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Enzyme-Catalyzed Synthesis of a Hybrid N-Linked Oligosaccharide using N-Acetylglucosaminyltransferase I

Rui Chen,[a,c] Mark A. Pawlicki,[b,c] Brian S. Hamilton,[b] and Thomas J. Tolbert[a,b,*]

[a] Department of Chemistry, Indiana University, 800 E. Kirkwood Ave., Bloomington, IN 47405, USA
[b] Indiana University Interdisciplinary Biochemistry Graduate Program, 800 E. Kirkwood Ave., Bloomington, IN 47405, USA
Fax: (+1)-812-855-8300; e-mail: tolbert@indiana.edu
[c] Contributed equally to this work.

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Dedicated to Prof. Chi-Huey Wong on the occasion of his 60th birthday.

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de/home/.

Abstract: The soluble catalytic domain of human N-acetylglucosaminyltransferase I was purified from *Escherichia coli* and utilized in the enzyme-catalyzed conversion of high mannose N-linked oligosaccharide **1** into the rare hybrid oligosaccharide **2**. Analysis of the reaction showed that the conversion of high mannose **1** into hybrid oligosaccharide **2** proceeded to 100% completion as assessed by MALDI-TOF-MS. Purification of the large polar oligosaccharide by gel filtration and silica gel chromatography afforded a 42% isolated yield of oligosaccharide **2**. This enzyme-catalyzed reaction can be utilized to produce rare hybrid oligosaccharides for biochemical and structural studies.

Keywords: enzyme catalysis; enzymes; glycosylation; oligosaccharides

The surfaces of cells are covered with glycoproteins and glycolipids that mediate cell–cell interactions important in development, immune responses, and disease.[1] Increased interest in understanding these interactions has led to synthetic efforts aimed at producing pure oligosaccharides for biochemical and structural studies. N-linked oligosaccharides are large, branched sugar structures attached to the amino acid asparagine on proteins that range from 5 to several hundred sugar residues in size. Three broad classes of N-linked oligosaccharides are produced in humans: high mannose, hybrid, and complex N-linked oligosaccharides (Figure 1). These N-linked oligosaccharide types differ in the sugars attached to the non-reducing ends of the glycan, where high mannose oligosaccharides have all mannose residues on their non-reducing ends, complex oligosaccharides have sugar residues other than mannose terminating their non-reducing ends, and hybrid oligosaccharides have both mannose and other types of sugars terminating their chains.

Although there have been many recent advances in chemical oligosaccharide synthesis, N-linked oligosaccharides still present large difficulties in synthesis due to their size, branched chains, and the wide variety of sugars and glycosidic linkages within their structures.[2] An alternative to total synthesis of N-linked oligosaccharides is to isolate them from natural sources. The difficulty with this strategy is that N-linked oligosaccharides are produced as multiple heterogeneous forms in biological systems.[3] This complicates the isolation of specific types of N-linked oligosaccharides from natural sources because the different types must be separated from one another chromatographically. Methods have been developed for the isolation of high mannose and complex type N-linked oligosaccharides using glycoproteins with a high percentage of those types of glycosylation. For instance, soybean agglutinin[4] and hen egg yolk sialylglycopeptide[5] are two glycoproteins that can be used to produce high mannose and complex type oligosaccharides, respectively. Unfortunately, no good method exists to produce hybrid N-linked oligosaccharides since they are generally present as a small percentage of the total oligosaccharides in glycoproteins.

Here we report the use of enzymatic synthesis to convert a high mannose oligosaccharide extracted from yeast (**1**) into the hybrid oligosaccharide $GlcNAcMan_5GlcNAc_2$ (**2**) (Figure 2). Enzyme-catalyzed synthesis of oligosaccharides and glycoproteins has the advantages of not requiring protecting groups to differentiate sugar hydroxy groups in glycosylation reactions, high stereoselectivity in the formation of glycosidic bonds, and generally high conversion

