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Preparation of Carbohydrate Arrays by Using Diels–Alder Reactions with Inverse Electron Demand

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Abstract: Carbohydrate microarrays are an emerging tool for the high-throughput screening of carbohydrate–protein interactions that represent the basis of many biologically and medicinally relevant processes. The crucial step in the preparation of carbohydrate arrays is the attachment of carbohydrate probes to the surface. We examined the Diels–Alder reaction with inverse-electron-demand (DARinv) as an irreversible, chemoselective ligation reaction for that purpose. After having shown the efficiency of the DARinv in solution, we prepared a series of carbohydrate–dienophile conjugates that were printed onto tetrazine-modified glass slides. Binding experiments with fluorescently labeled lectins proved successful and homogeneous immobilization was achieved by the DARinv. For immobilization of nonfunctionalized reducing oligosaccharides we developed a bifunctional chemoselective linker that enabled the attachment of a dienophile tag to the oligosaccharides through oxime ligation. The conjugates obtained were successfully immobilized on glass slides. The presented strategies for the immobilization of both synthetic carbohydrate derivatives and unprotected reducing oligosaccharides facilitate the preparation of high-quality carbohydrate microarrays by means of the chemoselective DARinv. This concept can be readily adapted for the preparation of other biomolecule arrays.

Keywords: carbohydrates · Diels–Alder reaction · high-throughput screening · immobilization · microarrays

Introduction

Carbohydrate–protein interactions play a fundamental role in various critical intra- and intercellular events, such as cell differentiation, immune response, inflammation, cancer metastasis, and pathogen adhesion.[1] Consequently, these interactions are of high biological and medicinal relevance and the development of efficient methods for their investigation is a crucial objective in the field of modern glycobiology.[2] Carbohydrate microarrays are an emerging technology that enables the high-throughput screening of such carbohydrate–protein interactions.[3] Many methods for the immobilization of carbohydrates onto a surface have been reported.[4] These methods include noncovalent strategies such as the adhesion of polysaccharides[5] or neoglycolipids[6] on nitrocellulose surfaces, or the adhesion of fluorous-tagged glycans[7] on fluorous surfaces. As an alternative, immobilization strategies that anchor glycans permanently onto surfaces by forming a covalent bond have been reported. Approaches for the covalent, site-nonspecific (i.e., randomly oriented immobilization) include, for example, the immobilization of carbohydrates onto photoreactive surfaces.[8] However, most methods aim for site-specific covalent immobilization that presents the glycans in their natural orientation towards their binding partners. To achieve this, the carbohydrate probes must be equipped with a suitable functional group that can react with the solid support. Examples include the attachment of thiols[9] or amines[10] for immobilization on electrophilic surfaces, the attachment of maleimide residues[11] for immobilization on thiol-coated surfaces, the attachment of dienes[12] for immobilization through classical Diels–Alder reactions, and application of copper-catalyzed azide–alkyne cycloaddition reactions.[13] These groups can be easily installed when the carbohydrates are of synthetic origin. In contrast, the site-specific covalent immobilization of nonfunctionalized reducing oligosaccharides isolated from biological samples remains a particular challenge. The aldehyde functionality of the reducing end of unprotected oligosaccharides can be used for chemoselective derivatization by reductive amination,[14] by reaction with alkoxyamines or hydrazides,[15] or by conversion into glycosyl

