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Neoglycolipid Probes Prepared via Oxime Ligation for Microarray Analysis of Oligosaccharide-Protein Interactions

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SUMMARY

Neoglycolipid technology is the basis of a microarray platform for assigning oligosaccharide ligands for carbohydrate-binding proteins. The strategy for generating the neoglycolipid probes by reductive amination results in ring opening of the core monosaccharides. This often limits applicability to short-chain saccharides, although the majority of recognition motifs are satisfactorily presented with neoglycolipids of longer oligosaccharides. Here, we describe neoglycolipids prepared by oxime ligation. We provide evidence from NMR studies that a significant proportion of the oxime-linked core monosaccharide is in the ring-closed form, and this form selectively interacts with a carbohydrate-binding protein. By microarray analyses we demonstrate the effective presentation with oxime-linked neoglycolipids of (1) Lewis^x trisaccharide to antibodies to Lewis^x, (2) sialyllactose analogs to the sialic acid-binding receptors, siglecs, and (3) *N*-glycans to a plant lectin that requires an intact *N*-acetylglucosamine core.

INTRODUCTION

Oligosaccharide chains of glycoproteins, glycolipids, and glycosaminoglycans can mediate important biological processes through their interactions with complementary proteins [1, 2]. However, detailed analysis of carbohydrate-protein interactions has been a challenging area due to the structural complexities of oligosaccharide sequences, the limited amounts that can be isolated, and the low affinities of the interactions. This has motivated developments in the design and synthesis of carbohydrate probes and analytical techniques for sensitive detection of their interactions with proteins [2, 3], culminating in the development of carbohydrate microarrays for high-throughput screening of carbohydrate-protein interactions [4–6].

With the exception of polysaccharides [7, 8] and natural glycolipids [2], chemical derivatizations are required for immobilizing carbohydrates for microarrays, as hydrophilicities of oligosaccharides preclude their direct noncovalent immobilization on solid matrices. One principle is to conjugate oligosaccharides to lipid by reductive amination to generate neoglycolipid (NGL) probes with amphipathic properties for arraying [9]. The use of reductive amination has also been described for preparing fluorescent oligosaccharide derivatives that contain a primary amine for array generation [10]. Most other mono- or oligosaccharide probes generated for printing have been chemically synthesized, requiring substantial chemical expertise for access to defined structures that incorporate specific functional groups, e.g., thiol [11], maleimide [12], amine [13, 14], azide [15], and cylcopentadiene [16] functionalities, or proteins as tags [11, 17] for covalent attachment to matrices. Oligosaccharides with lipid [18] or fluorous tags [19] for noncovalent immobilization have also been described. These methods are promising due to compatibilities with advanced synthetic chemistries [20, 21], but relatively few structures can be obtained by current synthetic approaches [22]. Moreover, multistep manipulations limit applications to the small quantities of oligosaccharides isolated from natural sources. As natural oligosaccharides are crucial for discoveries of hitherto unknown oligosaccharide ligands [2, 4], carbohydrate microarrays should ideally encompass oligosaccharides from both synthetic and natural sources. Approaches have been described for printing and covalent attachment of unmodified mono- and short oligosaccharides onto aminooxy- or hydrazide-modified glass slides [23, 24] and hydrazide-modified monolayers on gold [25]. Disadvantages

of direct surface immobilization are the high concentrations of saccharides required and the potential variation of the immobilization efficiencies depending on the nature of the saccharides.

The microarray platform based on NGL technology, which also encompasses glycolipids [2, 9, 26], is versatile. The facile synthesis of NGLs by microscale reductive amination allows minute amounts of oligosaccharides released from *O*- and *N*-glycosylated proteins, glycosaminoglycans, and polysaccharides to be converted into lipid-linked probes. These can be robustly immobilized on solid matrices in clustered display for direct binding experiments. It is particularly advantageous that NGL technology provides a way to resolve by high-performance (HP) TLC the mixtures of oligosaccharide probes, and to perform binding experiments on TLC plates in conjunction with oligosaccharide sequence determination by mass spectrometry in situ [27]. Over the years, the NGL principle has led to discoveries of unsuspected oligosaccharide sequences on glycoproteins [28] as well as new oligosaccharide ligands for carbohydrate-binding proteins [29] (other examples are reviewed in [30]). More recently, the NGL-based microarray system has been validated with known sequence-specific carbohydrate-binding proteins and sequence-defined oligosaccharide probes [2, 9], and it has served as the basis for "designer" oligosaccharide arrays generated from bacterial polysaccharides targeted by a novel carbohydrate-recognizing protein of the innate immune system, Dectin-1 [31].

In preparing conventional NGLs, the reducing ends of the oligosaccharides are reacted with an aminolipid, 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE), and are reduced with cyanoborohydride to form stable conjugates (referred to as DH-NGLs). Other than ring opening of the monosaccharide residues at reducing ends, oligosaccharides remain intact. DH-NGLs derived from tri- or larger oligosaccharides have performed well for the majority of carbohydrate-recognition systems that have the peripheral or backbone regions of oligosaccharides as recognition motifs [2]. However, ring opening of reducing-end monosaccharides may affect the biological activities of oligosaccharides. For instance, DH-NGL of Lewis^x (Le^x) trisaccharide is not bound by anti-Le^x antibodies [32], and that of sialyl-Le^x tetrasaccharide is not bound by the selectins [33]. It is highly desirable to overcome this limitation and enhance the applicabilities of NGL probes derived from short oligosaccharides that are the most accessible via chemical synthesis. In addition, certain plant lectins require both intact core and backbone regions of *N*-glycans to elicit strong binding [34]. To cater for recognition systems that have a requirement for intact core monosaccharides, and to enhance the applicabilities of NGLs, our aim is to develop NGLs with ring-closed monosaccharide cores.

Oxime ligation is an alternative to reductive amination for oligosaccharide derivatization [35–44]. It is highly selective toward the reducing end of carbohydrates, and the conjugates are stable within a wide pH range. Among recent applications are those involving the use of aminooxy-functionalized solid particles for capturing oligosaccharides from solutions [45, 46]. However, reports on the status of oxime-linked monosaccharide cores have been conflicting. Some investigators have provided evidence for the presence of ring-closed forms in equilibrium with the ring-opened forms in the sugar oximes [35, 37, 42], whereas others have reported that oxime-linked monosaccharides exist only in ring-opened forms [41, 47, 48]. This is a particularly important question to answer in the context of functional preservation and recognition of the core monosaccharides in oxime derivatives.

Here, we describe the preparation of oxime-linked NGLs (AO-NGLs) from various reducing sugars by ligation to an aminooxy-functionalized DHPE (AOPE). We provide evidence from NMR and carbohydrate-binding studies for the presence of a significant proportion of ring-closed, oxime-linked monosaccharide cores. We evaluate the oxime-linked NGLs in comparison with conventional DH-NGLs in carbohydrate microarrays for recognition by 12 carbohydrate-binding proteins that include anti-Le^x antibodies, plant lectins, and mammalian sialic acid-binding receptors known as siglecs [49].

RESULTS AND DISCUSSION

Synthesis of Oxime-Linked AO-NGLs

We modified DHPE, the lipid used for synthesizing conventional NGLs, to incorporate the aminooxy functionality in two steps (Figure 1A). The amino group of DHPE was first coupled to Boc-aminooxyacetic acid (Boc-AOAcOH) by activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The product Boc-AOPE was purified and deprotected by treatment with trifluoroacetic acid (TFA). The reaction yields in both steps were quantitative, as indicated by HPTLC analysis (see the Supplemental Data available with this article online). The final product, AOPE, was confirmed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and was used without purification.

AO-NGLs were prepared via a microscale oxime ligation reaction (Figure 1B) whereby the aldehyde group of a reducing sugar was conjugated to the aminooxy group of AOPE under mild conditions without reduction. A total of 28 reducing sugars that include 10 neutral mono- and disaccharides, a typical heparin disaccharide, 2 *N*-glycans, 3 Le^a- and Le^x-related oligosaccharides, 6 chemically synthesized sialyllactose analogs, a natural 4-*O*-acetylated analog, and 4 glucan fragments were converted into AO-NGLs (these and the abbreviations used are included in Table 1). HPTLC analysis showed that conjugation efficiencies were greater than 90% after 6–16 hr for most oligosaccharides (examples are given in the Supplemental Data). Conjugation was less efficient for glucan oligosaccharide fragments (Pust-7, Dex-7, Curd-11, and Curd-13), as was the case in their derivatization by reductive amination [31]. For these oligosaccharides, incubation was prolonged (24 hr) under acidic conditions by using a solvent with higher water content to increase their solubilities; conjugation efficiency was

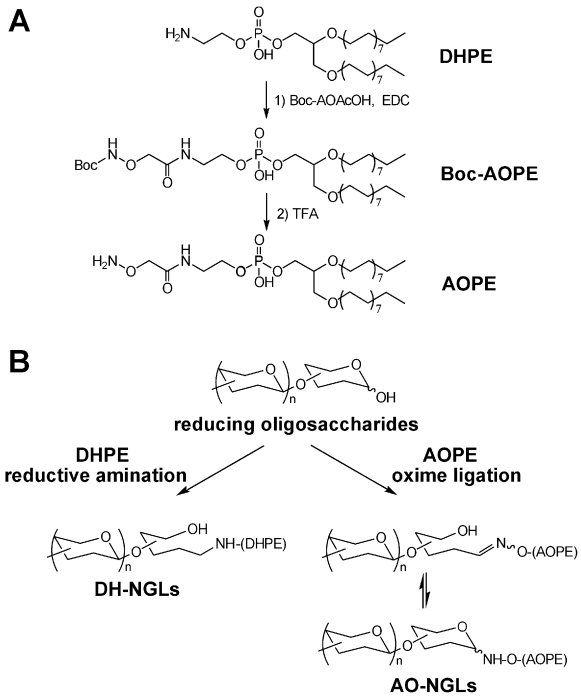

Figure 1. Reaction Schemes for Preparation of the Aminooxy-Functionalized Lipid AOPE and NGLs

(A) A two-step procedure for preparing AOPE.