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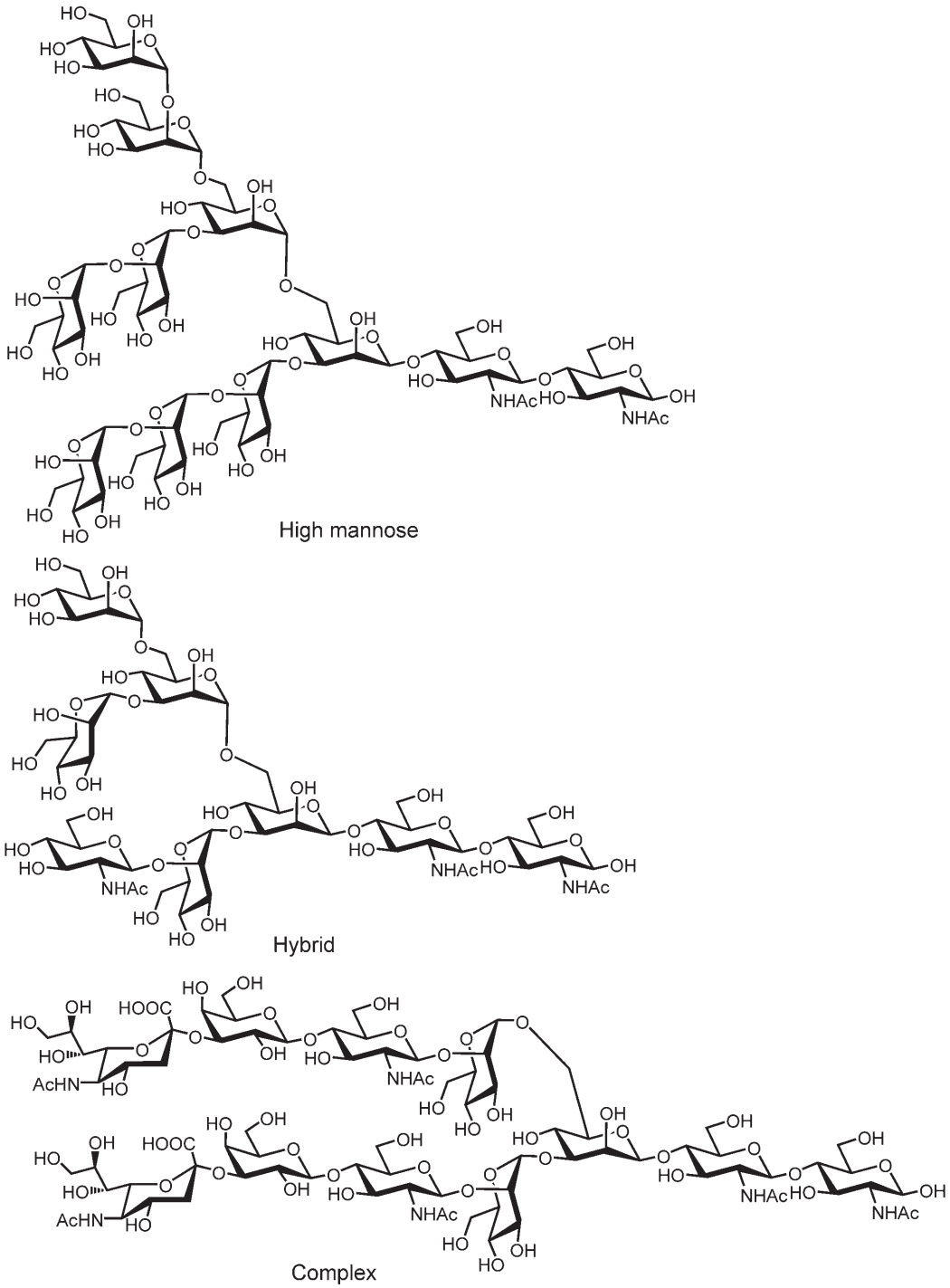

Figure 1. Types of human N-linked oligosaccharides.

yields.[6] The use of the enzyme N-acetylglucosaminyltransferase I (GnT-I) to produce the hybrid oligosaccharide **2** allows the regioselective and stereoselective addition of a β-1,2-*N*-acetylglucosamine onto the $Man_5GlcNAc_2$ high mannose N-linked oligosaccharide **1** in a single step in the absence of protecting groups.

