules such as proteins. Although oligosaccharides have been immobilized on microtiter plates with biotin-conjugated lectin-avidin complexes [15], few methods for direct and efficient immobilization of small peptides or oligosaccharides exist [16].

One such technique employs methyl vinyl ether-maleic anhydride copolymer (MMAC), which binds to the well surface through hydrophobic interactions stable in aqueous solutions [16]. However, wells must first be pretreated with MMAC followed by introduction of hydrazine groups before the polysaccharide can be attached. An immobilization scheme that was both general and free of interference with biological assays and did not rely on a multi-step process would be preferred. Exploitation of noncovalent interaction would also allow for straightforward removal under established wash conditions. With rapid advancement in carbohydrate combinatorial synthetic techniques and new insights into biological relevance, a small-molecule linker capable of displaying saccharides and exploiting noncovalent interaction was undertaken. The goal was to develop new microfabrication tools for preparation of

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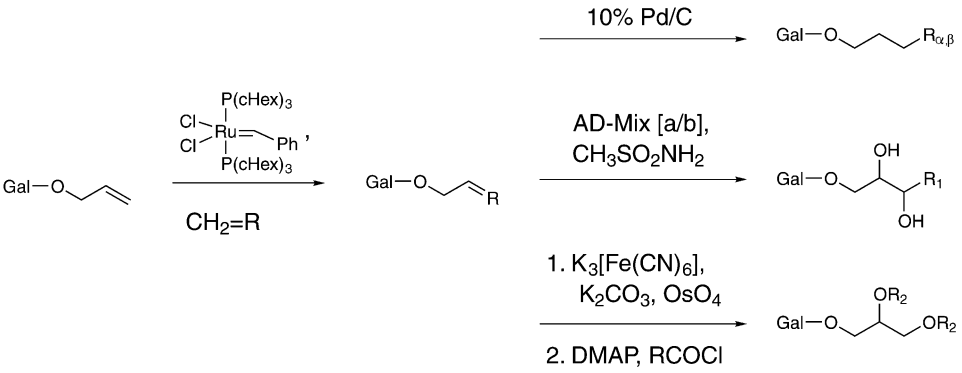


Figure 1. Olefin Metathesis with Subsequent Differentiation

Stereocontrol was maintained in both the dihydroxylation and epoxidation steps. The remaining library member synthesis relied on rudimentary glycosidic coupling with $BF_3{\cdot}OEt_2$ [17]. R_α = $-(CH_2)_nCH_3$ (n = 5, 7, 9, 11, 13, 15, 17) and $-(CH_2)_2C_6H_5$. R_β = $-(CH_2)_nCH_3$ (n = 11, 13). R_1 = $-(CH_2)_nCH_3$ (n = 9, 15). R_2 = $-CO(CH_2)_{13}CH_3$ and $-COCH_2C_6H_5$. R_3 = $-(CH_2)_nCH_3$ (n = 11, 12, 15) and $-(CH_2)_6C_6H_5$.

saccharide arrays giving high throughput screening capabilities to this most important and diverse class of molecules. In this study, we report the development of small-molecule linkers for the noncovalent attachment of sugars to the surface of microtiter plates.

Results and Discussion

Synthesis of Library Members

A variety of hydrophobic moieties ranging from 3 to 21 carbons in length were attached to the anomeric position of galactose via methodologies summarized in Figure 1. Special attention was paid to stereocontrol at the anomeric position. In the presence of participating neighboring groups, β-galactosyllipid synthesis is well established and proceeds with excellent regiochemistry [17]. Glycosylation of the lipid primary alcohol with peracetylated galactose gave individual β-linked members (compounds **15**, **16**, **18**, and **24**) as well as β-allyl galactose.

Anomerically pure α products are not possible with this mechanism without difficult separation of stereoisomers, however. Cross metathesis with allyl galactosides was therefore utilized. This method has proven suitable in several model studies and allows easy variation of the lipid moiety [18, 19]. Respectable *E*/*Z* ratios can be obtained, and glycoside dimerization can be suppressed with additional equivalents of the corresponding lipid. Perbenzylated α-allyl galactose gave only the pure *E* isomer after column chromatography, a prerequisite for subsequent asymmetric dihydroxylation. Olefin metathesis was therefore employed for all unsaturated α members (compounds **1–7**, **21**, and **23**) and the remaining unsaturated β member (compound **20**).

Unsaturation β to the galactose was in turn exploited for further library diversification. Hydrogenation of both α and β members gave library members **8–14**, **17**, **19**, and **22**. Dihydroxylation (**26—29**) was employed to study the effects of increased solubility on the retention of longer carbon chains. Glycolipids with two carbon chains, one containing two short aromatic residues (**25**) and the other with two medium-sized saturated hydrocarbon chains (**30**), were synthesized.

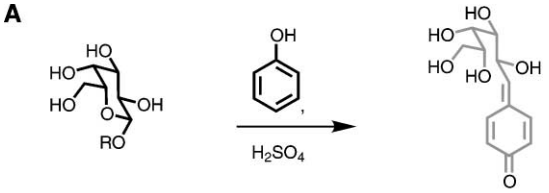
Sulfuric Acid-Phenol Assay

We then exploited galactose to analyze retention to the microtiter plate surface. Screening methods including an enzymatic assay, a galactose-specific labeled lectin, and mass spectral analysis were all considered. However, an inexpensive, facile, nonenzymatic assay independent of any linker effect was eventually used. Phenol in the presence of sulfuric acid (SAP) has previously been used in a colorimetric analysis for quantitative determination of saccharides [20]. Oligosaccharides, simple and methylated sugars, and glycopeptides can all be quantified with this technique, shown in Figure 2A [20, 21]. The SAP assay is inexpensive, rapid, stable, sensitive, and reproducible [21] and was therefore ideal for our purposes.

