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Convergent synthesis of a bisecting GlcNAc-containing *N*-glycan

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Abstract: The chemical synthesis of a bisecting *N*-acetylglucosamine (GlcNAc)-containing *N*-glycan was achieved by a convergent synthetic route via [4+2] and [6+2] glycosylations. This synthetic route reduced the number of reaction steps, although the key glycosylations were challenging in terms of yields and selectivities due to steric hindrance at the glycosylation site and a lack of neighboring group participation. The yields of these glycosylations were enhanced by stabilizing the oxocarbenium ion intermediate through ether coordination. Glycosyl donor protecting groups were explored in an effort to realize perfect α-selectivity by manipulating remote participation. We investigated the simultaneous glycosylations of a tetrasaccharide with two disaccharides to efficiently construct a bisecting GlcNAc-containing *N*-glycan.

Introduction

Glycosylation on protein is the most common post-translational modification. Asparagine-linked glycans (*N*-glycans) have a high structural diversity and are categorized as high-mannose-type, complex-type, or hybrid-type oligosaccharides. *N*-Glycans provide diverse physiological functions. They control protein quality,[1] protein dynamics,[2] cell development,[3] cancer invasion[4] and immunity,[5] depending on their type, modification, and position on a protein. *N*-acetylglucosaminyltransferase III (GnT-III) is an *N*-glycan processing enzyme that transfers GlcNAc to the branching mannose with a β1,4-linkage to form a bisecting *N*-acetylglucosamine (GlcNAc).[6] The bisecting GlcNAc moiety is a modification characteristic of complex-type *N*-glycans and has a variety of unique biological functions.[7]

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Bisecting GlcNAc appears to provide diverse functions via several mechanisms. For example, expression of bisecting GlcNAc suppresses tumor metastasis.[8] GnT-III acts on the same substrate with GnT-V and reduces the GnT-V product β1-6 GlcNAc branching structure, resulting in inhibition of metastasis. A bisecting GlcNAc group on a synthetic IgG glycoform significantly enhances binding between Fc and FcγRIIIa, thereby activating the Fcγ receptor and increasing the antibody-dependent cellular-cytotoxicity (ADCC) of IgG.[9] Alzheimer's disease (AD) patients have higher levels of bisecting GlcNAc on the β-site amyloid precursor protein cleaving enzyme-1 (BACE1). In GnT-III-deficient mice, the BACE1 lacking bisecting GlcNAc was localized to late endosomes/lysosomes and lysosomal degradation of BACE1 was accelerated. Since GnT-III-deficient mice showed the decrease in Aβ plaques and improved cognitive function, GnT-III may be a promising drug target for AD therapeutics.[10] Certain lectins can directly recognize bisecting GlcNAc moieties. For example, Calsepa and PHA-E lectins bound to the 'back-fold' conformation of the bisected *N*-glycan. The statistical conformational analysis suggests that bisecting GlcNAc restricts the conformations of branched structures in *N*-glycans.[11]

A sufficient supply of pure *N*-glycans containing bisecting GlcNAc is needed for the further investigation of the biological functions of these groups. Motivated by this need, we investigated the chemical synthesis of bisecting GlcNAc-containing *N*-glycans.

The syntheses of *N*-glycans have been extensively explored by several groups. Danishefsky et al. achieved the syntheses of various types of *N*-glycans, including *N*-glycans containing bisecting GlcNAc, multi-antennary *N*-glycans, and others.[12] Unverzagt et al. synthesized several *N*-glycans containing bisecting GlcNAc or core fucose.[13] We have reported the synthesis of *N*-glycan-containing core fucose and sialic acids.[14] Ito et al.,[15] Boons et al.,[16] Wang et al.,[17] and Wong et al.[18] reported the syntheses of *N*-glycan libraries using chemoenzymatic approaches. Solid-phase syntheses of complex-type *N*-glycans have been reported by Schmidt et al.[19] and by our group.[20]

The synthetic approach to obtain bisecting GlcNAc-containing *N*-glycan **1** is shown in Figure 1. An octasaccharide was constructed via two glycosylations at the 3- and 6-positions of mannose in the tetrasaccharide **2** using the disaccharides **3**–**7**.