(B) Generation of DH- and AO-NGLs from reducing oligosaccharides by reductive amination and oxime ligation reactions.

thus increased to ~70%. The water tolerance of oxime ligation contrasts with reductive amination (where water is an inhibitor) and is a potential advantage when preparing NGLs from large and highly acidic oligosaccharides that require water to assist solubilization.

AO-NGLs were purified by semipreparative TLC or silica cartridge, examined for purity by HPTLC, and quantified as described for conventional DH-NGLs [50]. The molecular masses determined by MALDI-MS were in accord with theoretical values. Examples of HPTLC and MS analysis of purified AO-NGLs are available in the Supplemental Data. AO-NGLs are stable for at least 12 months at −20°C.

Evaluation of the Status of the Oxime-Linked Core Monosaccharide

Bearing in mind that the carbohydrate-protein interactions in the microarray analyses with AO-NGLs are assayed in an aqueous environment and that NGLs have poor solubilities in water, we prepared a water-soluble analog of Fuc.AO (Fuc.AOAcOH) by conjugation of fucose with aminooxy-acetic acid and carried out NMR experiments in D_2O. We assigned both ring-opened (*E* and *Z*) and ring-closed forms of the oxime-linked sugar (^1H and ^{13}C-NMR assignments are listed in the Supplemental Data). Integration of the three H1 resonances indicated proportions of about 70:15:15 for the *E* form, the *Z* form, and the ring-closed β form, respectively.

Furthermore, in a novel approach to the study of the recognition of oxime-linked sugars within isomeric mixtures, we performed saturation transfer difference (STD) NMR experiments in the presence of a fucose-specific lectin from *Aleuria aurantia*. The interaction between fucose and this lectin has been shown to be suitable for STD NMR spectroscopy [51]. Our results show that the protein binds exclusively to the ring-closed form of Fuc.AOAcOH. Five STD NMR spectra were generated by using protein irradiation at different frequencies, and the patterns were very similar. One of the spectra is shown in Figure 2. The STD spectrum is dominated by a group of signals that correspond to relatively minor resonances in the control spectrum (15% of the isomeric mixtures of Fuc.AOAcOH); these are assigned as the oxime-linked, ring-closed β-fucose. All of the proton signals from the ring-closed β form of Fuc.AOAcOH are present in the STD spectrum, and a resonance at 4.15 ppm arising from the methylene of the aminooxy linker was also observed in the STD spectrum. There is precedent for the interaction of *Aleuria aurantia* lectin with a moiety in the vicinity of the fucose glycosidic linkage: in STD NMR studies on a glycoside of sialyl Le^x, the lectin was shown to interact with the *N*-acetyl group of the neighboring *N*-acetylglucosamine in addition to the fucose residue [51].

The behavior of AO- and DH-NGLs prepared from fucose were next investigated by using phenylboronic acid (PBA) chromatography under the controlled conditions [50, 52] that we have previously shown to fully retain neutral DH-NGLs with unbranched monosaccharide cores; details of the experiments with Fuc-derived NGLs are in the Supplemental Data. As predicted, there was partial retention of Fuc.AO, in contrast with full retention of the DH analog, which is in reduced form and thus exclusively has the open-chain vicinal diols. The results are consistent with the presence of both ring-opened and ring-closed forms of the fucose in the Fuc.AO, as depicted in Figure 1B.

The functional preservation of the oxime-linked monosaccharide was also shown in binding assay with immobilized NGLs probed with *Aleuria aurantia* lectin. We arrayed on nitrocellulose the AO- and DH-NGLs of fucose and the NGL pairs of six other monosaccharides for binding studies (Figure 3). Fucose was bound only in the form of the AO-NGL, not the DH-NGL. The binding was specific, as the AO- and DH-NGLs of the other monosaccharides were not bound.

Microarray Analyses of AO- and DH-NGL Probes with Antibodies, Plant Lectins, and Siglecs

A more detailed evaluation of the performance of AO-NGLs as probes for carbohydrate-protein interactions was carried out in a microarray format. Robotically printed microarrays of 64 lipid-linked saccharides (Table 1) were prepared. These included 24 AO-NGLs together with their respective DH-NGL counterparts, among which were those prepared from saccharides we knew to be unreactive or poorly reactive with particular recognition proteins when presented as DH-NGLs with the reduced open-chain

Table 1. Lipid-Linked Saccharide Probes Investigated

Abbreviation	Saccharide	Probe Type[a]	Probe Number
Glc	Glc	DH	**1**
		AO	**2**
Gal	Gal	DH	**3**
		AO	**4**
Man	Man	DH	**5**
		AO	**6**
Fuc	Fuc	DH	**7**
		AO	**8**
Rha	Rha	DH	**9**
		AO	**10**
GlcNAc	GlcNAc	DH	**11**
		AO	**12**
GalNAc	GalNAc	DH	**13**
		AO	**14**
Man6-P	HPO_3-6Man	DH	**15**
		AO	**16**
Lac	Galβ-4Glc	DH	**17**
		AO	**18**
LacNAc	Galβ-4GlcNAc	AO	**19**
Hep IS	ΔUA(2S)-4GlcNS(6S)[b]	AO	**20**
Malto5	Glcα-4Glcα-4Glcα-4Glcα-4Glc	DH	**21**
		AO	**22**
NA2	Galß-4GlcNAcß-2Manα-6 Manß-4GlcNAcß-4GlcNAc Galß-4GlcNAcß-2Manα-3	DH	**23**
NGA2	GlcNAcß-2Manα-6 Manß-4GlcNAcß-4GlcNAc GlcNAcß-2Manα-3	DH	**24**
NA2F	Galß-4GlcNAcß-2Manα-6 Fucα-6 Manß-4GlcNAcß-4GlcNAc Galß-4GlcNAcß-2Manα-3	DH	**25**
		AO	**26**
Man3GN2	Manα-6 Manß-4GlcNAcß-4GlcNAc Manα-3	DH	**27**
Man5GN2	Manα-6 Manα-3Manα-6 Manß-4GlcNAcß-4GlcNAc Manα-3	DH	**28**

Table 1. *Continued*

Abbreviation	Saccharide	Probe Type[a]	Probe Number
Man9GN2	Manα-2Manα-6 | Manα-2Manα-3Manα-6 | Manß-4GlcNAcß-4GlcNAc | Manα-2Manα-2Manα-3	DH	**29**
		AO	**30**
Le[a]-tri	Galß-3GlcNAc | Fucα-4	DH	**31**
		AO	**32**
Le[x]-tri	Galß-4GlcNAc | Fucα-3	DH	**33**
		AO	**34**
Le[x]-penta	Galß-4GlcNAcß-3Galß-4Glc | Fucα-3	DH	**35**
		AO	**36**
NeuAcα2-3Lac	NeuAcα-3Galβ-4Glc	DH	**37**
		AO	**38**
NeuAcα2-6Lac	NeuAcα-6Galβ-4Glc	DH	**39**
		AO	**40**
NeuAcβ2-3Lac	NeuAcβ-3Galβ-4Glc	DH	**41**
		AO	**42**
NeuAcβ2-6Lac	NeuAcβ-6Galβ-4Glc	DH	**43**
		AO	**44**
Neuα2-3Lac	Neuα-3Galβ-4Glc[b]	DH	**45**
		AO	**46**
Neuα2-6Lac	Neuα-6Galβ-4Glc[b]	DH	**47**
		AO	**48**
4-OAc-NeuAcα2-3Lac	4-OAc-NeuAcα-3Galβ-4Glc	DH	**49**
		AO	**50**
GM3	NeuAcα-3Galβ-4Glcβ	GL	**51**
GD1a	NeuAcα-3Galß-3GalNAcß-4Galß-4Glc | NeuAcα-3	GL	**52**
GD2	GalNAcß-4Galß-4Glc | NeuAcα-8NeuAcα-3	GL	**53**
A2	NeuAcα-6Galß-4GlcNAcß-2Manα-6 | Manß-4GlcNAcß-4GlcNAc | NeuAcα-6Galß-4GlcNAcß-2Manα-3	DH	**54**
Sial pg	NeuAcα-3Galβ-4GlcNAcβ-3Galβ-4Glcβ	GL	**55**
Curd-13	Glcβ-(3Glcβ)11-3Glc[c]	AD	**56**
		DH	**57**
		AO	**58**

(*Continued on next page*)

Table 1. *Continued*

Abbreviation	Saccharide	Probe Type[a]	Probe Number
Curd-11	Glcβ-(3Glcβ)9-3Glc[c]	AD	**59**
		DH	**60**
		AO	**61**
Curd-7	Glcβ-(3Glcβ)5-3Glc[c]	AD	**62**
Dex-7	Glcα-(6Glcα)5-6Glc[c]	AO	**63**
Pust-7	Glcβ-(6Glcβ)5-6Glc[c]	AO	**64**

[a] AD and DH, NGLs prepared by conjugating saccharides to ADHP and DHPE, respectively, by reductive amination; AO, NGLs prepared by conjugating saccharides to AOPE by oxime ligation; GL, glycolipids.
[b] ΔUA, 4,5-unsaturated hexuronic acid; Neu, de-*N*-acetylated *N*-acetylneuraminic acid.
[c] Major component in the oligosaccharide fraction.

monosaccharide cores ([32] and unpublished data). A total of 12 other NGLs and 4 glycolipids were included as reference compounds.