Sensitivity of the SAP assay for D-galactose was first probed in aqueous solution. Concentrations as low as 20 μM over background were observed consistently for the free sugar. The assay was then scaled down for microtiter plate application, when absorbance was still easily observable over background. Adsorption time varied among library members. After 6 hr, no compound showed more than 50% adsorption to the surface of the plate. After 24 hr, complete adsorption was observed among many library members, but a full 72 hr allowed the highest adsorption of all members.

Library Analysis

Retention of library members to the polystirene surface after washing is summarized in Table 1. Issues affecting both the adsorption and monosaccharide display were observed over the broad range of hydrophobic functionalities. Increased adsorption was expected with increasing tether length, although solubility became a major issue at these concentrations. Compounds with carbon chains longer than 19 (**7**, **14**) formed cottony clusters at 10 mM, as did **20**, which possesses an unsaturated 17-carbon tether. These were therefore loaded at 1 mM and analyzed accordingly. Solubility issues also plagued glycerol **30**. In this case, membranes formed on the surface rather than attaching to the polystirene. Although varying conditions may have allowed these more hydrophobic molecules to interact with the polystirene



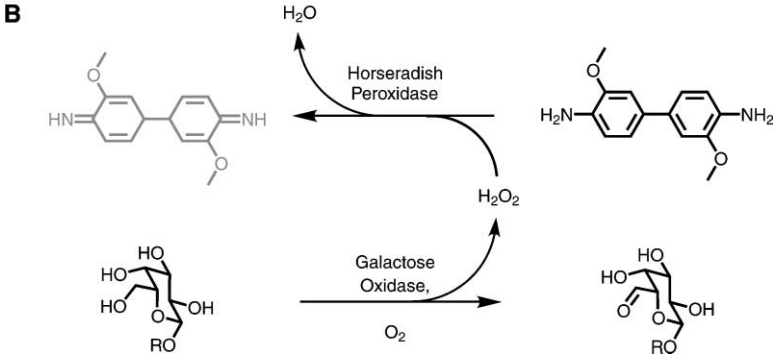


Figure 2. Sulfuric Acid-Phenol Assay and Galactose Oxidase-Peroxidase Assay

Figure 2A illustrates phenol in the presence of strong acid, generating the phenol adduct of the galactosyl lipid; this adduct is observable at 490 nm. Figure 2B shows the galactosyl lipid's oxidation by galactose oxidase as well as the indicator oxidized by the peroxidase. Both assays were performed in a microtiter plate.

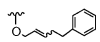
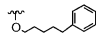
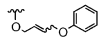
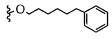
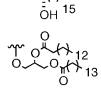
surface, their insolubility would also have hampered the tether's synthetic application. A minimum length for retention was also observed. When tether length dropped below 11 carbons (**1–2**, **8–9**), one wash released a majority from the well surface. The shorter length may have placed the polar saccharide and the nonpolar surface too close and therefore destabilized adsorption. Benzylation, though, gave some advantage to the shorter tethered molecules (**21–24**) but was still too inefficient for further consideration.

Increased solubility through dihydroxylation was also considered. Asymmetric introduction gave no additional benefit to adsorption or retention of the unsaturated 13 carbon tethers (**26–27**) but did aid in the solubility of **28** and **29**, which have 19-carbon chains. No relationship between stereochemistry at the anomeric position and

Table 1. Percent Galactosyl Lipid Bound after One and Five Wash Cycles

		1 Wash	5 Wash
1		12	
2		12	
3		66	
4		45	
5		100	23
6		100	21
7*		100	28
8		30	
9		43	
10		100	100

		1 Wash	5 Wash
11		56	
12		100	51
13		100	43
14*		70	
15		50	
16		100	68
17		100	100
18		100	48
19		100	42
20*		33	

		1 Wash	5 Wash
21		28	
22		46	
23		39	
24		47	
25		59	
26		32	
27		24	
28		100	100
29		91	
30		**	

Percent bound was calculated by dividing the absorbance of the washed wells by the absorbance of unwashed wells. [a]Because of solubility, this was analyzed at 1 mM in DMSO rather than 10 mM. [b]Membrane bilayer formed.

adsorption or retention was seen, with the exception of **5** and **20**. This is most likely an effect of the decreased solubility of the β member.

Additional Washing

We repeated the aqueous wash cycle up to five times to analyze the retention of members exhibiting total retention after one wash cycle; these were namely **5–7**, **10**, **12–13**, **16–19**, and **28**. Library members with tethers 17 carbons and longer showed retention preference for those fully saturated compounds (**12–13** and **18–19**) over their unsaturated counterparts (**5–7**), but none showed significant retention after five washes. Therefore, these long hydrocarbon chains may have formed micelles able to stick to the surface of the well through one wash but not several.

