DOI: 10.1002/chem.201001295

Construction of *N*-Glycan Microarrays by Using Modular Synthesis and On-Chip Nanoscale Enzymatic Glycosylation

Sonia Serna,[a] Juan Etxebarria,[a] Nerea Ruiz,[a] Manuel Martin-Lomas,[a, b] and Niels-Christian Reichardt*[a]

Abstract: An effective chemoenzymatic strategy is reported that has allowed the construction, for the first time, of a focused microarray of synthetic *N*-glycans. Based on modular approaches, a variety of *N*-glycan core structures have been chemically synthesized and covalently immobilized on a glass surface. The printed structures were then enzymatically diversified by the action of three different glycosyltransferases in nanodroplets placed on top of individual spots of the microarray by a printing robot. Conversion was followed by lectin binding specific for the terminal sugars. This enzymatic extension of surface-bound ligands in nanodroplets reduces the amount of precious glycosyltransferases needed by seven orders of magnitude relative to reactions carried out in the solution phase. Moreover, only those ligands that have been shown to be substrates to a specific glycosyltransferase can be individually chosen for elongation on the array. The methodology described here, combining focused modular synthesis and nanoscale on-chip enzymatic elongation, could open the way for the much needed rapid construction of large synthetic glycan arrays.

Keywords: glycosyltransferases · microarrays · nanodroplets · Glycans · oligosaccharides

Introduction

Microarrays of printed glycans have recently been established as powerful tools for the high-throughput screening of protein–carbohydrate interactions.[1] However, a serious handicap for an extensive use of arrays in glycomics studies continues to be the limited access to pure and well-characterized ligands. Recently, efforts have been made to include *N*-glycans, a particularly important class of carbohydrates accounting for about 90% of protein glycosylation,[2] into current array formats.[3]

Most ligands on these *N*-glycan arrays have been isolated from complex biological matrices in microgram amounts and are consequently far less well characterized than ligands on purely synthetic arrays.[4] The efficient tagging of the released natural glycans with a chromophoric linker, the fractionation of glycan mixtures into single compounds and the unambiguous identification by mass spectrometric methods are the major difficulties associated with this approach.

A concise study of *N*-glycan binding would ideally require a structurally diverse collection of synthetic oligosaccharides with systematic variations in degree and type of branching, terminal sugars and core modifications like fucosylation or xylosylation. Specific applications of focused *N*-glycan arrays could include the screening of the binding specificities of mammalian lectins involved in signalling,[3b,c] protein trafficking[5] and viral entry,[6] viral envelope proteins,[7] fungal,[8] and invertebrate lectins and glycan processing enzymes.[9]

In spite of extensive work on *N*-glycan synthesis during the last twenty years, the construction of a library of synthetic *N*-glycans constitutes still a serious challenge. Nevertheless, a wealth of effective methodologies and strategies has been reported and a great deal of valuable know-how exists for the chemo-enzymatic synthesis of *N*-glycan structures. The pioneering contributions of Ogawa[10] and Paulsen[11] and the further developments into modular strategies by Unverzagt,[12] Danishefsky[13] and Ito[14] among others provide a solid basis for the chemical synthesis of a library encompassing a diversity of *N*-glycan core structures. Effective enzymatic processing of these core structures to increase the structural diversity of the library could be per-

[a] Dr. S. Serna, Dr. J. Etxebarria, Dr. N. Ruiz, Prof. Dr. M. Martin-Lomas, Dr. N.-C. Reichardt
Biofunctional Nanomaterials Unit, CICbiomaGUNE
Paseo Miramon 182, 20009 San Sebastian (Spain)
Fax: (+34) 9-430-053-14
E-mail: nreichardt@cicbiomagune.es

[b] Prof. Dr. M. Martin-Lomas
CIBER-BBN, San Sebastian (Spain)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201001295.

formed if experimental conditions could be found to carry out the enzymatic reactions with minimum amounts of expensive glycosyltransferases and nucleotide donors, simultaneously improving the isolation of the final products.

Based on the on-chip chemical synthesis of oligonucleotides and peptides,[15] which paved the way for the widespread use of microarrays in genomics and peptidomics, Mrksich[16] recently reported the construction of an array of 21 disaccharides by on-chip chemical synthesis. The direct assembling of the carbohydrates on the biochip presents obvious advantages but the reported method lacks control of the stereochemistry of glycosidic bond formation and affords a low density of arrays relative to DNA chips.

The use of glycosyltransferases for the synthesis of oligosaccharides on solid supports was investigated over 15 years ago[17] by the group of Wong and later by Blixt and Norberg.[18] Other examples of enzyme catalysis on solid surfaces that have recently been reviewed[19] include the study of substrate specificities[20] and enzyme activity,[21] the on-chip modification of mono- and disaccharides[22] on surface-bound ligands or the extension of glycans on nanoparticles.[23] In these studies, the entire array was subjected to the action of glycosyltransferases with no possibility of selecting individual spots. This obviously excludes the acceptor compounds from subsequent studies in the same array. We now have found that immobilized carbohydrates on high-density arrays with spot diameters of 100–200 micrometers can be enzymatically processed with glycosyl transferases in nanodroplets placed on top of individual spots (Figure 1).

This methodology could open the way for the enzymatic on-chip construction of glycan arrays with very high ligand densities by using immobilized synthetic glycan scaffolds and recombinant glycosyltransferases that install glycosidic bonds with complete stereoselectivity. The on-chip elongation of ligands in nanodroplets promises the classical advantages of a solid-phase approach: Easy product clean-up by simply washing the slide and higher yields than solution-phase methods achieved by cycling and the use of excess reagents. In addition, the amount of glycosyl transferases and donors needed is reduced by over seven orders of magnitude (25 000 000-fold). And as hundreds of reactions can be carried out in parallel, the synthetic throughput is far higher than for previously reported methods. For reactions that do not go to completion, however, the purity can be lower than for compounds synthesized and purified in solution.

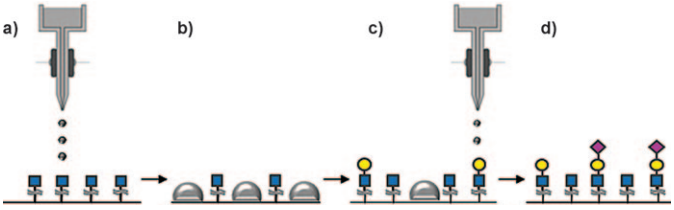


Figure 1. Schematic illustration of the nanodroplet method for spotwise elongation of printed arrays: a) droplet placement on individual spots, b) incubation and washing step, c) spotwise incubation with second glycosyltransferase, and d) finished high-density array.

In this paper, we report on an effective chemoenzymatic strategy that has allowed the construction, for the first time, of a focused microarray of synthetic *N*-glycans. Based on previously reported modular approaches, a variety of *N*-glycan structures have been chemically synthesized and covalently immobilized on a glass surface. The synthetic printed structures were then processed with glycosyl transferases first in well format and then in nanodroplets placed on top of the surface-bound oligosaccharides by the printing robot. By using this methodology, 26 new synthetic oligosaccharides have been prepared on the chip from only seven initial synthetic structures by the action of three enzymes within a few days. The printed high-mannose structures, which are not substrates for the enzymes employed, increase the structural diversity of the array to a total of 39 structures (Figure 2). To the best of our knowledge, this is the first application of recombinant enzymes in the synthesis of large surface-bound oligosaccharides and the first example of an enzymatic synthesis of core fucosylated *N*-glycans.

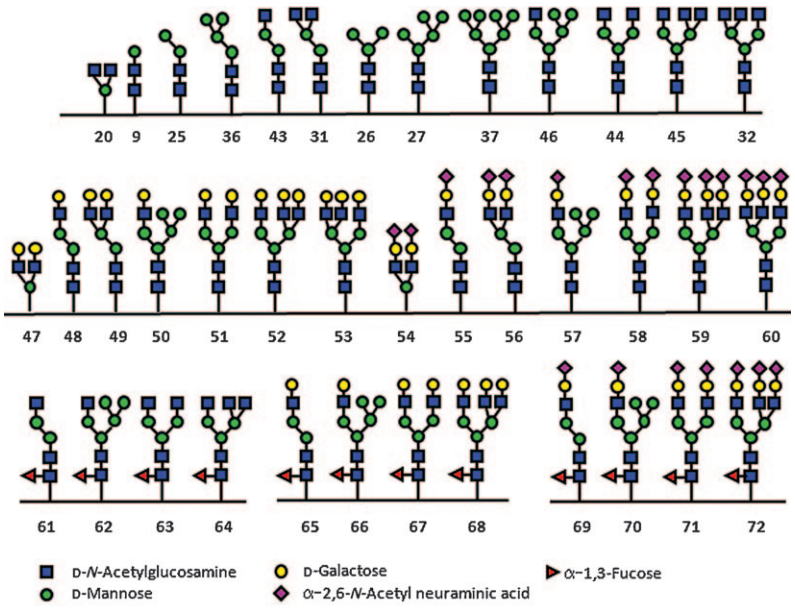


Figure 2. *N*-Glycan structures included on the microarray after enzymatic extension with GalT, SialT and FucT.

Results and Discussion

Analysis of the canonical *N*-glycan biosynthesis structures shows a limited number of variations for the 3″- and the 6″-arms of the conserved trisaccharide core.[24] With only 10 structurally different glycosyl donors, including a glucosamine donor for the bisecting GlcNAc, all natural *N*-glycan core structures of the complex and hybrid type with terminal GlcNAc would be accessible by assembly around a trisaccharidic scaffold. Subsequent enzymatic modifications for terminal galactose and neuraminic acids or core modifications, such as fucosylation or xylosylation, would increase substantially the number of structures accessible. As a proof of concept, *N*-glycans were synthesized by modular assembly of a trisaccharide scaffold and a panel of glycosyl donors for substitutions in 3-OH and 6-OH of the central β-mannoside. The overall strategy is exemplified for a hybrid-type oligosaccharide in Figure 3.

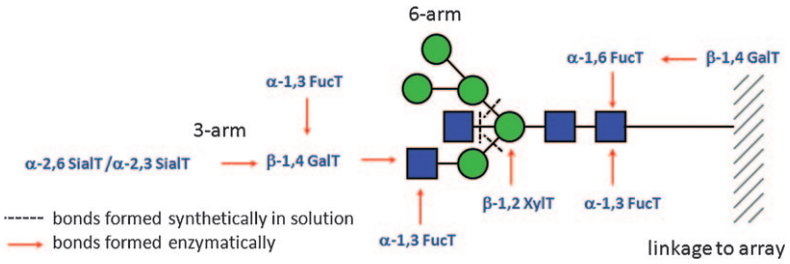
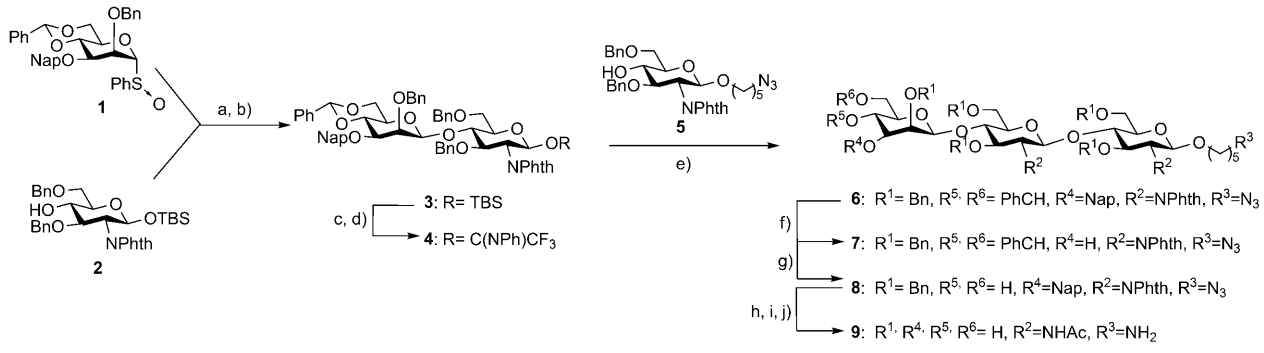


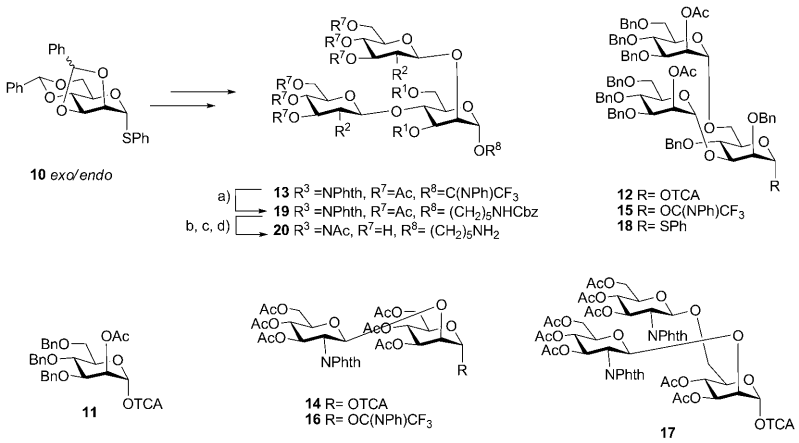
Figure 3. Proposed chemoenzymatic synthesis of *N*-glycans as ligands in microarrays exemplified for bisecting hybrid-type structures.

