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# Chemoenzymatic Approach for the Preparation of Asymmetric Bi-, Tri-, and Tetra-Antennary *N*-Glycans from a Common Precursor

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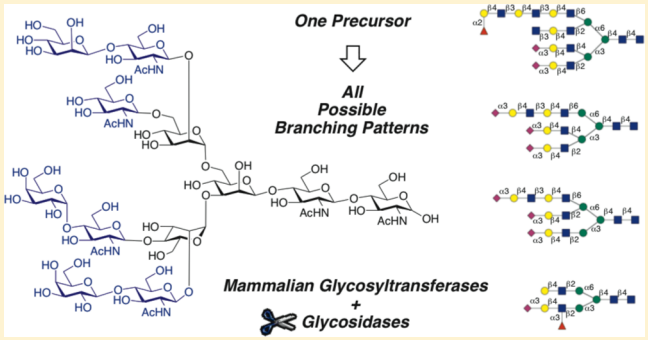
Supporting Information

**ABSTRACT:** Progress in glycoscience is hampered by a lack of well-defined complex oligosaccharide standards that are needed to fabricate the next generation of microarrays, to develop analytical protocols to determine exact structures of isolated glycans, and to elucidate pathways of glycan biosynthesis. We describe here a chemoenzymatic methodology that makes it possible, for the first time, to prepare any bi-, tri-, and tetra-antennary asymmetric *N*-glycan from a single precursor. It is based on the chemical synthesis of a tetra-antennary glycan that has *N*-acetylglucosamine (GlcNAc), *N*-acetyllactosamine (LacNAc), and unnatural Gal$\alpha$(1,4)-GlcNAc and Man$\beta$(1,4)-GlcNAc appendages. Mammalian glycosyltransferases recognize only the terminal LacNAc moiety as a substrate, and thus this structure can be uniquely extended. Next, the $\beta$-GlcNAc terminating antenna can be converted into LacNAc by galactosylation and can then be enzymatically modified into a complex structure. The unnatural $\alpha$-Gal and $\beta$-Man terminating antennae can sequentially be decaged by an appropriate glycosidase to liberate a terminal $\beta$-GlcNAc moiety, which can be converted into LacNAc and then elaborated by a panel of glycosyltransferases. Asymmetric bi- and triantennary glycans could be obtained by removal of a terminal $\beta$-GlcNAc moiety by treatment with $\beta$-*N*-acetylglucosaminidase and selective extension of the other arms. The power of the methodology is demonstrated by the preparation of an asymmetric tetra-antennary *N*-glycan found in human breast carcinoma tissue, which represents the most complex *N*-glycan ever synthesized. Multistage mass spectrometry of the two isomeric triantennary glycans uncovered unique fragment ions that will facilitate identification of exact structures of glycans in biological samples.




## INTRODUCTION

Of all post-translational modifications of proteins, complex *N*-linked glycans are the most prominent in terms of complexity and diversity.[1] All eukaryotic *N*-linked glycans have a common pentasaccharide core that can be modified by various *N*-acetyllactosamine ($\beta$-Gal(1,4)GlcNAc, LacNAc) moieties, which in turn can be elaborated by glycosyltransferases to give highly complex branched structures.[2] The structural diversity of *N*-glycans arises from the degrees and patterns of branching, various numbers of LacNAc repeating units, and further elaborations such as sialylation and fucosylation.[3] Structural studies have shown that the *N*-glycans are usually asymmetrically substituted having a unique appendage at each branching point. Variations in *N*-glycan structures occur during embryogenesis, cell activation, morphogenesis, cell cycle entry, and during oncogenesis.[4] These differences are largely based on cell-specific expressions of collection of glycosyltransferases. Furthermore, the degree of branching of *N*-glycans appears to be sensitive to the metabolic flux,[5] and it has been proposed that changes in branching cooperate with the regulation of cell proliferation and differentiation.[6]

Diverse libraries of well-defined complex glycans are urgently needed for the fabrication of the next generation of microarrays,[7] as standards for the development of analytical protocols to determine exact structures and quantities of isolated glycans, and for the elucidation of pathways of glycoconjugate biosynthesis. Often, well-defined oligosacchar-