Library members with shorter tethers showed much higher retention after the five-wash cycle independent of stereochemistry. Both **10** and **17** exhibited complete retention, and compound **16** showed a majority of the compound continued to be adsorbed. We believe this would be the case with additional washing as well. **28** also continued to show complete retention, whereas the olefin precursor **6** could not. All tethers are thought to be applicable to library screening because of their facile synthesis and inexpensive starting materials.

Wash Conditions

We studied various washing procedures to analyze not only what the linker could withstand but also how it could be easily removed. The retention of library member **10** under various wash conditions is summarized in Table 2. We tested a series of organic solvents to analyze solid phase synthetic applicability and removal. The addition of methanol (MeOH) to water only mildly lowered the ability of the linker to be retained to the surface, but MeOH alone was able to almost completely remove the linker. Organic solvents such as acetonitrile removed more than half of the library, although the addition of MeOH did not significantly increase linker removal. As

Table 2. Percent Galactosyl Lipid 10 Bound after One Wash

Wash Conditions	Percent Bound
Methanol	29
Methanol:water(1:1)	87
Methanol:acetonitrile (1:1)	40
Acetonitrile	46
Hexanes	73
Dimethyl sulfoxide	33
0.1 M Tris HCl	85
1 mM phosphate buffer	99
10 mM phosphate buffer	97
50 mM phosphate buffer	96
PBS buffer	99
50 mM citrate buffer	100
50 mM bicarbonate buffer	97
50 mM fumarate buffer	99
50 mM acetate buffer	100
TBS buffer	74
1% BSA in TBS buffer	86
0.1% Tween 20 in TBS buffer	71
1% Triton X-100 in TBS buffer	51

Tris: pH 8. Phosphate, citrate, bicarbonate, fumarate, acetate: pH 7. PBS buffer: 50 mM phosphate/150 mM NaCl. TBS buffer: 50 mM Tris HCl (pH 8)/150 mM NaCl. BSA: bovine serum albumin.

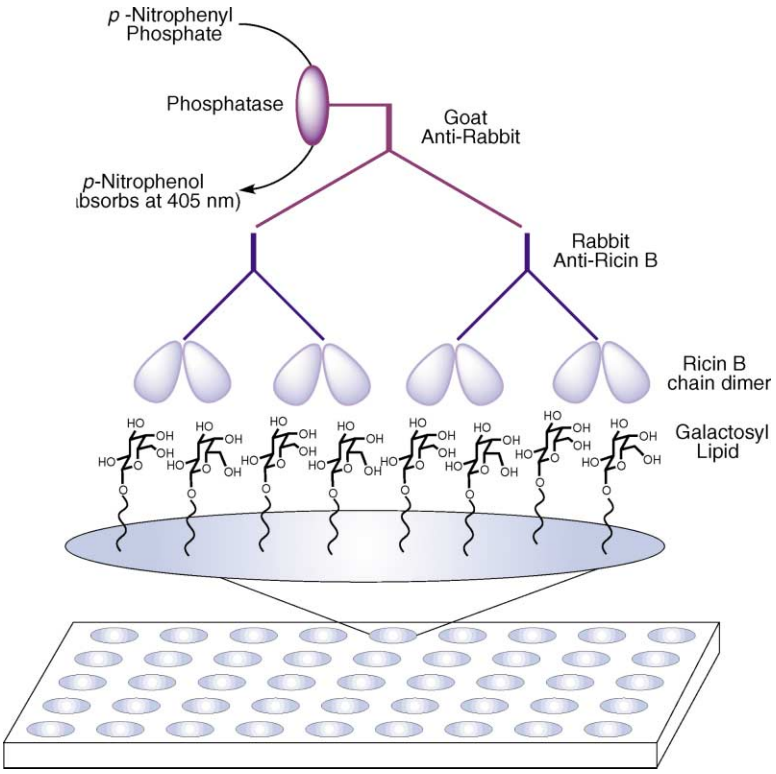


Figure 3. Ricin B Chain Binding

The alkaline phosphatase conjugated to the goat anti-rabbit immunoglobin converts p-nitrophenyl phosphate to p-nitrophenol, which is observable at 405 nm.