One of the key steps in the synthesis of **1** involved the introduction of a bisecting GlcNAc. Unverzagt et al. introduced the bisecting GlcNAc at the final stage of glycan construction.[13b, 13c, 13e] They achieved the efficient glycosylation of a bisecting GlcNAc at the 4-position of the branching mannose using trifluoroacetamide-protected *N*-glycans.[13e] Here, we instead elongated the bisecting GlcNAc to form a reducing end fragment

prior to introducing the branching disaccharides **3**–**7**. This approach is similar to Danishefsky's route.[12b]

Other important steps involved the construction of the branching structure. Previous studies explored two major strategies. The first strategy involved the introduction of glucosaminyl donors to acceptors containing a trimannosyl core that could be constructed via stereoselective α-mannosylation using the neighboring group participation of a 2-*O*-acyl-protected mannose residue. The other strategy involved a convergent synthetic route in which the branching structure was constructed via two glycosylations at the branching mannose residue. Although the latter strategy required fewer reaction steps than the former, neighboring group participation was not available in the glycosylations of the reducing end fragments and the branch fragments. Here, we employed the latter strategy. After thoroughly exploring the reaction conditions, the key glycosylations of the large fragments were achieved in good to moderate yields in the presence of ether as a solvent. The α-selectivity was improved by designing and synthesizing the mannosyl donors **3**–**7**. Per-acylated mannosyl donor **7** gave perfect α-selectivity by permitting remote group participation.[21] We also demonstrated the stepwise and simultaneous introduction of branching fragments at the 3- and 6-positions of mannose. The stepwise method enabled the syntheses of asymmetric *N*-glycans, whereas the simultaneous method reduced the number of reaction steps needed.

The other important feature of our synthesis involved the early-stage introduction of asparagine.[14] An asparagine residue was generally attached after deprotecting the glycan.[22] We achieved *N*-glycosylation between the glucosaminyl donor and the asparagine acceptor.[23] Here, The sugar chain was elongated from glucosaminyl asparagine which is obtained by *N*-glycosylation. Our method facilitated the preparation of an *N*-glycan-processing asparagine residue because conversion was not necessary after deprotection, and the protected glycosyl asparagines could be easily handled.

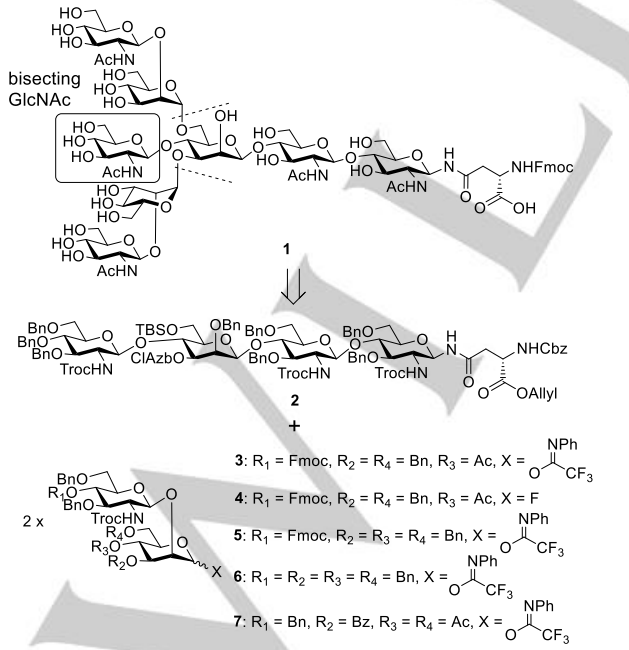
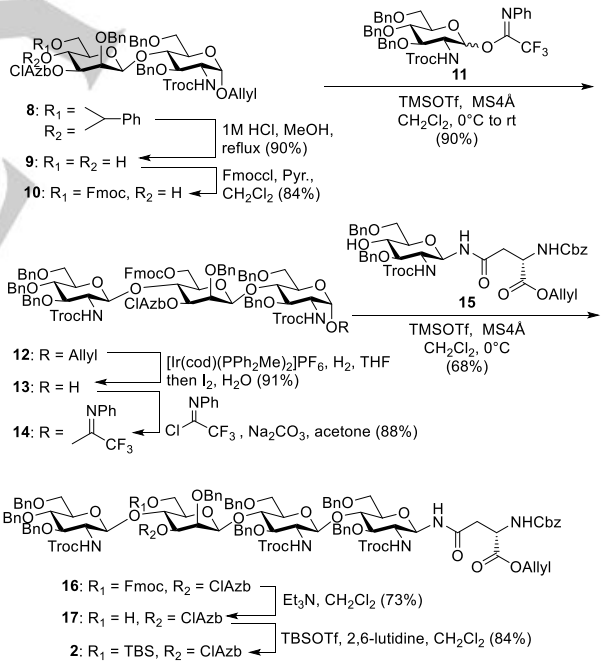


Figure 1. Synthetic plan of bisecting GlcNAc-containing *N*-glycan **1**.

