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Synthesis of *N*-glycan units for assessment of substrate structural requirements of *N*-acetylglucosaminyltransferase III

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ABSTRACT

N-Acetylglucosaminyltransferase (GnT) III is a glycosyltransferase which produces bisected *N*-glycans by transferring GlcNAc to the 4-position of core mannose. Bisected *N*-glycans are involved in physiological and pathological processes through the functional regulation of their carrier proteins. An understanding of the biological functions of bisected glycans will be greatly accelerated by use of specific inhibitors of GnT-III. Thus far, however, such inhibitors have not been developed and even the substrate-binding mode of GnT-III is not fully understood. To gain insight into structural features required of the substrate, we systematically synthesized four *N*-glycan units, the branching parts of the bisected and non-bisected *N*-glycans. The series of syntheses were achieved from a common core trimannose, giving bisected tetra- and hexasaccharides as well as non-bisected tri- and pentasaccharides. A competitive GnT-III inhibition assay using the synthetic substrates revealed a vital role for the Manβ(1–4)GlcNAc moiety. In keeping with previous reports, GlcNAc at the α1,3-branch is also involved in the interaction. The structural requirements of GnT-III elucidated in this study will provide a basis for rational inhibitor design.

Protein *N*-glycosylation takes place in the endoplasmic reticulum (ER) and influences many of characteristics and functions of glycoproteins including folding and degradation,[1] trafficking,[2] and cell adhesion.[3] Throughout the glycan processing pathway, one of the important modifications is the introduction of the bisecting *N*-acetylglucosamine (GlcNAc) residue. *N*-acetylglucosaminyltransferase (GnT) III is responsible for inserting bisecting GlcNAc at O4 of the core β-mannose via a β-linkage, using UDP-GlcNAc as donor substrate.[4] In general, it is thought that the bisecting GlcNAc is not further glycosylated under physiological conditions, which is in sharp contrast to the GlcNAc at the mannoses on both 1–3 and 1–6 branches.

Accumulating evidence suggests that bisecting GlcNAc regulates tumor progression and migration, and Alzheimer's disease. Bisecting GlcNAc suppresses tumor progression by an effect on growth factor signaling[5] induced through galectin-mediated cross-linking.[6] Cell adhesion molecules with bisecting GlcNAc on their *N*-glycans, particularly with respect to E-cadherin and integrins, retard tumor migration.[7–9] The mechanism of action may be due to the fact that the bisected *N*-glycan is a poor acceptor for GnT-V thereby arresting the formation of migration-related hyper branched *N*-glycans.[10,11] Bisecting GlcNAc is also involved in Alzheimer's disease, altering both the clearance and production of amyloid-β.[12,13] It also plays a pivotal role in suppression of *N*-glycan branching, that is, inhibition of β1,6-GlcNAc transfer by GnT-V and of core fucosylation by Fut8.[14–16]

Despite the synthetic challenge and because of its importance in cellular events, the chemical synthesis of *N*-glycans with bisecting GlcNAc has now been successfully achieved.[17–21] One example of an answer to overcoming some of the difficulties in the synthesis has been to couple bisecting GlcNAc before the introduction of the 1,3 and 1,6-branch mannose to avoid steric hindrance.

'Designed' bisected glycans through synthetic approaches are of great importance for analyses of structure, dynamics and interactions. The synthetic bisected hexasaccharide **1** has been used as a ligand for mouse dendritic cell inhibitory receptor 2 (DCIR2), which allowed the ligand recognition mode of DCIR2 to be revealed by X-ray crystallography and NMR.[22] Crystallographic analysis of a legume lectin PHA-E, which is widely used for detection of bisected glycans, was assisted using chemically synthesized ligands **1**.[23,24]

The substrate specificity of GnT-III has been studied using truncated substrates at the non-reducing terminus.[25] GnT-III requires a β-GlcNAc residue on the α1,3-Man which is transferred by GnT-I,

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while the other β-GlcNAc residue on the α1,6-Man moiety is not essential.[25] The addition of further β1,4-galactose units on the α1,6-Man has little effect on the activity of GnT-III while the addition of galactose onto α1,3-Man branch inhibits the GnT-III reaction.[26] Glycans with tri- or tetra-antenna are also accepted as substrates. In contrast, there are limited studies of the importance of the chitobiose unit.