Interactions of AO- and DH-NGLs of Le^a and Le^x Analogs with Anti-Le^x Antibodies and Aleuria aurantia Lectin

The AO- and DH-NGLs of Le^a and Le^x analogs were included in microarrays (probes **31**–**36**). These were probed with two anti-Le^x antibodies, anti-SSEA-1 and anti-L5, that require preservation of the core monosaccharide [32]. The AO- and DH-NGLs of Le^x pentasaccharide (probes **36** and **35**) and of Le^a trisaccharide (**32** and **31**) served as positive and negative controls, respectively. As predicted [32] neither anti-Le^x antibody bound to the DH-NGL of the Le^x trisaccharide (**33**) (Figure 4A; results with anti-L5 are in the Supplemental Data). Clearly, this is the result of loss of the pyranose structure of the core *N*-acetylglucosamine and the associated change in the orientation of the other residues linked to it. However, the AO-NGL (**34**) was bound by both anti-SSEA-1 and anti-L5, indicating the preservation of the correct ring form of the core monosaccharide. In striking contrast to the antibodies, *Aleuria aurantia* lectin, known to recognize peripheral fucose residues [53], bound to all but one of the fucose-containing probes in the array (Figure 4B). These included AO- and DH-NGLs of the core-fucosylated *N*-glycan NA2F (**26** and **25**), Le^a and Le^x analogs (**31**–**36**), and Fuc.AO (**8**). The reductively generated, and hence ring-opened, Fuc.DH (**7**) was not bound, in agreement with the initial experiment shown in Figure 3.

Interactions of AO- and DH-NGLs of Sialyllactose Analogs with Siglecs and Wheat Germ Agglutinin

The advantage of AO-NGLs for presenting short oligosaccharides is further illustrated in the results of microarray analyses with sialyllactose analogs and siglecs. The siglecs are important signaling and adhesion receptors, and they are known to recognize various sialyl oligosaccharide sequences with differing preferences for sialic acid linkage position and oligosaccharide backbone sequence [49]. The effects of sialic acid modifications on recognition by siglecs have been well documented in the literature. However, to our knowledge, the binding of siglecs to de-*N*-acetylated and 4-*O*-acetylated sialyl analogs has not been evaluated directly; both forms have been found in nature, although not in abundance. To extend knowledge of sialic acid variants for recognition by siglecs, we chemically synthesized the trisaccharides NeuAcα2-3Lac and NeuAcα2-6Lac in both the *N*-acetylated and de-*N*-acetylated forms together with non-natural β-linked anomers, namely, NeuAcβ2-3Lac and NeuAcβ2-6Lac (details of synthesis are available in the Supplemental Data). In addition, 4-OAc-NeuAcα2-3Lac purified from echidna milk [54] was examined for siglec binding. The AO- and DH-NGL pairs of these seven sialyllactose analogs (probes **37**–**50**) were probed with the human Siglec-7 and -9 and the murine Siglec-E and CD22. Whereas the DH-NGLs elicited little or no binding signals, with the exception of human Siglec-9 binding to NeuAcα2-6Lac.DH, various binding patterns were observed with the AO-NGLs, as summarized in Figure 5A (full microarray data are given in the Supplemental Data). These results extend the concept that certain siglecs recognize monosaccharides distant from the primary binding site [26, 55, 56]. NeuAcα2-3Lac.AO was bound by human Siglec-9 and murine Siglec-E. NeuAcα2-6Lac.AO was bound by all four siglecs, but not to the mutant siglec (mSn97A), which was included as a negative control (data not shown). The intensities of binding compared well with those to one or the other of the sialyl glycolipids and the DH-NGL of the disialylated biantennary *N*-glycan A2.

Interestingly, with the de-*N*-acetylated probes, DH- and AO-NGLs of Neuα2-3Lac and Neuα2-6Lac, no binding was observed by the human Siglec-7 and -9 and the murine Siglec-E, highlighting the importance of the *N*-acetyl substituent in NeuAc for binding by these three siglecs. This finding is supported by the recent observation on a crystal structure of the Siglec-7/DSLc4 complex [57] that the *N*-acetyl residue of NeuAc establishes contact with the protein via two hydrogen bonds arising from both the amide nitrogen and the carbonyl oxygen. In striking contrast, the de-*N*-acetylated analog Neuα2-6Lac.AO was bound by murine CD22, and the binding intensity was equivalent to that of the *N*-acetylated analog NeuAcα2-6Lac.AO. This is in agreement with previous ^{1}H-NMR

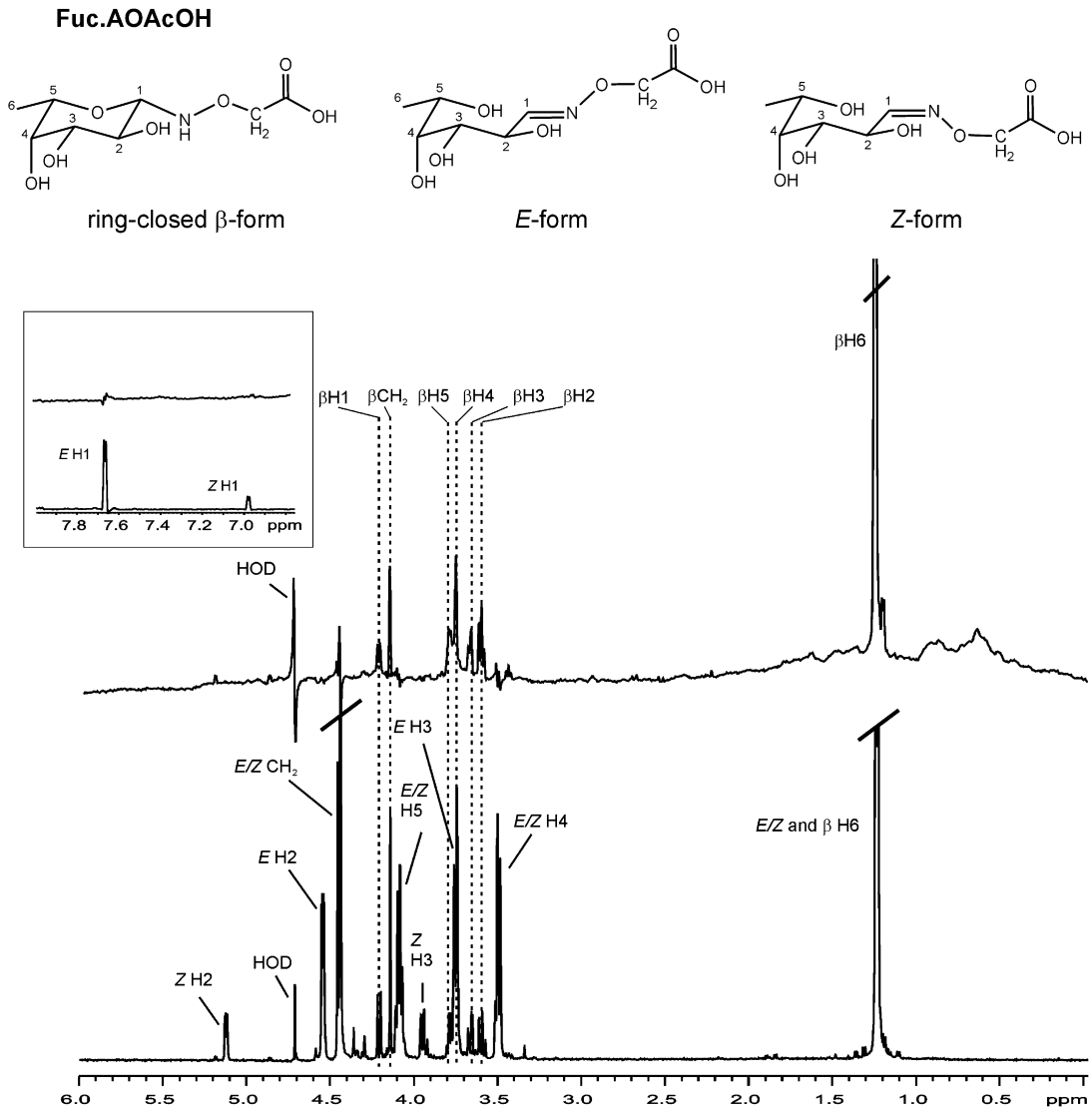

Figure 2. ^{1}H-NMR and STD NMR Spectra of Fuc.AOAcOH in the Presence of *Aleuria aurantia* Lectin

Upper spectrum: an expansion of one of the five saturation transfer difference (STD) spectra of Fuc.AOAcOH in the presence of lectin (molar ratio of 400:1). Lower spectrum: an expansion of the ^{1}H-NMR spectrum at 600 MHz of Fuc.AOAcOH in D_2O. Resonances arising from the ring-closed β-fucose form of Fuc.AOAcOH are clearly identifiable in the STD spectrum, as indicated. Two out-of-phase artifacts are seen coinciding with intense peaks; residual water at 4.72 ppm and the methylene singlet from the *E* form at 4.44 ppm. Some broad contributions from the protein can be seen, especially between 0 and 2 ppm. Insert: an expansion showing the open-ring H1 region of both of the above-mentioned spectra, not to scale.

data for murine CD22 that did not indicate an interaction between the *N*-acetyl group and the protein [58].