Results and Discussion

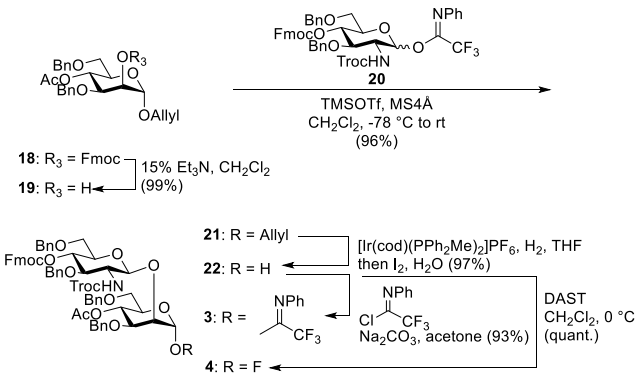
The synthesis of the asparagine-linked tetrasaccharide **2** was initiated from disaccharide **8**,[14, 20] which was synthesized via selective β-mannosylation, as reported by our group (Scheme 1). β-Selective mannosylation was achieved by activating 4,6-benzyliden protected mannosyl donor at low temperature (-78 ºC). Precise temperature control was the key of this glycosylation to achieve high β-selectivity (α/β≈1/4). After cleavage of the benzylidene group, an Fmoc group was selectively introduced at the 6-position of mannose to afford **10**. Glycosylation of **10** with *N*-pheny-2,2,2-trifluoroacetimidate[24] glucosaminyl donor **11** afforded trisaccharide **12** in 90% yield with perfect β-selectivity without formation of oxiazoline.[25] Trisaccharide **12** was then converted to glycosyl donor **14** via cleavage of the allyl group using an iridium complex, followed by imidation with *N*-phenyl-2,2,2-trifluoroacetimidoyl chloride.[24] Glycosylation between **14** and **15**,[14, 23, 25] synthesized by *N*-glycosylation, afforded tetrasaccharide **16** in 68% yield. The protecting group at the 6-position of mannose in **16** was converted from Fmoc to TBS to give **2**.



Scheme 1. Synthesis of asparagine-linked tetrasaccharide **2**.

Glycosyl donors **3** and **4** were synthesized to have different leaving groups (Scheme 2). After cleavage of the Fmoc group of **18**,[20] obtained **19** was glycosylated with glucosaminyl donor **20**[20] to give disaccharide **21** in 96% yield.[25] Cleavage of the

allyl group, followed by imidation or fluorination, afforded glycosyl donors **3** or **4**, respectively.



cheme 2. Syntheses of glycosyl donors **3** and **4**.

Prior to investigating the glycosylations of tetrasaccharide asparagine **2** with disaccharides **3** or **4**, a model study using trisaccharide **25** with **3** or **4** was carried out (Scheme 3, Table 1). After TBS protection of **9**, the resulting **23** was glycosylated with **11** to give **24**. Cleavage of the 4-azido-3-chlorobenzyl (ClAzb) group[26] in **24** via the Staudinger reaction, followed by DDQ treatment, afforded **25**. The glycosylations of **25** with **3** or **4** were then investigated. The solvents were examined using imidate **3** as a glycosyl donor and TMSOTf as an activator (Scheme 3, Table 1, entries 1–6). Although the reaction did not proceed in CH_2Cl_2, THF, or EtCN (Scheme 3, Table 1, entries 1, 4, 5), desired pentasaccharide **26** was obtained using Et_2O, CPME or toluene:dioxane = 10:1 (Scheme 3, Table 1, entries 2, 3, 4). Et_2O, in particular, gave higher yields compared to other solvents. The ether solvent probably stabilized the oxocarbenium ion intermediate generated from glycosyl donor **3** to give a better yield, as observed in previous studies of the synthesis of a core fucose-containing *N*-glycan.[14] Although ether solvent is generally expected to enhance α-selectivity, especially, in the case of mannosylation,[27] α-selectivity was poor in these glycosylations. The TBSOTf or TfOH activators in Et_2O gave lower yields compared to TMSOTf, although TfOH provided a higher α-selectivity (Scheme 3, Table 1, entries 2, 7, 8). The yields were improved dramatically at lower reaction temperatures: a 90% yield was obtained using TMSOTf, a 66% yield was obtained using TfOH (Scheme 3, Table 1, entries 2, 8, 9, 10). Next, glycosylation using glycosyl fluoride **4** was investigated. As with the investigation using glycosyl imidate **3**, ether gave a good yield compared to the other solvents (Scheme 3, Table 1, entries 11, 12). A variety of activators of glycosyl fluoride were investigated; however, the yields remained moderate to low (Scheme 3, Table 1, entries 12–17). To summarize these investigations, glycosylation with a sterically hindered acceptor gave good yields in Et_2O at low temperatures (Scheme 3, Table 1, entry 9), although α-selectivity was not satisfactory. After cleavage of the TBS group of **26**, the desired α-isomer was isolated to give pure **27**.