Chemical synthesis: The key trisaccharidic scaffold **6** was prepared in multigram amounts and in high yield as shown in Scheme 1. Applying methodology developed by Crich[25] and Kahne[26] the synthetically challenging β-mannopyranosidic linkage was formed with good stereoselectivity and in excellent yield by the condensation of nearly equimolar amounts of sulfoxide **1** and glucosamine acceptor **2** (Scheme 1). A 2-naphthylmethyl ether protection in 3′ provided stability under acidic conditions in later glycosylation and deprotection steps. The *tert*-butyldimethylsilyl ether (TBS) in **3** was removed with tetrabutylammonium fluoride (TBAF) and the resulting hemiacetal activated as *N*-phenyltrifluoroacetimidate **4**. Coupling **4** with **5**[27] under trimethylsilyl trifluoromethanesulfonate (TMSOTf) catalysis produced the trisaccharidic scaffold **6** in 89% yield. The orthogonality of protecting groups was demonstrated by treating **6** first with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), which cleaved the naphthylmethyl group (Nap) while leaving the acetal untouched (**7**). Treatment of **6** under transacetalisation conditions cleaved the benzylidene acetal while leaving the Nap group unaltered (**8**). From **6**, high-mannose, complex and hybrid-type *N*-glycans differing in the terminal sugar, type of branching and number of antennae were synthesized by using a choice of glycosyl donors (Scheme 2).

Donors **12**, **13**, **15** and **18** were synthesized by a convergent route based on the simultaneous selective reductive ring opening of dibenzylidene mannose thioglycosides **10**-*exo* and **10**-*endo*. The resulting products were either used directly as glycosyl donors or transformed into trichloro- or *N*-phenyl-trifluoroacetimidates, which allowed us to choose the best reactivity match between donor and acceptor for each glycosylation. Donors **11**[28] and **14**[12a] are known compounds, whereas **16** and **17** were prepared by following published procedures for analogous compounds.[29] From imidate **13**, glycoside **19** was obtained and then deprotected to provide **20** for surface immobilization. The symmetric pentasaccharide **23**, an early intermediate in *N*-glycan biosynthesis and thus a conserved motif in all *N*-glycans was prepared by glycosylation of **7** with imidate **11** at a low temperature to give tetrasaccharide **21** in 80% yield. After acid-catalysed transacetalisation the resulting diol **22** was regioselectively glycosylated with imidate **11** to afford **23** in 41% yield



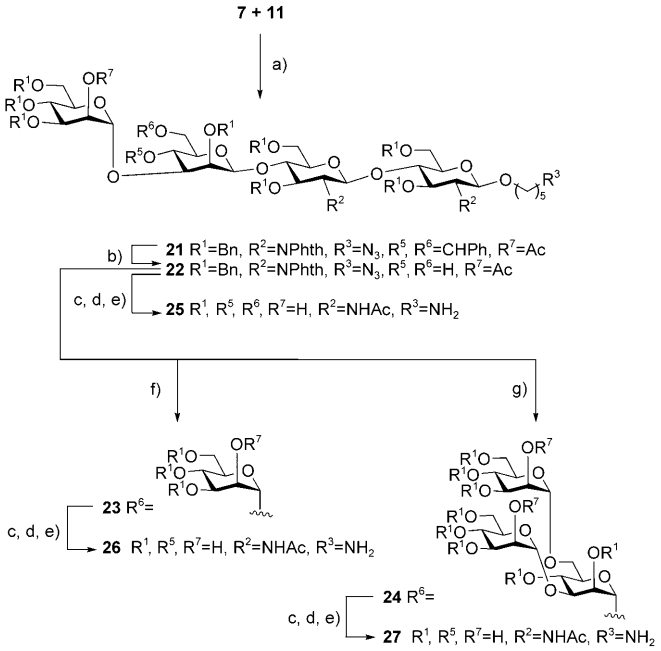
Scheme 1. a) Tf_2O, TTBP, 4 Å MS in CH_2Cl_2, −60 °C; b) **2**, −60 to −20 °C, 92%, (α/β 1:5); c) TBAF, AcOH, THF, 0 °C. d) $CF_3C(NPh)Cl$, K_2CO_3, acetone, RT, 78% (2 steps); e) TMSOTf, CH_2Cl_2, 0 °C to RT, 89%; f) DDQ, CH_2Cl_2, MeOH, H_2O, RT, 71%; g) *p*TsOH, EtSH, CH_2Cl_2, RT, 87%; h) $NH_2CH_2CH_2NH_2$, *n*BuOH, MW (120 °C); i) Ac_2O, pyridine, 0 °C to RT; j) Na, NH_3, THF, −78 °C, 52% (3 steps). TTBP: 2,4,6-tri-*tert*-butylpyrimidine.



Scheme 2. Panel of glycosyl donors employed in the assembly of *N*-glycan structures: a) $HO(CH_2)_5NHCbz$, TMSOTf, CH_2Cl_2, 0°C to RT, 91%; b) $NH_2CH_2CH_2NH_2$, *n*BuOH, MW (120°C); c) Ac_2O, pyridine, 0°C to RT; d) Na, NH_3, THF, −78°C, 72% (3 steps).

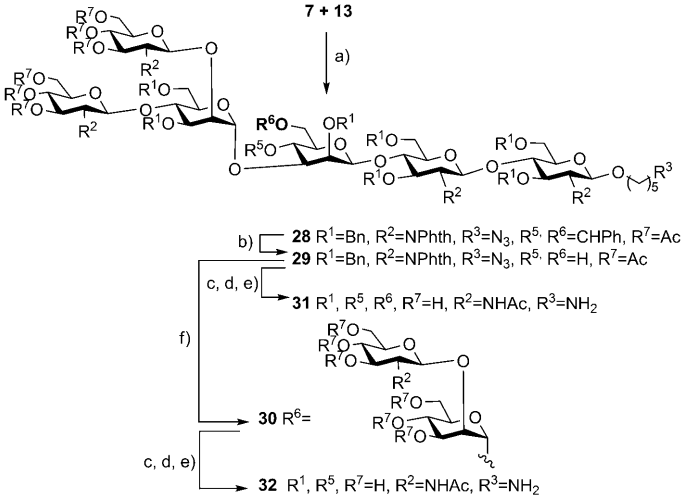
highly efficient for the glycosylation of the 3-arm with a series of linear and branched structures as will be shown for further examples later in the text. Hydrolysis of the benzylidene acetal in **28** afforded **29**, which was treated with trichloroacetimidate **14** to produce the complex protected *N*-glycan structure **30**.

The potential of this chemical approach was further shown by making the non-canonical structures **33** and **35**. The 3,6-branched trisaccharide donors **12**, **15** and **18** provide a usual (Scheme 3). Condensation of **22** with imidate **12** gave heptasaccharide **24**, an intermediate in GlcNAc-initiated branching in the Golgi apparatus[30] (Scheme 3), in a modest yield of 25%.



Scheme 3. Synthesis of high-mannose-type *N*-glycans **25**, **26** and **27**: a) TMSOTf, CH_2Cl_2, −40°C, 80%; b) EtSH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 87%; c) $NH_2CH_2CH_2NH_2$, *n*BuOH, MW (120°C); d) Ac_2O, pyridine, 0°C to RT; e) Na, NH_3, THF, −78°C, 56% for **25**, 56% for **26**, 47% for **27** (3 steps); f) **11**, TMSOTf, CH_2Cl_2, −40°C, 41%; g) **12**, TMSOTf, CH_2Cl_2, 0°C to RT, 25%.

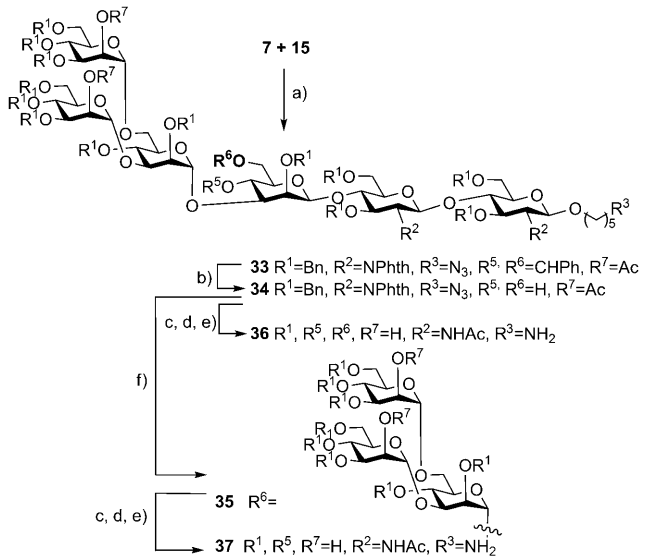
The synthesis of triantennary complex structure **30** started with the condensation of *N*-phentyltrifluoracetimidate **13** with trisaccharide **7** to give hexasaccharide **28** in 70% yield (Scheme 4). *N*-Phentyltrifluoracetimidate donors proved



Scheme 4. Synthesis of bi- and triantennary complex-type *N*-glycans **31** and **32**: a) TMSOTf, CH_2Cl_2, RT, 70%; b) EtSH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 69%; c) NH_2 $(CH_2)_2NH_2$, *n*BuOH, MW (120°C); d) Ac_2O, pyridine, 0°C to RT; e) Na, NH_3, THF, −78°C, 66% for **31**, 58% for **32** (3 steps); f) **14**, TMSOTf, CH_2Cl_2, 0°C to RT, 24%.

substitution pattern of the 6-arm in high mannose-type glycans. By using imidate **15**, this motif was first introduced on the 3-arm to form the non-natural homologue **33** in 76% yield with complete α-selectivity. After acid-catalysed thiolysis of the benzylidene acetal, imidate **12** was employed for the selective glycosylation of OH-6, giving rise to nonasaccharide **35** (Scheme 5).

Next the assembly of *N*-glycans of the complex and hybrid-type was approached. Condensation of **16** with trisaccharide **7** gave pentasaccharide **38** in 78% yield (Scheme 6). This compound was scaled up and served as an intermediate for the synthesis of **40**, **41** and **42**. Trichloroacetimidate **14** had been reported to be an efficient glycosylating agent in the construction of similar structures.[10,12a] However, when **39** was treated with **14** under $BF_3{\cdot}OEt_2$ catalysis heptasaccharide **40** was only isolated in a modest 29% yield



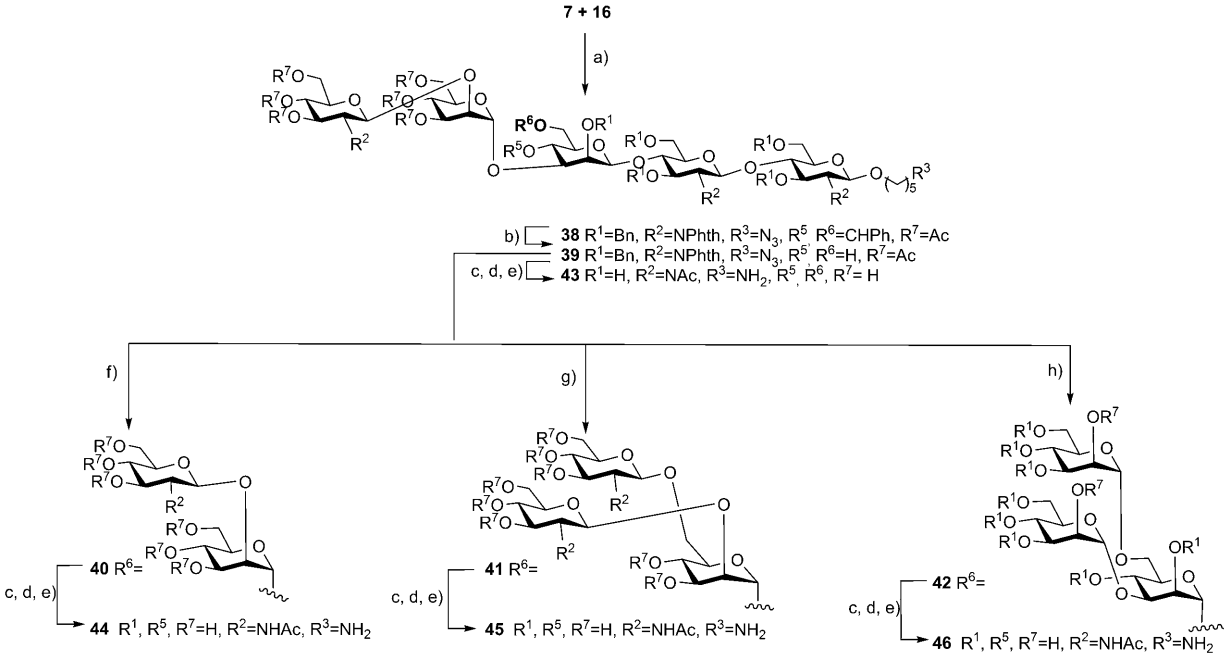
Scheme 5. Synthesis of unnatural high-mannose-type *N*-glycans **36** and **37**: a) **15**, TMSOTf, CH_2Cl_2, RT, 76%; b) $BF_3{\cdot}OEt_2$, EtSH, CH_2Cl_2, 76%; c) $NH_2CH_2CH_2NH_2$, *n*BuOH, MW (120°C); d) Ac_2O, pyridine, 0°C to RT; e) Na, NH_3, THF, −78°C; 39% for **36**, 60% for **37** (3 steps); f) **12**, TMSOTf, CH_2Cl_2, 0°C to RT, 33%.