GnT-I initiates the formation of hybrid oligosaccharides in the Golgi apparatus in mammals as part of the processing reactions that convert high mannose N-linked glycoproteins produced in the endoplasmic reticulum into hybrid and complex glycoforms.[7] GnT-I is a type II membrane protein with a short cytoplasmic N-terminus, a single pass transmembrane domain, and a C-terminal catalytic domain that resides in the lumen of the Golgi apparatus. Both rat and human GnT-I have been recombinantly expressed

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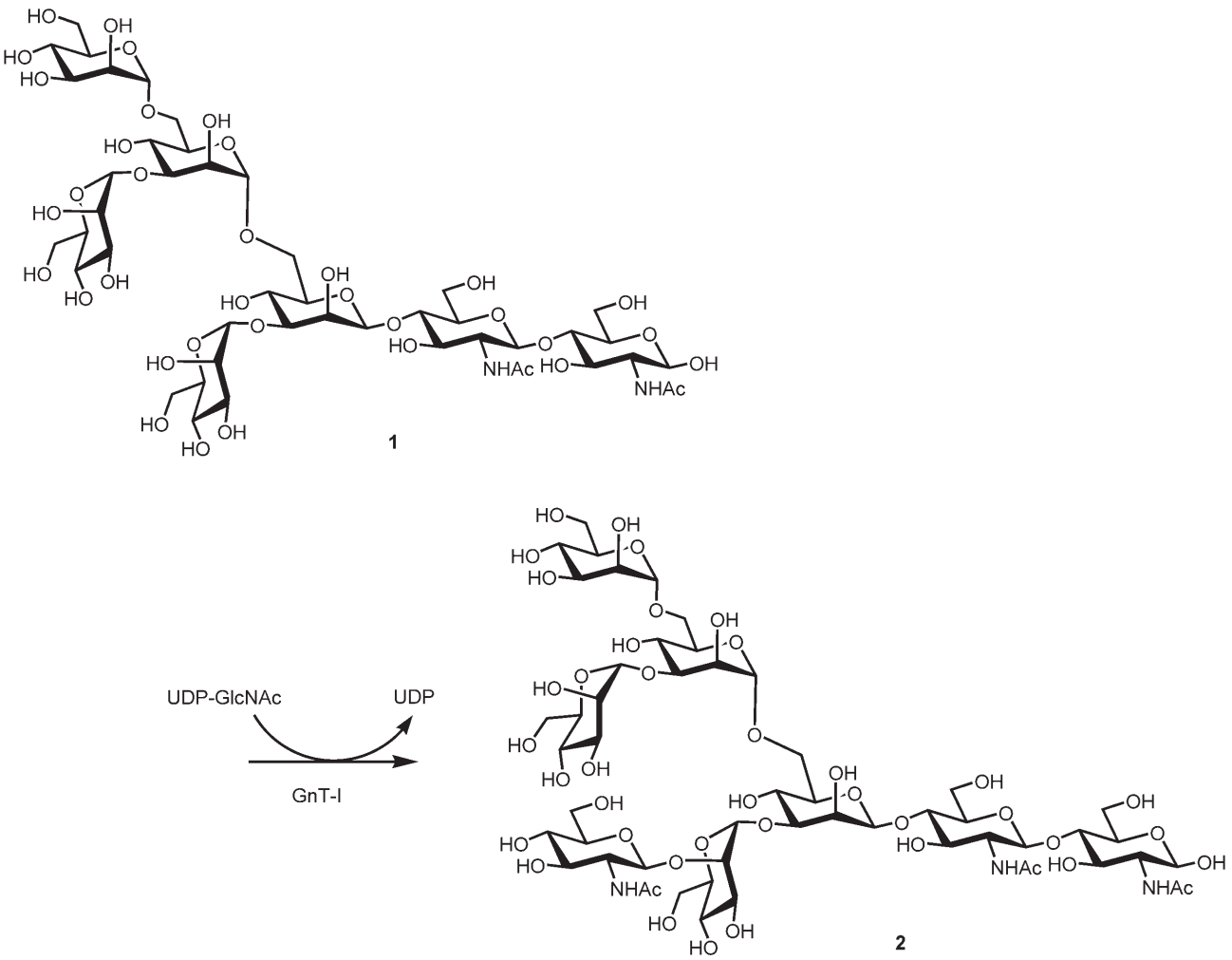

Figure 2. Enzyme-catalyzed synthesis of hybrid N-linked glycan **2** from high mannose **1** using GnT-I.

in *E. coli* as fusion proteins and characterized previously, with rat GnT-I[8] having been expressed as an N-terminally histidine-tagged fusion protein with a specific activity of 5.30 μmole/min mg^{-1} and human GnT-I[9] having been expressed as a maltose binding protein fusion with a specific activity of 0.101 μmole/min mg^{-1}.