Here we have investigated the structural requirements on the acceptor substrate of GnT-III using a series of synthetic glycans. To achieve the purpose, we designed branching structures **2** and **4** including core β-mannose having C4-OH which is the acceptor hydroxy group of bisecting GlcNAc, but excluding the chitobiose moiety at non-reducing termini (Fig. 1). We also synthesized compounds **1** and **3**, to investigate the effect of bisecting GlcNAc on GnT-III binding. The design focuses on the GlcNAc residues surrounding to the core trimannnose unit. The synthesis was achieved using common building blocks to construct bisectGN-Man_3GN_2 **1**, Man_3GN_2 **2**, bisectGN-Man_3 **3**, and Man_3 **4**, respectively. Using these synthetic glycans, we performed a competitive inhibition assay to reveal the key sugar units for the acceptor recognition by GnT-III.

The substrate specificity of glycosyltransferases including GnT-III has previously been studied using a series of glycans purified from natural sources. Such approaches, however, often have limitations in identifying minimum structural requirements especially when the enzyme acts on inner glycan residues. Since GnT-III is expected to recognize the sugar residues emanating from the β-mannose, we considered that studying individual parts of the whole glycan structure could be very rewarding.

Glycan units **1**, **2**, **3**, and **4** were systematically synthesized using a parallel approach through common intermediates **12a** and **12b** (Schemes 1 and 2).[17,27–29] Synthesis of tetrasaccharide **3** was initiated from diallyl protected mannoside **5**[17] (Scheme 1). To avoid difficulty of the introduction by steric reason, the bisecting GlcNAc was introduced at an early stage of the synthesis. It was inserted on the O4 of mannoside **5** using the imidate building block **6**[19] via an activation with catalytic amounts of TMSOTf[30] to give disaccharide **7**. Then, the allyl protection at the 3,6-positions was readily removed via a 2-step procedure involving treatment with Iridium complex and then I_2 in H_2O to give acceptor **8**. Mannosylation using imidate **9** by activation with TMSOTf in the presence of MS 4 Å gave core tetrasaccharide **10**. This was then treated with 1,2-ethylenediamine in 1-butanol at 100 °C followed by acetylation of amino and hydroxy groups to quantitatively yield **11**. Deacetylation of hydroxy groups using NaOMe provided **12a** in 82% yield (3 steps). Finally, removal of all benzyl ethers on **12a** using 20% $Pd(OH)_2/C$ under a hydrogen atmosphere gave the desired tetrasaccharide with bisecting GlcNAc **3** in 78% yield. The trisaccharide counterpart **4** was synthesized according to the previously reported procedure.[31]