(Scheme 6). This low yield may be partially attributed to loss of material during purification as LCMS showed a conversion of over 50%. The biantennary structure **40** is the smallest complete structure of complex *N*-glycans frequently found in serum glycoproteins and on the cell surface[31] Diol **39** was also reacted with imidate **17** affording the complex-type triantennary structure **41**. Again HPLC analysis of the crude suggested a conversion of more than 50%, the octasaccharide, however, could only be isolated in 27% yield.

Last in this series, the hybrid tetraantennary structure **42** was assembled by reacting pentasaccharide **39** with thioglycoside **18** since trichloroacetimidate **16** was found to be far too reactive for this condensation. Activation of **18** was carried out with the stable radical cation tris(4-bromophenyl)-ammoniumyl hexachloroantimonate in a one-electron transfer reaction as introduced by Sinay[32] and recently used by Danishefsky.[13c] This activation protocol proved sufficiently mild to cleanly conjugate the hindered acceptor **39** with the reactive donor **18** (Scheme 6).

All described compounds were deprotected in a three-step sequence to produce synthetic ligands for direct spotting on glass slides in good overall yields and with high purity. The removal of phthalimide protecting groups[12c] was carried out in *n*-butyl alcohol and with excess ethylenediamine by applying microwave heating to the pressure tight vials. After reacetylation of the free amines and purification, the intermediate neo-glycoconjugates were obtained in yields ranging from 61–81% for the two-step procedure. The remaining benzyl, acetate, and azide protecting groups were simultaneously removed by Birch reduction producing the deprotected ligands **9**, **20**, **25**, **26**, **27**, **31**, **36**, **37**, **43**, **44**, **45** and **46** in good yields and with high purity.

Enzymatic extension in well format: After complete characterization, the synthetic ligands were printed on NHS-acti-



Scheme 6. Synthesis of bi- and triantennary complex and triantennary hybrid-type *N*-glycans **44**, **45** and **46**: a) TMSOTf, CH_2Cl_2, RT, 78%; b) *p*TsOH, CH_3CN, 80%; c) $NH_2CH_2CH_2NH_2$, *n*BuOH, MW (120°C); d) Ac_2O, pyridine, 0°C to RT; e) Na, NH_3, THF, −78°C, 36% for **43**, 17% for **44**, 35% for **45**, 59% for **46** (3 steps); f) **14**, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −40°C, 29%; g) **17**, TMSOTf, CH_2Cl_2, 0°C to RT, 27%; h) **18**, $(BrC_6H_4)_3NSbCl_6$, CH_3CN, 15°C–RT, 51%.

vated glass slides and covalently immobilized by amide-bond formation by following standard procedures by a non-contact robotic printer. To find generally applicable conditions for the enzymatic processing of the surface-bound structures, the galactosylation and sialylation of some selected immobilized substrates was first investigated. Elongation of immobilized *N*-acetylglucosamine (GlcNAc), branched trisaccharide **20** and hexasaccharide **31** with β-1,4-galactosyl transferase (β-1,4-GalT) was studied by function of time of incubation, ligand surface density and enzyme concentration. Probing the arrays with cyanine-3-tagged[33] *Ricinus communis* agglutinin (Cy3-RCA), a terminal galactose/galactosamine specific lectin, at different time points showed saturation of fluorescence intensities after less than 4.5 h of incubation for the GlcNAc derivative, which suggests an end-point in the enzymatic glycosylation. By using the same conditions, galactosylation of the branched trisaccharide **20** and hexasaccharide **31** was achieved only after 20 h of incubation. Enzymatic galactosylation of surface-bound carbohydrates has been previously studied for simple monosaccharides,[21,34] disaccharides[16] and glycopeptides,[22a] but, to the best of our knowledge, not on larger structures.

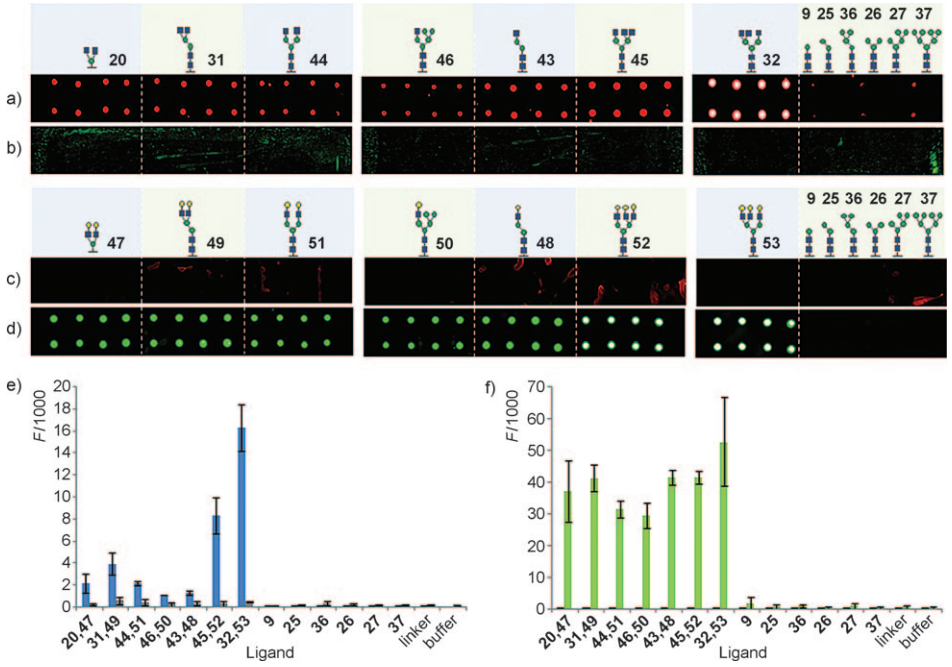


Figure 4. Enzymatic on-chip galactosylation with bovine β-1,4-galactosyl transferase. Images show eight spots of a total of 20 replicates for each ligand, all printed at 50 μM. a) Initial array probed with Cy5-BSL II for terminal GlcNAc recognition. b) Control incubation of initial array with Cy3-RCA specific for terminal galactose residues. c) Array after galactosylation probed with Cy5-BSL II for terminal GlcNAc recognition. d) Array after galactosylation probed with Cy3-RCA for terminal galactose recognition. e) Fluorescence intensities for incubation with Cy5-BSL II recognizing terminal GlcNAc residues before (blue columns) and after galactosylation (grey columns). f) Fluorescence intensity for unmodified (grey columns) and enzymatically galactosylated ligands (green columns) after incubation with Cy3-RCA recognizing terminal GlcNAc residues (histograms show average values for 20 spots).

The optimal conditions for the enzymatic sialylation with recombinant α-2,6-sialyl transferase (α-2,6-SialT) were explored in a similar manner on arrays of printed hexasaccharide **31**. Cyanine-5-tagged *Sambucus nigra* agglutinin (Cy5-SNA), specific for terminal *N*-acetylneuraminic acid (Neu5Ac) α-2,6-galactose/galactosamine structures,[35] clearly showed successful sialylation after incubation overnight but binding of Cy3-RCA to the remaining galactose residues was only lost completely after a second cycle of enzymatic extension.

In contrast to enzymatic galactosylation, very few authors have reported the use of sialyltransferases for the enzymatic extension of immobilized sugars. By starting from surface-bound GlcNAc, Shin reported the enzymatic synthesis of the sialyl LewisX tetrasaccharide by using three recombinant enzymes, including an α-2,3-SialT,[34a] whereas Blixt[20] determined the substrate specificities of a series of recombinant sialyltransferases on the Consortium of Functional Glycomics glycan array.[3a]

Once the experimental conditions for the enzymatic processing had been established, a panel of thirteen synthetic ligands (see the Supporting Information, Figure S17) was printed by using 50 μM spotting concentrations and replicates of five in a total of 14 subarrays (see the Supporting Information, Figure S4). All ligands with terminal glucosamine were printed in quadruplicate rows. Biofunctionality of the printed ligands was tested with the tagged lectins cyanine-3-tagged *Canavalia ensiformis* agglutinin (Cy3-ConA), cyanine-5-tagged *Wheat germ* agglutinin (Cy5-WGA), which binds strongly to the chitobiose core of *N*-glycans and interacts specifically with the Man β(1,4)GlcNAc β(1,4)GlcNAc trisaccharide[36] (see the Supporting Information, Figure S5) and cyanine-5 tagged *Griffonia (Bandeiraea) simplicifolia* (Cy5-BSL-II), the latter showing specific affinity for terminal β-linked *N*-acetylglucosamine-containing oligosaccharides (Figure 4a). Individual subarrays were incubated with a solution containing β-1,4-GalT and UDP-Gal for 48 h. After enzymatic elongation, tagged Cy3-RCA[37] was very strongly bound to the surface carbohydrates confirming the presence of new galactose residues (Figure 4b,d), whereas binding of Cy5-BSL-II (Figure 4c,e) to residual GlcNAc was no longer visible. A second subarray that had been subjected to enzymatic galactosylation but not incubation with the tagged lec-

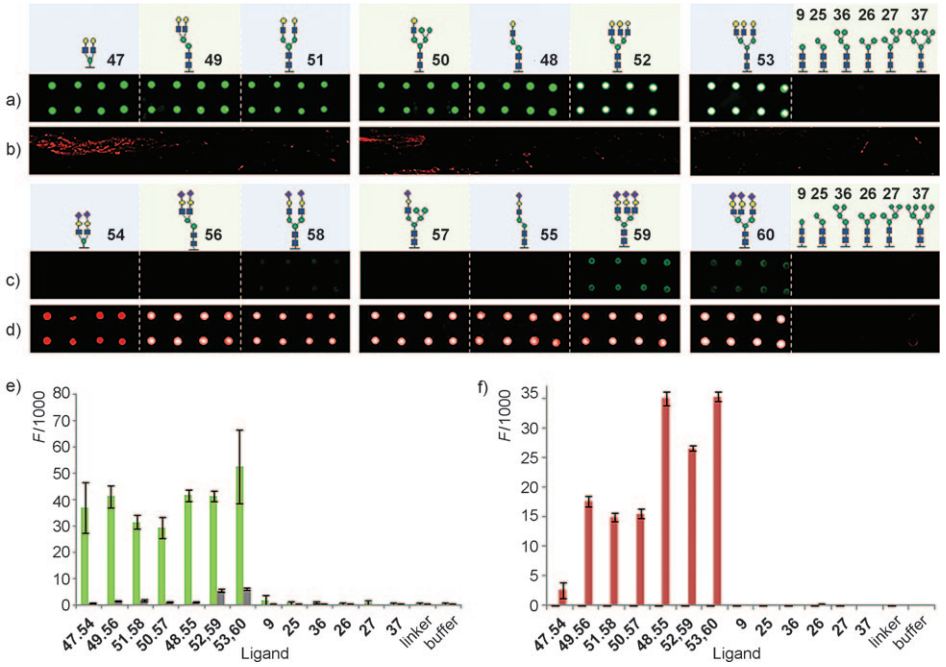


Figure 5. Enzymatic on-chip sialylation with recombinant human α-2,6-sialyl transferase. Images show eight spots of 20 replicates for each ligand printed at 50 μM. a) Galactosylated array probed with Cy3-RCA for terminal Gal recognition. b) Control incubation of initial array with Cy5-SNA specific for terminal Neu5Ac residues. c) Array after sialylation probed with Cy-3-RCA for terminal Gal recognition. d) Array after sialylation probed with Cy5-SNA for terminal Neu5Ac recognition. e) Fluorescence intensities for galactosylated (green columns) and enzymatically sialylated ligands (grey columns) after incubation with Cy3-RCA recognizing terminal Gal residues (histograms show average values for 20 spots). f) Fluorescence intensities for galactosylated (grey columns) and sialylated ligands (red columns) binding to Cy5-SNA, which recognizes terminal Neu5Ac residues (histograms show average values for 20 spots).

tins was then treated with a solution containing α-2,6-SialT and CMP-Neu5Ac donor for 48 h at 37 °C. Incubation of the array with Cy5-SNA[35] showed clearly the success of the on-surface sialylation procedure (Figure 5d,f).

After sialylation, binding of Cy3-RCA to carbohydrates was strongly reduced, as shown by fluorescence intensities below 10% of initial values for the majority of compounds tested (Figure 5c,e). Stronger residual fluorescence was observed for the sialylated triantennary octasaccharides **59** and **60**. Previous studies with a related sialyltransferase from bovine colostrum have shown a strong preference for galactose residues on the 3-arm and slower sialylation observed for residues on the 6-arm under the conditions studied.[38] This could help to explain the residual binding of tagged RCA after sialylation observed for the triantennary structures, particularly compound **59**. On the other hand, part of the residual fluorescence intensity observed, might be attributed to weak binding of Cy3-RCA to internal galactose residues.[39] Research is underway in our lab to investigate with the help of mass spectrometry the influence of surface density, number of antennae and enzyme concentration on the enzymatic extension, and, in particular, enzymatic sialylation of immobilized multiantennary *N*-glycans.