In a manner similar to the method reported by Nishiu et al.[8] for rat GnT-I, we have expressed a truncated form of human GnT-I consisting of the soluble catalytic domain as an N-terminally histidine-tagged fusion protein in *E. coli*. To improve the translation and folding of the human GnT-I protein in bacteria we used an *E. coli* strain that is supplemented with mammalian tRNA codons and with mutations to improve intracellular disulfide bond formation. After a single Ni^{2+}-NTA chromatography step, our histidine-tagged human GnT-I affords approximately 2.46 units of enzyme per liter with a specific activity of 0.48 μmole/min mg^{-1}. For comparison, Nishiu et al.[8] report obtaining a total of 1.11 units of rat GnT-I activity per liter with a specific activity of 5.30 μmole/min mg^{-1} after two chromatographic purification steps. Although the specific activity of our human GnT-I enzyme could be improved by additional chromatography steps, the purity of the enzyme after a single Ni^{2+}-NTA affinity chromatography step was found to be sufficient for oligosaccharide synthesis. The higher yield of total GnT-I enzyme activity allows for larger scale syntheses to be conducted, and the 2.46 units per liter of GnT-I activity produced by this method is sufficient for syntheses more than 50 times larger than the one reported here.

High mannose oligosaccharides for the production of hybrid N-linked oligosaccharides can be obtained from a variety of sources including soybean agglutinin, hen ovalbumin, and yeast.[4,10,11] Treatment of heterogeneous high mannose oligosaccharides derived from a variety of sources with α-1,2-mannosidases results in the formation of the truncated high mannose oligosaccharide **1**.[11,12] The high mannose glycan **1** used in this paper was extracted from yeast, but in practice any of the above sources of high mannose

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oligosaccharides could be utilized for hybrid N-linked oligosaccharide synthesis.

The enzyme-catalyzed synthesis of hybrid oligosaccharide **2** was conducted at room temperature with a 2.7-fold excess of the sugar donor UDP-GlcNAc over the oligosaccharide substrate **1**. The course of the reaction was monitored with MALDI-TOF mass spectrometry (Figure 3), which initially showed a relatively rapid conversion of **1** into **2** in the first day. The reaction slowed as more product was produced, probably due to GnT-1 inhibition by UDP, substrate depletion, or inactivation of the GnT-I enzyme over time. Additional enzyme was added on day 4 to drive the reaction to completion, and on day 5 MS analysis of the reaction showed complete conversion of the starting high mannose oligosaccharide **1** into the hybrid oligosaccharide **2**. Purification of **2** from the enzymatic reaction mixture was somewhat more difficult than the enzymatic reaction itself due to the highly polar nature of hybrid oligosaccharide **2**, which does not allow for reverse phase chromatography and which makes normal phase chromatography quite difficult. Isolation of **2** was accomplished using both size exclusion and silica gel chromatography. The isolated yield of hybrid oligosaccharide **2** was 42%. Given the observation of complete conversion of **1** into **2** by mass spectrometry (Figure 3), the low yield of **2** is believed to be due to losses that occurred during its purification. Nevertheless, a 42% yield of the rare hybrid oligosaccharide **2** from the more readily available high mannose oligosaccharide **1** on a 5.4 mg scale affords enough material to do a wide variety of biochemical experiments.

The hybrid N-linked oligosaccharide **2** was characterized by ^{1}H NMR spectroscopy to confirm the regioselectivity of the human GnT-I-catalyzed reaction. 1D and COSY ^{1}H NMR spectra were acquired for both the starting material **1** (Figure 4) and the hybrid product **2** (Figure 5). The anomeric H-1 and the well-resolved H-2 protons in the region between 5.3 and 4.0 ppm were assigned with reference to previous analyses of the high mannose **1** and hybrid **2** oligosaccharides (Table 1).[13] As can be seen from the NMR spectra, the purity of the product **2** is quite good, and it exists as a mixture of α- and β-anomers from the free reducing end of GlcNAc-1. A comparison of the NMR spectra of **1** and **2** in Figure 4 and Figure 5 shows the addition of a new doublet in the spectra of **2** at 4.580 ppm with a coupling constant of 8.5 Hz corresponding to the anomeric proton of GlcNAc-5 in a β-configuration. In addition, although most of the chemical shifts of the H-1 and H-2 protons of the high mannose oligosaccharide **1** do not change significantly in the hybrid oligosaccharide **2**, the H-1 and H-2 resonances of the Man-4 sugar residue shift significantly, indicating the GlcNAc-5 residue is linked to the Man-4 residue. These observations are in complete agreement with previous NMR characterizations of the hybrid oligosaccharide **2**, and in conjunction with previous combined NMR and degradation studies of **2** confirm the addition of a β-1,2-linked GlcNAc sugar to the Man-4 residue of **1**.[13]