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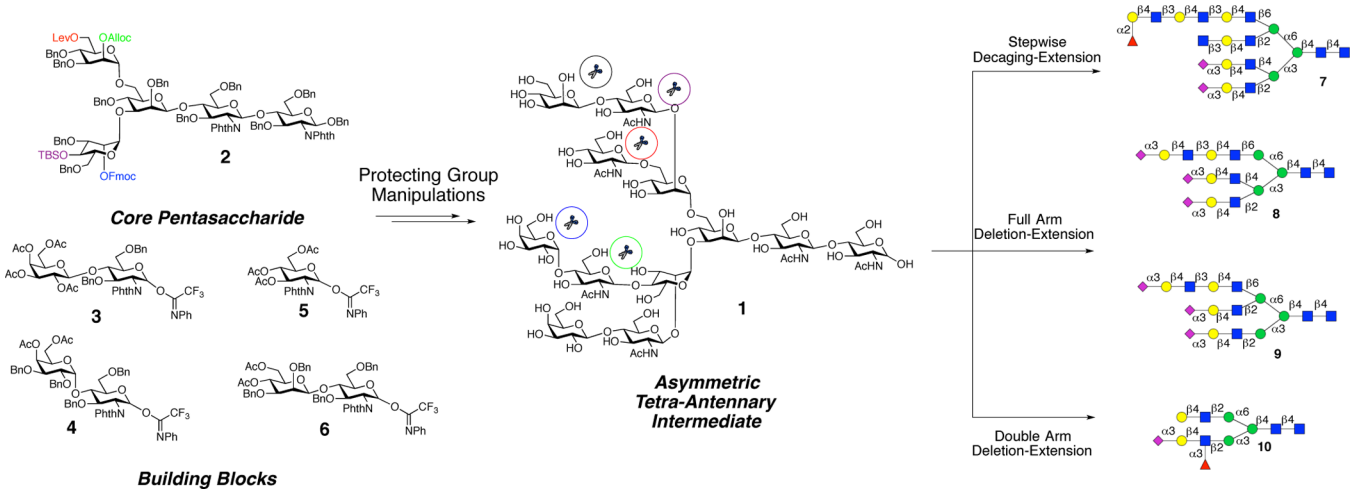
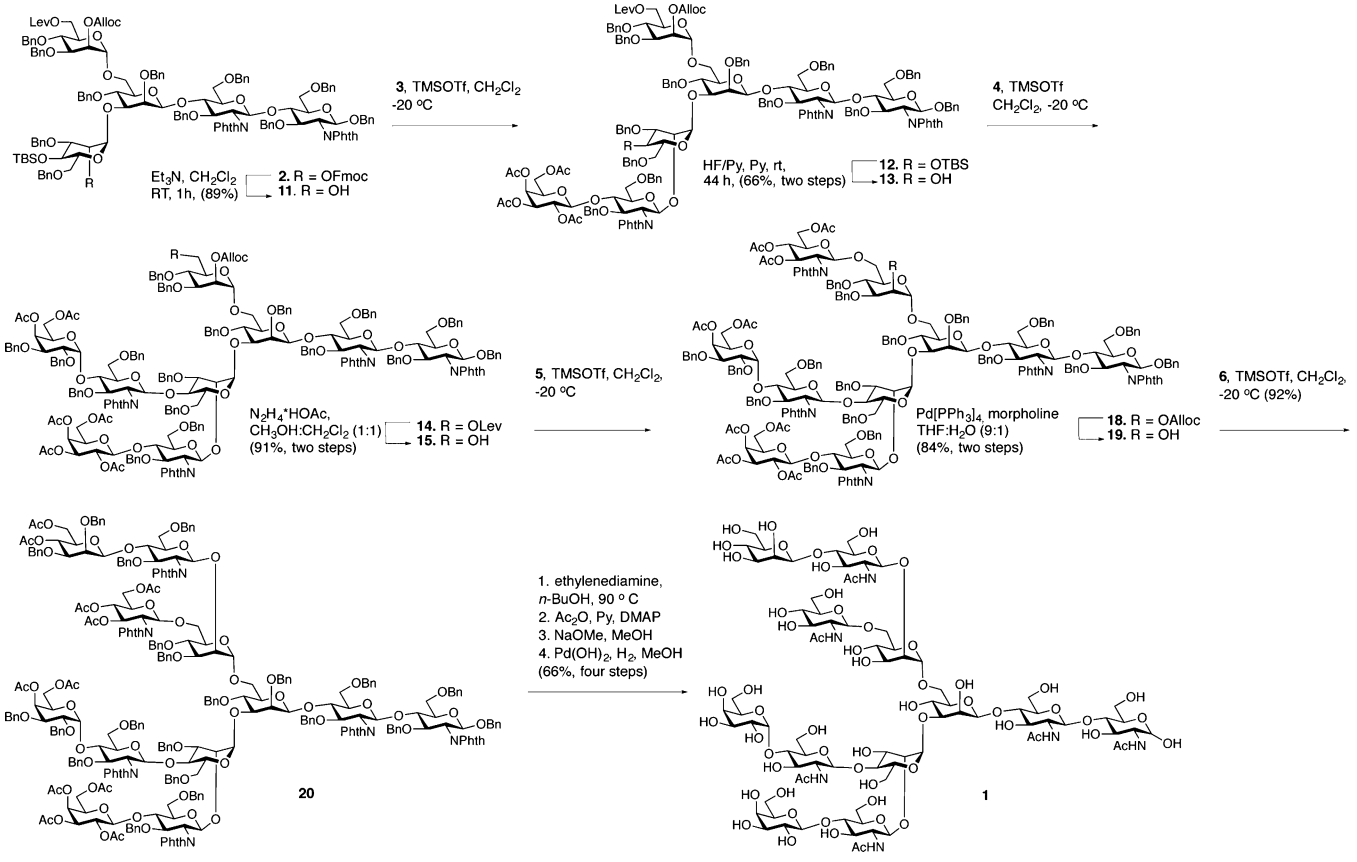



**Figure 1.** Asymmetric bi-, tri, and tetra-antennary *N*-glycans from common intermediate **1**.

**Scheme 1. Chemical Assembly of Common Intermediate 1**




ides can only be obtained by chemical or enzymatic approaches.[7,8] Despite that most complex *N*-glycans have asymmetrical architectures, synthetic efforts have almost exclusively focused on the preparation of incomplete or symmetrically branched structures.[9]

Recently, we reported a chemoenzymatic approach that for the first time can provide libraries of complex asymmetrically branched *N*-glycans.[10] It exploits chemically synthesized multiantennary *N*-glycans that at each antenna can selectively be extended by a panel of glycosyltransferases. The latter was possible because specific antennae were temporarily rendered inactive for enzymatic extension by acetylation of a GlcNAc and LacNAc appendage. At an appropriate stage of synthesis, the esters can be removed to give substrates that can selectively be extended by appropriate glycosyltransferases. The methodology made it possible to prepare a triantennary *N*-glycan having different complex oligosaccharide extensions at each antenna. Others laboratories are adopting this methodology for the preparation of panels of complex *N*-glycans.[11]

Although the chemoenzymatic approach gave entry into *N*-glycans of unprecedented complexity, it requires a unique precursor for each type of branched oligosaccharide. Furthermore, we have observed that acetyl esters are prone to hydrolysis during multiple enzymatic extensions, and could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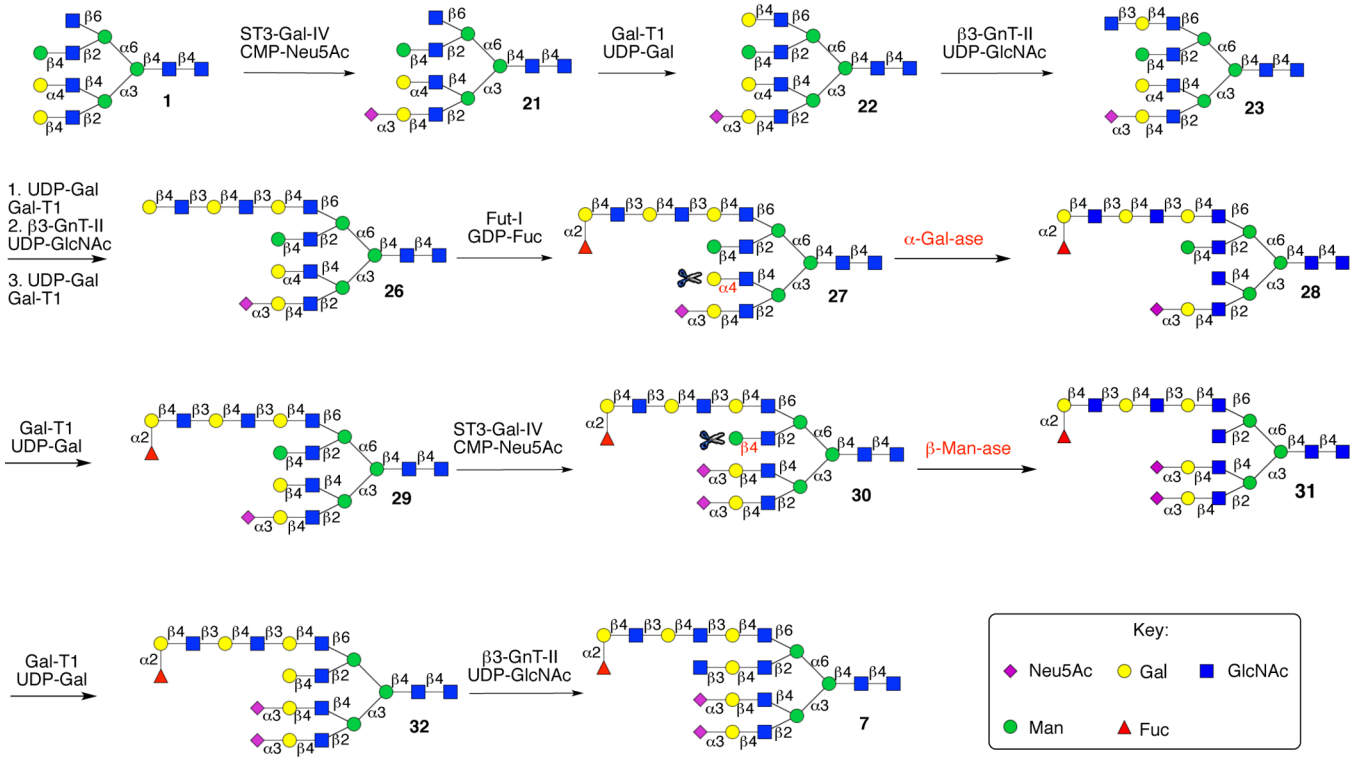
**Scheme 2. Synthesis of Asymmetrical Tetra-Antennary Glycan 7 from Precursor 1**