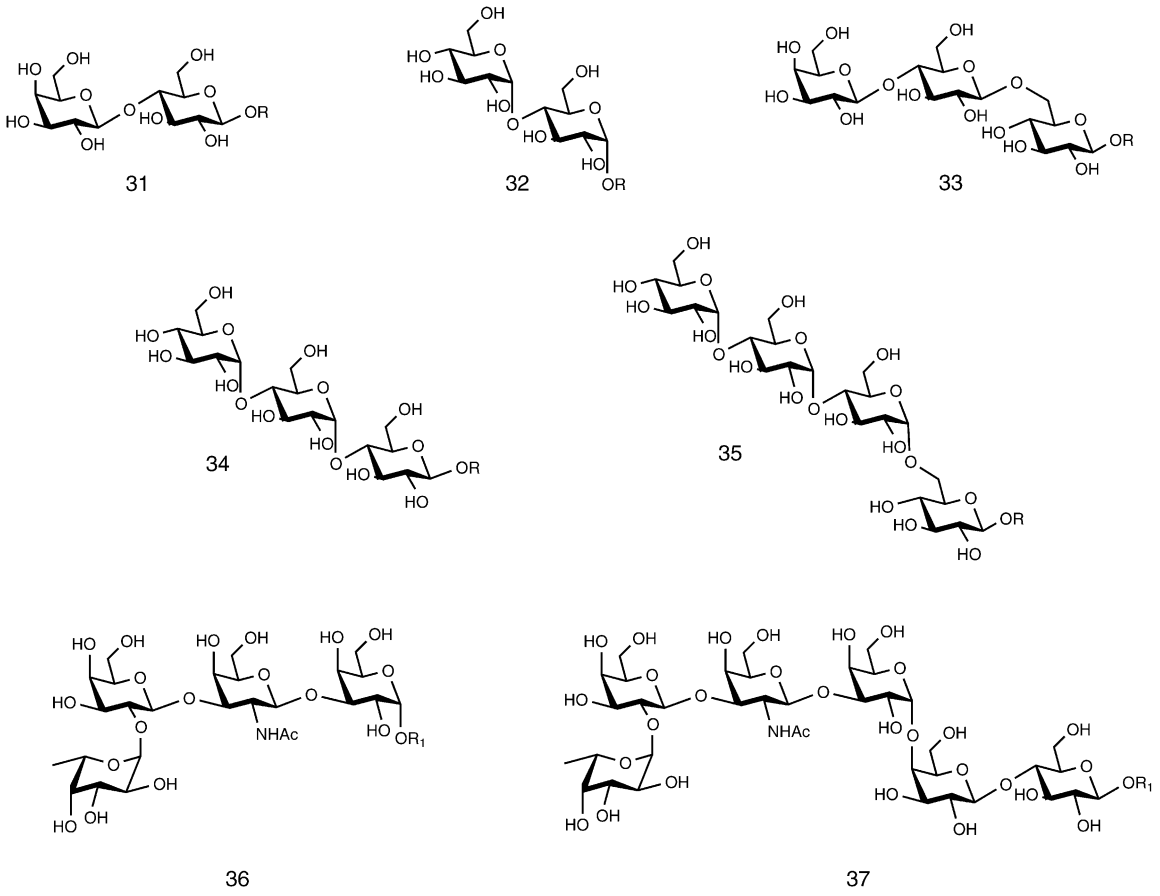


Figure 4. Selected Oligosaccharides
R = -$(CH_2)_{13}CH_3$ and R_1 = -$(CH_2)_5NHCO(CH_2)_{11}CH_3$.

expected, dimethyl sulfoxide (DMSO) was able to remove most of the linker, whereas hexanes did not.

For biological application, a variety of common buffer washes, illustrated in Table 2, were utilized. Although none significantly reduced the retention of library members, TBS buffer removed more than a quarter of the lipid and would not be recommended for biological assays. Citrate and bicarbonate buffers showed the highest linker retention. Increasing the salt concentration or decreasing the buffer concentration improved the retention of certain buffers, and it is therefore believed that the linker could withstand a variety of buffer conditions if these conditions were simply tailored.

The addition of bovine serum albumin (BSA) did not enhance removal. Decreased retention, however, was seen with Tween 20 and Triton X-100. This may hinder the applicability to certain ELISA-type assays, but the linker's stability to BSA washing should be sufficient for many biological applications.

Biological Screening

Effective saccharide display was analyzed with two biological assays. The first employed galactose oxidase (GAO), which is known to recognize certain glycolipids [22]. The coupled enzymatic assay illustrated in Figure 2B is commonly used for analysis of terminal galactose residues by GAO, which oxidizes the C-6 alcohol [23] and produces H_2O_2. Horseradish peroxidase (HRP) can then reduce the peroxide while oxidizing an indicator whose product is observable photometrically [24]. To prevent activation or inhibition of GAO by HRP and the indicator, we added these to the linker in buffer solution and then added GAO [23]. Both library members **10** and **16** were successfully analyzed in this way, proving the ability of these two linkers to display galactose effectively.

Ricin B chain, a lectin specific for β-galactose, was also utilized in a biological assay with **16** [25]. Ricin B presence can be analyzed photometrically via the pathway illustrated in Figure 3. When **16**'s binding to ricin B was compared to wells in which the ricin B chain was adsorbed directly to the surface, full retention of **16** could be seen, illustrating the linker's ability to display galactose effectively.

Oligosaccharides

Retention and display of larger saccharides was critical for further linker applications. The oligosaccharides selected for attachment and screening are illustrated in Figure 4, and the linker utilized in all cases was the fully saturated 14-carbon chain, with the exception of compounds **36** and **37**. The disaccharides lactose and