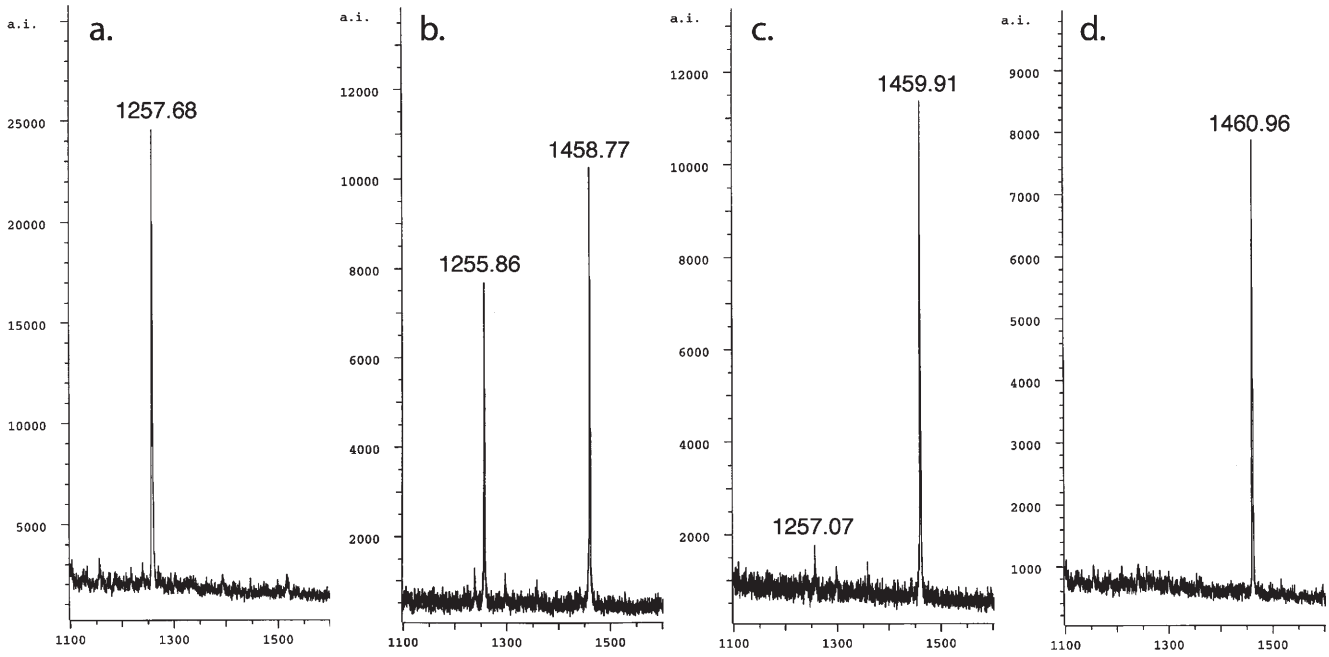

Figure 3. Enzyme-catalyzed synthesis of hybrid oligosaccharide **2** monitored by MALDI-TOF-MS. **a**) 0 h, **b**) 26 h. c) 94 h, **d**) 120 h. Expected masses: $Man_5GlcNAc_2$ (**1**), $M+Na^+$ 1257.42; $GlcNAcMan_5GlcNAc_2$ (**2**), $M+Na^+$ 1460.50.