not serve as an appropriate protecting group when complex tetra-antennary *N*-glycans were targeted.[12] To address these difficulties, we describe here a more versatile chemoenzymatic methodology that makes it possible to prepare any bi-, tri-, and tetra-antennary asymmetric *N*-glycans from a single precursor (**1**) through enzymatic transformations (Figure 1). The universal precursor could be obtained by sequential removal of the orthogonal protecting groups of core pentasaccharide **2** and glycosylation with glycosyl donors **3**−**6**. The resulting tetra-antennary glycan has a terminal GlcNAc, LacNAc, and unnatural Gal-$\alpha$(1,4)-GlcNAc and Man$\beta$(1,4)-GlcNAc moiety. It was anticipated that relevant glycosyltransferases would only modify LacNAc and not the other terminal structures allowing selective extension of this arm. At an appropriate stage of the synthesis, the $\beta$-GlcNAc terminating antenna can be converted into LacNAc by galactosylation using Gal-T1, which can then enzymatically be extended into a complex structure. Next, the unnatural $\alpha$-Gal and $\beta$-Man terminating antennae can sequentially be decaged by an appropriate glycosidase[13] to liberate a terminal $\beta$-GlcNAc moiety, which can then be converted into LacNAc and elaborated by our panel of glycosyltransferases. It was also envisaged that asymmetric bi- and triantennary glycans could be obtained by removal of a terminal $\beta$-GlcNAc moiety by treatment with $\beta$-*N*-acetylglucosaminidase. To demonstrate the power of the methodology, we describe here the synthesis of one of the glycoforms of a tetra-antennary *N*-glycan that was observed in human ductal invasive breast carcinoma tissue and has the potential to serve as a biomarker.[14] It represents the most complex *N*-glycan ever synthesized by chemoenzymatic means. It is the expectation that the availability of such synthetic standard will allow quantitation of the biomarker in biological samples by mass spectrometry. To illustrate further the flexibility of the methodology, the common precursor **1** was employed to synthesize triantennary positional isomers **8** and **9** and a biantennary glycan **10**. Analysis of the new compounds by multistage mass spectrometry, including $MS^3$, showed unique fragmentation patterns of the isomeric glycans. This data will facilitate the assignment of positional isomers in complex biological samples.

## RESULTS AND DISCUSSION

**Chemical Synthesis of the Common Intermediate.** Core pentasaccharide **2**, which is modified by the orthogonal protecting groups levulinoyl (Lev), fluorenylmethyloxycarbonyl (Fmoc), allyloxycarbonyl (Alloc), and *t*-butyl-dimethylsilyl (TBS) at positions where branching points may occur, was prepared from appropriate monosaccharide building blocks (see Scheme S1 for synthesis of **2**). Furthermore, *N*-phenyl trifluoroacetimidate-based[15] glycosyl donors **3**−**6** were synthesized for the stepwise extension of **2** to give target compound **1** (see Scheme S4 for synthesis of **3**−**6**). Treatment of **2** with the non-nucleophilic base triethylamine resulted in the selective removal of the Fmoc protecting group without affecting the other base sensitive functionalities to give glycosyl acceptor **11** in an excellent yield of 89% (Scheme 1). Glycosylation of **11** with *N*-phenyl trifluoroacetimidate **3** in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) at −20 °C gave heptasaccharide **12**, which was immediately treated with HF/pyridine to remove TBS ether to give acceptor **13** in a 66% overall yield. A TMSOTf promoted glycosylation of **13** with donor **4** proceeded efficiently to give, after purification by Biogel SX-1 (toluene:acetone, 1:1, v:v), the nonasaccharide **14** in a yield of 91%. The latter glycosylation required a high concentration of donor and acceptor (>100 mM); otherwise, a significant quantity of the glycosyl acceptor was recovered resulting in a modest yield of product. It was also found that the order of protecting group removal and glycosylation was critical



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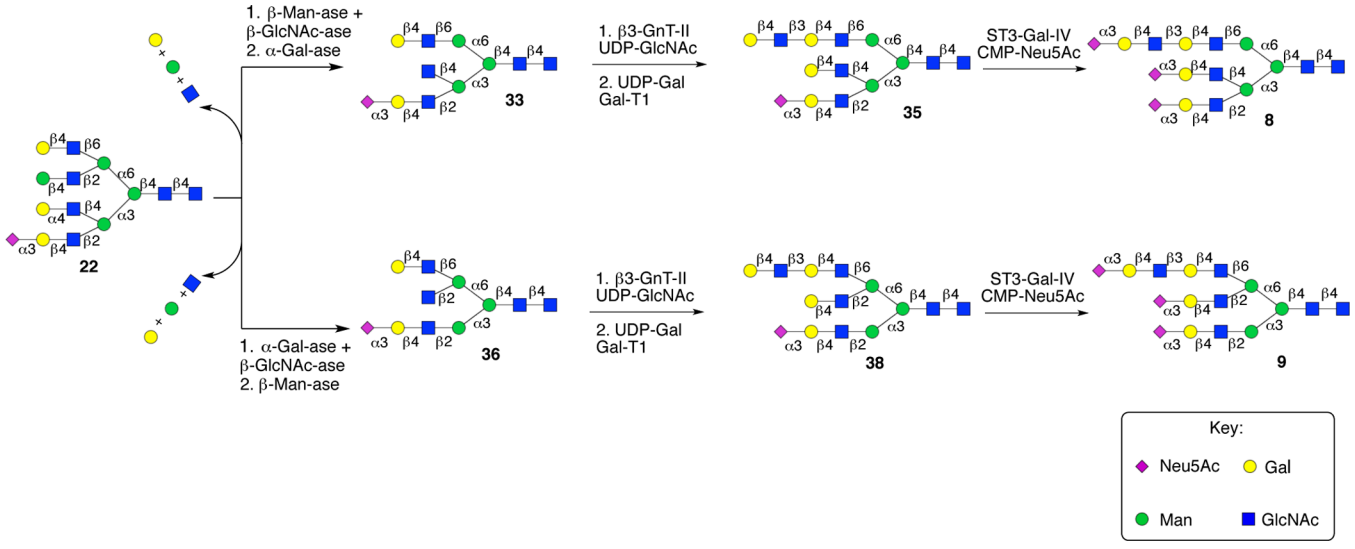
**Scheme 3. Preparation of Isomeric Tri-Antennary Glycans 8 and 9 from Common Precursor 22**