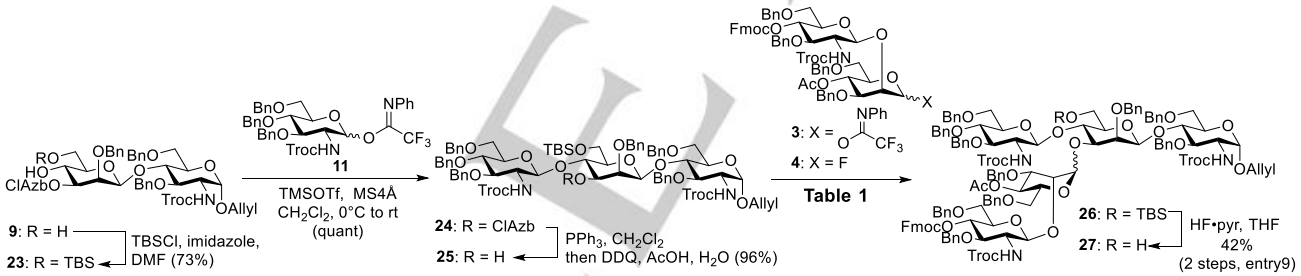


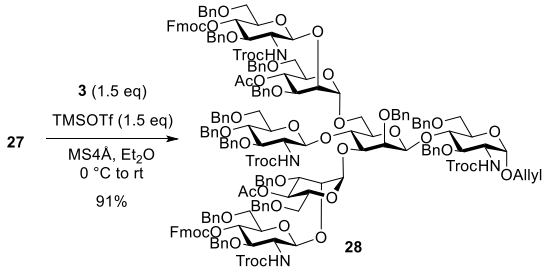
Table 1

entry	donor (1.5 eq)	solvent	activator (eq)	temperature	yield	α/β[a]
1	**3**	CH_2Cl_2	TMSOTf (1.5 eq)	0 °C to rt	ND	-
2	**3**	Et_2O	TMSOTf (1.5 eq)	0 °C to rt	52%	1.5/1
3	**3**	CPME	TMSOTf (1.5 eq)	0 °C to rt	37%	1.5/1
4	**3**	Toluene/dioxane = 10/1	TMSOTf (1.5 eq)	0 °C to rt	27%	1.7/1
5[b]	**3**	THF	TMSOTf (1.5 eq)	0 °C to rt	ND	-
6	**3**	EtCN	TMSOTf (1.5 eq)	0 °C to rt	trace	-
7	**3**	Et_2O	TBSOTf (1.5 eq)	0 °C to rt	37%	1.6/1
8	**3**	Et_2O	TfOH (1.5 eq)	0 °C to rt	37%	2.3/1
9	**3**	Et_2O	TMSOTf (1.5 eq)	-40 °C to rt	90%	1.1/1
10	**3**	Et_2O	TfOH (1.5 eq)	-40 °C to rt	66%	1.3/1
11	**4**	CH_2Cl_2	Cp_2HfCl_2 (1.5 eq), AgOTf (1.5 eq)	0 °C to rt	ND	-
12	**4**	Et_2O	Cp_2HfCl_2 (1.5 eq), AgOTf (1.5 eq)	0 °C to rt	51%	1.7/1
13	**4**	Et_2O	Cp_2ZrCl_2 (1.5 eq), AgOTf (1.5 eq)	0 °C to rt	40%	1.3/1
14	**4**	Et_2O	$Cp_2Zr(OTf)_2$ • THF (1.5 eq)	0 °C to rt	36%	1.3/1
15	**4**	Et_2O	Cp_2HfCl_2 (1.5 eq), $AgClO_4$ (1.5 eq)	0 °C to rt	ND	-
16	**4**	Et_2O	Cp_2ZrCl_2 (1.5 eq), $AgClO_4$ (1.5 eq)	0 °C to rt	37%	1.8/1
17[b]	**4**	Et_2O	TfOH (1.5 eq)	0 °C to rt	ND	-

[a] estimated by NMR analysis, [b] MS5A was used

Scheme 3. Model study of the glycosylations of **25** with **3** and **4**.

A model study of the second mannosylation was conducted (Scheme 4). Glycosylation at the 6-position of mannose of **27** with glycosyl imidate **3** was carried out using TMSOTf as an activator in Et_2O to give desired octasaccharide **28** in 91% yield with perfect α-selectivity.