The results of our microarray analyses have also shown that acetylation of NeuAc at the 4-hydroxyl has no perceptible effect on recognition by human Siglec-9 and murine Siglec-E, as these two siglecs gave binding signals with the AO-NGL of 4-OAc-NeuAcα2-3Lac that were almost equivalent to the signals with the 4-hydroxyl analog. Our observations suggest that the 4-hydroxyl group of NeuAc is not involved in the interactions of the human Siglec-9 and the murine Siglec-E. This is different in the case of murine sialoadhesin (Siglec-1), the 1.85 Å crystal structure of which shows that the 4-hydroxyl group makes a hydrogen bond with a main chain carbonyl of a serine residue [59]. The crystal structure of Siglec-7 in complex with sialosides recently published [57] shows that this interaction is missing. It would be predicted, therefore, that the 4-*O*-acetylated analog with α2-6 sialyl linkage (not currently available to us) would be bound by Siglec-7.

A further observation is the lack of recognition of NeuAc in the unnatural β-anomeric configuration by the human Siglec-7 and -9 and the murine Siglec-E and CD22, as indicated by the lack of binding to the AO-NGLs of NeuAcβ2-3Lac and NeuAcβ2-6Lac. This is a property shared by murine sialoadhesin [58] and MAG ([myelin-associated glycoprotein]/Siglec-4) of the rat [60]. In contrast, the plant

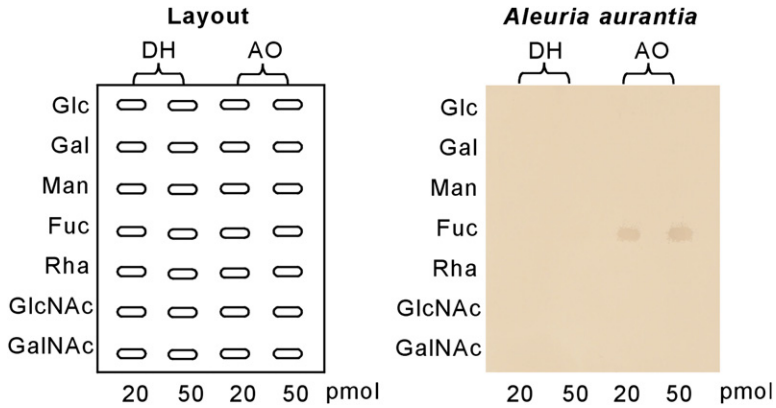

Figure 3. Evaluation of the Interactions of DH- and AO-NGLs of Seven Monosaccharides, Printed on a Nitrocellulose Membrane, with the Plant Lectin *Aleuria aurantia*

The NGL probes were arrayed by jet spray at 20 and 50 pmol/spot as shown in the layout. Binding of the lectin was detected by using a colorimetric biotin-streptavidin-peroxidase system.

lectin wheat germ agglutinin (WGA) showed binding to both α- and β-linked sialyllactose-derived NGLs either in DH- or AO-NGL forms, but not to the NGLs of the de-*N*-acetylated analogs or the 4-*O*-acetylated analog. This is in accord with previous knowledge that WGA recognizes both the 5-*N*-acetyl group and the 4-hydroxyl group of NeuAc as binding motifs [61].

Collectively, the results with the AO-NGLs show that sialyl trisaccharides are suitably presented to siglecs in the form of AO-NGLs, rather than DH-NGLs. The findings complement previous structural and ligand-binding studies on the modes of interaction of siglecs with sialic acids, and they provide novel information on the effects of *N*-deacetylation and 4-*O*-acetylation of NeuAc. Discovering hitherto unknown specificities for the siglecs provides novel biological insights into their functions and information for future drug designs.

Interactions of N-Glycan-Derived NGLs with Plant Lectins

Reduction of the fucosylated core *N*-acetylglucosamine in *N*-glycans such as NA2F (Table 1) impairs the affinity of binding of the glycans in solution by the plant lectins from *Pisum sativum* (PSA, pea lectin) and *Lens culinaris* agglutinin (lentil lectin), which recognize both the intact core and the backbone region [62]. Moreover, when NA2F was biotinylated via reductive amination and immobilized on streptavidin wells, it elicited no binding signals with these two lectins [34]. PSA is particularly fastidious in this regard; results with this lectin are illustrated here. NA2F.AO and NA2F.DH were included among the eight *N*-glycan-derived NGLs (probes **23**–**30**) in the microarrays. As summarized in Figure 5B (full microarray data are in the Supplemental Data), PSA gave binding signals that were clearly above background with the NA2F.AO, but not with NA2F.DH. Interestingly, a weak binding signal was observed with the nonfucosylated probe Man_9GN_2.AO. This is most likely due to the highly clustered display of the lipid-linked probe that has resulted in detectable binding to the lower-affinity ligand.

The microarrays were probed with five other plant lectins whose specificities do not involve the core *N*-acetylglucosamine of *N*-glycans [53] (Figure 5B; full microarray data in the Supplemental Data). Concanavalin A (Con A) showed, as predicted, similar binding to both DH- and AO-NGLs of various high-mannose and biantennary *N*-glycans. *Ricinus communis* agglutinin 120 (RCA_{120}) and *Erythrina cristagalli* lectin (EC) bound to both DH- and AO-NGLs derived from *N*-glycans with terminal galactose.

Microarray Analysis of *N*-Methylated AO-NGL Derived from Le^x Trisaccharide

It has been suggested that if reducing sugars react with *N*-methylated aminooxy derivatives rather than primary alkoxyamine, the equilibrium for the core monosaccharide can be forced in favor of the ring-closed form [41]. A recent application of this principle involves the preparation of glycan probes by using a bifunctional spacer containing a *N*-methylated aminooxy functionality for glycan arrays [44]. In exploratory experiments, we prepared NGL of Le^x trisaccharide (referred as Le^x-tri.(Me)AO) by oxime ligation to *N*-methylated aminooxy-functionalized lipid, and we carried out comparative binding studies with Le^x-tri.AO probed with anti-SSEA-1 and anti-L5 (Supplemental Data). In contrast with Le^x-tri.AO, which was bound by both antibodies, Le^x-tri.(Me)AO was bound by anti-L5, but not anti-SSEA-1. This observation suggests that introducing a hydrophobic substituent, such as the methyl group used here, in close proximity to a recognition motif at the anomeric position may interfere with some recognition systems. Therefore, we have not pursued further the (Me)AO-NGL approach.

SIGNIFICANCE

This is, to our knowledge, the first time that the ring-closed, oxime-linked sugar has been identified, via protein recognition by STD NMR, from a mixture that also contains the ring-opened forms. In solution, the ring-closed form of the oxime-linked fucose is present at 15% in the absence of binding protein. Nothing is known about the equilibrium when the saccharide probes are immobilized on nitrocellulose as AO-NGLs; it is possible that binding to the protein can drive the equilibrium toward the ring-closed isomer that is recognized.