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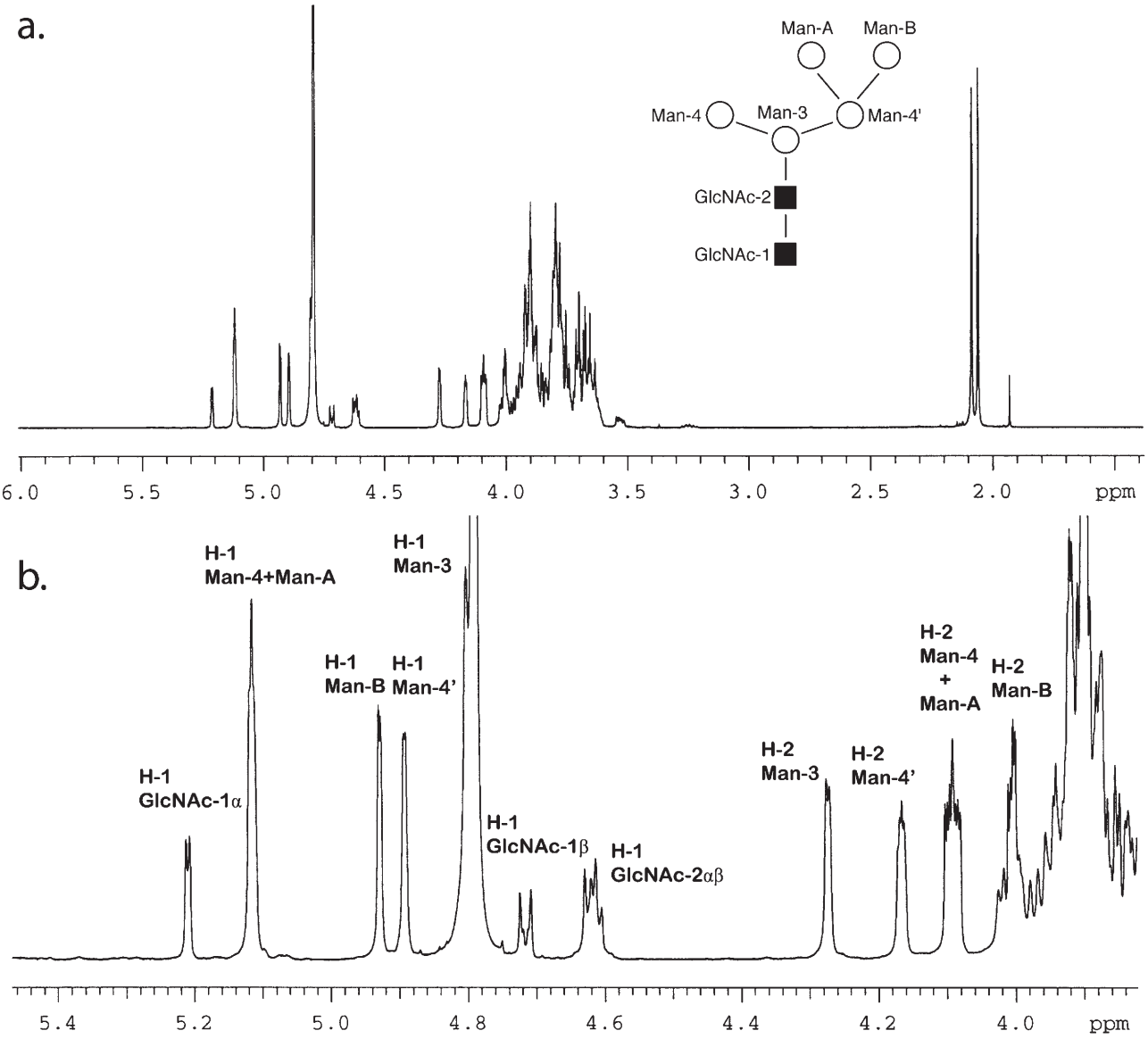

Figure 4. a) 500 MHz ^{1}H NMR spectra of $Man_5GlcNAc_2$ (**1**) in D_2O, the inset shows the sugar labeling nomenclature for **1.** **b**) Expansion of the ^{1}H NMR spectra to show the anomeric fingerprint region of **1** with H-1 and H-2 assignments labeled.

In summary, human GnT-I purified from *E. coli* has been utilized in the preparative enzymatic synthesis of the hybrid N-linked oligosaccharide **2** from more readily available high mannose oligosaccharides. The conversion of the high mannose **1** into hybrid **2** was accomplished in a single enzymatic step that was observed to go to completion by MS analysis of the reaction. Purification of the large polar oligosaccharide presented some difficulties, resulting in a 42% isolated yield of hybrid oligosaccharide **2** even though the reaction was observed to go to completion by MS. This enzyme-catalyzed reaction can be readily scaled up to provide a source of hybrid N-linked oligosaccharides for biochemical and structural studies.