As a third enzyme for probing the enzymatic elongation of surface-bound *N*-glycans, we chose a recombinant α-1,3-fucosyltransferase from *Arabidopsis thaliana* expressed in *Pichia pastoris*, which specifically glycosylates the 3-OH of the reducing end GlcNAc residue. The α-1,3-core fucosylated *N*-glycans are immunogenic and allergenic epitopes specific to plants and invertebrates.[40] Only four of the thirteen immobilized synthetic structures were core fucosylated after overnight incubation with α-1,3-FucT, highlighting the tight substrate specificities of glycosyltransferases. Successful fucosylation was determined by probing the array with the fucose binding Cy3-tagged lectin from *Aleuria aurantia* (Cy3-AAL), which recognizes fucosylated structures[39a] (Figure 6a,b).

Compounds **31** and **32** with two terminal GlcNAc-residues on the 3-arm were not substrates for the enzyme. In contrast to the enzymatic galactosylation and sialylations, we could not follow the fucosylation progress due to lack of a lectin recognizing specifically the non-fucosylated starting compounds. To maximize yields for the enzymatic extension, however, two cycles of 16 h each were applied.

To further increase the number of ligands on the slide to a total of 39 ligands, fucosylated wells were treated sequentially with GalT and SialT and incubated with Cy3-RCA and Cy5-SNA recognizing galactosylation and sialylation, respectively (Figure 6c–f, for control experiments see the Supporting Information, Figures S8–S10). To our knowledge, this is the first example of an enzymatic synthesis of core fucosylated *N*-glycans.

Enzymatic extension in nanodroplets: Next, the enzymatic processing on individual spots was investigated. By transferring the enzymatic reaction from a microlitre-based well format into nanodroplets placed on top of the printed glycans, the amount of glycosyl transferases and donors needed could be decreased by up to 100000-fold. To avoid rapid evaporation of droplets, glycerol was added in varying amounts to the printing solutions. We found that addition of 30% of glycerol to the buffer was sufficient for the formation of well-defined droplets without compromising the enzymatic activity. Droplet volumes of enzyme spotting solutions were optimized to assure complete coverage of the underlying sugar array with the enzyme cocktail (Figure 7). Sugars containing terminal GlcNAc, were printed in quadru-

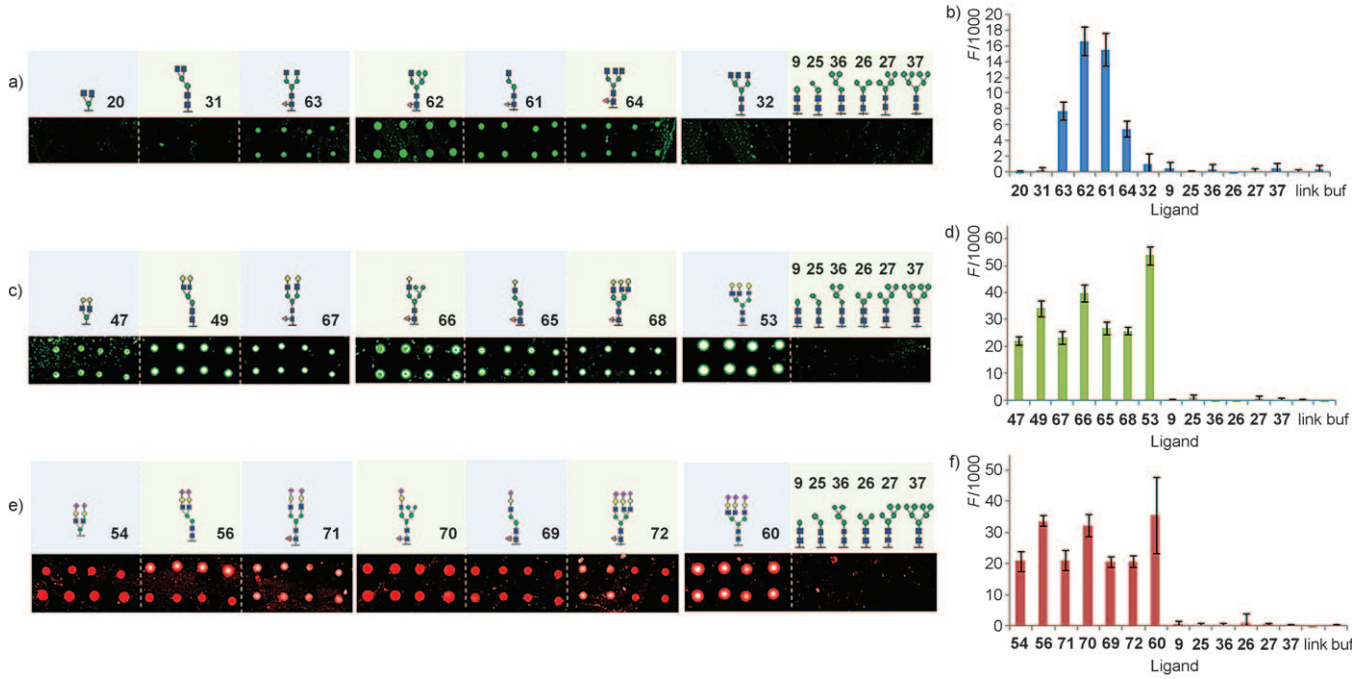


Figure 6. Enzymatic on-chip fucosylation with α-1,3-fucosyl transferase, β-1,4-galactosyl transferase and α-2,6-sialyl transferase. a) Array after fucosylation probed with Cy3-AAL for fucose recognition. b) Analysis of the fluorescence values of the fucosylated ligands incubated with 50 nM solution of Cy3-AAL. c) Array after galactosylation probed with Cy3-RCA for galactose recognition. d) Analysis of the fluorescence values of the galactosylated ligands incubated with 50 nM solution of Cy3-RCA. e) Array after sialylation probed with Cy5-SNA for terminal Neu5Ac recognition. f) Analysis of the fluorescence values of the sialylated ligands incubated with a 50 nM solution of Cy5-SNA (histograms show an average of 20 spots for every ligand, eight spots for every ligand shown in the images).

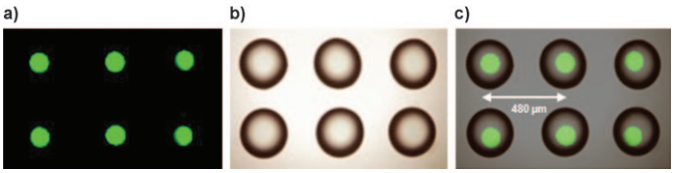


Figure 7. a) Fluorescence scan showing spotsize of immobilized glycans. b) Micrograph showing size of nanodroplets. c) Overlay of a and b demonstrating complete coverage of spots with enzyme-containing nanodroplets and absence of cross-contamination between vicinal spots.

plicate rows to be able to accommodate both extended and non-extended compounds in the same subarray and a GalT- and UDP-Gal-containing solution was placed on top of selected individual spots by the robotic printer. After 48 h of incubation, all droplets had maintained their size and position, due to the low volatility of glycerol drops on glass slides. The subsequent incubation of subarrays with a mixture of Cy5-BSL-II and Cy3-RCA showed loss of binding to residual GlcNAc and intense binding to newly incorporated galactose residues for all treated structures (Figure 8a), except for octasaccharide **32**, which showed 17% of residual fluorescence for binding to Cy5-BSL-II. In an attempt to drive reactions to completion for all ligands, we incubated an array containing octasaccharides with twice the enzyme concentration. After an incubation of 48 h, we observed complete loss of BSL-II binding to any residual GlcNAc for all ligands including octasaccharide **32** (see the Supporting Information, Figure S16).

We expected the sialylation in nanodroplets to be more difficult due to the reported lower in vitro activity of the α-2,6-SialT relative to the bovine β-1,4-GalT,[12e] The first results with HEPES buffer led to disappointing results, even after extended reaction times of 72 h and increasing enzyme or nucleotide donor concentrations. Changing the buffer to a cacodylate system resulted in a far higher incorporation of sialic acid residues in nearly all compounds, as demonstrated by probing the arrays with Cy3-RCA and Cy5-SNA (Figure 8b).

Octasaccharides **52** and **53** were sialylated in around 80% yield as suggested by residual fluorescence values, very similar to the results obtained in the sialylation in well-formats. Incubation with tagged ECA (*Erythrina cristagalli* agglutinin; Figure 8b, lower row), which is reported not to tolerate 6-O-sialyation of galactose,[39b] showed some residual binding as well, confirming incomplete sialylation of the triantennary structures under the tested conditions. Finally, we proceeded with the spotwise fucosylation of ligands, which had been shown to be substrates for the α-1,3-FucT in previous experiments. Enzymatic extension in nanodroplets resulted in a clearly visible core fucosylation of the tested substrates **43**, **44**, **45** and **46** giving rise to compounds **61**, **63**, **64** and **62** as seen by probing with Cy3-AAL. Additional incubation cycles with fucosyltransferase did not increase the fluorescence intensities (Figure 8c).

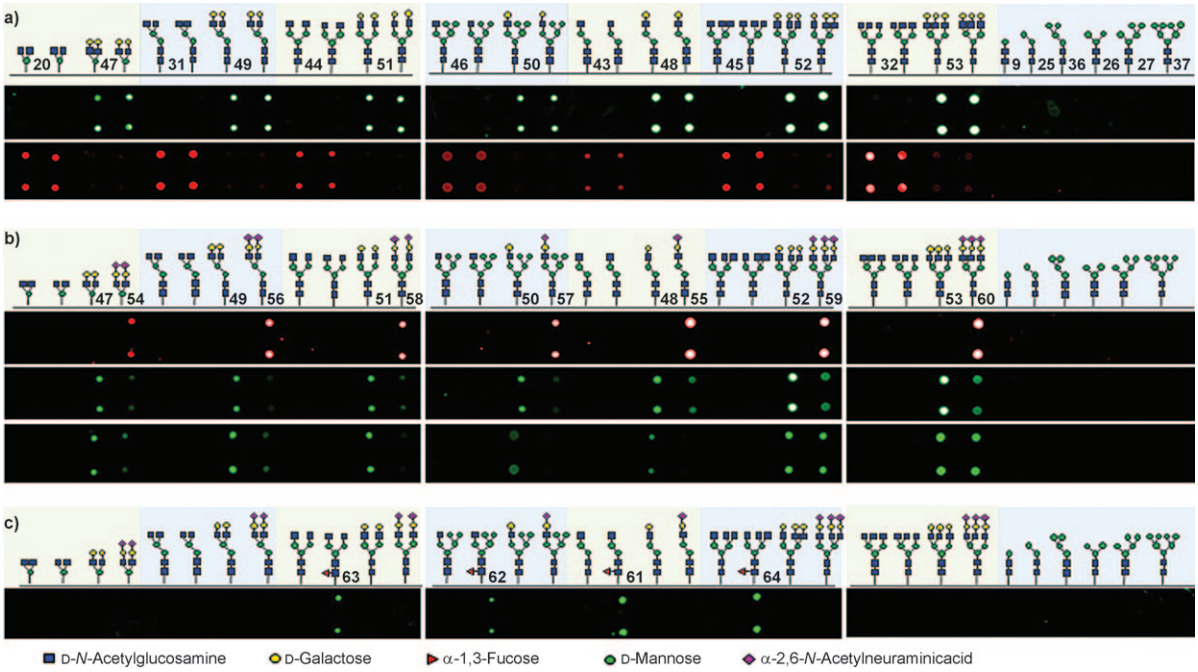


Figure 8. Spotwise enzymatic glycosylation by glycosyltransferases: a) Spot by spot galactosylation of glycan array with GalT. Upper row: Cy3-RCA showing saturated signals for terminal galactose. Lower row: Cy5-BSL-II to visualize residual GlcNAc. b) Spotwise sialylation of glycan array with α-2,6-SialT. Upper row: Cy5-SNA showing saturated signals for terminal sialic acid. Middle row: Array probed with Cy3-RCA to visualize residual galactose. Lower row: Array probed with biotinylated ECA/Cy3-Neutravidine to visualize residual galactose. c) Spot by spot fucosylation with α-1,3-FucT probed with Cy3-AAL.

Conclusion

Microarrays focused on specific classes of carbohydrates are more easily accomplished than more heterogeneous glycan arrays: The synthetic effort is minimized by taking advantage of modular strategies, whereas structural similarity of ligands permits the general application of class-specific transferases exhibiting relatively tight substrate specificities. According to this working hypothesis, we have prepared the first microarray of fully synthetic *N*-glycan structures by using a chemoenzymatic approach. A modular chemical synthesis based on a versatile trisaccharidic scaffold has afforded a series of natural and non-natural high-mannose, complex and hybrid-type *N*-glycans functionalized with an anomeric spacer. Overall short synthetic routes and generally good yields combined with a straightforward deprotection protocol have afforded pure compounds on a scale suited for microarray preparation. The low yields encountered for some selective glycosylations are most probably due to mismatched reactivities of donor and acceptor and to the difficult purification of compound mixtures of similar polarity. After immobilization on glass slides, these synthetic core structures were extended by enzymatic reactions first in wells and then, for the first time, in nanodroplets placed on top of individual spots. This has permitted us to reduce the amount of precious glycosyltransferases by several orders of magnitude relative to reactions in solution and in well-formats. Three recombinant glycosyltransferases have been employed to extend an array of 13 synthetic ligands to nearly 40 well-defined *N*-glycans in a few days. Start and endpoints of enzymatic reactions were defined where possible by incubating untreated and processed subarrays with fluorescently tagged lectins that show an affinity for a specific terminal sugar, a validation method that has been used by others before.[20,21,34a]

In any case, we are well aware that a general use of tagged lectin pairs for monitoring conversion of enzymatic reactions is limited and we are currently working on the direct analysis of spot compositions by mass spectrometry techniques to confirm the high conversion rates we observed by probing the arrays with lectins. As glycosyltransferases have narrow substrate specificities, some ligands might not be completely glycosylated, even after repeated cycles when subjecting the entire array to the enzymatic extension conditions.[20–21] To avoid compound mixtures coming from incomplete reactions. the usefulness of a glycosyltransferase would have to be established for every acceptor individually and ideally confirmed by a mass spectrometric readout of spot compositions. For this reason, we propose the enzymatic extension in well format as a first screen for potential acceptors of a particular glycosyltransferase to test its synthetic usefulness. For the fabrication of arrays, the enzymatic extension in nanodroplets could then be used to produce pure ligands on the chip, choosing only spots occupied by validated acceptors of the enzyme used. This feature of the nanodroplet approach allows the preparation of arrays of higher diversity and density than by classical enzymatic on-chip elongation.