Scheme 4. Model study of glycosylation between **27** and **3**.

In these model studies, a branching structure was constructed in good yield; however, the stereoselectivity of glycosylation between **25** and **3** was low. The low selectivity was considered to be attributed to remote participation, as reported by Kim et al.[21] (Figure 2). To improve the α-selectivity of these key mannosylations, we designed new glycosyl donors **5** and **6**, which did not include an acyl group at the 4-position, to avoid remote participation. Compound **5** enabled further sugar elongation after cleavage of the Fmoc group, whereas compound **6** was readily synthesized and required a simpler deprotection process compared to **5**.

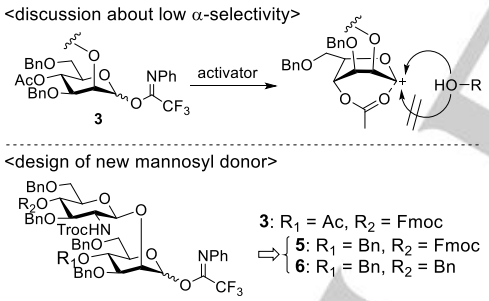
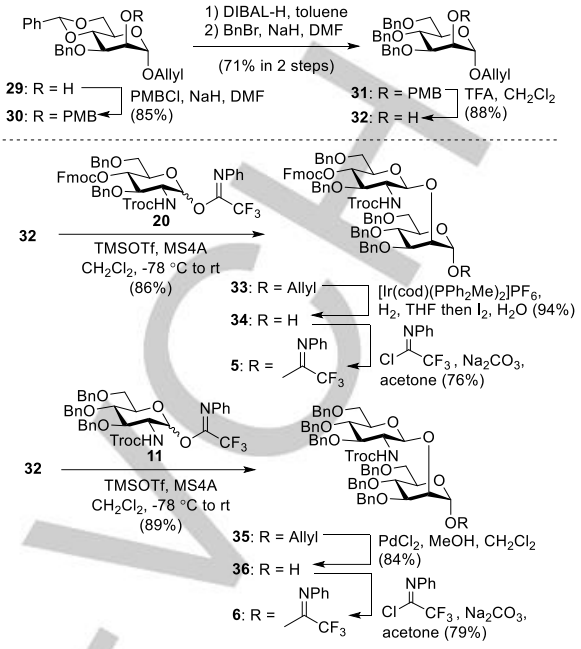


Figure 2. The α-selectivity of the key mannosylation was low, motivating the design of new mannosyl donors **5** and **6**.

Newly designed mannosyl donors **5** and **6** were synthesized (Scheme 5). After PMB protection of **29**,[20] reductive cleavage of the benzylidene of **30** was followed by benzylation to afford **31**. The PMB group was cleaved to give **32** for further glycosylation. Glycosylation between **32** and **20** gave the disaccharide **33** in 86% yield.[25] Cleavage of the allyl group of **33**, followed by imidation, afforded glycosyl imidate **5**. Glycosyl imidate **6** was synthesized in the same manner. After glycosylation between **32** and **11**, the allyl group was converted to the imidate to give **6**.

The two glycosylations at the 3- and 6-positions of the branching mannose constructed a bisecting GlcNAc-containing *N*-glycan skeletal structure. These glycosylations were investigated using



Scheme 5. Syntheses of glycosyl donors and **6**.

mannosyl donors **3**, **5**, and **6** (Scheme 6). Cleavage of the ClAzb group of **2** afforded **38** and the [4+2] glycosylations between **38** and **3**, **5**, or **6** were investigated. The use of **3**, which was expected to have undesired remote participation, provided desired hexasaccharide **39** in 70% yield with low α-selectivity (α/β = 1.5/1, Scheme 6, Table 2, entry 1). Glycosylations using newly designed **5** or **6** showed moderate yields and improved α-selectivities (**5**: 38%, α only, **6**: 53%, α/β = 3/1, Scheme 6, Table 2, entries 2, 3). After cleavage of the TBS group with HF·pyridine, the [6+2] glycosylations of **42**, **43**, and **44** were investigated. In these reactions, glycosyl donors **3** and **5** both provided perfect α-selectivity (**42**+**3**: 34%, α only, **43**+**5**: 52%, α only, Scheme 6, Table 3, entries 1, 2). On the other hand, glycosylation using **6** did not give the desired octasaccharide **47** because the oxocarbenium ion generated from **6** decomposed smoothly, even at low temperatures, due to its high reactivity (Scheme 6, Table 3, entry 3). The employment of milder conditions using TBSOTf provided **47** in 55% yield with a low α-selectivity (α/β = 1/1, Scheme 6, Table 3, entry 4). In this case, S_N2-like reaction from the α-isomer of glycosyl imidate **6** probably competed to afford a substantial amount of the undesired β-product.