Experimental Section

Enzyme-Catalyzed Synthesis of Hybrid Oligosaccharide 2 from High Mannose 1

High mannose oligosaccharide **1** (11.1 mg, 9.0 μmole) was placed into a 25-mL round-bottom flask and dissolved in 10 mL of a solution containing 20 mM Hepes pH 7.5, 150 mM NaCl, 20 mM $MnCl_2$, 2.5 mM UDP-GlcNAc, and 32 milliunits of GnT-I. The resulting reaction mixture was incubated at room temperature for approximately 5 days. The reaction was monitored with MALDI-TOF mass spectrometry using a Bruker Biflex III MALDI-TOF mass spectrometer. The technique used for oligosaccharide ionization was similar to that described by Harvey.[14] The machine was

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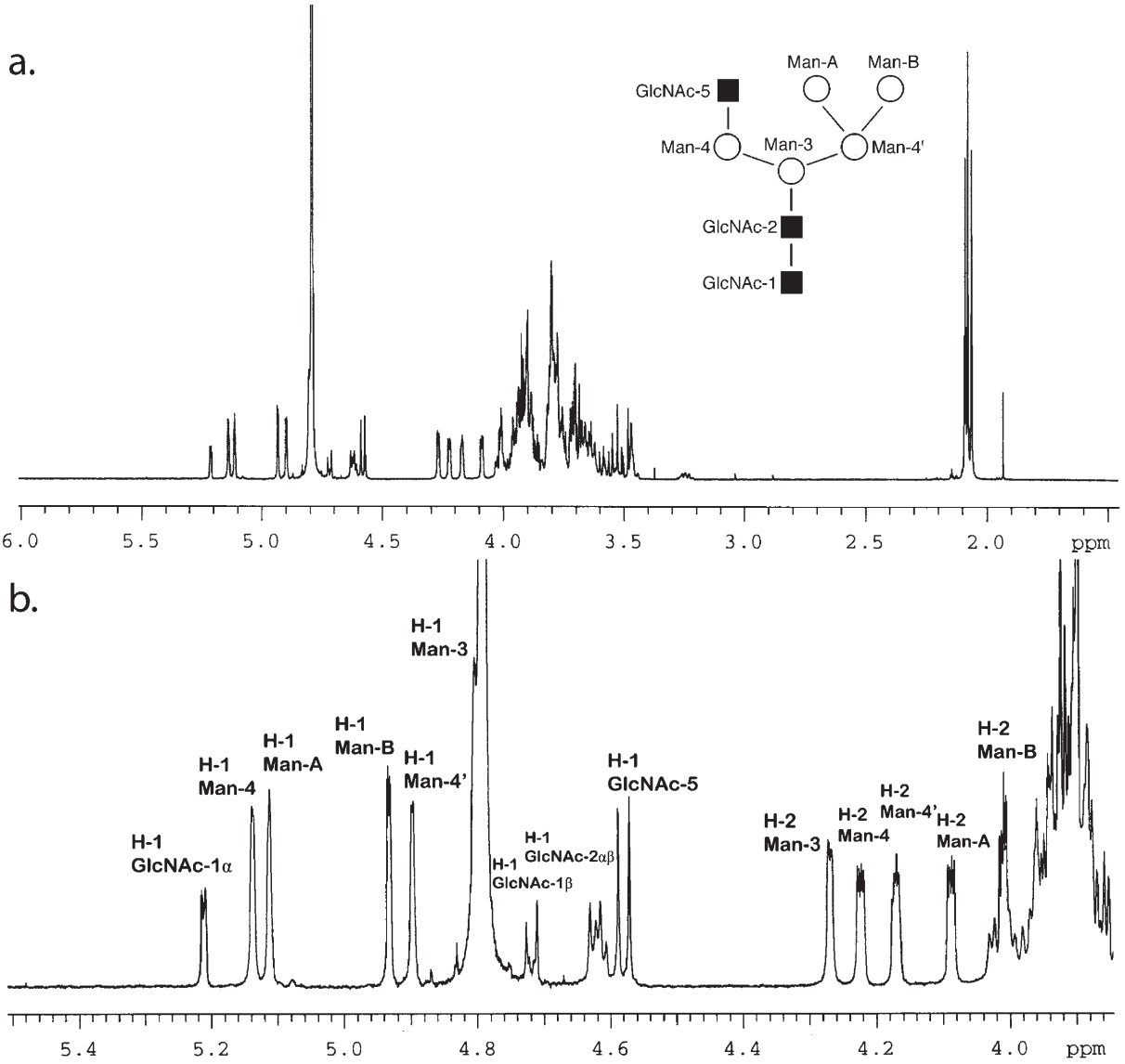