The methodology here described could open the way for the rapid production of large synthetic glycan arrays. However, alternative methods for spot analysis have to be developed to confirm enzymatic conversions. Work in process includes the investigation of these analytical methods and the preparation of a large and representative *N*-glycan array by increasing both the number of synthetic core structures and available glycosyl transferases.

Experimental Section

General methods: General methods and spectroscopic data for the synthesis of the oligosaccharides, commercial sources and detailed information for the microarray experiments can be found in the Supporting Information.

***tert*-Butyldimethylsilyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-(2-naphthylmethyl)- β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (3)**: Tf_2O (200 μL, 1.18 mmol) was added to a stirred solution of donor **1** (714 mg, 1.18 mmol), TTBP (539 mg, 2.18 mmol) and 4 Å activated molecular sieves in dry CH_2Cl_2 (10 mL) at −60 °C under argon atmosphere. After five minutes at −60 °C, a solution of acceptor **2** (550 mg, 0.91 mmol) in dry CH_2Cl_2 (3 mL) by syringe. The reaction mixture was allowed to warm to −20 °C over 1 h and was then quenched by the addition of aqueous saturated $NaHCO_3$ solution (75 mL), filtered over a plug of Celite and extracted with CH_2Cl_2 (3× 75 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The crude product was purified by medium-pressure flash chromatography (toluene/EtOAc 100:0 to 96:4) to yield the β-anomer as a white foam (760 mg, 71 %). R_f = 0.32 (toluene/EtOAc 19:1); $[\alpha]_D^{20}$ = +4.0 (c = 0.21 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.90–7.70 (m, 8H; Ar), 7.60–7.24 (m, 18H; Ar), 7.05–7.04 (m, 2H; Ar), 6.97–6.91 (m, 3H; Ar), 5.64 (s, 1H; C*H*Ph), 5.44 (d, J = 8.1 Hz, 1H; H-1), 5.02–4.94 (m, 4H; CH_2Ar, CH_aH$_{a'}$Ar, CH_bH$_{b'}$Ar), 4.84 (d, J = 12.7 Hz, 1H; CH$_b$H$_{b'}$Ar), 4.72–4.62 (m, 2H; CH_cH$_{c'}$Ar, H-1′), 4.58 (d, J = 12.4 Hz, 1H; CH$_a$H$_{a'}$Ar), 4.46 (d, J = 12.1 Hz, 1H; CH$_c$H$_{c'}$Ar), 4.39 (dd, J = 8.6, 10.8 Hz, 1H; H-3), 4.29 (dd, J = 4.7, 10.4 Hz, 1H; H-6$_a$′), 4.25–4.19 (m, 2H; H-4′, H-2), 4.13 (t, J = 9.2 Hz, 1H; H-4), 3.92 (d, J = 2.6 Hz, 1H; H-2′), 3.73–3.55 (m, 5H; H-6$_a$, H-6$_b$, H-6$_b$′, H-3′, H-5), 3.29 (td, J = 4.9, 9.7 Hz; 1H; H-5′), 0.76 (s, 9H; *t*Bu), 0.13 (s, 3H; CH$_3$), −0.00 ppm (s, 3H; CH$_3$); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.20, 167.50 (CO), 138.77, 138.54, 137.71, 137.58, 135.88 (qC, Ar), 133.59 (CH, Ar), 133.17, 132.81, 131.52 (qC, Ar), 128.74, 128.32, 128.19, 128.09, 127.93, 127.77, 127.69, 127.54, 127.46, 126.80, 126.05, 125.98, 125.92, 125.72, 125.33, 123.05, 122.91 (CH, Ar), 101.87 (C-1′, J_{C1-H1} = 158.7 Hz), 101.31 (*C*HPh), 93.36 (C-1), 79.46 (C-4), 78.58 (C-3′), 78.17 (C-4′), 76.86 (C-2′), 76.67 (C-3), 74.94 (*C*H$_2$Ar), 74.65 (C-5), 74.32, 73.35, 72.30 (*C*H$_2$Ar), 68.60, 68.48 (C-6, C-6′), 67.26 (C-5′), 57.79 (C-2), 25.24 (CH$_3$, *t*Bu), 17.45 (qC, *t*Bu), −4.31 (CH$_3$), −5.54 ppm (CH$_3$); HRMS: m/z: calcd for $C_{65}H_{69}NO_{12}SiNa$: 1106.4487 $[M+Na]^+$; found: 1106.4537.

2-*O*-Benzyl-4,6-*O*-benzylidene-3-*O*-(2-naphthylmethyl)-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl *N*-phenyl trifluoroacetimidate (4): TBAF (1 M in THF, 1.2 mL, 1.16 mmol) and acetic acid (66 μL, 1.16 mmol) were added to a solution of compound **3** (785 mg, 0.72 mmol) in dry THF (3.6 mL) at 0 °C and the resulting mixture was stirred for 2 h at 0 °C. The reaction was quenched with saturated $NaHCO_3$ solution (75 mL) and extracted with EtOAc (3× 50 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The crude product was dissolved in acetone (7.2 mL) and then ClC(NPh)CF_3 (299 mg, 1.44 mmol) and K_2CO_3 (199 mg, 1.44 mmol) were added and the mixture stirred at room temperature overnight. The reaction mixture was diluted with EtOAc, filtered over Celite and the solvent was evaporated. The crude product was purified by medium-pressure flash chromatography (hexane/EtOAc 94:6 to 60;40) to give the title compound as a white foam (642 mg, 78 % over two steps). R_f = 0.16 (hexane/EtOAc 3:1); ^{1}H NMR (500 MHz, $CDCl_3$ at 50 °C): δ = 7.93–7.67 (m, 8H; Ar), 7.62–6.89 (m, 23H; Ar), 6.74 (d, J = 7.3 Hz, 1H; Ar), 6.35 (s, 1H; H-1), 5.61 (s, 1H; C*H*Ph), 5.02–4.94 (m, 4H; CH_aH$_{a'}$Ar, CH_bH$_{b'}$Ar), 4.85 (d, J = 12.6 Hz, 1H; CH$_b$H$_{b'}$Ar), 4.67–4.62 (m, 2H; CH_cH$_{c'}$Ar, H-1′), 4.52–4.37 (m, 4H; CH$_a$H$_{a'}$Ar, CH$_c$H$_{c'}$Ar, H-2, H-3), 4.28–4.16 (m, 3H; H-4, H-4′, H-6$_a$′), 3.88 (d, J = 2.4 Hz, 1H; H-2), 3.75–3.41 (m, 5H; H-3′, H-6$_b$′, H-5, H-6$_a$, H-6$_b$), 3.24 ppm (td, J = 4.8, 9.7 Hz, 1H; H-5′); ^{13}C NMR (126 MHz, $CDCl_3$ at 50 °C): δ = 167.47 (CO), 143.06, 138.64, 137.78, 137.57, 136.06 (qC, Ar), 133.82 (CH, Ar), 133.36, 133.00, 131.56 (qC, Ar), 128.77, 128.54, 128.45, 128.23, 128.14, 128.10, 127.99, 127.94, 127.81, 127.76, 127.66, 127.63, 127.53, 126.93, 126.16, 126.04, 125.78, 125.44, 124.36, 123.32, 119.40 (CH, Ar), 115.93 (q, J = 284.5 Hz; *C*F$_3$), 101.63, 101.50 (C-1′, *C*HPh), 93.66 (C-1), 78.78, 78.50, 78.44 (C-3′, C-4, C-4′), 77.18 (C-2′), 76.78 (C-3), 75.64 (C-5), 75.14, 74.64, 73.53, 72.58 (CH$_2$Ar), 68.55, 68.05 (C-6′, C-6), 67.44 (C-5′), 54.89 ppm (C-2); HRMS (ESI): m/z: calcd for $C_{67}H_{59}F_3N_2O_{12}Na$: 1163.3917 $[M+Na]^+$; found: 1163.3882.

5-Azidopentyl 2-*O*-Benzyl-4,6-*O*-benzylidene-3-*O*-(2-naphthylmethyl)-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (6): Trimethylsilyltrifluoromethanesulfonate (4.2 μL, 0.023 mmol) was added to a mixture of donor **4** (631 mg, 0.55 mmol), acceptor **5** (277 mg, 0.46 mmol) and activated molecular sieves 4 Å in dry CH_2Cl_2 (0.9 mL). The reaction mixture was stirred at room temperature for 40 min and then quenched by the addition of Et_3N (100 μL), diluted with CH_2Cl_2, filtered over Celite and concentrated. The crude product was purified by flash chromatography (hexane/EtOAc 3:2) to give the title compound as a white foam (636 mg, 89 %). R_f = 0.27 (hexane/EtOAc 3:2); $[\alpha]_D^{20}$ = +9.1 (c = 0.12 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.92–7.67 (m, 12H; Ar), 7.53–7.15 (m, 23H; Ar), 7.00–6.96 (m, 4H; Ar), 6.90–6.89 (m, 3H; Ar), 6.80–6.79 (m, 3H; Ar), 5.55 (s, 1H; C*H*Ph), 5.30 (d, J = 8.1 Hz, 1H; H-1), 4.98–4.83 (m, 6H; H-1′, CH$_2$Ar, CH_aH$_{a'}$Ar, CH_bH$_{b'}$Ar, CH_cH$_{c'}$Ar), 4.76 (d, J = 12.8 Hz, 1H; CH$_a$H$_{a'}$Ar), 4.63–4.41 (m, 6H; H-1″, CH$_b$H$_{b'}$Ar, CH$_c$H$_{c'}$Ar, CH$_2$Ar, CH_dH$_{d'}$Ar), 4.36–4.05 (m, 9H; CH$_d$H$_{d'}$Ar, H-2, H-2′, H-3, H-3′, H-4, H-4′, H-4″, H-6$_a$″), 3.81 (d, J = 2.2 Hz, 1H; H-2″), 3.70 (dt, J = 6.2, 9.9 Hz, 1H; OC*H*H(CH$_2$)$_4$N$_3$), 3.63–3.55 (m, 3H; H-6$_a$, H-6$_a$′, H-6$_b$″), 3.50 (dd, J = 2.7, 9.9 Hz, 1H; H-3″), 3.46 (dd, J = 3.6, 11.1 Hz, 1H; H-6$_b$′), 3.40 (dd, J = 1.9, 10.9 Hz, 1H; H-6$_b$), 3.34 (dd, J = 2.4, 9.5 Hz, 1H; H-5′), 3.28 (dt, J = 6.6, 9.8 Hz, 1H; OCH*H*-(CH$_2$)$_4$N$_3$), 3.23 (d, J = 9.6 Hz, 1H; H-5), 3.15 (td, J = 4.8, 9.5 Hz, 1H; H-5″), 2.96–2.82 (m, 2H; O(CH$_2$)$_4$CH_2N$_3$), 1.47–1.23 (m, 4H; 2×CH$_2$), 1.18–1.02 ppm (m, 2H; CH$_2$); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.40, 167.49 (CO), 138.80, 138.57, 138.42, 137.69, 137.60, 135.91 (qC, Ar), 133.92, 133.72, 133.55 (CH, Ar), 133.21, 132.86, 131.71, 131.59, 131.40 (qC, Ar), 128.77, 128.38, 128.24, 128.14, 128.11, 127.98, 127.81, 127.72, 127.65, 127.60, 127.55, 127.23, 126.83, 126.78, 126.08, 126.03, 126.00, 125.79, 125.40, 123.55, 123.04 (CH, Ar), 101.73 (C-1″), 101.34 (*C*HPh), 98.04 (C-1′), 97.03 (C-1), 79.19, 78.56, 76.96, 76.93, 76.71, 75.83 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-2″), 78.18 (C-3″), 75.07, 74.60 (*C*H$_2$Ar), 74.48 (C-5, C-5′), 74.31, 73.11, 72.59, 72.35 (*C*H$_2$Ar), 68.80 (O*C*H$_2$(CH$_2$)$_4$N$_3$), 68.47 (C-6″), 68.18 (C-6′), 67.85 (C-6), 67.23 (C-5″), 56.51 (C-2), 55.66 (C-2′), 51.00 (O(CH$_2$)$_4$*C*H$_2$N$_3$), 28.59, 28.19, 22.92 ppm (CH$_2$ linker); HRMS (ESI): m/z: calcd for $C_{92}H_{89}N_5O_{18}Na$: 1574.6100 $[M+Na]^+$; found: 1574.6102.