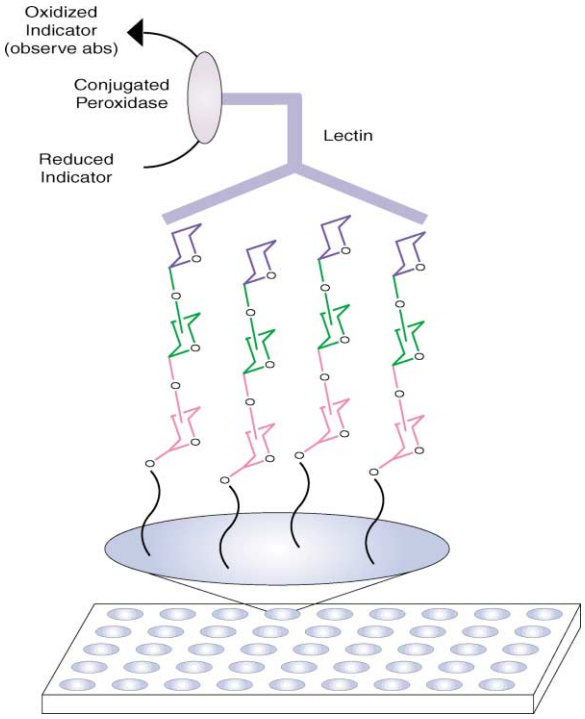


Figure 5. Oligosaccharide-Lectin Binding Assays

Oligosaccharides **32**, **34**, and **35** were analyzed with ConA for α-glucose, whereas **36** and **37** were evaluated with TP for α-fucose. Both lectins were conjugated with a peroxidase, and lectin binding was observed by ABTS oxidation at 405 nm.

maltose were initially investigated. Tetradecyl-α-D-maltoside (**31**) is commercially available (Sigma), and glycosylation of acetylated lactose with tetradecanol with subsequent deprotection gave tetradecyl-β-D-lactoside (**32**). The trisaccharide maltotriose was attached to the linker by glycosidic coupling to yield **33**. Oligosaccharides **34** and **35** were synthesized via electrophilic substitution of bromine at the anomeric position of peracetylated oligosaccharides and coupling with 1,2,3,4-tetraacetyl glucose at the C-6 position. The linker could then be connected as before. Oligosaccharides **36** and **37** were synthesized with a programmable reactivity-based one-pot strategy [26].

When these oligosaccharides were evaluated with the SAP assay, complete retention was seen. Analysis of biological applicability utilized the previously mentioned GAO and ricin B lectin assays as well as the two lectin binding assays illustrated in Figure 5. Those oligosaccharides with terminal galactosyl residues were also observed with both the coupled GAO-HRP assay and the ricin B assay. Concanavalin A (ConA), which binds α-glucosyl residues, shows binding to compounds **32**, **34**, and **35**, whereas Tetragonolobus purpureas (TP), which recognizes α-fucose, was capable of interacting with **36** and **37**. These illustrate the potential for this noncovalent attachment strategy with larger oligosaccharides as well as monosaccharides.

Significance

That carbohydrates are biologically important is recognized, but their mechanism of involvement is still largely elusive. Increased synthetic and screening capabilities are therefore critical. With the advent of rapid oligosaccharide synthesis, complex oligosaccharide libraries are now within reach. Analytical tools for their investigation are now therefore more important than ever. An ideal strategy would involve attachment of library members to microtiter plates and examination of their interaction with a variety of biological components. By screening a small library of galactosyllipids, we discovered noncovalent anchoring strategies for carbohydrate display. Broad application would require cost-effective, facile attachment. Glycosylation or olefin-metathesis with subsequent hydrogenation or dihydroxylation were found to be well suited in gaining access to anomerically well-defined libraries that could withstand a variety of wash conditions and effectively display saccharides for biological analysis. The saccharide microarrays prepared in this study are stable and functional, as illustrated in the various assay conditions involving multiple proteins. In light of these findings, attachment of short-chain (C_{13}–C_{15}) lipids during oligosaccharide synthesis should be highly advantageous to the high-throughput analysis of carbohydrates in biological assays. These simple, hydrophobic linkers may be applicable to other highly hydrophilic compounds, and this strategy is currently being studied.

Experimental Procedures

General

All nonaqueous reactions were run in oven-dried glassware under an inert argon atmosphere. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride prior to use. Unless otherwise noted, reagents and materials were obtained from commercial sources and used as provided. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AMX-400 and AMX-500 MHz spectrometers and were referenced to residual solvent peaks ($CDCl_3$: ^{1}H δ 7.26, ^{13}C δ 77.0; CD_3OD δ 3.30, ^{13}C δ 49.0). All materials for biological assays were purchased from Sigma except for the Costar brand 96-well half-area high binding polystirene plates utilized in all multi-well assays purchased from Fisher Scientific. A Ceres UV 900 HDi plate reader operating the KC-Jr. Curve Fit Program was utilized for absorbance measurements.

Preparation of Unsaturated Galactosyllipids by Olefin Metathesis

To α-D-allyl-2,3,4,6-tetra-*O*-acetyl-galactoside (1 mmol) and the coupling olefin (4 mmol) in dry CH_2Cl_2 (8 ml) was added a solution of Grubb's catalyst (0.07 mmol, Strem) in CH_2Cl_2 (2 ml). The reaction mixture was heated to reflux over 20 hr, concentrated in vacuo, and purified by column chromatography (hexanes:ethyl acetate [EtOAc], 3:1) to give the metathesis product as an *E*/*Z* mixture. This was dissolved in MeOH, and 5% sodium methoxide (NaOMe) in MeOH (3 ml) was added. The reaction mixture was stirred for 2 hr, and ion exchange resin (Amberlyst 15, H^+ form) was added. After being stirred for an additional 5 min, the reaction mixture was diluted with MeOH and filtered. Evaporation gave the unsaturated metathesis products **1–7**, **20**, **21**, and **23**.