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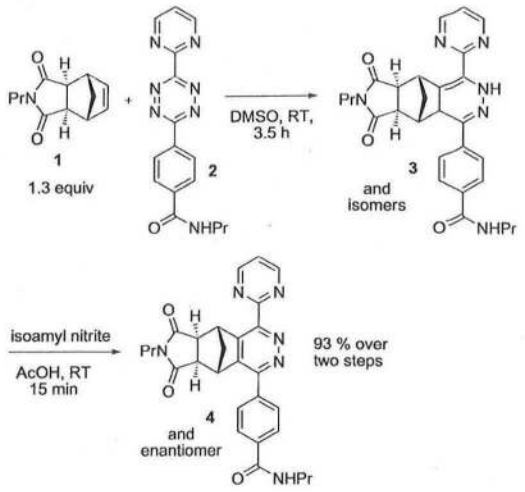
amines followed by acylation.[11,16] Although the direct immobilization of reducing carbohydrates onto aminooxy- and hydrazide-coated slides has been reported,[17] a two-step procedure employing bifunctional linkers offers advantages. Typically, glycans isolated from natural sources, such as glycoproteins, are obtained as complex mixtures due to the microheterogeneity of the glycoconjugates. These mixtures have to be separated prior to immobilization, which usually requires attachment of a label to allow detection in chromatographic processes. A suitably designed bifunctional linker that can be attached to the reducing end of the glycans may first serve as a label for purification, then, in a second step, the obtained carbohydrate conjugates could be covalently immobilized through the second functionality of the linker. For this purpose, common nucleophile-based immobilization methods such as thiol-maleimide addition[11,16a] or the reaction of amines[14–15,16b] with epoxides or activated esters have been reported. The use of an inert but chemoselectively addressable functionality instead of amines or thiols would be beneficial in terms of handling and purification of such conjugates. In addition, it should be compatible with biologically relevant amino sugars. To the best of our knowledge, such an approach to the immobilization of nonfunctionalized carbohydrates based on a chemoselective ligation has not been reported.

Recently, we[18] and others[19] recognized the potential of Diels–Alder reactions with inverse-electron-demand (DARinv) as promising bioorthogonal ligation reactions. The irreversible reaction of tetrazines as electron-deficient dienes with electron-rich dienophiles proceeds without the need for any additives, such as cell-toxic metal ions, and is driven by a high thermodynamic force. Subsequently, the DARinv was used for labeling of various biomolecules and affinity probes.[20] Due to its outstanding characteristics, we anticipated that the DARinv would also be an ideal chemoselective reaction for the preparation of biomolecule arrays. Here, we report efficient methods for the immobilization of both synthetic carbohydrate derivatives and unprotected reducing oligosaccharides by DARinv.

Results and Discussion

DARinv in solution: We identified *exo*-norbornenedicarboxylic imides (such as **1**, cf. Scheme 1) as appropriate dienophiles. Norbornenes exhibit an exceptionally high reactivity in the DARinv.[21] Additionally, norbornenedicarboxylic imides are stable dienophiles that are easily accessible from the corresponding, commercially available anhydride (see the Supporting Information for details) and, due to their symmetry, do not give rise to the formation of regioisomers. Beside norbornene dienophiles, we assumed that simple terminal alkenes might also be attractive dienophilic tags. Although alkenes are expected to exhibit a lower DARinv reactivity compared with norbornenes, they are smaller and the reaction products are less complex. As electron-deficient dienes, we chose tetrazine derivatives such as **2** (see the Supporting Information for synthetic details), the amide bond of which can be employed for convenient attachment to molecular entities.

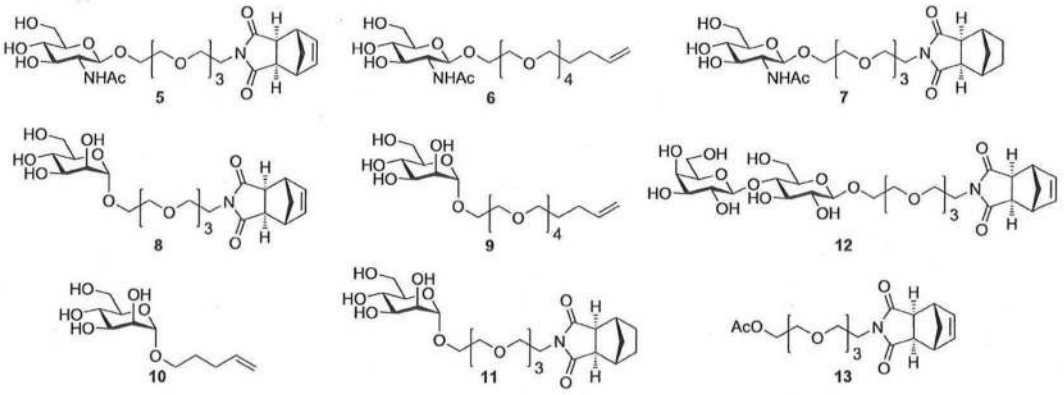


Scheme 1. DARinv of tetrazine **2** with *exo*-norbornene derivative **1** in solution. The resulting tautomeric dihydropyridazine adducts **3** were oxidized to pyridazine **4** to facilitate characterization.