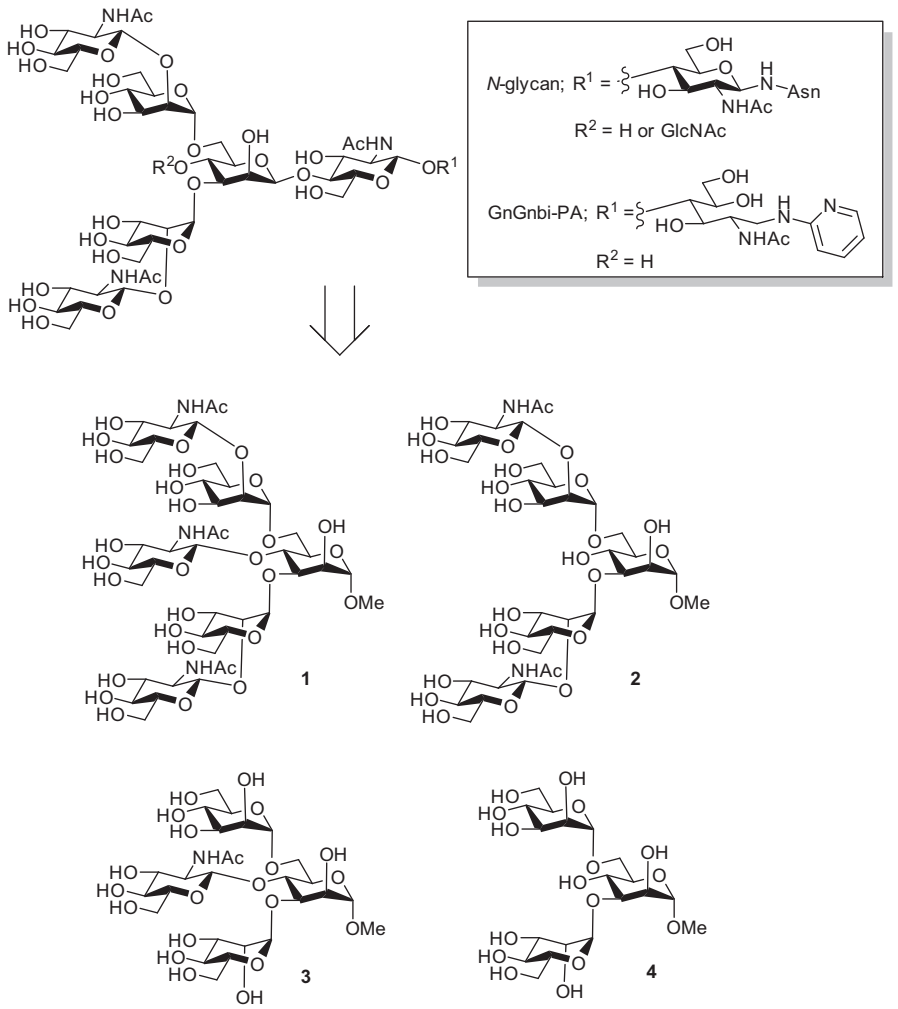
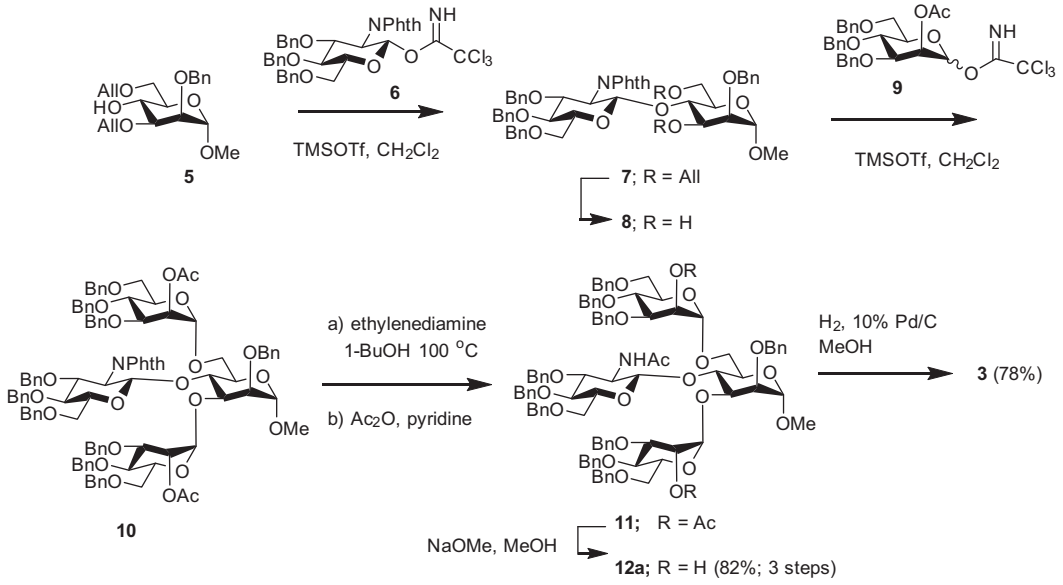
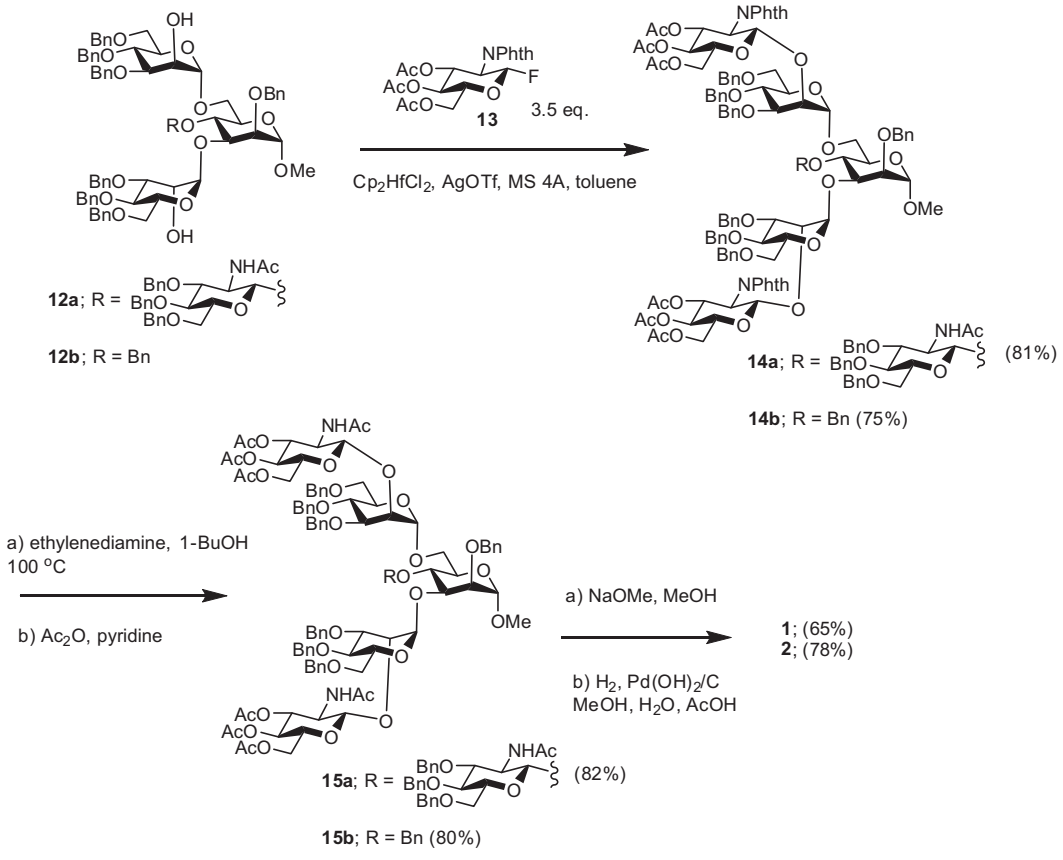