for the successful preparation of **14**. First removal of the TBS ether of **2** followed by glycosylation with **4** gave the corresponding heptasaccharide in a low yield of 14% (see Scheme S2). Probably, the electron withdrawing Fmoc protecting group at C-2 significantly reduces the glycosyl acceptor reactivity of the C-4 hydroxyl of **16**. The observations indicate that the C-4 hydroxyl has a much high reactivity when the C-2 hydroxyl is glycosylated.

The Lev ester of **14** was selectively removed by treatment with hydrazine acetate to give alcohol **15**, which was coupled with glycosyl donor **5** using TMSOTf as a promoter to provide the desired decasaccharide **18**. Next, the Alloc protecting group of **18** was removed by treatment with $Pd[PPh_3]_4$ and the resulting acceptor **19** was coupled with donor **6** using standard conditions to give dodecasaccharide **20** in high yield. Global deprotection of **20** was accomplished in four steps by first treatment with ethylenediamine in *n*-butanol at 90 °C to remove the phthaloyl-protecting groups, which was followed by reacetylation of the resulting free amines and hydroxyls by acetic anhydride in pyridine. Finally, the esters were selectively cleaved by catalytic sodium methoxide and hydrogenation over $Pd(OH)_2$ afforded the required tetra-antennary glycan **1**. The orthogonal protecting groups described here are more convenient than the previously employed set of protecting groups,[10] and in particular the replacement of a 2-naphthylmethyl (NAP) by TBS ether was advantageous because cleavage of the former by DDQ required great care and could result in side reactions such as cleavage of primary benzyl ethers. Also, the use of phthaloyl instead of Troc for protection of amino functions gave a more robust precursor.

**Enzymatic Diversification of Common Intermediate.** The stage was now set for regioselective extension of each antenna of **1** to give entry into highly complex asymmetric tetra-antennary *N*-linked glycans (Scheme 2). Compounds of such complexity have not been synthesized before and would demonstrate the power of the new methodology. Toward this end, glycan **7** was selected as a target which is a putative compound observed in a glycoproteomic study of human ductal invasive breast carcinoma tissue samples and may be attractive for biomarker development for early disease diagnosis.[14]

The use of a bacterial $\alpha(2,3)$-sialyltransferase (PmST1),[16] cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), and calf intestine alkaline phosphatase (CIAP) resulted in sialylation of the LacNAc moiety and terminal $\alpha$-Gal moiety of the C-2 and C-3 arms. Fortunately, this lack of selectivity could be addressed by using the mammalian $\alpha(2,3)$-sialyltransferase ST3-Gal-IV[17] and this enzyme exhibited absolute selectivity for the LacNAc antenna, and could selectively convert compound **1** into monosialylated derivative **21**. A downfield shift of the $\beta$-Gal H-1 supported the site of transfer of the Neu5Ac residue. The GlcNAc moiety of the $\beta(1,6)$-antenna of **21** was converted into LacNAc by using recombinant mammalian $\beta(1,4)$-galactosyltransferase (Gal-T1), uridine-5′-diphosphogalactose (UDP-Gal) and CIAP, and complete conversion to **22** was observed after an incubation time of 10 h. Surprisingly, the use of bovine Gal-T1 led only to partial galactosylation of **21**. Treatment of **22** with $\beta(1,3)$-*N*-acetylglucosaminyltransferase ($\beta$3-GnT-II),[18] uridine-5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) and CIAP resulted in the introduction of a GlcNAc residue at the $\beta(1,6)$-branch to give **23**. Full assignment of $^1$H NMR spectra (900 MHz) combined with a band-selective 2D TOCSY experiments confirmed that only the terminal $\beta$-Gal of the $\beta(1,6)$-arm was modified by GlcNAc. In this respect, only one new doublet at 4.69 ppm corresponding to H-1 of the anomeric $\beta$-GlcNAc moiety was observed. In addition, an upfield shift of the H-1 and the downfield shift of H-4 protons of the $\beta$-Gal residue (4.31 and 4.0 ppm, respectively) supported that only the LacNAc moiety of the $\beta(1,6)$-arm was extended and that the $\alpha$-Gal residue at the $\beta(1,4)$-arm was unaffected by the enzymatic transformation. Permethylation of the product followed by mass spectrometric (MS) analysis confirmed the attachment of a single GlcNAc moiety ($m/z$ 3574.7880). Controlled extension of the $\beta(1,6)$-arm to give a tri-LacNAc residue was accomplished by a repetition of galactosylation by Gal-T1 and *N*-acetylglucosaminylation with $\beta$3-GnT-II to give the octadecasaccharide **26**. After each step, the product was purified by size-exclusion chromatography, and the progress of the reactions was monitored by MS of permethylated derivatives. If any starting material was observed, the reaction was prolonged until a homogeneous product was obtained.