Hydrogenation of Unsaturated Galactosyllipids

10% Pd on charcoal (20 mg) suspended in MeOH (20 ml) was set under hydrogen for 15 min, and a solution of the unsaturated saccharide (0.5 mmol) in MeOH (5 ml) was added. The reaction mixture was stirred overnight, diluted with MeOH and EtOAc, and filtered through Celite. Evaporation of the solvent gave **8–14**, **17**, **19**, and **22**. Compound **10**—^{1}H-NMR (500 MHz, CD_3OD): 4.77 (1H); 3.86 (d, 1H, J = 1.9 Hz); 3.77 (t, 1H, J = 6.0 Hz); 3.74–3.64 (m, 5H,); 3.40 (dt, 1H, J = 9.7, 6.5 Hz); 1.60 (m, 2H); 1.35 (m, 2H); 1.33–1.88 (m, 18H); 0.86 (t, 3H); ^{13}C-NMR (125 MHz, CD_3OD): 100.27; 72.24; 71.53; 71.01; 70.25; 69.20; 62.64; 33.03; 30.75; 30.62; 30.59; 30.45; 27.31; 23.70;

14.45; MALDI-FTMS: calculated ($C_{19}H_{39}O_6Na^+$) = 385.256, found 385.2558. Compound **11**—^{1}H-NMR (500 MHz, CD_3OD): 4.77 (1H); 3.86 (d, 1H, J = 1.6 Hz); 3.77 (t, 1H, J = 6.0 Hz); 3.73-3.63 (m, 5H,); 3.40 (dt, 1H, J = 9.5, 6.4 Hz); 1.60 (m, 2H); 1.34 (m, 2H); 1.31–1.20 (m, 26 H); 0.8 (t, 3H); ^{13}C-NMR (125 MHz, CD_3OD): 100.27; 72.21; 71.52; 71.00; 70.24; 69.20; 62.64; 33.04; 30.78; 30.76; 30.74; 30.64; 30.59; 30.45; 27.32; 23.70; 14.47; MALDI-FTMS: calculated ($C_{21}H_{42}O_6Na^+$) = 413.2873, found 413.2865. Compound **17**—^{1}H-NMR (500 MHz, CD_3OD): 4.19 (d, 1H, 7.3 Hz); 3.87 (m, 1H, J = 1.6 Hz); 3.83 (d, 1H, J = 2.2 Hz); 3.73 (dd, 1H, J = 5.7, 2.0 Hz); 3.55–3.41 (m, 4H); 1.60 (m, 2H); 1.38 (m, 2H); 1.38–1.20 (m, 26 H); 0.87 (t, 3H); ^{13}C-NMR (125 MHz, CD_3OD): 104.51; 76.03; 74.64; 72.21; 70.69; 69.81; 62.03; 32.75; 30.49; 30.45; 30.33; 30.17; 26.79; 23.44; 14.39; MALDI-FTMS: calculated ($C_{21}H_{42}O_6Na^+$) = 413.2873, found 413.2869.

Preparation of Saturated α-Galactosyl Glycerols

To α-D-allyl-2,3,4,6-tetra-*O*-benzyl-galactoside (300 mg, 0.52 mmol) suspended in *t*-butanol/H_2O (1:1; 10 ml) was added $K_3[Fe(CN)_6]$ (990 mg, 3 mmol), K_2CO_3 (420 mg, 3 mmol), and OsO_4 (0.08 M in *t*-butanol, 7 ml). The reaction was stirred vigorously, cooled to 0°C, and quenched by the addition of solid sodium sulfite (1.7 g, 13.5 mmol). The mixture was partitioned between H_2O and EtOAc, and the aqueous layer was extracted with EtOAc (3×). The combined organic layers were washed with brine, dried over sodium sulfate (Na_2SO_4), concentrated in vacuo, and purified by column chromatography to give the two diastereomers (1.6:1). This mixture was dissolved in pyridine (10 ml) and catalytic DMAP, and the acid chloride (3 equivalents) was added. After 12 hr, the reaction mixture was poured on ice and extracted with EtOAc (3×). The combined organic layers were washed with saturated $NaHCO_3$ (3×) and brine, dried over Na_2SO_4, concentrated in vacuo, and purified by column chromatography. Hydrogenation was performed as before to give the free galactosyl glycerols **25** and **30**.

Sharpless Dihydroxylation of Galactosyllipids

The unsaturated starting material for this reaction was prepared by cross metathesis as described in the synthesis of compounds **1–7** with α-D-allyl-2,3,4,6-tetrabenzylgalactoside as the glycosidic component. The isomerically pure compound was accessible by column chromatography. AD-Mix [α/β] (1.4 g) was dissolved in *t*-butanol:H_2O (4:5; 9 ml). The mixture was stirred until phases went clear. Methane sulfonamide (95 mg, 1 mmol) was added, and the solution was cooled to 4°C. The starting material (0.5 mmol) dissolved in *t*-butanol (1 ml) was added to the reaction mixture, and stirring continued until complete conversion was seen by TLC. Sodium sulfite (1.4 g, 11.1 mmol) was added, the reaction mixture was raised to room temperature and diluted with EtOAc, and the aqueous layer was extracted with EtOAc (3×). The combined organic layers were extracted with brine and dried over Na_2SO_4. After evaporation of the solvent, the major diastereomer (**26–29**) was obtained in pure form by column chromatography. Compound **28**—^{1}H-NMR (500 MHz, $CDCl_3/CD_3OD$): 4.91 (d, 1H, J = 3.7); 3.97 (d, 1H, J = 2.2 Hz); 3.92–3.72 (m, 5H); 3.68 (m, 1H,); 3.63 (m, 2H); 1.53 (m, 3H); 1.43–1.25 (m, 28 H); 0.92 (t, 3H); ^{13}C-NMR (125 MHz, $CDCl_3/CD_3OD$): 99.46; 72.72; 71.94; 71.30; 70.56; 70.12; 69.78; 69.37; 61.98; 42.74; 33.25; 32.16; 29.98; 29.92; 29.88; 29.58; 26.11; 22.88; 13.96; MALDI-FTMS: calculated ($C_{25}H_{50}O_8Na^+$) = 501.3398, found 501.3389.