Figure 5. a) 500 MHz ^{1}H NMR spectra of GlcNAcMan$_5$GlcNAc$_2$ (**2**) in D_2O, the inset shows the sugar labeling nomenclature for **2. b**) Expansion of the ^{1}H NMR spectra to show the anomeric fingerprint region of **2** with H-1 and H-2 assignments labeled.

calibrated using Dextrin 10. 3.5 μL of sample or calibrant were mixed with 1.5 μL matrix (100 mg mL^{-1} 2,5-dihydroxybenzoic acid in EtOH) and spotted onto the sample plate. After approximately 4 days additional GnT-I enzyme (12 milliunits) was added to push the reaction to completion. MALDI-TOF MS analysis of the reaction on day 5 showed complete conversion of **1** into **2** (Figure 3). The enzymatic reaction mixture was then evaporated to dryness, and the white residue was taken up into water and loaded onto a P4 size exclusion column (Bio-Rad, 1.5 × 50 cm, approximately 89 mL column volume), equilibrated and eluted with water. Fractions containing product were detected positively by phenol-sulfuric acid test,[15] then pooled together and evaporated to dryness to give 20 mg of a white solid which still contained some UDP-GlcNAc from the reaction as determined by NMR spectroscopy. Next, the white solid was purified twice by silica gel flash chromatography (ethyl acetate/methanol/water, 12:3:3 to 7:3:3) and the fractions containing the hybrid oligosaccharide **2** (R_f = 0.46, ethyl acetate/methanol/water, 5:3:3) were collected and then lyophilized to give **2** as a pure product; yield: 5.4 mg (3.8 μmole, 42%). ^{1}H NMR (500 MHz, D_2O): δ = 5.212 (d, J = 2.5 Hz, 0.6 H), 5.139 (s, 1 H), 5.112 (s, 1 H), 4.932 (d, J = 1.5 Hz, 1 H), 4.898 (d, J = 1.5 Hz, 1 H), 4.803 (br, 1 H), 4.718 (d, J = 8.0 Hz, 0.4 H), 4.630–4.608 (m, 1 H), 4.580 (d, J = 8.5 Hz, 1 H), 4.270 (d, J = 2.5 Hz, 1 H), 4.223 (dd, J = 3.0, 1.5 Hz, 1 H), 4.176–4.167 (m, 1 H), 4.088 (dd, J = 3.5, 1.5 Hz, 1 H), 4.031–3.470 (m, 44 H), 2.089 (s, 3 H), 2.078(s, 3 H), 2.062 (s, 3 H); HR-ESI-TOF MS: m/z = 1460.5000, calcd. for $C_{54}H_{91}N_3O_{41}Na$ $[M+Na]^+$: 1460.5026

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Table 1. NMR chemical shift assignments for $Man_5GlcNAc_2$ (**1**) and $GlcNAcMan_5GlcNAc_2$ (**2**).

	1[a]	**2**[a]
	H-1	
GlcNAc-1α	5.210	5.212
GlcNAc-1β	4.716	4.718
GlcNAc-2	4.617	4.619
Man-3	4.803	4.803
Man-4	5.116	5.139
Man-4′	4.892	4.898
Man-A	5.116	5.112
Man-B	4.928	4.932
GlcNAc-5	–	4.580
	H-2	
Man-3	4.274	4.270
Man-4	≈4.092	4.223
Man-4′	4.166	4.170
Man-A	≈4.092	4.088
Man-B	≈4.006	≈4.010
	NHAc	
CH_3CO	2.086	2.089
CH_3CO	2.060	2.062
CH_3CO	–	2.078

[a] Chemical shifts in ppm.

Supporting Information

Procedures used for the subcloning of a human GnT-I expression plasmid, expression and purification of human GnT-I in *E. coli*, and GnT-I enzyme assay are given in the Supporting Information.

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