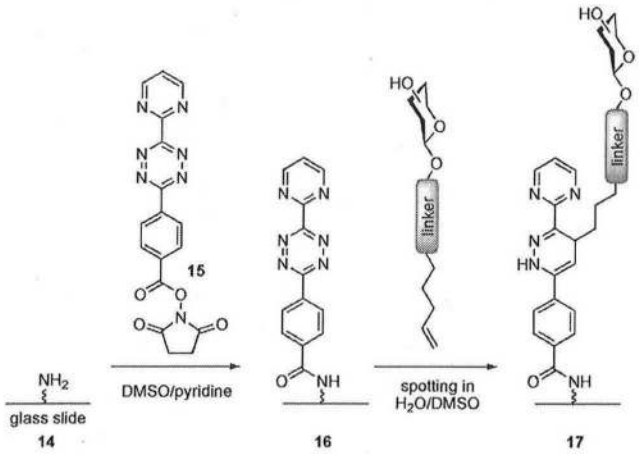
An initial DARinv experiment in solution is shown in Scheme 1. The reaction of tetrazine **2** with norbornenedicarboxylic imide **1** proceeded smoothly at room temperature. As expected, the intermediate dihydropyridazine **3** was isolated as a mixture of several interconverting tautomeric forms. Although this is not an obstacle for the application of the DARinv on solid support, in this case, the tautomers of **3** were oxidized to the homogeneous pyridazine **4**, which was isolated in an excellent yield over two steps and fully characterized.

Immobilization of synthetically derived carbohydrate–dienophile conjugates: Encouraged by this initial experiment in solution, we prepared a range of carbohydrate–dienophile conjugates as probes for array experiments (Scheme 2). Oligo(ethylene glycol)-tethered norbornenedicarboxylic imides or terminal alkenes were attached to GlcNAc, mannose, and lactose by glycosylation (see the Supporting Information for synthetic details). Norbornane conjugates **7** and **11**, which were obtained by hydrogenation of the corresponding norbornenes, cannot undergo Diels–Alder reactions. They were designed as probes for unspecific immobilization of the carbohydrate conjugates. Compound **13** carries no carbohydrate epitope and was used as a probe for unspecific binding of the lectins to the Diels–Alder adduct or the oligo(ethylene glycol) tether.

For the preparation of the carbohydrate arrays, commercially available amine-coated glass slides **14** were used (Scheme 3). The slides were diene-functionalized in one step by reaction with tetrazine active ester **15**. Solutions of carbohydrate–dienophile conjugates and controls **5**–**13** in water/dimethyl sulfoxide (DMSO) (4:1) were spotted onto the tet-



Scheme 2. Carbohydrate–dienophile conjugates.



Scheme 3. Preparation of carbohydrate microarrays.

razine-functionalized slides **16** using an automated array printer. For each concentration, nine identical replicates were printed in a block (3×3). By carrying out the immobilization in a humidity chamber under controlled conditions under which the spots do not dry out, we were able to obtain very homogeneous spots. Immobilized carbohydrates were detected by incubating the slides **17** with a rhodamine-conjugated lectin followed by fluorescence scanning. Employed lectins include wheat germ agglutinin (WGA), which is specific for *N*-acetylglucosamine (GlcNAc) residues,[22] concanavalin A (Con A), which is specific for mannose residues, and peanut agglutinin (PNA), which is specific for terminal galactose residues, such as those occurring in the T-antigen or in lactose.[23]

The fluorescence images are shown in Figure 1a. Selective binding of the lectins to their specific carbohydrate epitopes was observed (GlcNAc conjugates **5** and **6** for WGA, mannose conjugates **8**–**10** for Con A and lactose conjugate **12** for PNA). For spots in which linker **13** had been immobilized (which carries no carbohydrate epitope), only fluorescence near the background level was measured. This result indicates that the lectins do not bind to the DARinv adduct. Lack of fluorescence at positions for which the hydrogenated conjugates **7** and **11** had been spotted indicates that immobilization does not occur by simple adhesion but by covalent immobilization of the carbohydrate–dienophile conjugates.