The microarray analyses illustrate the key value of AO-NGLs in overcoming the limitations of the conventional

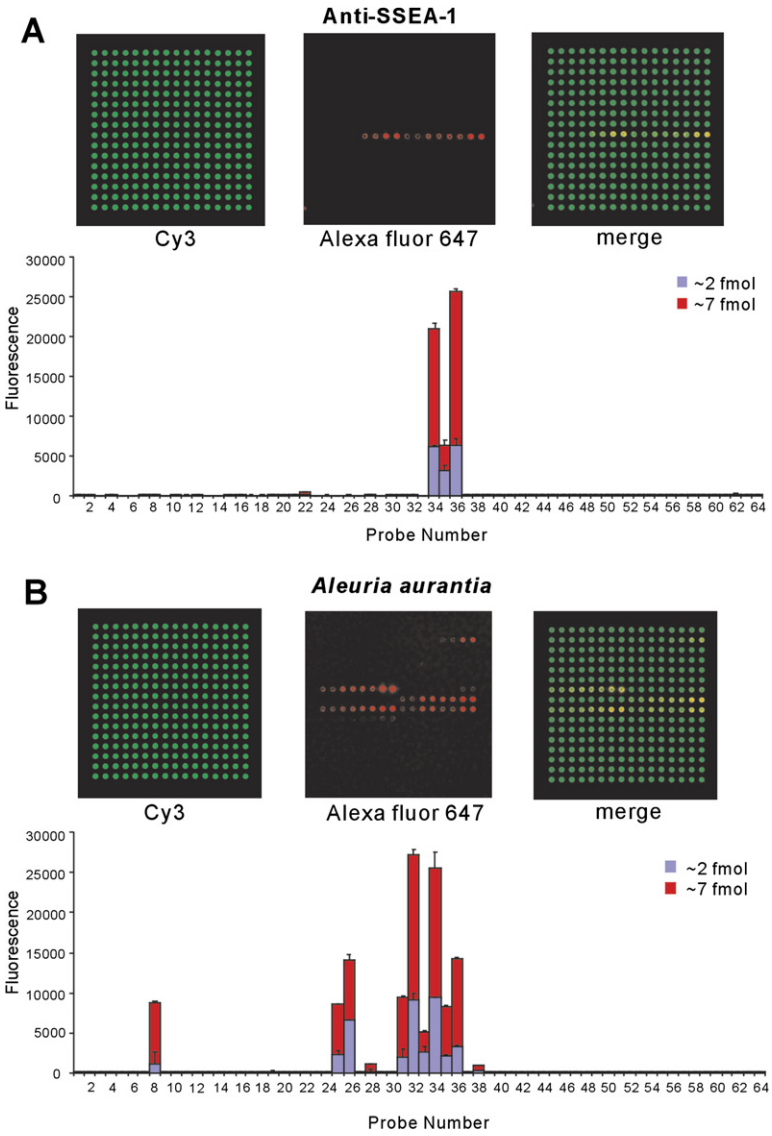

Figure 4. Microarray Analyses of 64 Lipid-Linked Saccharide Probes Printed on Nitrocellulose-Coated Glass Slides

(A and B) Results are shown for the (A) anti-Le^x antibody anti-SSEA-1 and (B) *Aleuria aurantia* lectin, each including three scan images and a bar graph. Each of the 64 probes (Table 1) was printed at 2 and 7 fmol/spot in duplicate with Cy3 dye included as a marker (green emission, left images); binding was detected with Alexa Fluor 647-labeled streptavidin (red emission, middle images); merged images of arrayed and bound spots show bound spots as yellow (right images); numerical scores of binding are measured as fluorescence intensity (means of duplicate spots with error bars, which indicate half of the difference of the two values) in blue for 2 fmol/spot and in red for 7 fmol/spot.

DH-NGLs for presenting short-chain oligosaccharides and some fucosylated *N*-glycans to recognition systems that require an unmodified core monosaccharide. The binding of siglecs to the AO-NGLs of sialyllactoses contrasts with the lack of binding to the DH analogs, in which the core glucose is ring opened, and it extends the concept that certain siglecs recognize monosaccharides distant from the primary sialic acid-binding site. Our observations with AO-NGLs have added to knowledge on the binding specificities of siglecs toward modified forms of NeuAc, particularly the effects of *N*-deacetylation and 4-*O*-acetylation, which have not been directly evaluated so far. An adverse effect observed upon introducing a methyl group to the aminooxy linker attached to short oligosaccharides (namely, the hindrance or masking of the core monosaccharide for recognition) provides a cautionary note for the application of *N*-methylated, oxime-linked oligosaccharide probes in microarray analyses.

As AO-NGLs can be presented and probed in the same microarray platform as conventional DH-NGLs and glycolipids, they have broadened the scope of the oligosaccharide microarrays. Thus, AO-NGLs of short oligosaccharides have a place in screening studies in parallel with long oligosaccharides in microarrays. AO-NGLs of *N*-glycans are of potential value as probes in future studies of novel carbohydrate-recognition systems.

EXPERIMENTAL PROCEDURES

Saccharides

Saccharides were investigated as lipid-linked probes, and their abbreviations are in Table 1. The 2,3- and 2,6-linked sialyllactose analogs with α or β configurations (NeuAcα2-3Lac, NeuAcα2-6Lac, NeuAcβ2-3Lac, NeuAcβ2-6Lac, Neuα2-3Lac, and Neuα2-6Lac) were synthesized chemically; details are provided in the Supplemental Data. The 4-*O*-acetyl-α2,3-sialyllactose (4-OAc-NeuAcα2-3Lac) purified

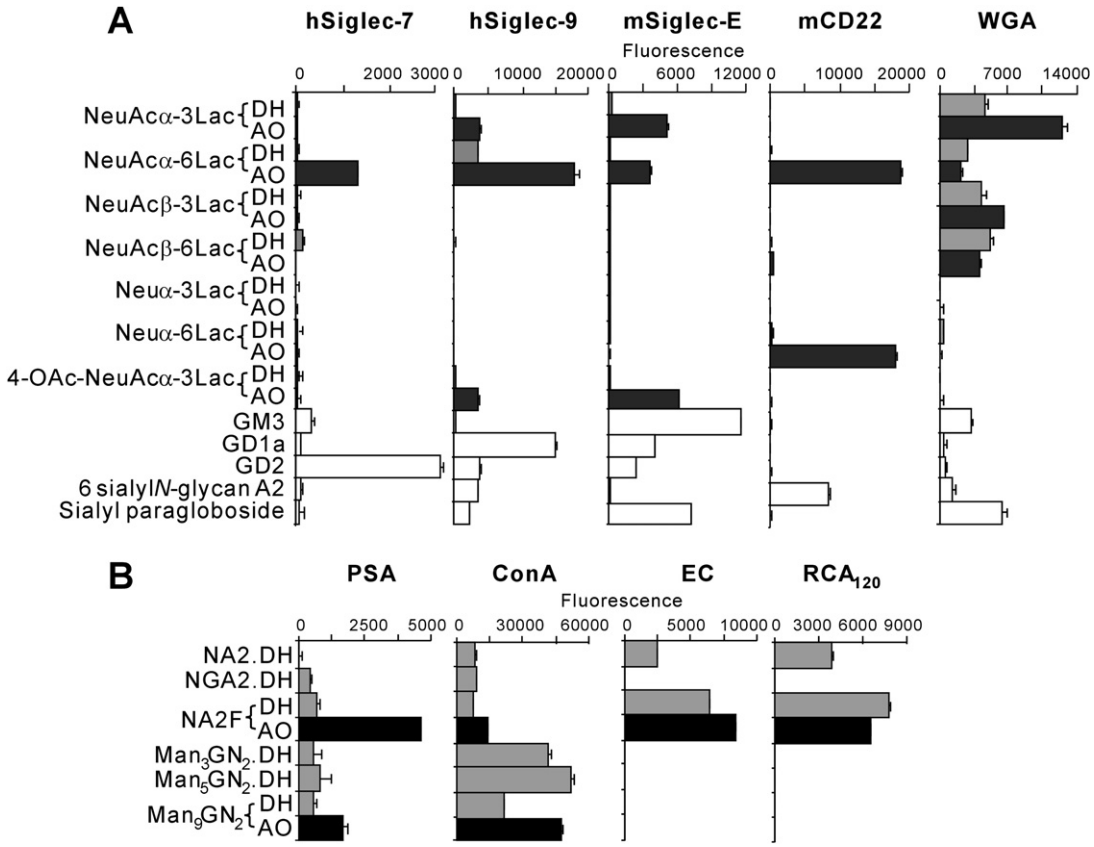

Figure 5. Summary of Microarray Analyses of Four Siglecs and Five Plant Lectins with DH- and AO-NGLs

(A) Microarray analyses of interactions of DH- (gray) and AO-NGLs (black) of sialyllactose analogs and controls (white) with the human Siglec-7 and -9, the murine Siglec-E and CD22, and WGA.

(B) Microarray analyses of interactions of DH- (gray) and AO-NGLs (black) of *N*-glycans with plant lectins PSA, ConA, EC, and RCA_{120}. Signals at 7 fmol/spot are shown (means of duplicate spots with error bars).

from echidna milk [54] was a gift from Dr. Michael Messer (University of Sydney) and Dr. Tadasu Urashima (Obihiro University of Agriculture and Veterinary). Curdlan oligosaccharide fragments 7-mer, 11-mer, and 13-mer (Curd-7, Curd-11, and Curd-13), pustulan fragment 7-mer (Pust-7), and dextran 7-mer (Dex-7) were isolated [31] from the partially depolymerized polysaccharides. Monosaccharides, lactose (Lac), *N*-acetyllactosamine (LacNAc), maltopentaose (Malto5), and heparin disaccharide IS (Hep IS) were purchased from Sigma (Dorset, UK). *N*-glycans (NA2, NGA2, NA2F, Man_3GN_2, Man_5GN_2, Man_9GN_2, A2), $Lewis^a$ and $Lewis^x$ trisaccharides, and $Lewis^x$ pentasaccharide (Le^a-tri, Le^x-tri, and Le^x-penta, respectively) were purchased from Dextra Laboratories (Reading, UK). DH-NGLs were prepared by conjugating saccharides to DHPE by reductive amination and were corroborated by MALDI-MS [50]. Three of the NGLs (probes **56**, **57**, and **58**), designated AD, had been prepared [31] by conjugating glucan oligosaccharides to anthracenyl DHPE (ADHP) by reductive amination. The glycolipid sialyl paragloboside (Sial pg) was a gift of Professor Peter Hanfland (University of Bonn). The glycolipids GM3 and GD1a were purchased from Sigma, and GD2 was purchased from HyTest (Turku, Finland).