Next, the terminal LacNAc moiety of the $\beta(1,6)$-arm was converted into an H-type antigen by treatment with mammalian $\alpha(1,2)$-fucosyltransferase (Fut-I)[19] in the presence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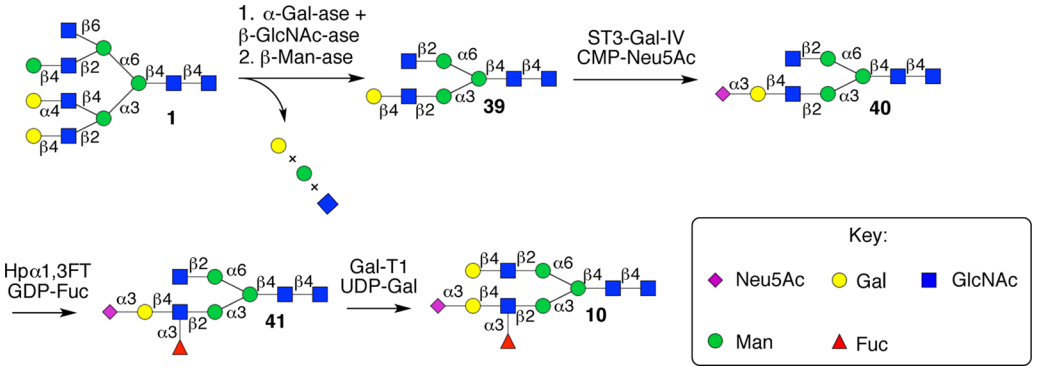
**Scheme 4. One-Pot Multiple-Enzyme Approach for the Preparation of Biantennary Glycans from Common Precursor 1**




of GDP-Fuc to furnish **27**. This intermediate was purified by semipreparative HPLC using an amide HILIC column (10 × 250 mm, Waters Inc.) under isocratic conditions (55% $CH_3CN$:100 mM ammonium formate, pH 3.4) with the UV (210 nm) detection to give the homogeneous nonadecasaccharide **27** in a sufficient quantity for further enzymatic modification of the $\beta$(1,2)- and $\beta$(1,4)-antennae.

At this point in the synthesis, the $\beta$(1,4)- and the $\beta$(1,2)-antenna were sequentially decaged by treatment with either $\alpha$-galactosidase or $\beta$-mannosidase, respectively followed by elaboration of the resulting terminal GlcNAc moieties into complex structures. Thus, the $\alpha$-Gal moiety was removed by treatment with the $\alpha$-galactosidase from green coffee beans, which proceeded smoothly to give glycan **28**. The resulting terminal GlcNAc residue of **28** could easily be converted into a sialyl LacNAc moiety by subsequent treatment with Gal-T1 and ST3-Gal-IV to give the disialylated glycan **30**. Finally, the $\beta$(1,2)-arm was elaborated by first removal of the capping $\beta$-mannoside using *Helix pomatia* $\beta$-mannosidase. Mass spectrometric analysis of the product revealed that in addition to the $\beta$-mannoside, also the $\alpha$-fucoside had been cleaved, which probably was due to contamination with a fucosidase. Fortunately, the use of the inhibitor 1-deoxyfuconojirimycin abolished the loss of the fucosyl residue to give reliable entry into glycan **31**. Finally, the targeted asymmetric 21-mer **7** was obtained by galactosylation of **31** with Gal-T1 to give **32**, which was further extended by $\beta$(1,3)GlcNAc unit by employing $\beta$3-GnT-II. Homogeneity of glycan **7** was confirmed by LC-MS using an amide HILIC column (55% $CH_3CN$:100 mM ammonium formate, pH 3.4).

Next, attention was focused on the synthesis of triantennary glycans from the universal precursor. It was expected that selective removal of the terminal $\beta$-Man or $\alpha$-Gal of the $\beta$(1,2)- or $\beta$(1,4)-antenna of **22** followed by treatment with $\beta$-*N*-acetylglucosaminidase to remove the revealed terminal GlcNAc moiety would provide triantennary structures **33** and **36**, respectively (Scheme 3). The latter compounds can then be elaborated to the triantennary glycans **8** and **9**, which are positional isomers. Thus, treatment of compound **22** with a mixture of $\alpha$-galactosidase and $\beta$-*N*-acetylglucosaminidase from Jack bean resulted in deletion of the $\beta$(1,4)-arm as shown by MS. Next, the reaction mixture was heat deactivated and subsequent addition of $\beta$-mannosidase and incubation for additional 2 h afforded homogeneous triantennary glycan **36**. Alternatively, exposure of **22** to $\beta$-mannosidase and $\beta$-*N*-acetylglucosaminidase resulted in the removal of the $\beta$(1,2) appendage, and after the addition of the $\alpha$-galactosidase and further incubation for 2 h, glycan **33** was obtained. Detailed inspection of NMR data as well as the permethylation followed by MALDI-TOF MS analysis confirmed that the trimming process had proceeded with the expected regioselectivity. For both compounds, the anomeric proton for $\alpha$-Gal (5.44 ppm) had disappeared while a similar signal for the $\beta$-Gal (4.47 ppm) was still present, which confirmed that the $\beta$(1,6)-arm had remained intact. The one-pot multiple-enzyme procedure[16b] made the transformation very efficient.

Treatment of **33** and **36** with $\beta$3-GnT-II and UDP-GlcNAc followed by bis-galactosylation with Gal-T1 and UDP-Gal afforded triantennary glycans **35** and **38**, respectively containing a terminal $\beta$(1,6)-di-LacNAc moiety. Sialylation of **35** and **38** with ST3-Gal-IV in the presence of CMP-Neu5Ac gave the positional isomers **8** and **9**, respectively.

The final goal was to synthesize a biantennary glycan from common tetra-antennary precursor **1** by a one-pot multiple-enzyme approach (Scheme 4). Sequential treatment of **1** with the $\alpha$-galactosidase and $\beta$-*N*-acetylglucosaminidase followed by heat-deactivation and further processing with $\beta$-mannosidase lead to clean formation of biantennary glycan **39** (LC-MS on a HILIC column, 65% $CH_3CN$:100 mM ammonium formate, pH 3.4). Sialylation of the LacNAc moiety of the $\beta$(1,2)-arm with ST3-Gal-IV resulted in the formation of glycan **40**. Antenna-selective installation of a sialyl-Le$^x$ moiety was easily accomplished by exposing the newly formed $\alpha$(2,3)-sialyl LacNAc to GDP-Fuc and *Helicobacter pylori* $\alpha$(1,3)-fucosyltransferase (Hp$\alpha$1,3FT)[20] providing **41**. The final step involved galactosylation of the $\beta$ (1,2)-arm of **41** by employing Gal-T1 and UDP-Gal to furnish biantennary glycan **10**.