The measured average fluorescence intensities are shown in Figure 1b. Spots of norbornene-tagged carbohydrates generally exhibited higher fluorescence intensities than spots of carbohydrates tagged with a terminal alkene (cf. **5** vs. **6** and **8** vs. **9**). This is likely due to the lower dienophilic reactivity of terminal alkenes compared with norbornenedicarboxylic imides. Much weaker fluorescence intensity was observed for compound **10**, which lacks the spacer between the carbohydrate and the dienophile (cf. **9** vs. **10**). This highlights the importance of a spacer for the efficient interaction of lectins with carbohydrates on a solid support, as previously observed for other immobilization reactions.[4b,24] In general, very homogeneous spots were obtained, as exemplified by the cross section through a row of spots shown in Figure 1c. This is a very important prerequisite if quantitative data such as dissociation constants or IC_{50} values are to be determined.[25]

Immobilization of unfunctionalized carbohydrates: After the preparation of carbohydrate arrays using synthetically prepared carbohydrate–dienophile conjugates had been established, we investigated methods for the specific immobilization of unprotected carbohydrates as they are typically obtained from biological samples. Such methods involve the specific attachment of a dienophile handle to the unprotected carbohydrate, followed by immobilization of the formed conjugate on a tetrazine chip. We selected oxime ligation as a powerful tool for the selective functionalization of unprotected carbohydrates that proceeds without additives.[26] As dienophile, we chose the reactive *exo*-norbornenedicarboxylic imide, which was connected to the oxyamine by an oligo(ethylene glycol) tether, resulting in bifunctional linker **18** (Scheme 4, see the Supporting Information for details).

N,*N*′-Diacetylchitobiose **19** and mannotriose **20** were incubated with linker **18** in acetate buffer at 37 °C (Scheme 4).

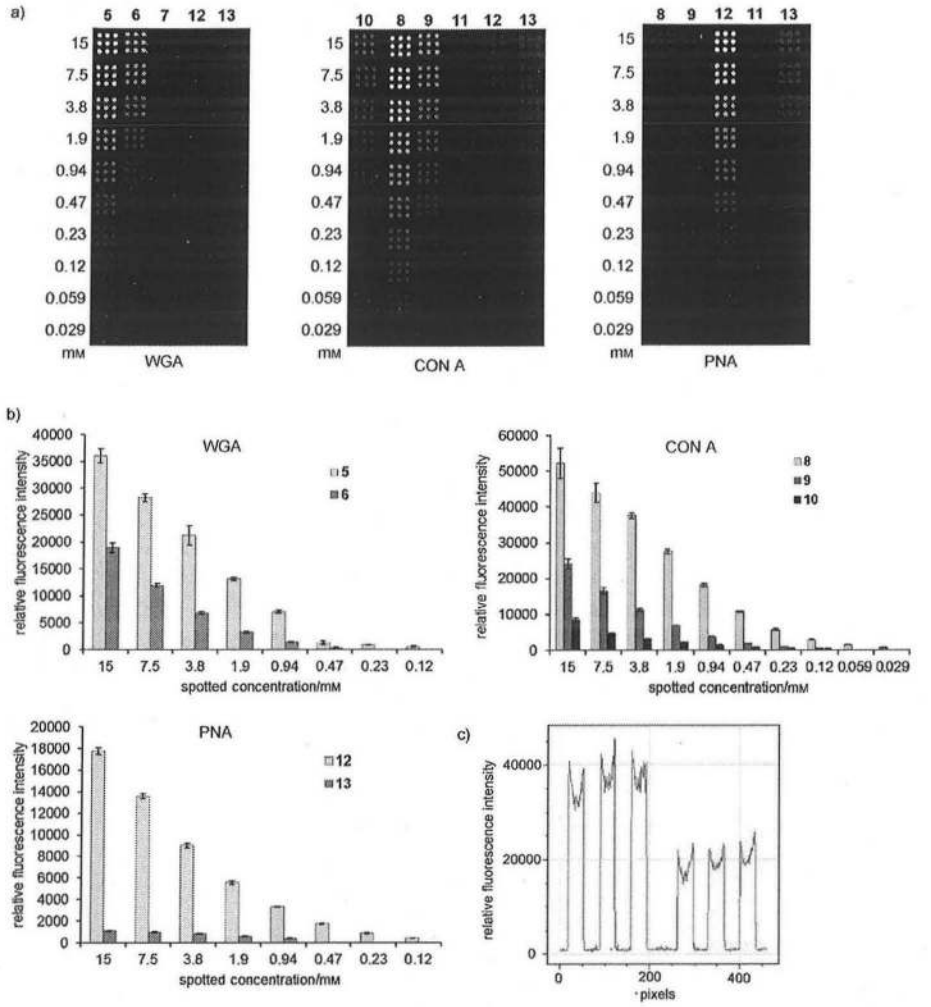


Figure 1. a) Fluorescence images of carbohydrate arrays after incubation with rhodamine-labeled WGA, Con A, and PNA. b) Fluorescence intensities obtained from these fluorescence images. c) Cross section through a row of spots of the array incubated with WGA.