Preparation of Saturated Galactosyllipids by Glycosylation

The saccharide (0.4 mmol) was dissolved in pyridine (2 ml) and brought to 0°C, and acetic anhydride (1 ml) was added. When acetylation was complete, the reaction was quenched with MeOH, concentrated, and purified by column chromatography (1:1–3:1 EtOAc:hexanes). The fully acetylated β-D-saccharide (0.26 mmol) was dissolved in CH_2Cl_2 (2.6 ml), and the reaction was brought to 0°C. The primary alcohol of the linker (0.52 mmol) and boron trifluoride diethyl etherate (0.39 mmol) were added. After 4 hr, the reaction was quenched with saturated $NaHCO_3$ (5 ml) and extracted with EtOAc. The organic phase was dried over Na_2SO_4, concentrated in vacuo, and purified by flash chromatography (EtOAc:hexanes, 1:3) to give the acetylated β-D-linked glycolipid. This (0.1 mmol) was dissolved in MeOH (1 ml), and NaOMe in H_2O (1 M) was added dropwise until a basic pH was reached. Upon deprotection, DOWEX 50WX4-50 ion-exchange resin was added, and the reaction mixture was filtered and concentrated to give **15–16**, **18**, **24**, **32**, and **33**. Compound **16**—^{1}H-NMR (400 MHz,$CDCl_3$): 4.95 (d, 1H, J = 2.0 Hz); 4.10 (s, 1H); 3.98–3.94 (m, 1H); 3.87–3.77 (m, 4H,); 3.74–3.70 (m, 1H); 3.48–3.43 (m, 1H); 2.34–2.09 (s, br, 4 H); 1.26 (s, 26H); 0.88 (m, 3H); ^{13}C-NMR (400 MHz, CD_3OD): 100.32; 72.34; 71.55; 71.18; 70.29; 69.21; 33.08; 30.82; 30.77; 30.66; 30.61; 30.50; 27.36; 23.75; 14.45; MALDI-FTMS: calculated ($C_{20}H_{40}O_6Na^+$) = 399.271, found 399.272. Compound **31**—^{1}H-NMR (400 MHz,CD_3OD): 4.66 (d, 1H, J = 3.8 Hz); 4.25 (d, 1H, J = 7.36); 3.77–3.65 (m, 5H); 3.61–3.58 (m, 3H,); 3.50–3.48 (m, 1H); 3.46–3.45 (m, 1H); 3.44–3.43 (m, 1H); 3.39 (d, 1H, J = 3.2); 3.37–3.36 (m, 1H); 3.35–3.34 (m, 1H); 1.20 (s, 26H); 0.82 (m, 3H); ^{13}C-NMR (400 MHz, CD_3OD): 105.11; 99.90; 80.95; 77.09; 74.82; 73.50; 73.25; 72.56; 72.04; 70.30; 69.32; 62.49; 61.84; 33.08; 30.82; 30.77; 30.63; 30.50; 27.36; 23.74; 14.45; MALDI-FTMS: calculated ($C_{26}H_{50}O_{11}Na^+$) = 561.325, found = 561.325. Compound **34**—^{1}H-NMR (400 MHz, CD_3OD): 5.06 (m, 2H); 4.66 (d, 0.5H, J = 3.5 Hz); 4.16 (d, 1H, J = 7.9); 3.80–3.64 (m, 8H); 3.60–3.49 (m, 5H,); 3.42–3.38 (m, 4H); 3.35–3.32 (m, 2H); 3.16–3.09 (m, 1H); 1.53–1.50 (m, 2H); 1.20 (s, 22H); 0.80 (m, 3H); ^{13}C-NMR (400 MHz, CD_3OD): 101.05; 100.9; 98.13; 79.92; 79.4; 79.34; 75.97; 74.69; 73.21; 73.08; 73.04; 72.9; 72.82; 73.39; 72.03; 71.91; 71.45; 71.34; 70.40; 69.61; 69.10; 67.47; 60.85; 60.35; 60.27; 31.22; 28.93; 28.7; 28.62; 25.3; 21.88; 12.60; MALDI-FTMS: calculated ($C_{32}H_{60}O_{16}Na^+$) = 723.3773, found = 723.3783.