Chemical and chromatographic materials used for AO-NGL preparation are provided in the Supplemental Data.

Biochemical and Immunochemical Reagents and Matrices

Murine monoclonal anti-Le^x antibody, anti-SSEA-1 [63], was purchased as ascites from Developmental Studies Hybridoma Bank (University of Iowa). Rat monoclonal anti-Le^x, anti-L5 [32], purified from culture supernatant was a gift from Dr. Andrea Streit (Kings College, London). Human Siglec-7, murine Siglec-E, and murine CD22 (Siglec-2) were examined as recombinant, soluble IgG Fc chimeras [26]. These consist of the extracellular Ig-like domains 1–3 of human Siglec-7, murine Siglec-E, and CD22, stably secreted by transfected Chinese hamster ovary cells harvested in X-VIVO-10 serum-free medium and quantified by ELISA. Also included was a sialoadhesin mutant (designated mSnR97A) with substitution $Arg^{97} \rightarrow Ala$, which abrogates interaction with NeuAc [58]. Human Siglec-9 was examined as a chimera, fused to enhanced green fluorescent protein (EGFP) via Gateway technology (to be described in detail elsewhere). Biotinylated plant lectins WGA, PSA, ConA, EC, RCA_{120}, and *Aleuria aurantia*, as well as the nonbiotinylated *Aleuria aurantia* lectin, were purchased from Vector Laboratories. Biotinylated anti-human-IgG, goat anti-GFP, and anti-rat IgG (H^+L, crossreactive with IgM, designated anti-rat immunoglobulins) were purchased from Vector Laboratories (Burlingame, CA); biotinylated anti-mouse immunoglobulins were purchased from Sigma (Dorset, UK). Cy3 dye and Alexa Fluor-647-labeled Streptavidin were purchased from Molecular Probes (Invitrogen, UK). 16-Pad nitrocellulose-coated (FAST) glass slides (Whatman International Ltd., Folkestone, UK) were used in conjunction with a 16-pad gasket and slide holder (Molecular Probes). Nitrocellulose membranes were purchased from Bio-Rad (Hemel, Hempstead, UK). Bovine serum albumin (BSA), Tris-HCl buffer, and HEPES buffer were purchased from Sigma.

Preparation of *N*-Aminooxyacetyl-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine, AOPE

Step 1: Preparation of Boc-AOPE

EDC (8 mg, 42 μmol) at 0°C was added to a solution of DHPE (13.3 mg, 20 μmol) and Boc-AOAcOH (11.5 mg, 60 μmol) in $CHCl_3$ (2 ml), and the reaction mixture was stirred for 1 hr at 0°C, followed by 4 hr at ambient temperature. HPTLC of an aliquot of the reaction mixture (~1 nmol lipid) revealed a major product, R_f 0.4 (developed with $CHCl_3$:EtOH:H_2O, 50:50:1), visualized under longwave UV light after primulin staining [50]. The reaction mixture was washed twice with 2 ml water, the organic layer was collected, and the solvent was evaporated under a nitrogen stream. The product was purified by silica gel chromatography (CH_2Cl_2:MeOH:25% NH_4OH, 100:10:1); the yield was ~80% and gave an MNa^+ ion at m/z 860 by MALDI-MS corresponding to that of Boc-AOPE.

Step 2: Preparation of AOPE

TFA (50 μl) at 0°C was added to a solution of Boc-AOPE (10 μmol) in anhydrous dichloromethane (0.5 ml). The reaction mixture was stirred for 5 hr at 0°C. Toluene (50 μl) was added, and the solvents were evaporated in vacuo to give the deprotected product in quantitative yield; R_f 0.1 in HPTLC (developed with $CHCl_3$:EtOH:H_2O, 50:50:1). The product gave an MNa^+ ion at m/z 760 corresponding to that of AOPE. The reaction mixture was evaporated, and the residue was dissolved in 2 ml $CHCl_3$:MeOH (1:1) and was used without purification.

For a figure of HPTLC analysis of lipid products, see the Supplemental Data.

Preparation of Oxime-Linked NGLs, AO-NGLs

Typically, saccharides (10–100 nmol) were incubated with two equivalents of AOPE. For example, 100 nmol AOPE (20 μl 5 mM in $CHCl_3$:MeOH, 1:1) was added to 50 nmol dried saccharide in a glass microvial. The mixture was evaporated to dryness under a nitrogen stream and was dissolved in 20–50 μl $CHCl_3$:MeOH:H_2O, 10:10:1 or 25:25:8 (depending on the solubility of saccharide). The mixture was incubated at ambient temperature for 6–16 hr and was evaporated slowly in a heating block at 60°C over the course of 1 hr to dryness. For glucan oligomeric fragments (Pust-7, Dex-7, Curd-11, and Curd-13), ten equivalents of AOPE were applied, and incubation was conducted for 24 hr under acidic conditions ($CHCl_3$:MeOH:H_2O:AcOH, 25:25:8:1). Aliquots of the reaction mixtures (containing ~1 nmol NGL) were analyzed by HPTLC (developed with $CHCl_3$:MeOH:H_2O, 60:35:8) by using primulin and orcinol staining. AO-NGLs derived from neutral mono- and disaccharides were isolated from reaction mixtures by semipreparative TLC by using one of the following solvents: (1) $CHCl_3$:MeOH:H_2O, 60:35:8; (2) 130:50:9; (3) 50:25:1; (4) $CHCl_3$:EtOH:H_2O, 50:50:1. AO-NGLs derived from larger oligosaccharides as well as acidic mono- and disaccharides were purified by using silica cartridges. For these, reaction mixtures were dissolved in up to 200 μl $CHCl_3$:MeOH:H_2O (130:50:9), applied to prewashed cartridges as described for DH-NGLs [50]. Excess AOPE was removed by washing with the same solvent (4 × 300 μl), and the NGLs were eluted with 4 × 300 μl $CHCl_3$:MeOH:H_2O (60:35:8) and 4 × 300 μl $CHCl_3$:MeOH:H_2O (25:25:8). Purified AO-NGLs were analyzed by HPTLC with primulin and orcinol staining. The AO-NGLs were quantified as for conventional DHPE-NGLs [50], and they were stored at −20°C in $CHCl_3$:MeOH:H_2O (25:25:8).

For a figure of HPTLC analysis of AO-NGL products, see the Supplemental Data.

Mass Spectrometry

All oligosaccharides and NGLs were analyzed by MALDI-MS on a Tof Spec-2E instrument (Waters, Manchester, UK); they were dissolved in methanol or $CHCl_3$:MeOH:H_2O (25:25:8), respectively, at a concentration of 10–20 pmol/μl, and 0.5 μl was deposited on the sample target together with a matrix of 2-(4-hydroxyphenylazo)benzoic acid. Laser energy was 20% (coarse) and 60% (fine); resolution was at 1000. DH-NGLs of the de-*N*-acetylated sialyllactose analogs (probes **45** and **47**) were analyzed by negative-ion electrospray tandem mass spectrometry with collision-induced association to verify the preservation of the free amine in the sialic acid residue. The experiments were carried out on a Q-TOF instrument (Waters, Manchester, UK) essentially as described [64], but by using the solvent $CHCl_3$:MeOH:2 mM NH_4HCO_3 (25:25:8).

NMR Experiments

NMR spectra (500 and 600 MHz) were recorded on Varian Inova spectrometers, for samples of Fuc.AOAcOH in D_2O, 30°C, at concentrations of 1–5 mM. Assignment of the ^1H and ^{13}C spectra was based on TOCSY, HSQC, and HMBC spectra (Supplemental Data available). STD spectroscopy was carried out for a 2 mM solution of Fuc.AOAcOH in deuterated phosphate buffer (10 mM, pH 7.0) with the addition of a solution of *Aleuria aurantia* lectin (10 μg/μl, resulting in a protein-binding site concentration of 10 μM). Selective shaped pulses 200 Hz in bandwidth were used to irradiate the protein spectrum away from Fuc.AOAcOH signals, at −0.3 ppm, 0.55 ppm, 2.7 ppm, 6.23 ppm, and 6.6 ppm (saturation time of 1.9 s), with a control spectrum in which the irradiation was well away from any protein or ligand resonances. For each irradiation frequency, 2048 transients were collected, in an interleaved protocol for improved subtraction. These conditions for STD spectroscopy were validated by using a mixture of free fucose and glucose with the *Aleuria aurantia* lectin (results not shown).