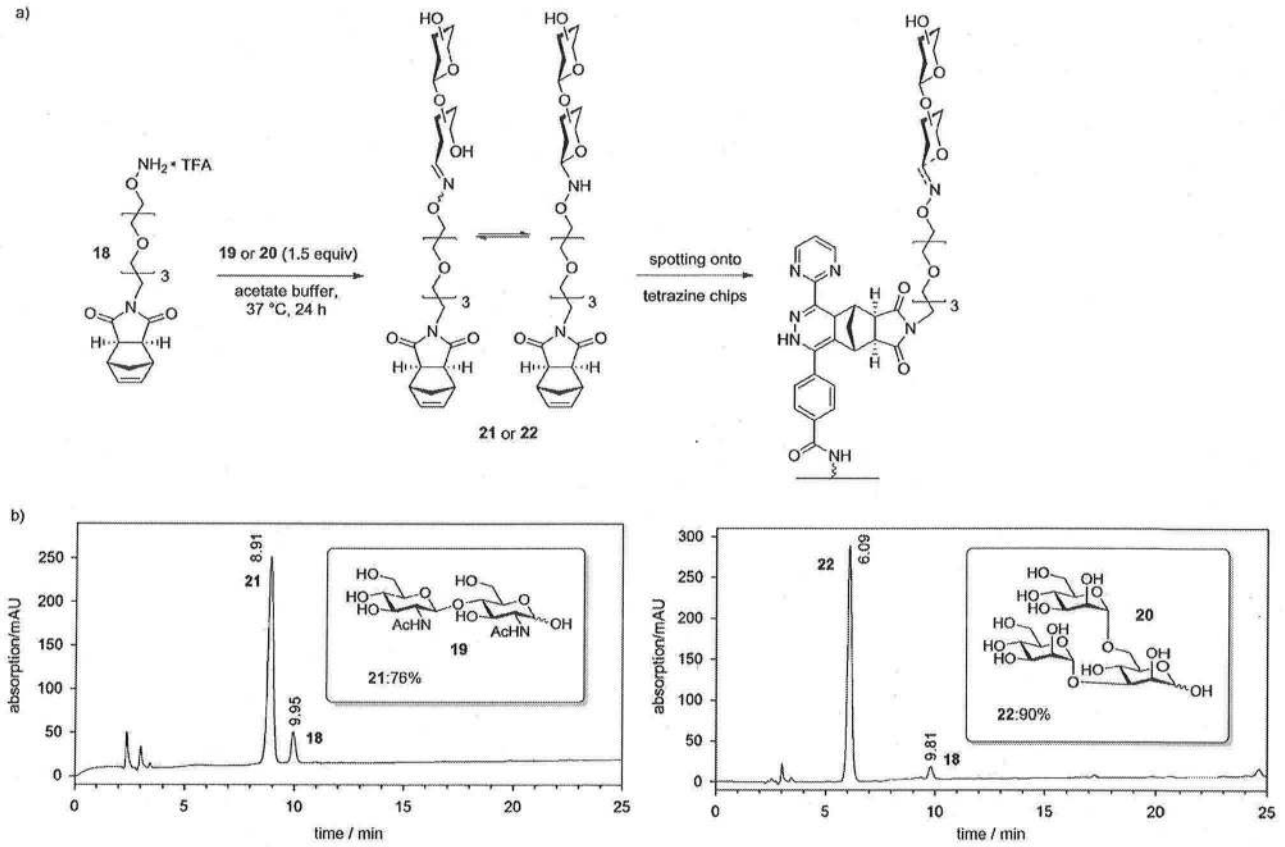
After 24 h, HPLC analysis indicated a yield of 76% for conjugate **21** and 90% for conjugate **22**. Dilution series of these reaction mixtures were directly spotted onto tetrazine chips as described above. Again, the successful immobilization of both oligosaccharides was verified by incubation with rhodamine-labeled lectins and subsequent fluorescence scanning. After incubation with WGA, spots of the tagged *N,N'*-diacetylchitobiose **21** exhibited fluorescence as well as spots of the tagged mannotriose **22** after incubation with Con A (Figure 2). These results indicate that bifunctional linker **18** is a valuable tool for dienophile-tagging and subsequent immobilization of unfunctionalized oligosaccharides. This two-step process is experimentally straightforward and does not require any additives or special equipment. Because most oligosaccharide samples from biological sources are obtained as complex mixtures, a separation is often necessary prior to immobilization on the array. Due to the UV absorbance of the imide, **18** facilitates HPLC separations. In terms of inertness, it is superior to previously reported amine or thiol tags. Further developments could include the incorporation of a fluorescent tag and/or an isotopic label into the bifunctional linker providing additional means for high-sensitivity purifications as well as for mass spectrometry analyses.