Figure 1. Structures of bisected *N*-glycans and synthetic bisected *N*-glycan units (bisectGN-Man_3GN_2) **1** and (bisectGN-Man_3) **3**, and corresponding bibranched glycan (Man_3GN_2) **2** and pauch-mannose (Man_3) **4**. PA; pyridylamino.



Scheme 1. Synthesis of the bisected tetrasaccharide **3**.



Scheme 2. Parallel syntheses of bisected hexasaccharide **1** and bibranched pentasaccharide **2**.

The syntheses of bisecting hexasaccharide **1** and non-bisecting pentasaccharide **2** are shown in Scheme 2. Synthetic intermediates **12a** and **12b** were employed as the glycosyl acceptors. The tetrasaccharide **12a** and trisaccharide **12b**[31] were coupled with fluoride **13**[32] following activation with Cp_2HfCl_2 and AgOTf[33,34] in toluene in the presence of MS 4 Å, to give corresponding **14a** and **14b** in 81% and 75% yield, respectively. Global deprotection gave desired glycans **1** and **2**. Phthalimide groups were removed by treatment with 1,2-ethylenediamine in 1-butanol at 100 °C, and re-acetylation provided **15a** and **15b** in 82% and 80% yield, respectively. Then hydrogenolysis with 20% $Pd(OH)_2/C$ in methanol produced **1** and **2** in 65% and 78% yield, respectively.

Possible interaction of the synthetic glycans **1**, **2**, **3**, and **4** with GnT-III was studied through the use of a competitive inhibition

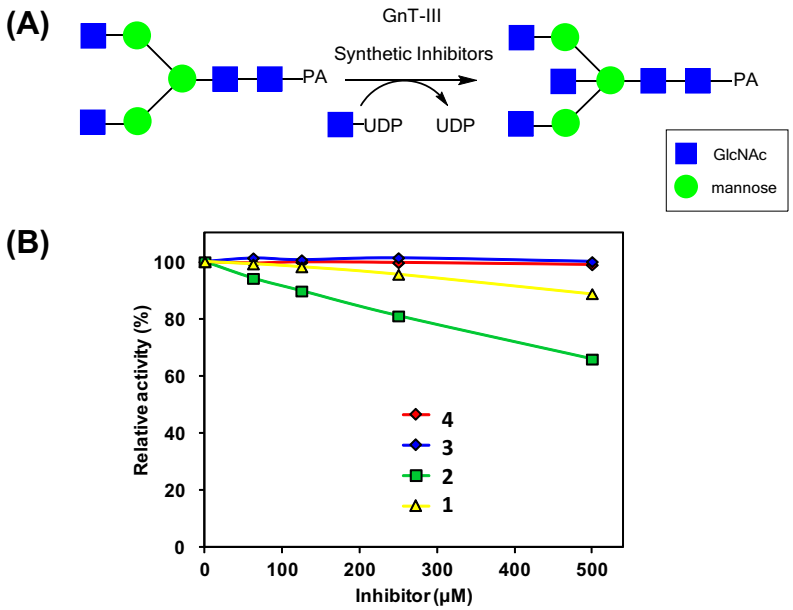


Figure 2. Substrate specificity of GnT-III by observing inhibition with the synthetic substrates **1**, **2**, **3**, and **4**. (A) GlcNAc transfer scheme by GnT-III. (B) The relative sugar transfer rate by GnT-III at each inhibitor concentration. The model substrate GnGnbi-PA was used at 5 μM, and synthetic substrates added to 62.5, 125, 250, and 500 μM, respectively. The time-course quantification of substrate and product was performed by HPLC analysis with fluorescence detection.