**Multistage Mass Spectrometry of Isomeric Glycans.** The use of MS in glycomics is driven by the need to obtain quantitative and structural information on glycans of glycoproteins present in biological samples to understand metabolic or disease processes and to discover and validate putative new biomarkers.[21] In general, glycans are enzymatically or chemically released from glycoproteins, separated by chromatography and analyzed by MALDI-TOF or ESI-TOF MS. Although powerful, such studies do often not provide exact structures of glycans because of their isobaric nature (i.e., different structures with identical molecular weights). It is anticipated that the use of well-defined glycan standards will make it possible to examine chromatographic behavior and fragmentation patterns in multistage mass spectrometry, which may reveal unique features of glycan isomers that may facilitate exact structure identification.[22]

$[M + 3H]^{3+}$ precursor ions corresponding to native/nonpermethylated glycan **8** and glycan **9** were isolated and subjected to CID to obtain fragments that may distinguish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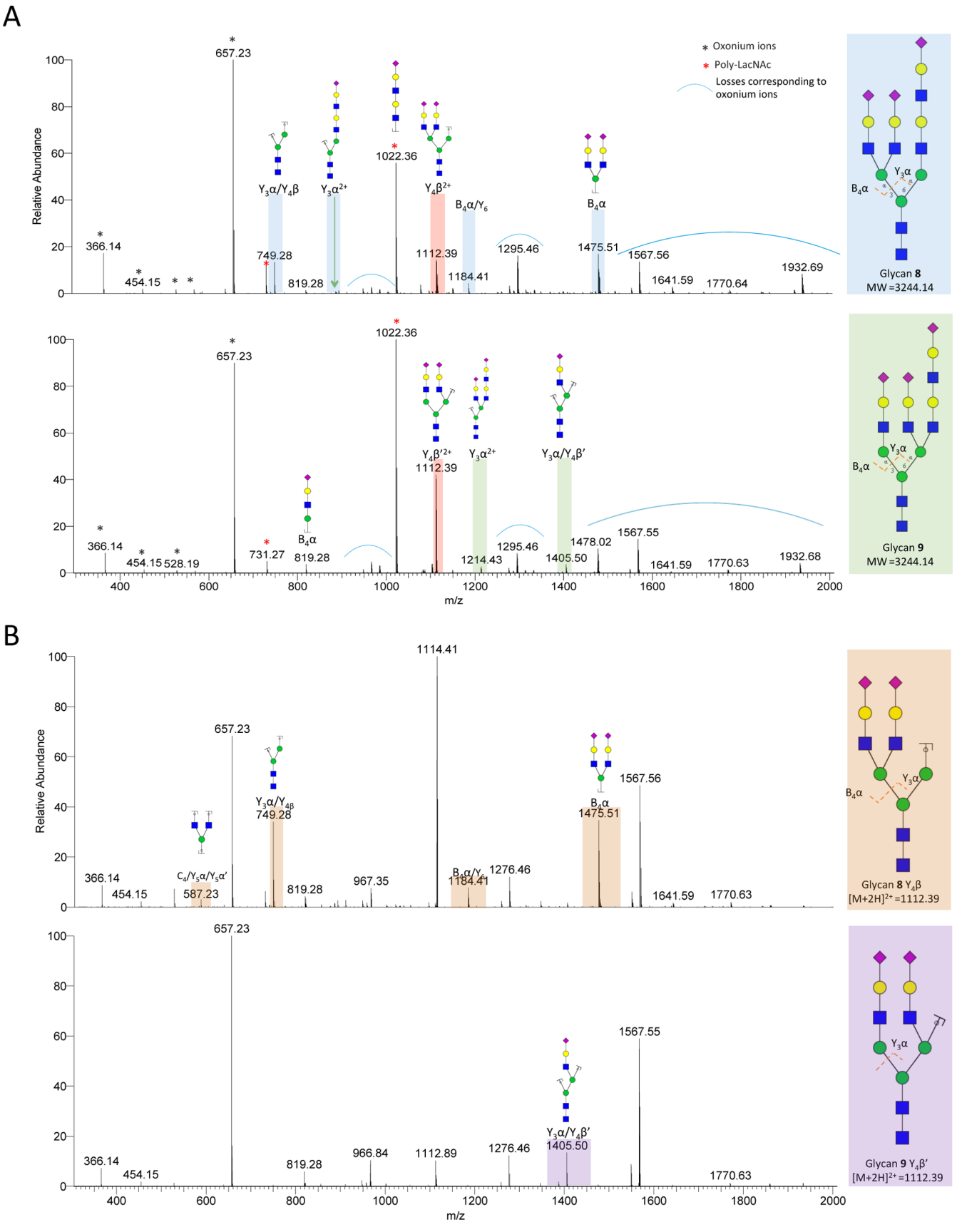


**Figure 2.** (A) Multistage mass spectrometry can distinguish the isomer glycans **8** and **9**. CID $MS^2$ of the triply charged precursor ion at $m/z$ 1082.39. Fragment ions indicative for distinguishing the two isomers are color-coded. (B) CID-$MS^3$. The $MS^2$ fragment ion at $m/z$ 1112.39 was subjected to $MS^3$ to reveal the structural differences between these two isobaric fragment ions. Fragment ions that can distinguish the two isomers are color-coded.



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these two isomers. Oxonium ions were observed in the low mass range as well as the corresponding fragments resulting from the losses of oxonium ions from precursor ions in the higher mass range. A peak at $m/z$ 1022 is indicative of di-*N*-acetyllactosamine containing sialic acid (Neu5Ac-di-LacNAc), which can further lose Neu5Ac as observed by the presence of peak at $m/z$ 731. To confirm the branching structures of glycans **8** and **9**, we searched for unique and structurally informative peaks in each spectrum, mainly the branching specific $B_4\alpha$ and $Y_3\alpha$ fragment pairs. For glycan **8**, $MS^2$ CID branching specific fragments (Figure 2, top panel, shaded in blue) were observed as $B_4\alpha$ at $m/z$ 1475 and its Y counterpart fragment $Y_3\alpha^{2+}$ at $m/z$ 885 confirmed the presence of two Neu5Ac-LacNAc units on $\alpha(1,3)$-branch and Neu5Ac-di-LacNAc on $\alpha(1,6)$-branch, respectively. The presence of two Neu5Ac-LacNAc units on the $\alpha(1,3)$-branch is further supported by the $B_4\alpha/Y_6$ $m/z$ 1184 peak (loss of one Neu5Ac from $B_4\alpha$). Doubly protonated fragment $Y_4\beta^{2+}$ at $m/z$ 1112 (shaded in red) resulting from the loss of Neu5Ac-di-LacNAc from precursor ion. $Y_4\beta^{2+}$ can further lose the entire $\alpha(1,3)$branch and give rise to a $Y_3\alpha$/ $Y_4\beta$ fragment at $m/z$ 749. Although $Y_3\alpha^{2+}$ is barely visible in the spectra, it can still be confidently assigned with signal-to-noise ratio (S/N) of 45. The low abundance thereof is due to the lability of Neu5Ac-di-LacNAc unit in the gas phase resulting once again in $Y_3\alpha/Y_4\beta$ fragment.