The global deprotection of **47** was carried out (Scheme 7). Allyl cleavage of **47** with palladium gave carboxylic acid **48**.[28] After removal of all acyl and Troc groups under basic conditions,[29] acetyl groups were introduced to the amino groups. Hydrogenation, followed by Fmoc protection of the amino group of asparagine afforded desired bisecting GlcNAc-containing *N*-glycan **1** in 53% yield in 4 steps. Fmoc group was introduced to purify the deprotected octasaccharide by HPLC using an ODS column.

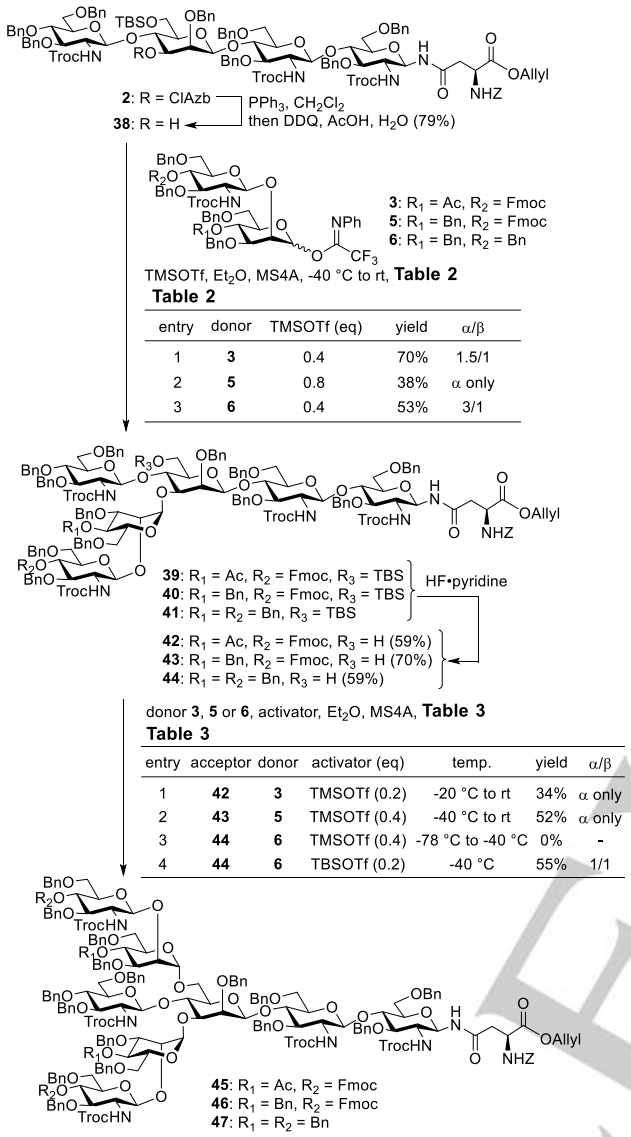


Table 2

entry	donor	TMSOTf (eq)	yield	α/β
1	**3**	0.4	70%	1.5/1
2	**5**	0.8	38%	α only
3	**6**	0.4	53%	3/1

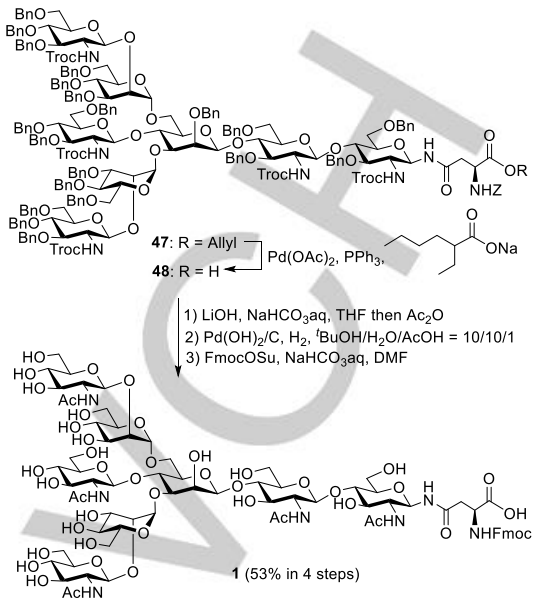
Table 3

entry	acceptor	donor	activator (eq)	temp.	yield	α/β
1	**42**	**3**	TMSOTf (0.2)	-20 °C to rt	34%	α only
2	**43**	**5**	TMSOTf (0.4)	-40 °C to rt	52%	α only
3	**44**	**6**	TMSOTf (0.4)	-78 °C to -40 °C	0%	-
4	**44**	**6**	TBSOTf (0.2)	-40 °C	55%	1/1

Scheme 6. Investigations of the [4+2] glycosylations and [6+2] glycosylations.