assay (Fig. 2). The bi-branched *N*-glycan carrying a pyridylamino (PA) group at the reducing terminus (GnGnbi-PA) served as a typical acceptor substrate and UDP-GlcNAc as a donor substrate.[35,36] GnT-III activity and GlcNAc transfer was evaluated by a shift in HPLC retention time from GnGnbi-PA to bisectGlcNAc-GnGnbi-PA. Results indicated that GN_2Man_3 **2** has a significant inhibition activity. The bisected GlcNAc-GN_2Man_3 **1**, a product of GnT-III, showed less but significant competition activity. Mannose-terminated **3** and **4** were not inhibitory, even in 100-fold excess.

Evidently, the bibranched structure **2** is a substrate of GnT-III. This finding is consistent with a previous study using synthetic glycan with a β-*O*-octyl group at the anomeric position of the core-mannose.[37] The relative catalytic efficiencies can now be understood. Compound **1** is significantly inhibitory, indicating that GnT-III is sensitive to product inhibition. Furthermore, the GlcNAc of the Manβ(1–4)GlcNAc moiety is highly recognized by GnT-III. Clearly, GnGnbi-PA is a good substrate for GnT-III and can be usefully assayed at a concentration of 5 μM. The synthetic glycans **1** and **2** were moderately competitive at rather high concentrations (62.5–500 μM). Structural differences between substrates GnGnbi-PA and **2** are the sugar units at the reducing termini, the GlcNAc residues on Manβ(1–4)GlcNAc and the PA tag. The penultimate GlcNAc residue moiety is important for recognition by GnT-III. Additionally, previous data showed that β-alkyl linked pentasaccharides are a substrate of GnT-III.[37] Our data, taken together with the previous studies, suggests that GnT-III likely recognizes sugar residues between GlcNAc residues at the α1,3-Man branch and Manβ(1–4)GlcNAc. Unfortunately, 3D structural information on GnT-III is not available and further details of the role of the key residues must await atomic structures.

The inhibition constant (K_i) of the synthetic compounds **1** and **2** were determined by kinetic assay (Table 1). The substrates of GnT-III, either GnGnbi-PA or UDP-GlcNAc is used in a single concentration, and the concentrations of the other substrate and the synthetic compound were changed. The apparent K_i values in the presence of a fixed concentration (5 μM) of the acceptor GnGnbi-PA are similar in hexasaccharide **1** (1.5 mM) and pentasaccharide **2** (1.4 mM). In contrast, the apparent K_i values in the presence of a fixed concentration (20 mM) of the donor UDP-GlcNAc are 14 mM in hexasaccahride **1** and 1.2 mM in pentasaccharide **2**, which causes the higher inhibition efficiency of **2**. One-order difference of K_i in **1** and **2** in the presence of 20 mM UDP-GlcNAc is explained by the differences in competition efficiency toward the preferential substrate, GnGnbi-PA.

In summary, we have investigated the substrate structural requirements of GnT-III using a series of putative synthetic substrates and products. Syntheses of the individual branching parts of *N*-glycans were achieved through systematic coupling of core tri/tetrasaccharide acceptors and GlcNAc donor. A competitive inhibition assay suggests that GlcNAc residues connecting the core-trimannose play a vital role in recognition by GnT-III. An understanding of the substrate structural requirements of GnT-III will assist in uncovering how *N*-glycan biosynthesis is controlled and help design specific glycosyltransferase inhibitors.[38]

Table 1
Apparent K_i values of the synthetic compounds **1** and **2** in the inhibition of GnT-III

Compound	K_i (mM)	
	UDP-GlcNAc[a]	GnGnbi-PA[b]
1	1.5	14
2	1.4	1.2

[a] GnGnbi-PA (5 μM), UDP-GlcNAc (0.5, 1, 2, 4 mM), and **1** or **2** (0, 125, 250, 500 μM).
[b] GnGnbi-PA (2.5, 5, 10 μM), UDP-GlcNAc (20 mM), and **1** or **2** (0, 125, 250, 500 μM).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.074.

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