The CID $MS^2$ spectrum of glycan **9** provided two unique fragment peaks (Figure 2A, bottom panel, shaded in green). A unique peak is observed for the $Y_3\alpha^{2+}$ $m/z$ 1214 fragment ion, resulting from the loss of Neu5Ac-LacNAc-Man from $\alpha(1,3)$-branch, which can be found as $B_4\alpha$ fragment ion at $m/z$ 819. Loss of Neu5Ac-LacNAc-Man from $Y_4\beta'^{2+}$ $m/z$ 1112 results in second unique peak $Y_3\alpha/Y_4\beta'$ found at $m/z$ 1405. These two peaks corroborate glycan **9** branching pattern consisting of one Neu5Ac-LacNAc unit on the $\alpha(1,3)$-branch, and Neu5Ac-LacNAc Neu5Ac-di-LacNAc on the $\alpha$6-branch.

To validate the annotation of the $m/z$ 1112, which appears in both $MS^2$ spectra, $MS^3$ CID analyses were performed for each glycan (Figure 2B). Fragmentation of the $Y_4\beta^{2+}$ $m/z$ 1112 fragment, belonging to glycan **8** $MS^2$ CID (Figure 2B, top panel shaded in orange) produced $B_4\alpha$ fragment at $m/z$ 1475 as well as the $Y_3\alpha/Y_4\beta$ $m/z$ 749 ion. These informative fragments can be formed if both Neu5Ac-LacNAc blocks are present on the $\alpha(1,3)$ branch. In contrast, the $MS^3$ CID fragmentation of the $Y_4\beta'^{2+}$ $m/z$ 1112 ion isolated from glycan **9** $MS^2$ CID (Figure 2B, bottom panel shaded in purple) resulted in a distinct $Y_3\alpha/Y_4\beta'$ $m/z$ 1405 peak which can only be formed if the two Neu5Ac-LacNAc units are found on different branches within **9**. These results clearly demonstrate the importance of $MS^3$ to distinguish isobaric fragments of glycans that cannot be distinguished based on mass measurements alone.

Although it is possible to achieve richer fragmentation spectra of glycans by permethylation or measuring them as adducts of alkali metals, the resulting spectra require expert knowledge of glycan fragmentation for unambiguous annotation. We have measured glycans **8** and **9** as potassium and sodium adducts in positive mode as well as chlorinated glycans in negative mode, and although the resulting fragmentation spectra are rich in information, we have found that CID of protonated glycans provides sufficient details to distinguish isobaric glycans **8** and **9** and result in a simple fragmentation pathway consisting mainly of glycosidic bond cleavages and thus simplifying spectral annotation.

## CONCLUSION

Although naturally occurring complex *N*-linked oligosaccharides are usually asymmetrically substituted having a unique saccharide appendage at each branching point, previous synthetic efforts have almost exclusively been directed to the preparation of simpler symmetrical structures.[9a,d−j,l,m,o] This stems from the difficulties of controlling diversification at the various sites of branching, especially when several different complex terminal structures need to be appended. To address this challenge, we describe here a chemoenzymatic methodology that makes it possible to prepare any bi-, tri-, and tetra-antennary asymmetric *N*-glycan from a common precursor (**1**). This latter precursor is a tetra-antennary glycan that at positions where branching may occur is modified by LacNAc, GlcNAc, and unnatural $\alpha$-Gal(1,4)GlcNAc and $\beta$-Man(1,4)GlcNAc. We have found that relevant mammalian glycosyltransferases will only recognize the LacNAc containing antenna of the common precursor as a substrate, therefore allowing unique extension of this arm. At an appropriate stage of synthesis, the antenna containing a terminal GlcNAc residue can be "armed" by conversion into LacNAc by a galactosyltransferase, which can then be further extended by our panel of glycosyltransferases into a complex structure. The antenna that are modified by the unnatural hexoses $\alpha$-Gal and $\beta$-Man can be decaged by an appropriate glycosidase to reveal a terminal $\beta$-GlcNAc moiety, and thus the process of conversion into LacNAc and extension by glycosyltransferases can be repeated to give entry into asymmetrical tetra-antennary glycan. The use of unnatural distal hexoses to temporarily block an antenna from enzymatic modification is much more robust than our previously reported approach[10] based on acetylation of terminal GlcNAc or LacNAc residues. The new strategy made it possible, for the first time, to prepare a tetra-antennary *N*-glycan that at each branching point has a different appendage. We also describe that the common precursor can easily be converted into bi- and triantennary glycans by deletion of an antenna by the action $\beta$-*N*-acetylglucosaminidase, which selectively cleaves a terminal GlcNAc residue. This process can be performed by an efficient one-pot multiple-enzyme procedure. The latter strategy made it possible to prepare two isomeric triantennary *N*-glycans. As proof of principle to demonstrate the usefulness of the methodology, the isomers were subjected to multistage mass spectrometry to discover diagnostic ions that can determine the branching pattern of *N*-glycans in complex biological samples.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12080.

Synthetic protocols, compounds characterization of glycans, and NMR spectra (PDF)

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### Notes

The authors declare no competing financial interest.