5-Azidopentyl 2-*O*-benzyl-4,6-*O*-benzylidene-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (7): 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (153 mg, 0.68 mmol) was added in one portion to a stirred solution of compound **6** (350 mg, 0.22 mmol) in wet CH_2Cl_2/MeOH (4:1, 1.1 mL). The resulting mixture was stirred for 1 h, diluted with CH_2Cl_2 (50 mL), washed with saturated $NaHCO_3$ and extracted with CH_2Cl_2 (2×100 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated. The crude product was purified by medium-pressure flash chromatography (hexane/EtOAc 9:1 to 1:4) to give the title compound (220 mg, 71 %). R_f = 0.22 (hexane/EtOAc 3:2); $[\alpha]_D^{20}$ = +3.5 (c = 0.062 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.90–7.69 (m, 8H; Ar), 7.51–7.24 (m, 20H; Ar), 7.06–6.99 (m, 2H; Ar), 6.97–6.95 (m, 2H; Ar), 6.92–6.88 (m, 3H; Ar), 6.82–6.81 (m, 3H; Ar), 5.45 (s, 1H; C*H*Ph), 5.31 (d, J = 7.6 Hz,

1H; H-1), 5.02 (d, J=11.6 Hz, 1H; CH_aH$_{a'}$Ar), 4.97 (d, J=7.8 Hz, 1H; H-1′), 4.94–4.88 (m, 2H; CH_bH$_{b'}$Ar, CH_cH$_{c'}$Ar), 4.71–4.69 (m, 2H; H$_1$″, CH$_a$$H_{a'}$Ar), 4.63 (d, J=12.0 Hz, 1H; CH_dH$_{d'}$Ar), 4.57–4.54 (m, 3H; CH$_c$$H_{c'}Ar, CH_2$Ar), 4.48 (d, J=12.1 Hz, 1H; CH$_d$$H_{d'}$Ar), 4.42 (d, J=12.2 Hz, 1H; CH$_b$$H_{b'}$Ar), 4.32–4.09 (m, 7H; H-2, H-2′, H-3, H-3′, H-4, H-4′, H-6$_a$″), 3.75–3.66 (m, 4H; H-2″, H-4″, H-6$_a$, OCHH(CH$_2$)$_4$N$_3$), 3.62–3.57 (m, 2H; H-3″, H-6$_{a'}$), 3.54–3.44 (m, 3H; H-6$_b$″, H-6$_b$, H-6$_{b'}$), 3.35 (dd, J=3.2, 9.9 Hz, 1H; H-5′), 3.28 (dt, J=6.6, 10.0 Hz, 1H; OCHH-(CH$_2$)$_4$N$_3$), 3.23 (d, J=9.8 Hz, 1H; H-5), 3.14 (td, J=4.8, 9.5 Hz, 1H; H-5″), 2.97–2.82 (m, 2H; O(CH$_2$)$_4$CH_2N$_3$), 2.37 (d, J=8.6 Hz, 1H; OH), 1.46–1.26 (m, 4H; 2×CH$_2$), 1.15–1.03 ppm (m, 2H; CH$_2$); ^{13}C NMR (126 MHz, CDCl$_3$): δ=168.45, 167.50 (CO), 138.72, 138.60, 138.45, 138.14, 137.66, 137.21 (qC, Ar), 133.97, 133.76, 133.60 (CH, Ar), 131.72, 131.62, 131.39 (qC, Ar), 129.01, 128.55, 128.47, 128.17, 128.14, 127.99, 127.95, 127.90, 127.85, 127.77, 127.51, 127.24, 126.92, 126.83, 126.22, 123.59, 123.07 (CH, Ar), 101.88 (*C*HPh, C-1″), 98.07 (C-1′), 97.02 (C-1), 79.32, 79.11, 78.92, 77.04, 76.75, 75.81 (C-2″, C-4″, C-3, C-3′, C-4, C-4′), 75.75, 74.61 (*C*H$_2$Ar), 74.55, 74.49 (C-5, C-5′), 74.32, 73.36, 72.59 (*C*H$_2$Ar), 70.88 (C-3″), 68.84 (O*C*H$_2$(CH$_2$)$_4$N$_3$), 68.42 (C-6″) , 68.22 (C-6′), 67.74 (C-6), 66.80 (C-5″), 56.48 (C-2), 55.69 (C-2′), 51.03 (O-(CH$_2$)$_4$*C*H$_2$N$_3$), 28.61, 28.21, 22.94 ppm (CH$_2$ linker); HRMS (ESI): m/z: calcd for $C_{81}H_{81}N_5O_{18}Na$: 1434.5475 $[M+Na]^+$; found: 1434.5571.

5-Azidopentyl *O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-β-D-mannopyranosyl-(1→3)-*O*-(2-*O*-benzyl-4,6-*O*-benzylidene-β-D-mannopyranosyl)-(1→4)-*O*-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-*O*-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (38): Trimethylsilyltrifluoromethanesulfonate (1.7 μL, 0.01 mmol) was added to a stirred mixture of donor **16** (100 mg, 0.11 mmol), acceptor **7** (135 mg, 0.10 mmol) and activated molecular sieves 4 Å in dry CH_2Cl_2 (2 mL) and the reaction mixture was stirred at room temperature for 40 min. The reaction was quenched by adding Et_3N (100 μL), diluted with CH_2Cl_2, filtered over Celite and evaporated. The crude product was purified by flash column chromatography (hexane/EtOAc 1:1) to give the title compound as clear oil (159 mg, 78%). $[\alpha]_D^{20}=-19.9$ (c=1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, CDCl$_3$): δ=7.90–7.86 (m, 3H; Ar), 7.75–7.69 (m, 9H; Ar), 7.56–7.44 (m, 3H; Ar), 7.43–7.22 (m, 16H; Ar), 7.01 (m, 1H; Ar), 6.92–6.89 (m, 4H; Ar), 6.82–6.80 (m, 2H; Ar), 5.47 (dd, J=9.0, 11.0 Hz, 1H; H-3^E), 5.43 (s, 1H; C*H*Ph), 5.30 (d, J=7.8 Hz, 1H; H-1^A), 5.05 (dd, J=3.4, 10.4 Hz, 1H; H-4^D), 5.01–4.84 (m, 6H; H-1^D, H-1^B, CH_aH$_{a'}$Ar, CH_bH$_{b'}$Ar, H-4^E, H-1^E), 4.79 (s, 2H; CH$_2$Ar), 4.66 (d, J=12.8 Hz, 1H; CH_cH$_{c'}$Ar), 4.55–4.49 (m, 3H; CH$_2$Ar, CH$_a$$H_{a'}$Ar), 4.44 (s, 1H; H-1^C), 4.39 (d, J=12.8 Hz, 1H; CH$_b$$H_{b'}$Ar), 4.33 (d, J=12.8 Hz, 1H; CH$_c$$H_{c'}$Ar), 4.29–4.08 (m, 9H; H-2^A, H-2^B, H-2^E, H-3^A, H-3^B, H-3^D, H-4^A, H-4^B, H-6$_b^C$), 3.96 (dd, J=4.1, 12.2 Hz, 1H; H-6$_a^E$), 3.92 (t, J=10.1 Hz,1H; H-4^C), 3.69–3.66 (m, 3H; OCHH(CH$_2$)$_4$N$_3$, H-6$_b^E$, H-6$_a^A$), 3.57–3.40 (m, 9H; H-2^C, H-6$_a^D$, H-6$_b^D$, H-3^C, H-2^D, H-5^D, H-6$_a^B$, H-6$_b^B$, H-6$_b^C$), 3.57–3.33 (m, 1H; H-5^B), 3.27–3.24 (2H, m; H-5^A, OCHH(CH$_2$)$_4$N$_3$), 2.98 (m, 1H; H-5^C), 2.88 (m, 2H; O-(CH$_2$)$_4$CH_2N$_3$), 2.07, 2.06, 2.02, 2.01, 1.86 (s, 18H; 6×OCH$_3$), 1.40–1.22 (m, 4H; 2×CH$_2$), 1.10–1.05 ppm (m, 2H; CH$_2$); ^{13}C NMR (126 MHz, CDCl$_3$): δ=170.5, 170.4, 170.3, 170.1, 169.2, 169.1, 168.5, 167.9, 167.8, 167.7, 167.5, 138.8, 138.7, 138.5, 138.1, 137.7, 137.6, 134.1, 134.0, 133.8, 133.6, 131.7, 131.6, 131.5, 131.4, 131.3, 130.2, 129.8, 129.0, 128.9, 128.7, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3, 127.2, 127.0, 126.9, 126.8, 123.6, 123.5, 123.1, 102.4, 100.9 (C-1^C, J_{C1-H1}= 158.0 Hz), 98.2 (C-1^D, J_{C1-H1}=176.1 Hz), 98.1, 97.2, 95.5, 78.5, 78.1, 77.8, 76.1, 75.0, 74.6, 74.5, 73.5, 72.7, 70.9, 70.3, 69.2, 68.9, 68.5, 68.4, 68.2, 67.5, 66.6, 66.0, 62.9, 61.0, 56.5, 55.7, 54.1, 51.0, 28.6, 28.2, 23.0, 20.7, 20.6, 20.5, 20.4 ppm; HRMS: m/z: calcd for $C_{113}H_{116}N_6O_{35}Na$: 2140.7412 $[M+Na]^+$; found: 2140.7908.

5-Azidopentyl *O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl]-(1→3)-*O*-(2-*O*-Benzyl-β-D-mannopyranosyl)-(1→4)-*O*-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-*O*-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (39): *p*-Toluenesulfonic acid monohydrate (23 mg, 0.12 mmol) was added to a stirred solution of compound **38** (100 mg, 47.2 μmol) in CH_3CN (2 mL) at room temperature. After 3 h, the reaction mixture was neutralized with pyridine and evaporated. The residue was diluted with CH_2Cl_2, washed with 1 M HCl and $KHCO_3$, dried over anhydrous $MgSO_4$, filtered and evaporated. The crude product was purified by flash column chromatography (hexane/EtOAc 1:1) to afford the title compound as a transparent oil (77 mg, 80%). ^{1}H NMR (500 MHz, CDCl$_3$): δ=7.90–7.63 (m, 12H; Ar), 7.52 (m, 2H; Ar), 7.40–7.21 (m, 14H; Ar), 7.01 (m, 2H; Ar), 6.91–6.85 (m, 3H; Ar), 6.81–6.79 (m, 4H; Ar), 5.62 (dd, J=9.4, 10.6 Hz, 1H; H-3^E), 5.28 (d, J=8.3 Hz, 1H; H-1^A), 5.20 (s, 1H; H-1^D), 5.12 (d, J=8.3 Hz, 1H; H-1^E), 5.08 (dd, J=3.0, 9.8 Hz, 1H; H-4^D), 5.61–4.98 (m, 4H; H-4^E, CH_aH$_{a'}$Ar, C$H_{2'}$Ar), 4.95 (d, J=7.6 Hz, 1H; H-1^B), 4.89 (m, 2H; CH_bH$_{b'}$Ar, CH_cH$_{c'}$Ar), 4.69 (d, J=12.4 Hz, 1H; CH$_a$$H_{a'}$Ar), 4.62–4.47 (m, 4H; CH$_{2'}$Ar, CH$_b$$H_{b'}$Ar, H-1^C), 4.34–4.26 (m, 2H; H-2^E, CH$_c$$H_{c'}$Ar), 4.23–4.11 (m, 8H; H-2^A, H-2^D, H-2^B, H-3 ^{A}, H-3^B, H-3^D, H-4^B, H-6$_a^E$), 4.05 (t, J=9.9 Hz, 1H; H-4^A), 3.99 (t, J=9.9 Hz, 1H; H-4^C), 3.92 (m, 1H; H-6$_b^E$), 3.81–3.67 (m, 5H; H-6$_a^C$, H-6$_a^D$, H-2^C, H-6$_a^A$, OCHH(CH$_2$)$_4$N$_3$), 3.61–3.52 (m, 4H; H-6$_b^C$, H-6$_a^B$, H-6$_b^D$, H-6$_b^A$), 3.45–3.41 (m, 2H; H-3^C, H-6$_b^B$), 3.34 (m, 2H; H-5^B, H-5^D), 3.26 (m, 2H; H-5^A, OCHH(CH$_2$)$_4$N$_3$), 3.08 (m, 1H; H-5^C), 2.93–2.85 (3H, m; H-5^E, O(CH$_2$)$_4$CH_2N$_3$), 2.11, 2.06, 2.03, 2.00, 1.88, 1.86 (s, 18H; 6×OCH$_3$), 1.40–1.27 (m, 4H; 2×CH$_2$), 1.12–1.06 ppm (m, 2H; CH$_2$); ^{13}C NMR (126 MHz, CDCl$_3$): δ=170.7, 170.6, 170.1, 170.0, 169.3, 169.2, 168.4, 167.8, 167.7, 167.6, 138.7, 138.5, 137.7, 134.2, 134.1, 133.8, 133.6, 132.5, 131.7, 131.6, 131.5, 131.4, 130.9, 128.8, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.6, 127.3, 127.2, 127.0, 126.9, 126.6, 123.6, 123.5, 123.1, 123.0, 100.8 (C-1^C, J_{C1-H1}=158.5 Hz), 98.1, 97.0, 96.4 (C-1^A, C-1^B , C-1^E), 95.7 (C-1^D, J_{C1-H1}=170.2 Hz), 81.3, 78.6, 76.0, 75.5, 74.5, 74.5, 74.4, 74.3, 73.4, 72.7, 71.3, 70.2, 69.5, 68.9, 68.7, 68.6, 68.2, 67.5, 65.8, 65.5, 62.9, 62.5, 61.6, 60.4, 56.5, 55.7, 54.2, 51.0, 30.3, 28.9, 28.6, 28.3, 23.7, 23.0, 20.8, 20.7, 20.6, 20.5 ppm; HRMS: m/z: calcd for $C_{106}H_{112}N_6O_{35}Na$: 2051.7065 $[M+Na]^+$; found: 2051.5115.