Microarray Analyses

Binding of the Lipid-Linked Saccharide Probes Was Studied in Two Assay Formats

In the first assay format, 14 monosaccharide-derived NGLs were arrayed by nitrogen-assisted jet spray as 2 mm bands at 20 and 50 pmol onto a nitrocellulose membrane [9]. The membrane was blocked with 3% w/v BSA in HEPES-buffered saline (HBS, 10 mM HEPES buffer [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$, 0.01 mM $MnCl_2$) for 1 hr. Biotinylated *Aleuria aurantia* lectin was warmed to 37°C before dilution to 20 μg/ml in HBS containing 1% w/v BSA. The membrane was overlaid at ambient temperature for 90 min with the diluted lectin. After washing in HBS (4 × 2 min), binding was detected by overlaying with streptavidin-peroxidase (10 μg/ml), followed by color development.

In the second assay format, microarrays of 64 lipid-linked saccharide probes (Table 1) were robotically generated on nitrocellulose-coated glass slides by noncontact arrayer (Piezorray; PerkinElmer, Wellesley, USA) and were probed essentially as described [31]. In brief, the lipid-linked probes were robotically arrayed onto 16-pad nitrocellulose-coated glass slides as duplicate spots (2 and 7 fmol/spot) together with Cy3 dye as a marker to monitor sample application [31]. Microarray slides were blocked at ambient temperature for 60 min with 1% w/v BSA in Pierce Casein Blocker solution (BSA/casein) for antibody and siglec-binding assays and with 3% w/v BSA in HBS for lectin binding. Slides were overlaid, at ambient temperature for 90 min, with 10 μg/ml biotinylated plant lectins (warmed to 37°C for 20 min before dilution) or with anti-Le^x antibodies, anti-SSEA-1 at 1:200 and anti-L5 at 1:50 dilution, in the respective blocking solutions, or they were overlaid with siglecs precomplexed with biotinylated anti-human-IgG in a ratio of 1:3 (w/w). In the experiments in which human Siglec-9-EGFP was used, precomplexing was performed with goat anti-GFP. The precomplexed siglecs were applied at a final concentration of 1.2 μg/ml. Binding of anti-SSEA-1 was detected with biotinylated anti-mouse immunoglobulins, 1:500, and anti-L5 with biotinylated anti-rat immunoglobulins, 1:500, followed by streptavidin-Alexa Fluor-647 reagent at 1 μg/ml, in blocking solution. Pads were washed with HBS with a final rinse in deionized water, dried, and scanned with a ProScanArray (Perkin Elmer). Alexa Fluor-647-binding signals were quantified with ScanArrayExpress software (Perkin Elmer); microarray data analysis was carried out by using in-house software (M.S. Stoll, unpublished data). Results illustrated are representative of at least two overlay experiments with probes arrayed on two occasions.

Supplemental Data

Supplemental Data include chemical and chromatographic materials used for AO-NGL preparation, a table and a figure of MS analyses of AO-NGLs, figures of HPTLC analyses of lipid and AO-NGL products, and microarray data of ten of the proteins investigated that were summarized in the main text. Also included are the method for preparing *N*-methylated AO-NGL; figures of HPTLC, MS, and microarray analyses of Le^x-tri.(Me)AO; results of PBA affinity chromatography experiments; additional text and a table detailing ^{1}H- and ^{13}C-NMR assignments of Fuc.AOAcOH; and chemical synthesis procedures and characterization data for sialyllactose analogs. These data can be found with this article online at http://www.chembiol.com/cgi/content/full/14/7/847/DC1/.

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REFERENCES

1. Taylor, M.E., and Drickamer, K. (2006). Introduction to Glycobiology (New York: Oxford University Press).
2. Feizi, T., and Chai, W. (2004). Oligosaccharide microarrays to decipher the glyco code. Nat. Rev. Mol. Cell Biol. *5*, 582–588.
3. Lee, R.T., and Lee, Y.C. (2000). Affinity enhancement by multivalent lectin-carbohydrate interaction. Glycoconj. J. *17*, 543–551.
4. Feizi, T., Fazio, F., Chai, W., and Wong, C.H. (2003). Carbohydrate microarrays - a new set of technologies at the frontiers of glycomics. Curr. Opin. Struct. Biol. *13*, 637–645.
5. Ratner, D.M., Adams, E.W., Disney, M.D., and Seeberger, P.H. (2004). Tools for glycomics: mapping interactions of carbohydrates in biological systems. ChemBioChem *5*, 1375–1383.
6. Shin, I., Park, S., and Lee, M.R. (2005). Carbohydrate microarrays: an advanced technology for functional studies of glycans. Chem. Eur. J. *11*, 2894–2901.
7. Wang, D., Liu, S., Trummer, B.J., Deng, C., and Wang, A. (2002). Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. Nat. Biotechnol. *20*, 275–281.
8. Willats, W.G., Rasmussen, S.E., Kristensen, T., Mikkelsen, J.D., and Knox, J.P. (2002). Sugar-coated microarrays: a novel slide surface for the high-throughput analysis of glycans. Proteomics *2*, 1666–1671.
9. Fukui, S., Feizi, T., Galustian, C., Lawson, A.M., and Chai, W. (2002). Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nat. Biotechnol. *20*, 1011–1017.
10. Xia, B.Y., Kawar, Z.S., Ju, T.Z., Alvarez, R.A., Sachdev, G.P., and Cummings, R.D. (2005). Versatile fluorescent derivatization of glycans for glycomic analysis. Nat. Methods *2*, 845–850.
11. Adams, E.W., Ratner, D.M., Bokesch, H.R., McMahon, J.B., O'Keefe, B.R., and Seeberger, P.H. (2004). Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology; glycan-dependent gp120/protein interactions. Chem. Biol. *11*, 875–881.
12. Park, S., and Shin, I. (2002). Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. Angew. Chem. Int. Ed. Engl. *41*, 3180–3182.
13. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M.E., Alvarez, R., Bryan, M.C., Fazio, F., Calarese, D., Stevens, J., et al. (2004). Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. USA *101*, 17033–17038.
14. de Paz, J.L., Noti, C., and Seeberger, P.H. (2006). Microarrays of synthetic heparin oligosaccharides. J. Am. Chem. Soc. *128*, 2766–2767.
15. Fazio, F., Bryan, M.C., Blixt, O., Paulson, J.C., and Wong, C.H. (2002). Synthesis of sugar arrays in microtiter plate. J. Am. Chem. Soc. *124*, 14397–14402.
16. Houseman, B.T., and Mrksich, M. (2002). Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem. Biol. *9*, 443–454.
17. Manimala, J.C., Roach, T.A., Li, Z.T., and Gildersleeve, J.C. (2006). High-throughput carbohydrate microarray analysis of 24 lectins. Angew. Chem. Int. Ed. Engl. *45*, 3607–3610.
18. Bryan, M.C., Plettenburg, O., Sears, P., Rabuka, D., Wacowich-Sgarbi, S., and Wong, C.H. (2002). Saccharide display on microtiter plates. Chem. Biol. *9*, 713–720.
19. Mamidyala, S.K., Ko, K.S., Jaipuri, F.A., Park, G., and Pohl, N.L. (2006). Noncovalent fluorous interactions for the synthesis of carbohydrate microarrays. J. Fluor. Chem. *127*, 571–579.
20. Plante, O.J., Palmacci, E.R., and Seeberger, P.H. (2001). Automated solid-phase synthesis of oligosaccharides. Science *291*, 1523–1527.
21. Koeller, K.M., and Wong, C.H. (2000). Complex carbohydrate synthesis tools for glycobiologists: enzyme-based approach and programmable one-pot strategies. Glycobiology *10*, 1157–1169.
22. Seeberger, P.H., and Werz, D.B. (2005). Automated synthesis of oligosaccharides as a basis for drug discovery. Nat. Rev. Drug Discov. *4*, 751–763.
23. Lee, M., and Shin, I. (2005). Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. Org. Lett. *7*, 4269–4272.
24. Zhou, X.C., and Zhou, J.H. (2006). Oligosaccharide microarrays fabricated on aminooxyacetyl functionalized glass surface for characterization of carbohydrate-protein interaction. Biosens. Bioelectron. *21*, 1451–1458.
25. Zhi, Z.L., Powell, A.K., and Turnbull, J.E. (2006). Fabrication of carbohydrate microarrays on gold surfaces: direct attachment of nonderivatized oligosaccharides to hydrazide-modified self-assembled monolayers. Anal. Chem. *78*, 4786–4793.
26. Campanero-Rhodes, M.A., Childs, R.A., Kiso, M., Komba, S., Le Narvor, C., Warren, J., Otto, D., Crocker, P.R., and Feizi, T. (2006). Carbohydrate microarrays reveal sulphation as a modulator of siglec binding. Biochem. Biophys. Res. Commun. *344*, 1141–1146.
27. Chai, W., Cashmore, G.C., Carruthers, R.A., Stoll, M.S., and Lawson, A.M. (1991). Optimal procedure for combined high-performance thin-layer chromatography/high-sensitivity liquid secondary ion mass spectrometry. Biol. Mass Spectrom. *20*, 169–178.
28. Yuen, C.-T., Chai, W., Loveless, R.W., Lawson, A.M., Margolis, R.U., and Feizi, T. (1997). Brain contains HNK-1 immunoreactive *O*-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose). J. Biol. Chem. *272*, 8924–8931.
29. Yuen, C.-T., Lawson, A.M., Chai, W., Larkin, M., Stoll, M.S., Stuart, A.C., Sullivan, F.X., Ahern, T.J., and Feizi, T. (1992). Novel sulfated

ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among *O*-linked oligosaccharides on an ovarian cystadenoma glycoprotein. Biochemistry *31*, 9126–9131.