Conclusion

We have established the efficient immobilization of synthetic as well as unmodified mono- and oligosaccharides on tetrazine-derivatized glass slides using a Diels–Alder reaction with inverse-electron-demand (DARinv). Two different dienophilic tags, terminal alkenes and ring-strained norbornene derivatives were incorporated into synthetically derived carbohydrate–dienophile conjugates, the latter of which proved to be more efficient for immobilization. By using various probes, we demonstrated that the carbohydrate–dineophile conjugates were covalently immobilized through the DARinv. Bifunctional linker **18** enables the convenient attachment of a dienophile to unprotected, reducing oligosaccharides through chemoselective oxime ligation. The resulting conjugates can be directly immobilized on tetrazine-derivatized glass slides as we demonstrated in proof-of-principle experiments. The methods reported herein facilitate the preparation of high-quality carbohydrate microarrays and are expected to be of great value in the emerging field of glycomics. Additionally, this chemoselective immobilization procedure can be readily adapted for the preparation of other biomolecule arrays.

Experimental Section

General procedure for carbohydrate functionalization using bifunctional linker 18: A stock solution of bifunctional linker **18** (19 mM) was prepared in acetate buffer (100 mM, pH 4.0) and the pH was readjusted to pH 4. An aliquot of the stock solution of **18** (typically 200 µL, 1 equiv) was added to the reducing carbohydrate (1.5 equiv) and the mixture was held for 24 h at 37 °C. Subsequently, the mixture was analyzed by HPLC



Scheme 4. a) Application of bifunctional linker **18** for immobilization of reducing oligosaccharides. b) HPLC chromatograms (UV detection at 220 nm) after 24 h of oxime ligation and calculated yields of ligation products.

and HRMS and the turnover was estimated by comparing the peak areas (220 nm) of **18** in the mixture and in the stock solution.

Conjugate 21: Bifunctional linker **18** and *N,N'*-diacetylchitobiose **19** were reacted according to the general procedure. RP-HPLC (20–40% B in 20 min): $t_R = 8.91$ min; HRMS (ESI-IT): *m/z* calcd for $C_{33}H_{52}N_4O_{16}$: 761.3451 $[M+H]^+$; found: 761.3437; calcd for $C_{33}H_{51}N_4O_{16}+Na^+$: 783.3271 $[M+Na]^+$; found: 783.3255.

Conjugate 22: Bifunctional linker **18** and 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannopyranose **20** were reacted according to the general procedure. RP-HPLC (20–40% B in 20 min): $t_R = 6.09$ min; HRMS (ESI-IT): *m/z* calcd for $C_{35}H_{56}N_2O_{21}$ 841.3448 $[M+H]^+$; found: 841.3428; calcd for $C_{35}H_{55}N_2O_{21}+Na$: 863.3268 $[M+Na]^+$; found: 863.3251.

Tetrazine-functionalization of glass slides: Aminopropylsilanilized glass slides (Nexterion Slide A+ from Schott, Mainz, Germany) were immersed in a solution of tetrazine active ester **15** (5 mM in anhydrous DMSO containing 5% pyridine) for 72 h. Subsequently, the slides were washed once with DMSO and twice with acetone for 5 min each. The slides were dried in a stream of nitrogen and stored under nitrogen.