5-Azidopentyl *O*-[3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)]-*O*-{(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→3)-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-3,6-di-*O*-benzyl-α-D-mannopyranoside-(1→6)}-*O*-(2-*O*-benzyl-β-D-mannopyranosyl)-(1→4)-*O*-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-*O*-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (42): A mixture of donor **18** (79.5 mg, 56.7 μmol) with activated 4 Å molecular sieves in dry CH_3CN (0.4 mL) was stirred at room temperature for 20 min. The resulting mixture was cooled to 15 °C, tris(4-bromophenyl)ammoniumyl hexachloroantimonate (TBAP; 88.5 mg, 108.4 μmol) was added followed by a solution of acceptor **39** (58 mg, 28.3 μmol) in CH_3CN (0.3 mL). The reaction mixture was stirred at 15 °C for 40 min and then a second portion of TBAP (21 mg, 26.4 μmol) was added. The reaction mixture was allowed to warm to room temperature and was then stirred overnight. The solvents were evaporated and the crude product was purified by flash column chromatography (hexane/EtOAc 1:1 to 1:2) to give the title compound as a white solid (48 mg, 51%). R_f=0.11 (hexane/EtOAc 1:1); $[\alpha]_D^{20}=+17.8$ (c=1.21 in $CHCl_3$); ^{1}H NMR (500 MHz, CDCl$_3$): δ=7.89–6.59 (m, 137H), 5.72 (dd, J=10.7, 9.1 Hz, 1H), 5.54–5.45 (m, 2H), 5.36 (d, J=8.5 Hz, 1H), 5.25 (d, J=7.8 Hz, 1H), 5.20–5.05 (m, 4H), 5.03–4.77 (m, 9H), 4.76–4.35 (m, 18H), 4.33–4.07 (m, 8H), 4.06–3.78 (m, 13H), 3.76–3.36 (m, 14H), 3.35–3.20 (m, 4H), 3.10 (dt, J=9.4, 3.4 Hz, 1H), 2.97–2.81 (m, 2H), 2.10 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 1.92 (s, 3H), 1.87 (s, 3H), 1.42–1.26 (m, 4H), 1.13–1.03 ppm (m, 2H); ^{13}C NMR (126 MHz, CDCl$_3$): δ=170.69, 170.39, 170.34, 170.21, 170.07, 170.04, 169.34, 169.18, 168.22, 167.39, 138.73, 138.63, 138.52, 138.41, 138.25, 138.09, 137.91, 137.74, 134.20, 133.58, 131.40, 129.73, 128.98, 128.86, 128.59, 128.36, 128.30, 128.26, 128.21, 128.14, 128.09, 128.01, 127.76, 127.65, 127.58, 127.52, 127.49, 127.39, 127.31, 127.25, 127.06, 126.84, 123.47, 123.10, 101.60 (C-1, J_{C1-H1}= 158.3 Hz), 99.54 (C-1, J_{C1-H1}=170.9 Hz), 98.31 (C-1, J_{C1-H1}=171.9 Hz), 98.08 (C-1, J_{C1-H1}=172.4 Hz), 98.02 (C-1), 97.09 (2×C-1, J_{C1-H1}= 170.1 Hz), 96.71 (C-1), 79.51, 78.19, 78.07, 77.52, 76.01, 75.04, 74.89, 74.73, 74.61, 74.52, 74.45, 74.37, 74.19, 74.11, 74.05, 73.43, 73.30, 73.25, 73.17, 72.61, 72.18, 72.01, 71.74, 71.53, 71.45, 71.11, 70.55, 69.72, 68.83, 68.74, 68.65, 68.30, 68.15, 66.17, 65.86, 62.68, 61.64, 56.49, 55.70, 54.38, 51.06, 28.64, 28.23, 22.96, 21.08, 21.00, 20.75, 20.67, 20.58, 20.47, 20.43 ppm; HRMS (ESI): m/z: calcd for $C_{184}H_{192}N_6O_{52}Na_2$: 1683.6292 $[M+2Na]^{+2}$; found: 1683.6140.

5-Aminopentyl [2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl]-(1→3),[di-(α-D-mannopyranosyl)-(1→3),(1→6)-α-D-mannopyranosyl](1→6)-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (46): A mixture of compound **42** (48 mg, 14.4 μmol) and ethylenediamine (1.2 mL) in *n*BuOH (4.8 mL) was heated at 120 °C under microwave irradiation for 3 cycles of 30 min each. The reaction mixture was concentrated and co-evaporated with toluene (3×) and EtOH (3×). The crude product was dissolved in anhydrous pyridine (5 mL), cooled to 0 °C and Ac_2O (2.5 mL) was added dropwise. The reaction was warmed to room temperature and stirred overnight. The mixture was concentrated and co-evaporated with toluene (3×). The crude product was purified by flash column chromatography (SiO_2, EtOAc) to give the title compound as a transparent oil (35 mg, 78 % over two steps). Sodium (12 mg) was added to liquid ammonia (15 mL) at −78 °C and the resulting blue mixture was stirred for 5 min. A solution of the protected product (35 mg, 11.3 μmol) in THF (2 mL) was added dropwise and the resulting mixture was stirred at −78 °C until the blue colour disappeared (10 min). The reaction was quenched by adding solid NH_4Cl until a neutral pH was reached and was then evaporated to dryness. The crude product was purified by size-exclusion column chromatography (Biorad P2 gel, aqueous 40 mM NH_4HCO_3). The fraction was lyophilized to give the title compound as a white solid (13 mg, 75 %). ^{1}H NMR (500 MHz, D_2O): $\delta = 5.05$ (brs, 1H; H-1), 5.02 (brs, 1H; H-1), 4.84 (d, $J = 1.1$ Hz, 1H; H-1), 4.80 (d, $J = 1.3$ Hz, 1H; H-1), 4.71 (brs, 1H; H-1), 4.52 (dd, $J = 4.2$, 3.5 Hz, 1H; H-1), 4.49 (d, $J = 8.4$ Hz, 1H; H-1), 4.42 (d, $J = 7.6$ Hz, 1H; H-1), 4.18 (s, 1H), 4.13 (s, 1H), 4.08 (s, 1H), 4.00 (s, 1H), 3.95–3.36 (m, 41H), 2.91 (t, $J = 7.7$ Hz, 2H; CH_2 linker), 2.00 (s, 3H; $NHCOCH_3$), 1.98 (s, 3H; $NHCOCH_3$), 1.96 (s, 3H; $NHCOCH_3$), 1.66–1.43 (m, 4H; 2×CH_3 linker), 1.38–1.29 (m, 2H; CH_3 linker); ^{13}C NMR from HSQC experiment (500 MHz, D_2O): $\delta = 102.21$ (C-1, $J_{C1\text{-}H1} = 171.5$ Hz), 101.33 (C-1), 100.98 (C-1), 100.24 (C-1, $J_{C1\text{-}H1} = 160.0$ Hz), 99.66 (C-1, $J_{C1\text{-}H1} = 171.5$ Hz), 99.52 (C-1), 99.47(C-1, $J_{C1\text{-}H1} = 170.2$ Hz), 99.18 (C-1, $J_{C1\text{-}H1} = 171.9$ Hz), 80.32, 79.61, 79.50, 79.24, 79.22, 78.55, 76.28, 75.74, 74.49, 74.35, 73.29, 73.19, 72.47, 71.78, 70.58, 70.06, 69.97, 69.82, 69.80, 69.30, 67.18, 66.61, 65.75, 65.55, 65.41, 65.16, 65.13, 64.97, 61.65, 60.83, 59.96, 54.97, 54.73, 39.20, 27.86, 26.31, 22.17, 21.95 ppm; HRMS (ESI): *m/z*: calcd for $C_{59}H_{102}N_4O_{41}$: 1522.610 $[M+H]^+$; found: 1523.622.

Enzymatic on-chip galactosylation with β-1,4-galactosyl transferase. (well-format): The microarrays were separated into wells containing single subarrays by a 16-well gasket (Fast Frame incubation chambers from Whatman). A solution (100 μL) containing bovine β-1,4-galactosyltransferase (5 mU), UDP-Gal (800 μM), $MnCl_2$ (5 mM), BSA (0.02 %) and alkaline phosphatase (16 mU) in HEPES buffer (50 mM, pH 7.4) was added to each well and the slide was incubated at 37 °C for 48 h and with 70 % humidity. The slide was then sonicated in SDS solution (35 mM in PBS buffer, 40 °C) to reduce background fluorescence, washed (10 min with PBST; 2×10 min with PBS and 2×10 min with water) and dried under a stream of argon. Conversion of the acceptors to the galactosylated species was confirmed by incubation of one subarray with labelled *Ricinus communis* agglutinin (Cy3-RCA, 50 nM in PBS, 0.5 % Tween-20) and another with a solution of tagged *Griffonia (Bandeiraea) simplicifolia* lectin (Cy5-BSL-II, 50 nM in PBS, 0.5 % Tween-20) for 1 h at 25 °C. The slide was washed with PBST, PBS and water, dried and analyzed in a fluorescence scanner.

Enzymatic on-chip sialylation with α-2,6-sialyl transferase. (well-format): For sialylation of terminal LacNAc functions, a solution (100 μL) containing human α-2,6-Sialyltransferase (2 mU), CMP-Neu5Ac (200 μM), $MnCl_2$ (5 mM), BSA (0.02 %) and alkaline phosphatase (20 μU) in HEPES buffer (50 mM, pH 6.8) was added into individual wells and the slide incubated at 37 °C for 48 h and with 75 % humidity. The slide was washed as described in the galactosylation protocol. Sialylation was confirmed by incubation of one subarray with labelled *Sambucus nigra* agglutinin (Cy5-SNA, 50 nM in PBS, 0.5 % Tween-20) and another with a solution of tagged *Ricinus communis* agglutinin (Cy3-RCA, 50 nM in PBS, 0.5 % Tween-20) for 1 h at 25 °C. The slide was washed with PBST, PBS and water, dried and analyzed in a fluorescence scanner.

Enzymatic on-chip fucosylation with core α-1,3-fucosyl transferase. (well-format): A solution (150 μL) containing α-1,3-FucT-core type recombinant from *Arabidopsis thaliana* (3 μg), GDP-Fuc (500 μM), $MnCl_2$ (30 mM) and alkaline phosphatase (20 μU) in MES buffer (50 mM, pH 6.5) was added into each well and the slide was placed into a humid chamber at 30 °C for 16 h. The slide was washed with the above-mentioned protocol previous to examination. To examine successful fucosylation, one well after incubation with Fuc-T was treated with a 100 nM solution labelled Cy3-AAL (*Aleuria aurantia* lectin, core GlcNAc α-1,3-Fuc recognition) in PBS containing 0.5 % Tween-20 for 1 h in an incubation chamber at 25 °C. The slide was washed with PBS and water and analyzed.

Spotwise elongation with β-1,4-Galactosyltransferase: Nanoliter volumes (2.0–5.0 nL) from a solution (100 μL) containing β-1,4-GalT (4 mU), UDP-Gal (800 μM), $MnCl_2$ (5 mM), BSA (0.02 %) and alkaline phosphatase (160 μU) in HEPES buffer (50 mM, pH 7.4, 30 % glycerol) were spotted onto selected ligands of the glycan array by a programmed piezoelectric printing robot. With this buffer the average diameter of the microdroplets measured by brightfield microscopy was 300 μm (3.3 nL volume). The slide was incubated (48 h, 37 °C, 75 % humidity) then sonicated in a SDS solution (35 mM in PBS buffer, 40 °C) to remove reagents and proteins that could interfere in the fluorescence assay. After washing with PBST (10 min ×1), PBS (10 min ×2) and water (10 min ×2) the slide was dried under a stream of argon and was ready for use. The successful galactosylation was confirmed by probing one subarray of the slide with a solution of Cy3-RCA and Cy5-BSL-II (both at 50 nM in PBS, 0.1 % Tween-20) for 1 h in an incubation chamber at 25 °C. The slide was washed with PBST, PBS and water, dried under a stream of argon and analyzed in a fluorescence scanner.

Spotwise elongation with α-2,6-sialyltransferase: Nanoliter volumes (2.0–5.0 nL) from a solution (100 μL) of human α-2,6-sialyltransferase (4 mU), CMP-Neu5Ac (1 mM), $MnCl_2$ (5 mM), BSA (0.02 %) and alkaline phosphatase (100 μU) in cacodylate•HCl buffer (50 mM, pH 6.0, 30 % glycerol) were spotted onto selected spots of the glycan array. After incubation (75 % humidity, 24 h, 37 °C), the slide was sonicated with a SDS solution (35 mM in PBS buffer) to reduce background fluorescence, washed (10 min with PBST; 2×10 min with PBS and 2×10 min with water) and dried under a stream of argon. A second cycle was performed to assure complete reaction. Sialylation was tested by treating one subarray of the slide in an incubation chamber with a solution of *Sambucus nigra* lectin and *Ricinus comunis* lectin (Cy5-SNA and Cy3-RCA both at 100 nM in PBS, 0.1 % Tween-20) for 1 h at 25 °C. The slide was washed with PBST, PBS and water, dried under a stream of argon and analyzed in a fluorescence scanner.