Tetradecyl β-D-Gal(1→4)β-D-Glc((1→6)D-Glucose (33) and Tetradecyl α-D-Glc(1→4)α-D-Glc(1→4)D-Glc(1→6) β-D-Glucose (35)

33% HBr in acetic acid (5 ml) was added to the fully acetylated β-D-saccharide (1.5 mmol) in acetic anhydride (2 ml) and acetic acid (2 ml). The bromide (1.2 mmol) was dissolved in CH_2Cl_2 (2.4ml) and 1,2,3,4-tetraacetyl β-D-glucose (1.3 mmol) was added. Silver carbonate (1.2 mmol) was added and the reaction was covered with aluminum foil and stirred. After 1h, the reaction was filtered through celite, concentrated and purified by column chromatography (1:1 to 3:1 EtOAc: hexanes). Coupling with tetradecanol, deprotection and purification followed previously discussed procedures to give **33** and **35**. Compound **33**: ^{1}H-NMR (400 MHz, CD_3OD): 4.33–4.03 (m, 3H); 3.81–3.76 (m, 2H); 3.74–3.73 (d, 2H, J = 2.9); 3.70–3.69 (m, 1H,); 3.67 (s, .5H); 3.63–3.59 (m, 1.5H); 3.51–3.42 (s, br, 5H); 3.41–3.40 (d, 1H, J = 2.9); 3.38–3.33 (m, 2H); 3.27–3.18 (m, 2H); 3.12–3.06 (m, 1H); 1.55–1.51 (m, 2H) 1.20 (s, 22H); 0.81 (m, 3H). ^{13}C-NMR (400 MHz, CD_3OD): 105.13; 104.68; 104.43; 80.54; 77.97; 77.11; 76.98; 76.56; 76.39; 75.09; 74.84; 72.57; 71.42; 71.10; 70.32; 69.78; 62.51; 61.88; 33.10; 30.82; 30.79; 30.67; 30.50; 27.16; 23.75; 14.45. MALDI-FTMS: calculated ($C_{32}H_{60}O_{16}Na^+$) = 723.377; found = 723.369.

Sulfuric Acid-Phenol Assay

Library compounds (10 mM in DMSO, 14 μl) were added to wells. After 72 hr, excess solvent was removed from all wells. This procedure was the same in all assays. Exchange of dH_2O (50 μl) with an automatic pipetman three times constituted one wash. When other wash conditions were utilized, the general procedure was the same. The SAP method is that described by Saha et al. [20]. A 5% phenol solution in dH_2O (14 μl) followed rapidly by concentrated H_2SO_4 (70 μl) was added to each well. After 30 min, absorbance was measured at 490 nm. Blank absorbance measurements contained dH_2O, phenol solution, and concentrated H_2SO_4. Positive controls were not washed.

Galactose Oxidase-Horseradish Peroxidase Coupled Assay

The enzymatic method is that described by Amaral and coworkers [24]. After excess solvent was removed, the wells were washed with dH_2O as before. Peroxidase (EC 1.11.1.7, type II from Horseradish, 100 U/ml, 1 μl) in Tris buffer (0.1 M, pH 8.0) and *o*-dianisidine (1 mg/ml MeOH, 1 μl) were then added. Galactose oxidase in buffer (EC 1.1.3.9, from *Dactylium dendroides*, 200 U/ml, 1 μl) was added last. The plate was read at 490 nm over 30 min.

Ricin B Assay

Library compound **16** was loaded and washed as before. Wells were then blocked with TBS buffer (50 mM Tris-HCl pH 7.5/150 mM NaCl; 100 μl) containing 1% bovine albumin (BSA) (buffer A). Buffer A was removed after an hour. This wash procedure was repeated between

all incubation steps. The ricin B chain from *Ricinus communis* (castor bean)(0.1 mg/ml buffer A; 50 μl) was incubated in the well for 1 hr, followed by incubation with the anti-lectin for *Ricinus communis* from rabbit (5 μg/ml buffer A, 50 μl). Monoclonal anti-rabbit immunoglobulin conjugated with an alkaline phosphatase (5 μg/ml in 0.05 M bicarbonate buffer at pH 9.6; 50 μl) was incubated last. Wells were subsequently washed with buffer A (100 μl) overnight. *p*-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer [pH 9.8], 0.5 mM $MgCl_2$, 100 μl) was added, and absorbance was read at 405 nm over 30 min.

Concanavalin A (ConA) Lectin Assay

The lectin binding method is based on research described by Leriche and coworkers [27]. Linker incubation and aqueous washing proceeded as before. After the oligosaccharide was incubated in the well, the wells were washed with 100 μl TBS buffer (50 mM Tris-HCl [pH 7.5]/150 mM NaCl) containing 1% bovine albumin (BSA) (buffer A) over an hour. The buffer was then removed, and ConA conjugated with a phosphatase (10 μg/ml buffer A, 50 μL) was incubated in the well over an hour. The wash procedure with buffer A was then repeated over an hour. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was then prepared according to the manufacturer and added to the well (50 μl), and the plate was allowed to develop in the dark for 15 min. The plate was then read at 405 nm.

Tetragonolobus Purpureas (TP) Lectin Assay

Linker incubation and aqueous washing proceeded as before. After the oligosaccharide was incubated in the well, the wells were washed with 100 μl TBS buffer (50 mM Tris-HCl [pH 7.5]/150 mM NaCl) containing 1% bovine albumin (BSA) (buffer A) over an hour. The buffer was then removed, and TP conjugated with a phosphatase (0.1 mg/ml buffer A; 50 μl) was incubated in the well over an hour. The wash procedure with buffer A was then repeated over an hour. ABTS was then prepared according to the manufacturer's instructions and was added to the well (50 μl), and the plate was allowed to develop in the dark for 15 min. The plate was then read at 405 nm.

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