Immobilization of carbohydrate probes: Dilution series of carbohydrate–dienophile conjugates in water (or in acetate buffer in the case of functionalized reducing carbohydrates) with 20% DMSO were spotted onto tetrazine-functionalized glass slides (4 nL per spot) using an automated array printer (Nano-Plotter 2 from GeSiM, Grosserkmannsdorf, Germany) that was equipped with a thermostat (Unistat Tango, Huber Kältemaschinenbau, Offenburg, Germany) and a Humidity Control II (Lucky Reptile, Waldkirch, Germany) connected to a mist generator (Hobby Hygro Plus, Dohse Aquaristik, Grafschaft-Gelsdorf, Germany). During spotting, the relative humidity was adjusted to 68–70% and the slide tray was cooled to 10°C. Every probe was spotted in nine replicates (3×3). After spotting, the slides were stored at RT in a closed petri dish on a tray over NaCl solution (0.5 M) for 24 h. Subsequently, the slides were briefly rinsed with MeOH followed by shaking gently in MeOH (3× 5 min).

Incubation with fluorescence-labeled lectins: The carbohydrate arrays were immersed in a solution of rhodamine-labeled lectins (1 $\mu g\,mL^{-1}$) in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.5, 0.1 mM $CaCl_2$, 0.01 mM $MnCl_2$ for WGA and Con A; 10 mM HEPES, 150 mM NaCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$ for PNA) containing BSA (1% w/v). Utilized lectins rhodamine–Con A, rhodamine–PNA, and rhodamine–WGA were all purchased from Vector Laboratories (Burlingame, USA). After gently shaking in the dark at RT for 1 h, the slides were gently shaken in HEPES buffer with 0.1% Tween 20 (2×5 min) followed by two very quick washing steps in water. Finally the slides were dried in a stream of nitrogen.

Fluorescence readout: Fluorescence of rhodamine–lectin conjugates bound to the arrays was quantified by an array scanner (GenePix Personal 4100 A from Axon, now Molecular Devices, Sunnyvale, USA; excitation 532 nm, detection filters 550–600 nm). Microarray image data were analyzed by using GenePix 4.1 software. Median fluorescence of the spot and of the surrounding background was determined for every spot. The spot-fluorescence and the background-fluorescence were averaged over the nine replicates. Given error values were calculated based on the standard deviations of the nine replicates.

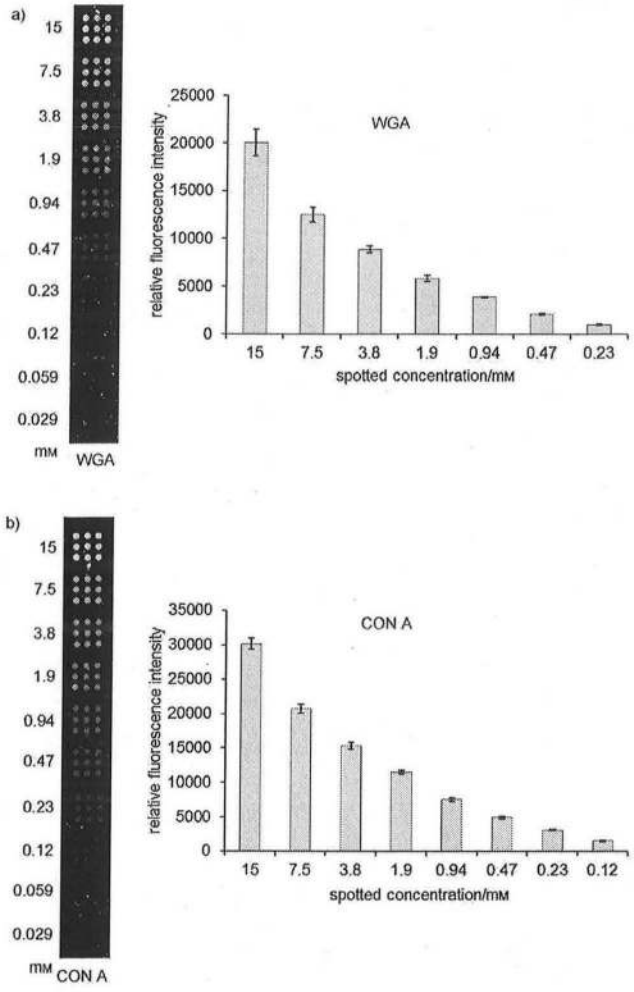


Figure 2. Fluorescence images and intensities of carbohydrate arrays after immobilization of conjugates a) **19** and b) **20** and incubation with rhodamine-labeled WGA and Con A, respectively.

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