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## ■ REFERENCES

(1) Hart, G. W.; Copeland, R. J. *Cell* **2010**, *143*, 672−676.
(2) Moremen, K. W.; Tiemeyer, M.; Nairn, A. V. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 448−462.
(3) Spik, G.; Debruyne, V.; Montreuil, J.; van Halbeek, H.; Vliegenthart, J. F. G. *FEBS Lett.* **1985**, *183*, 65−69.
(4) (a) Freeze, H. H. *Nat. Rev. Genet.* **2006**, *7*, 537−551. (b) Ohtsubo, K.; Marth, J. D. *Cell* **2006**, *126*, 855−867.
(5) Abdel Rahman, A. M.; Ryczko, M.; Nakano, M.; Pawling, J.; Rodrigues, T.; Johswich, A.; Taniguchi, N.; Dennis, J. W. *Glycobiology* **2015**, *25*, 225−240.
(6) Lau, K. S.; Partridge, E. A.; Grigorian, A.; Silvescu, C. I.; Reinhold, V. N.; Demetriou, M.; Dennis, J. W. *Cell* **2007**, *129*, 123−134.
(7) Lepenies, B.; Yin, J.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2010**, *14*, 404−411.
(8) (a) Boltje, T. J.; Buskas, T.; Boons, G. J. *Nat. Chem.* **2009**, *1*, 611−622. (b) Zhu, X.; Schmidt, R. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 1900−1934. (c) Palcic, M. M. *Curr. Opin. Chem. Biol.* **2011**, *15*, 226−233. (d) Schmaltz, R. M.; Hanson, S. R.; Wong, C. H. *Chem. Rev.* **2011**, *111*, 4259−4307.
(9) (a) Unverzagt, C. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2350−2353. (b) Seifert, J.; Lergenmüller, M.; Ito, Y. *Angew. Chem., Int. Ed.* **2000**, *39*, 531−534. (c) Ratner, D. M.; Swanson, E. R.; Seeberger, P. H. *Org. Lett.* **2003**, *5*, 4717−4720. (d) Hanashima, S.; Manabe, S.; Ito, Y. *Angew. Chem., Int. Ed.* **2005**, *44*, 4218−4224. (e) Jonke, S.; Liu, K. G.; Schmidt, R. R. *Chem. - Eur. J.* **2006**, *12*, 1274−1290. (f) Eller, S.; Schuberth, R.; Gundel, G.; Seifert, J.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2007**, *46*, 4173−4175. (g) Sun, B.; Srinivasan, B.; Huang, X. *Chem. - Eur. J.* **2008**, *14*, 7072−7081. (h) Unverzagt, C.; Gundel, G.; Eller, S.; Schuberth, R.; Seifert, J.; Weiss, H.; Niemietz, M.; Pischl, M.; Raps, C. *Chem. - Eur. J.* **2009**, *15*, 12292−12302. (i) Serna, S.; Etxebarria, J.; Ruiz, N.; Martin-Lomas, M.; Reichardt, N. C. *Chem. - Eur. J.* **2010**, *16*, 13163−13175. (j) Serna, S.; Yan, S.; Martin-Lomas, M.; Wilson, I. B. H.; Reichardt, N. C. *J. Am. Chem. Soc.* **2011**, *133*, 16495−16502. (k) Sakamoto, I.; Tezuka, K.; Fukae, K.; Ishii, K.; Taduru, K.; Maeda, M.; Ouchi, M.; Yoshida, K.; Nambu, Y.; Igarashi, J.; Hayashi, N.; Tsuji, T.; Kajihara, Y. *J. Am. Chem. Soc.* **2012**, *134*, 5428−5431. (l) Walczak, M. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 16430−16433. (m) Walczak, M. A.; Hayashida, J.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2013**, *135*, 4700−4703. (n) Maki, Y.; Okamoto, R.; Izumi, M.; Murase, T.; Kajihara, Y. *J. Am. Chem. Soc.* **2016**, *138*, 3461−3468. (o) Mönnich, M.; Eller, S.; Karagiannis, T.; Perkams, L.; Luber, T.; Ott, D.; Niemietz, M.; Hoffman, J.; Walcher, J.; Berger, L.; Pischl, M.; Weishaupt, M.; Wirkner, C.; Lichtenstein, R. G.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2016**, *55*, 10487−10492. (p) Nagasaki, M.; Manabe, Y.; Minamoto, N.; Tanaka, K.; Silipo, A.; Molinaro, A.; Fukase, K. *J. Org. Chem.* **2016**, *81*, 10600−10616.
(10) Wang, Z.; Chinoy, Z. S.; Ambre, S. G.; Peng, W.; McBride, R.; de Vries, R. P.; Glushka, J.; Paulson, J. C.; Boons, G. J. *Science* **2013**, *341*, 379−383.
(11) (a) Echeverria, B.; Etxebarria, J.; Ruiz, N.; Hernandez, Á.; Calvo, J.; Haberger, M.; Reusch, D.; Reichardt, N. C. *Anal. Chem.* **2015**, *87*, 11460−11467. (b) Li, L.; Liu, Y.; Ma, C.; Qu, J.; Calderon, A. D.; Wu, B.; Wei, N.; Wang, X.; Guo, Y.; Xiao, Z.; Song, J.; Sugiarto, G.; Li, Y.; Yu, H.; Chen, X.; Wang, P. G. *Chem. Sci.* **2015**, *6*, 5652−5661. (c) Shivatare, S. S.; Chang, S. H.; Tsai, T. I.; Tseng, S. Y.; Shivatare, V. S.; Lin, Y. S.; Cheng, Y. Y.; Ren, C. T.; Lee, C. C. D.; Pawar, S.; Tsai, C. S.; Shih, H. W.; Zeng, Y. F.; Liang, C. H.; Kwong, P. D.; Burton, D. R.; Wu, C. Y.; Wong, C. H. *Nat. Chem.* **2016**, *8*, 338−346.
(12) Li, T.; Huang, M.; Liu, L.; Wang, S.; Moremen, K. W.; Boons, G. J. *Chem.−Eur. J.* **2016**, *22*, 18633−18980.
(13) (a) Koizumi, A.; Matsuo, I.; Takatani, M.; Seko, A.; Hachisu, M.; Takeda, Y.; Ito, Y. *Angew. Chem., Int. Ed.* **2013**, *52*, 7426−7431. (b) Fujikawa, K.; Koizumi, A.; Hachisu, M.; Seko, A.; Takeda, Y.; Ito, Y. *Chem. - Eur. J.* **2015**, *21*, 3224−3233.
(14) Abbott, K. L.; Aoki, K.; Lim, J. M.; Porterfield, M.; Johnson, R.; O'Regan, R. M.; Wells, L.; Tiemeyer, M.; Pierce, M. *J. Proteome Res.* **2008**, *7*, 1470−1480.
(15) (a) Yu, B.; Tao, H. *J. Org. Chem.* **2002**, *67*, 9099−9102. (b) Yang, Y.; Zhang, X.; Yu, B. *Nat. Prod. Rep.* **2015**, *32*, 1331−1355.
(16) (a) Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q.; Chen, X. *J. Am. Chem. Soc.* **2005**, *127*, 17618−17619. (b) Yu, H.; Chen, X. *Org. Biomol. Chem.* **2016**, *14*, 2809−2818.
(17) Kono, M.; Ohyama, Y.; Lee, Y. C.; Hamamoto, T.; Kojima, N.; Tsuji, S. *Glycobiology* **1997**, *7*, 469−479.
(18) Ujita, M.; Misra, A. K.; McAuliffe, J.; Hindsgaul, O.; Fukuda, M. *J. Biol. Chem.* **2000**, *275*, 15868−15875.
(19) Chang, W. W.; Lee, C. H.; Lee, P.; Lin, J.; Hsu, C. W.; Hung, J. T.; Lin, J. J.; Yu, J. C.; Shao, L. E.; Yu, J.; Wong, C. H.; Yu, A. L. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11667−11672.
(20) Wang, W.; Hu, T.; Frantom, P. A.; Zheng, T.; Gerwe, B.; del Amo, D. S.; Garret, S.; Seidel, R. D.; Wu, P. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16096−16101.
(21) North, S. J.; Hitchen, P. G.; Haslam, S. M.; Dell, A. *Curr. Opin. Struct. Biol.* **2009**, *19*, 498−506.
(22) Mechref, Y.; Novotny, M. V. *Mass Spectrom. Rev.* **2009**, *28*, 207−222.


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