Although we achieved the chemical synthesis of bisecting GlcNAc-containing *N*-glycan **1**, the α/β-selectivities of the key [4+2] and [6+2] glycosylations were unsatisfactory. To achieve α-selective mannosylation, we redesigned new mannosyl donor **7** (Figure 3). The acyl groups introduced at the 3- and 6-positions of **7** were expected to enhance α-selectivity by remote participation.[21]

Compound **7** was synthesized, as shown in Scheme 8. Selective benzylation at the 3-position of **49**[20] using Me_2SnCl_2 afforded **50**.[30] Glycosylation between **50** and **11** gave disaccharide **51**. After converting the protecting groups from benzylidene to acetyl groups, allyl deprotection of **52**, followed by imidation of **53**, gave **7**.



Scheme 7. Global deprotection of bisecting GlcNAc-containing *N*-glycan **47**.

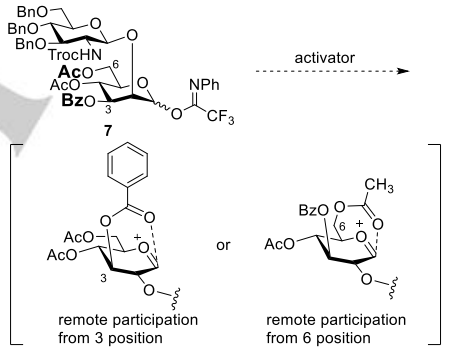
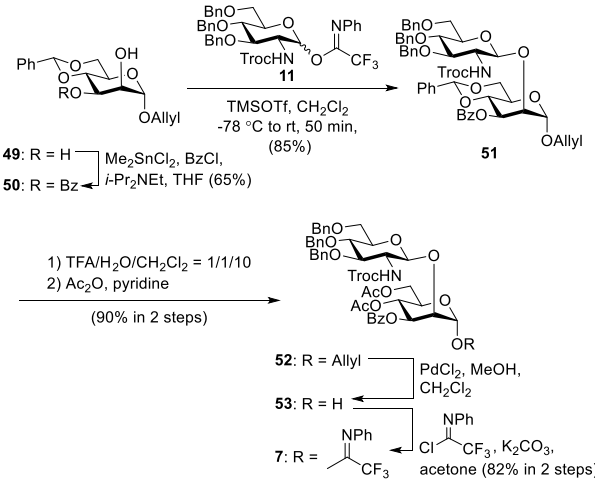


Figure 3. Redesign of glycosyl donor **7**.



Scheme 8. Synthesis of glycosyl donor **7**.