30. Feizi, T., Lawson, A.M., and Chai, W. (2003). Neoglycolipids: identification of functional carbohydrate epitopes. In Carbohydrate-Based Drug Discovery: From the Laboratory to the Clinic, C.-H. Wong, ed. (Weinheim: Wiley-VCH), pp. 747–760.
31. Palma, A.S., Feizi, T., Zhang, Y., Stoll, M.S., Lawson, A.M., Diaz-Rodríguez, E., Campanero-Rhodes, A.S., Costa, J., Brown, G.D., and Chai, W. (2006). Ligands for the β-glucan receptor, Dectin-1, assigned using 'designer' microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. J. Biol. Chem. *281*, 5771–5779.
32. Streit, A., Yuen, C.-T., Loveless, R.W., Lawson, A.M., Finne, J., Schmitz, B., Feizi, T., and Stern, C.D. (1996). The Le^x carbohydrate sequence is recognized by antibody to L5, a functional antigen in early neural development. J. Neurochem. *66*, 834–844.
33. Leteux, C., Stoll, M.S., Childs, R.A., Chai, W., Vorozhaikina, M., and Feizi, T. (1999). Influence of oligosaccharide presentation on the interactions of carbohydrate sequence-specific antibodies and the selectins. Observations with biotinylated oligosaccharides. J. Immunol. Methods *227*, 109–119.
34. Leteux, C., Childs, R.A., Chai, W., Stoll, M.S., Kogelberg, H., and Feizi, T. (1998). Biotinyl-L-3-(2-naphthyl)-alanine hydrazide derivatives of *N*-glycans: versatile solid-phase probes for carbohydrate-recognition studies. Glycobiology *8*, 227–236.
35. Ramsay, S.L., Freeman, C., Grace, P.B., Redmond, J.W., and MacLeod, J.K. (2001). Mild tagging procedures for the structural analysis of glycans. Carbohydr. Res. *333*, 59–71.
36. Zhao, Y., Kent, S.B., and Chait, B.T. (1997). Rapid, sensitive structure analysis of oligosaccharides. Proc. Natl. Acad. Sci. USA *94*, 1629–1633.
37. Cervigni, S.E., Dumy, P., and Mutter, M. (1996). Synthesis of glycopeptides and lipopeptides by chemoselective ligation. Angew. Chem. Int. Ed. Engl. *35*, 1230–1232.
38. Langenhan, J.M., and Thorson, J.S. (2005). Recent carbohydrate-based chemoselective ligation applications. Curr Org Synth *2*, 59–81.
39. Vondeyn, W., York, W.S., Albersheim, P., and Darvill, A.G. (1990). 1-Alkoxyamino-1-deoxy alditols, useful Uv-absorbing derivatives of neutral and acidic oligosaccharides. Carbohydr. Res. *201*, 135–144.
40. Pauly, M., York, W.S., Guillen, R., Albersheim, P., and Darvill, A.G. (1996). Improved protocol for the formation of N-(p-nitrobenzyloxy)aminoalditol derivatives of oligosaccharides. Carbohydr. Res. *282*, 1–12.
41. Peri, F., Dumy, P., and Mutter, M. (1998). Chemo- and stereoselective glycosylation of hydroxylamino derivatives: a versatile approach to glycoconjugates. Tetrahedron *54*, 12269–12278.
42. Hatanaka, Y., Kempin, U., and Jong-Jip, P. (2000). One-step synthesis of biotinyl photoprobes from unprotected carbohydrates. J. Org. Chem. *65*, 5639–5643.
43. Vila-Perello, M., Gallego, R.G., and Andreu, D. (2005). A simple approach to well-defined sugar-coated surfaces for interaction studies. ChemBioChem *6*, 1831–1838.
44. Bohorov, O., Andersson-Sand, H., Hoffmann, J., and Blixt, O. (2006). Arraying glycomics: a novel bi-functional spacer for one-step microscale derivatization of free reducing glycans. Glycobiology *16*, 21C–27C.
45. Niikura, K., Kamitani, R., Kurogochi, M., Uematsu, R., Shinohara, Y., Nakagawa, H., Deguchi, K., Monde, K., Kondo, H., and Nishimura, S.I. (2005). Versatile glycoblotting nanoparticles for high-throughput protein glycomics. Chem. Eur. J. *11*, 3825–3834.
46. Lohse, A., Martins, R., Jorgensen, M.R., and Hindsgaul, O. (2006). Solid-phase oligosaccharide tagging (SPOT): validation on glycolipid-derived structures. Angew. Chem. Int. Ed. Engl. *45*, 4167–4172.
47. Peluso, S., and Imperiali, B. (2001). Asparagine surrogates for the assembly of N-linked glycopeptide mimetics by chemoselective ligation. Tetrahedron Lett. *42*, 2085–2087.
48. Sadamoto, R., Niikura, K., Ueda, T., Monde, K., Fukuhara, N., and Nishimura, S.I. (2004). Control of bacteria adhesion by cell-wall engineering. J. Am. Chem. Soc. *126*, 3755–3761.
49. Varki, A., and Angata, T. (2006). Siglecs - the major subfamily of I-type lectins. Glycobiology *16*, 1R–27R.
50. Chai, W., Stoll, M.S., Galustian, C., Lawson, A.M., and Feizi, T. (2003). Neoglycolipid technology - deciphering information content of glycome. Methods Enzymol. *362*, 160–195.
51. Haselhorst, T., Weimar, T., and Peters, T. (2001). Molecular recognition of sialyl Lewis(x) and related saccharides by two lectins. J. Am. Chem. Soc. *123*, 10705–10714.
52. Stoll, M.S., and Hounsell, E.F. (1988). Selective purification of reduced oligosaccharides using a phenylboronic acid bond elut column: potential application in HPLC, mass spectrometry reductive amination procedures and antigenic/serum analysis. Biolmed. Chromatogr. *2*, 249–253.
53. Sharon, N., and Lis, H. (2003). Lectins (Dordrecht, The Netherlands: Kluwer Academic Publishers).
54. Messer, M. (1974). Identification of N-acetyl-4-O-acetylneuraminyl-lactose in echidna milk. Biochem. J. *139*, 415–420.
55. Attrill, H., Imamura, A., Sharma, R.S., Kiso, M., Crocker, P.R., and van Aalten, D.M.F. (2006). Siglec-7 undergoes a major conformational change when complexed with the α(2,8)-disialylganglioside GT1b. J. Biol. Chem. *281*, 32774–32783.
56. Strenge, K., Schauer, R., Bovin, N., Hasegawa, A., Ishida, H., Kiso, M., and Kelm, S. (1998). Glycan specificity of myelin-associated glycoprotein and sialoadhesin deduced from interactions with synthetic oligosaccharides. Eur. J. Biochem. *258*, 677–685.
57. Attrill, H., Takazawa, H., Witt, S., Kelm, S., Isecke, R., Brossmer, R., Ando, T., Ishida, H., Kiso, M., Crocker, P.R., et al. (2006). The structure of Siglec-7 in complex with sialosides: leads for rational structure-based inhibitor design. Biochem. J. *397*, 271–278.
58. Crocker, P.R., Vinson, M., Kelm, S., and Drickamer, K. (1999). Molecular analysis of sialoside binding to sialoadhesin by NMR and site-directed mutagenesis. Biochem. J. *341*, 355–361.
59. May, A.P., Robinson, R.C., Vinson, M., Crocker, P.R., and Jones, E.Y. (1998). Crystal structure of the N-terminal domain of sialoadhesin in complex with 3′ sialyllactose at 1.85 Å resolution. Mol. Cell *1*, 719–728.
60. Kelm, S., Brossmer, R., Isecke, R., Gross, H.J., Strenge, K., and Schauer, R. (1998). Functional groups of sialic acids involved in binding to siglecs (sialoadhesins) deduced from interactions with synthetic analogues. Eur. J. Biochem. *255*, 663–672.
61. Peters, B.P., Ebisu, S., Goldstein, I.J., and Flashner, M. (1979). Interaction of wheat-germ agglutinin with sialic-acid. Biochemistry *18*, 5505–5511.
62. Yamamoto, K., Tsuji, T., and Osawa, T. (1982). Requirement of the core structure of a complex-type glycopeptide for the binding to immobilized lentil- and pea-lectins. Carbohydr. Res. *110*, 283–289.
63. Gooi, H.C., Feizi, T., Kapadia, A., Knowles, B.B., Solter, D., and Evans, M.J. (1981). Stage specific embryonic antigen SSEA-1 involves α1–3 fucosylated type 2 blood group chains. Nature *292*, 156–158.
64. Chai, W., Piskarev, V., and Lawson, A.M. (2001). Negative-ion electrospray mass spectrometry of neutral underivatized oligosaccharides. Anal. Chem. *73*, 651–657.