Additionally a second sialylated subarray was incubated with biotinylated ECA (100 nM in HEPES 20 mM. pH 7.7) under the same conditions as described above, washed as before with PBST, PBS, water and incubated with tagged Cy3-neutravidine (30 nM PBS, 0.5 % Tween) for 1 h, and then washed again as before, dried and analyzed with a fluorescence scanner.

Spotwise elongation with α-1,3-fucosyltransferase: For core fucosylation of *N*-glycans. nanoliter volumes (2.0–5.0 nL) from a solution (100 μL) containing a α-1,3-fucosyltransferase (3 μg) from *Arabidopsis thaliana,* GDP-Fuc (1 mM), $MnCl_2$ (30 mM) and alkaline phosphatase (100 μU) in MES buffer (50 mM, pH 6.5, 30 % glycerol), were spotted onto selected spots of the glycan array by a printing robot. After incubation (75 % humidity, 24 h, 30 °C), the slide was sonicated with a solution containing SDS (35 mM in PBS buffer, 40 °C) to reduce background fluorescence, washed (10 min with PBST; 2×10 min with PBS and 2×10 min with water) and dried under a stream of argon. To examine fucosylation, one subarray of the slide was incubated with a solution of labelled *Aleuria aurantia* lectin (Cy3-AAL, 100 nM in PBS, 0.1 % Tween-20) at 25 °C. The slide was washed with PBST, PBS and water, dried under a stream of argon and analyzed in a fluorescence scanner.

Acknowledgements

We are grateful to Prof. I. B. H. Wilson, BOKU, Vienna for providing the clones and assisting in the expression of a recombinant α-1,3-fucosyltransferase from *Arabidopsis thaliana.* We thank D. Padró for help with NMR spectroscopic experiments and C. Márquez and J. Calvo (MDRenal Lifesciences) for acquisition of mass spectra. Funding from Ministerio de Ciencia e Innovación, CTQ2008–04444/BQA grant, Government of the Basque Country, Etortek grant, and the European Union, RTN-Euroglycoarrays grant are gratefully acknowledged.

[1] a) S. Park, M. R. Lee, I. Shin, *Chem. Commun.* **2008**, 4389–4399; b) T. Horlacher, P. H. Seeberger, *Chem. Soc. Rev.* **2008**, *37*, 1414–1422; c) Y. Liu, A. S. Palma, T. Feizi, *Biol. Chem.* **2009**, *390*, 647–656; d) N. Laurent, J. Voglmeir, S. L. Flitsch, *Chem. Commun.* **2008**, 4400–4412; e) P. H. Liang, C. Y. Wu, W. A. Greenberg, C. H. Wong, *Curr. Opin. Chem. Biol.* **2008**, *12*, 86–92.

[2] R. Apweiler, H. Hermjakob, N. Sharon, *Biochim. Biophys. Acta, Gen. Subj.* **1999**, *1473*, 4–8.

[3] a) O. Blixt, S. Head, T. Mondala, C. Scanlan, M. E. Huflejt, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C. H. Wong, J. C. Paulson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17033–17038; b) T. Schallus, C. Jaeckh, K. Feher, A. S. Palma, Y. Liu, J. C. Simpson, M. Mackeen, G. Stier, T. J. Gibson, T. Feizi, T. Pieler, C. Muhle-Goll, *Mol. Biol. Cell* **2008**, *19*, 3404–3414; c) X. Z. Song, B. Y. Xia, S. R. Stowell, Y. Lasanajak, D. F. Smith, R. D. Cummings, *Chem. Biol.* **2009**, *16*, 36–47; d) A. R. de Boer, C. H. Hokke, A. M. Deelder, M. Wuhrer, *Anal. Chem.* **2007**, *79*, 8107–8113; e) X. Song, Y. Lasanajak, C. Rivera-Marrero, A. Luyai, M. Willard, D. F. Smith, R. D. Cummings, *Anal. Biochem.* **2009**, *395*, 151–160.

[4] a) C. C. Wang, Y. L. Huang, C. T. Ren, C. W. Lin, J. T. Hung, J. C. Yu, A. L. Yu, C. Y. Wu, C. H. Wong, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 11661–11666; b) F. Kamena, M. Tamborrini, X. Liu, Y.-U. Kwon, F. Thompson, G. Pluschke, P. H. Seeberger, *Nat. Chem. Biol.* **2008**, *4*, 238–240; c) S. E. Tully, M. Rawat, L. C. Hsieh-Wilson, *J. Am. Chem. Soc.* **2006**, *128*, 7740–7741.

[5] A. Helenius, M. Aebi, *Annu. Rev. Biochem.* **2004**, *73*, 1019–1049.

[6] E. W. Adams, D. M. Ratner, H. R. Bokesch, J. B. McMahon, B. R. O'Keefe, P. H. Seeberger, *Chem. Biol.* **2004**, *11*, 875–881.

[7] a) J. Stevens, O. Blixt, T. M. Tumpey, J. K. Taubenberger, J. C. Paulson, I. A. Wilson, *Science* **2006**, *312*, 404–410; b) J. Stevens, O. Blixt, L. Glaser, J. K. Taubenberger, P. Palese, J. C. Paulson, I. A. Wilson, *J. Mol. Biol.* **2006**, *355*, 1143–1155.

[8] M. A. Wälti, P. J. Walser, S. Thore, A. Grünler, M. Bednar, M. Künzler, M. Aebi, *J. Mol. Biol.* **2008**, *379*, 146–159.

[9] K. Paschinger, E. Staudacher, U. Stemmer, G. Fabini, I. B. H. Wilson, *Glycobiology* **2004**, *15*, 463–474.

[10] T. Ogawa, M. Sugimoto, T. Kitajima, K. K. Sadozai, T. Nukada, *Tetrahedron Lett.* **1986**, *27*, 5739–5742.

[11] a) H. Paulsen, M. Heume, H. Nurnberger, *Carbohydr. Res.* **1990**, *200*, 127–166; b) H. Paulsen, M. Heume, Z. Gyorgydeak, R. Lebuhn, *Carbohydr. Res.* **1985**, *144*, 57–70; c) H. Paulsen, *Chem. Soc. Rev.* **1984**, *13*, 15–45.

[12] a) C. Unverzagt, S. Eller, S. Mezzato, R. Schuberth, *Chem. Eur. J.* **2008**, *14*, 1304–1311; b) S. Eller, R. Schuberth, G. Gundel, J. Seifert, C. Unverzagt, *Angew. Chem.* **2007**, *119*, 4251–4253; *Angew. Chem. Int. Ed.* **2007**, *46*, 4173–4175; c) C. Unverzagt, *Angew. Chem.* **1997**, *109*, 2078–2081; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1989–1992; d) H. Weiss, C. Unverzagt, *Angew. Chem.* **2003**, *115*, 4389–4392; *Angew. Chem. Int. Ed.* **2003**, *42*, 4261–4263; e) C. Unverzagt, *Carbohydr. Res.* **1997**, *305*, 423–431.

[13] a) B. Wu, Z. H. Hua, J. D. Warren, K. Ranganathan, Q. Wan, G. Chen, Z. P. Tan, J. H. Chen, A. Endo, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 5577–5579; b) M. Mandal, V. Y. Dudkin, X. Geng, S. J. Danishefsky, *Angew. Chem.* **2004**, *116*, 2611–2615; *Angew. Chem. Int. Ed.* **2004**, *43*, 2557–2561; c) V. Y. Dudkin, J. S. Miller, A. S. Dudkina, C. Antczak, D. A. Scheinberg, S. J. Danishefsky, *J. Am. Chem. Soc.* **2008**, *130*, 13598–13607.

[14] a) Y. Ohnishi, H. Ando, T. Kawai, Y. Nakahara, Y. Ito, *Carbohydr. Res.* **2000**, *328*, 263–276;b) I. Matsuo, T. Kashiwagi, K. Totani, Y. Ito, *Tetrahedron Lett.* **2005**, *46*, 4197–4200; c) I. Matsuo, Y. Ito, *Trends Glycosci. Glycotechnol.* **2005**, *17*, 85–95.

[15] a) R. Frank, *Tetrahedron* **1992**, *48*, 9217–9232;b) S. P. A. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, D. Solas, *Science* **1991**, *251*, 767–773.

[16] L. Ban, M. Mrksich, *Angew. Chem.* **2008**, *120*, 3444–3447; *Angew. Chem. Int. Ed.* **2008**, *47*, 3396–3399.

[17] M. Schuster, P. Wang, J. C. Paulson, C. H. Wong, *J. Am. Chem. Soc.* **1994**, *116*, 1135–1136.

[18] O. Blixt, T. Norberg, *J. Carbohydr. Chem.* **1997**, *16*, 143–154.

[19] N. Laurent, R. Haddoub, S. L. Flitsch, *Trends Biotechnol.* **2008**, *26*, 328–337.

[20] O. Blixt, K. Allin, O. Bohorov, X. F. Liu, H. Andersson-Sand, J. Hoffmann, N. Razi, *Glycoconjugate J.* **2008**, *25*, 59–68.

[21] S. Park, I. Shin, *Org. Lett.* **2007**, *9*, 1675–1678.

[22] a) N. Laurent, J. Voglmeir, A. Wright, J. Blackburn, N. T. Pham, S. C. C. Wong, S. J. Gaskell, S. L. Flitsch, *ChemBioChem* **2008**, *9*, 883–887; b) M. C. Bryan, F. Fazio, H. K. Lee, C. Y. Huang, A. Chang, M. D. Best, D. A. Calarese, C. Blixt, J. C. Paulson, D. Burton, I. A. Wilson, C. H. Wong, *J. Am. Chem. Soc.* **2004**, *126*, 8640–8641.

[23] N. Nagahori, S. I. Nishimura, *Chem. Eur. J.* **2006**, *12*, 6478–6485.

[24] H. Schachter, in *Comprehensive Glycoscience* (Ed.: J. P. Kamerling), Elsevier, Oxford, **2007**, pp. 11–32.

[25] a) D. Crich, P. Jayalath, T. K. Hutton, *J. Org. Chem.* **2006**, *71*, 3064–3070; b) D. Crich, S. Sun, *J. Am. Chem. Soc.* **1998**, *120*, 435–436.

[26] S. H. Kim, D. Augeri, D. Yang, D. Kahne, *J. Am. Chem. Soc.* **1994**, *116*, 1766–1775.

[27] S. Serna, B. Kardak, N. C. Reichardt, M. Martin-Lomas, *Tetrahedron: Asymmetry* **2009**, *20*, 851–856.

[28] F. Yamazaki, S. Sato, T. Nukada, Y. Ito, T. Ogawa, *Carbohydr. Res.* **1990**, *201*, 31–50.

[29] a) B. G. Davis, A. J. Fairbanks, N.-C. Reichardt, V. U. Angardiparambil (Glycoform Ltd., GB), WO2007/091040A2, **2007**; b) B. Yu, H. C. Tao, *Tetrahedron Lett.* **2001**, *42*, 2405–2407.

[30] R. Kornfeld, S. Kornfeld, *Annu. Rev. Biochem.* **1985**, *54*, 631–664.

[31] C. Unverzagt, *Angew. Chem.* **1994**, *106*, 1170–1173; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1102–1104.

[32] A. Marra, J. M. Mallet, C. Amatore, P. Sinay, *Synlett* **1990**, 572–574.

[33] Cy3 and Cy5 are used as descriptors for the scanner channels operated in the imaging of microarray slides. See the Supporting Information for the cyanine-analogue fluorophores used for tagging the lectins.

[34] a) S. Park, M. R. Lee, S. J. Pyo, I. Shin, *J. Am. Chem. Soc.* **2004**, *126*, 4812–4819; b) J. Su, M. Mrksich, *Angew. Chem.* **2002**, *114*, 4909–4912; *Angew. Chem. Int. Ed.* **2002**, *41*, 4715–4718.

[35] N. Shibuya, I. J. Goldstein, W. F. Broekaert, M. Nsimba-Lubaki, B. Peeters, W. J. Peumans, *J. Biol. Chem.* **1987**, *262*, 1596–1601.

[36] K. Yamamoto, T. Tsuji, I. Matsumoto, T. Osawa, *Biochemistry* **1981**, *20*, 5894–5899.

[37] S. K. Podder, A. Surolia, Bachhawa. Bk, *Eur. J. Biochem.* **1974**, *44*, 151–160.

[38] D. H. Joziasse, W. E. Schiphorst, D. H. van den Eijnden, J. A. van Kuik, H. van Halbeek, J. F. Vliegenthart, *J. Biol. Chem.* **1985**, *260*, 714–719.

[39] a) T. Iskratsch, A. Braun, K. Paschinger, I. B. H. Wilson, *Anal. Biochem.* **2009**, *386*, 133–146; b) Y. Itakura, S. Nakamura-Tsuruta, J. Kominami, N. Sharon, K.-I. Kasai, J. Hirabayashi, *J. Biochem.* **2007**, *142*, 459–469.

[40] C. Jin, B. Hantusch, W. Hemmer, J. Stadimann, F. Altmann, *J. Allergy Clin. Immunol.* **2008**, *121*, 185–190.

Received: May 13, 2010
Published online: September 28, 2010