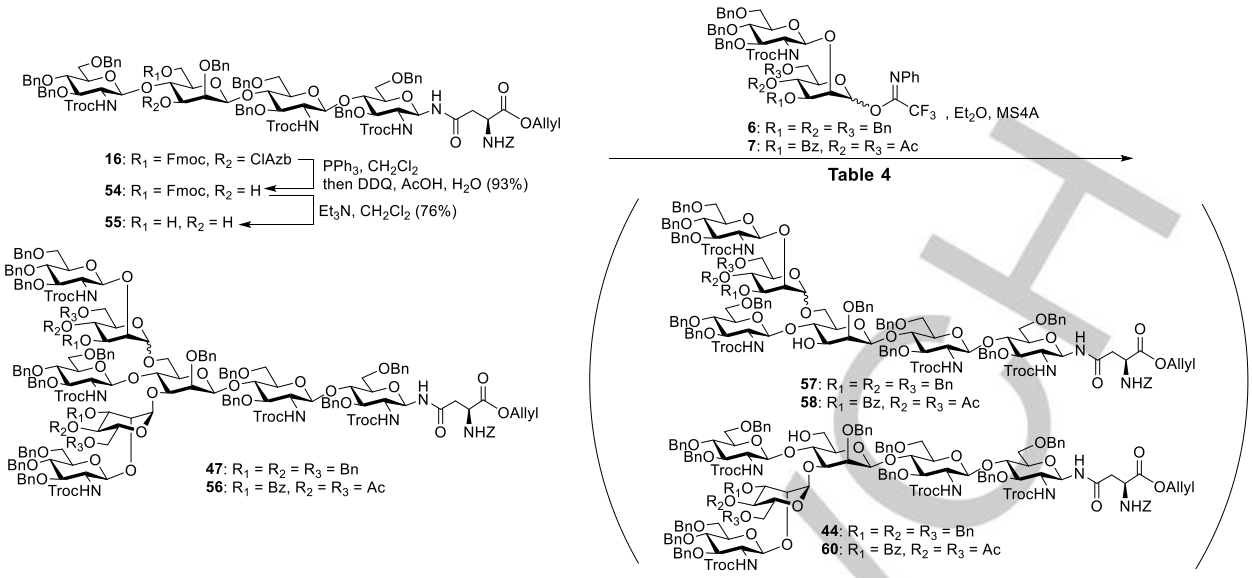


Table 4

entry	donor (eq)	activator (eq)	temp.	yield(%)		
				47 or **56**	**57** or **58**	**44** or **60**
1	**6** (5.0)	TMSOTf (0.8)	-40 °C	27 (α/β = 3/1)	30	13
2	**6** (5.0)	TMSOTf (0.8)	0 °C	50 (α/β = 2/1)[2]	50	nd[1]
3	**6** (5.0)	TfOH (0.8)	0 °C	62 (α/β = 3/1)	15	15
4	**7** (5.0)	TfOH (0.8)	0 °C	52 (α only)[3]	23	nd[1]
5	**7** (5.0)	TMSOTf (5.0)	0 °C	78 (α only)[3]	18	nd[1]

[1]not detected, [2]estimated by TLC, [3]determined by HPLC

Scheme 9. Investigation of simultaneous glycosylation between **55** and **6** or **7**

In addition to utilizing newly designed mannosyl donor **7**, we investigated the simultaneous glycosylations at the 3- and 6-positions of branching mannose to achieve the efficient construction of a bisecting GlcNAc-containing *N*-glycan skeletal structure (Scheme 9). After cleavage of the ClAzb and Fmoc groups of **16**, glycosylations between **55** and **6** or **7** were investigated. When **6** was used as a glycosyl donor, relatively harsh conditions gave good results (Scheme 9, Table 4, entries 1–3). Activation by TfOH at 0 ºC gave desired octasaccharide **47** in 62% yield (Scheme 9, Table 4, entry 3). In this case, the undesired β-isomer at the 6-position was also obtained (α/β = 3/1). Activation of **7** with TfOH provided octasaccharide **56** in 52% yield with perfect α-selectivity (Scheme 9, Table 4, entry 4). Activation of **7** by TMSOTf gave excellent results: octasaccharide **56** was obtained in 78% yield with perfect α-selectivity (Scheme 9, Table 4, entry 5). As shown here, an α-selective mannosylation was achieved by manipulating the protecting group of the glycosyl donor.

Conclusions

In summary, the chemical synthesis of a bisecting GlcNAc-containing *N*-glycan was achieved. We developed a convergent synthetic route in which a branching structure was constructed via two glycosylations at the 3- and 6-positions of a branching mannose. This synthetic strategy reduced the number of reaction steps. High yields of the key glycosylations were realized by stabilizing the oxocarbenium ion intermediate through coordination of the ether solvent. α-Selective mannosylations were achieved by manipulating the mannosyl donor protection pattern to ensure remote participation. This study provides a universal synthetic strategy for the efficient synthesis of various types of *N*-glycans in an effort to accelerate functional studies of synthetic *N*-glycans.

Experimental Section

The synthetic procedures and characterization of the compounds studied herein can be found in the Supporting Information.

Acknowledgements

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Keywords: *N*-glycan • oligosaccharides • bisecting GlcNAc • glycosylation

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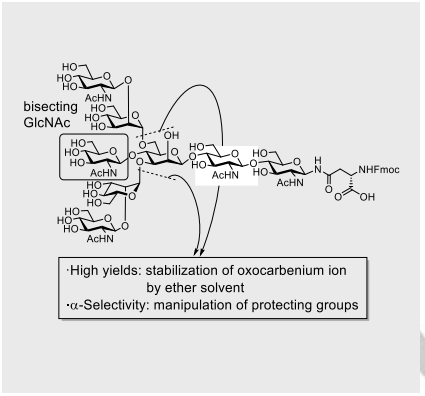
FULL PAPER

Synthesis of a bisecting GlcNAc-containing *N*-glycan: The convergent synthesis of a bisecting GlcNAc-containing *N*-glycan was achieved via two glycosylations at branching positions. In these key glycosylations, high yields were obtained by stabilizing the oxocarbenium ion intermediate in ether, and perfect α-selectivity was achieved by manipulating the protecting groups.



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