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Efficient synthesis of antigenic trisaccharides containing *N*-acetylglucosamine: protection of NHAc as NAc_2

Masato Tsutsui,[†[a]] Julinton Sianturi, [†§[a]] Seiji Masui,[[a]] Kento Tokunaga,[[a]] Yoshiyuki Manabe,*[[a, b]] and Koichi Fukase*[[a, b]] ([†]equal contribution)

Abstract: The antigenic trisaccharides, α-gal epitope and H antigen, containing *N*-acetyl-D-glucosamine (GlcNAc) were synthesized using a diacetyl strategy, in which NHAc is tentatively converted to NAc_2 during oligosaccharide construction. Acetylation of NHAc in GlcNAc significantly improved the reactivity in glycosylation reactions. The diacetyl strategy allowed to achieve the efficient synthesis of α-gal via a sequential one-pot and one-flow procedure. Meanwhile, H antigen was synthesized by stepwise elongation from the reducing end side of GlcNAc. The enhancement of the reactivity by NAc_2 protection was observed in both glycosylations at proximal and distal positions to GlcNAc. This diacetyl strategy is expected to be applicable to the synthesis of a wide range of glycans.

Introduction

Glycans are involved in various biological phenomena including self and nonself recognition, viral and bacterial infection, immunoregulation, cancer invasion, and cell development.[1] Bacterial glycoconjugates such as lipopolysaccharide and peptidoglycan are known to activate innate immunity.[2] More than 60% of proteins are posttranslationally modified with glycans, which control protein folding and quality,[3] *in vivo* biological activity,[4] circulatory residence,[5] and receptor function[6]. Since natural glycans have high diversity and heterogeneity, the production of pure glycans by chemical synthesis has been a powerful tool for the elucidation of the biological function of glycans.

Many glycans act as antigens, among which ABO blood group antigens stand out. ABO blood types are classified according to the glycan structure on erythrocytes. O blood type individuals express H antigen (Figure 1), which is also a precursor of ABO blood antigens. The interaction between ABO blood antigens and their natural antibodies causes blood agglutination.[7] They are also closely related to various diseases, since these glycans are widely expressed on various organs including epithelium, gaster, intestine, and pancreas.[8] Meanwhile, α-gal (Figure 1) is another important antigenic glycan that is abundantly expressed in nonprimate mammals, prosimians, and New World monkeys. In contrast, humans and Old World monkeys do not have α-gal due to mutation of α-1,3-galactosyltransferase (α1,3GT) during evolution.[9] Instead, humans produce a large amount of anti-Gal antibodies, which specifically interact with α-gal.[10] Thus, α-gal induces an acute immune response, i.e., hyperacute rejection caused by xenotransplantation from pig to baboon.[11] These findings prompted the use of α-gal as an adjuvant in vaccine therapy[12] or immune therapy using antibody-recruiting molecules.[13] Herein, we describe a new approach for the efficient synthesis of H antigen and α-gal.

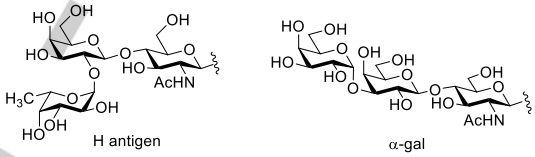

Figure 1. Structure of H antigen and α-gal.

Amino sugars, including *N*-acetylglucosamine (GlcNAc) and sialic acid (Neu5Ac), are contained in many glycans such as H antigen and α-gal. When synthesizing glycan containing amino sugars, the selection of the protecting group on amines is important because it affects the glycosylation reactivity (Figure 2a). Various protecting groups including 2,2,2-trichloroethoxycarbonyl,[14] allyloxycarbonyl,[15] trichloroacetyl,[16] trifluoroacetyl,[17] and phthaloyl[18] have been reported for the protection of amino sugars. The azide group is also a useful precursor of the amine group.[19] On the other hand, since most amino groups are acetylated in natural glycans, protected amino groups have to be converted to acetamide (NHAc) by an acetylation reaction. These steps can be omitted when glycans bearing the NHAc group are directly used for the construction of glycan chains. However, low reactivity of fragments having NHAc in glycosylations has been reported,[20] with the exception of the work by Tamura that describes that the formation of interglycosidic *O*-imidates with NHAc in GalNAc increases the glycosylation yields.[21] Crich et al. reported that intermolecular hydrogen bonding formed by NHAc in GlcNAc acceptor decreases the reactivity, which can be increased by converting NHAc to NAc_2.[20b] Auzanneau et al. observed by NMR spectroscopy the presence of aggregation of a tetrasaccharide through the formation of hydrogen bonds involving the NHAc group in GlcNAc.[20f] Boons et al. reported that a NAc_2 sialyl donor showed significantly higher reactivity than the NHAc donor.[20a] Moreover, the presence of NHAc hydrogen bonds was detected in a Neu5Ac donor by Kononov et al. More recently, the reactivity has been improved by protection of NHAc as NAc_2 or addition of external amides/imides to cleave the mentioned hydrogen bonds.[20c-e] We previously demonstrated that NAc_2

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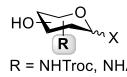
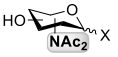
protection of Neu5Ac can greatly improve the reactivity in glycosylation at a position away from the Neu5Ac residue in the synthesis of a Neu5Ac-containing *N*-glycan and a tetrasaccharide having two Neu5Ac.[20g, 20h] On the basis of these reports, we envisioned that a "diacetyl strategy," in which the NHAc group is tentatively protected as NAc_2 during sugar elongation to enhance the reactivity in glycosylation (Figure 2b). Subsequent conversion of NAc_2 to NHAc was readily accomplished by simple removal of one Ac group under mild basic conditions. This strategy avoids handling of polar and reactive amine intermediates. Herein, we applied this strategy to the synthesis of the GlcNAc-containing glycans α-gal and H antigen. Since GlcNAc is the most abundant amino sugar, this study can greatly expand the applicability of diacetyl strategy.

a) Reactivity in glycosylations and conversion process to NHAc for each amino sugar structure

Substrate	Reactivity in glycosylations	Conversion to NHAc
 R = NHTroc, NHAlloc, NPhth, N_3, etc.	High	Deprotection and acetylation via highly reactive amine
	Low	Not necessary
	High	Simple removal of one Ac

b) Diacetyl strategy

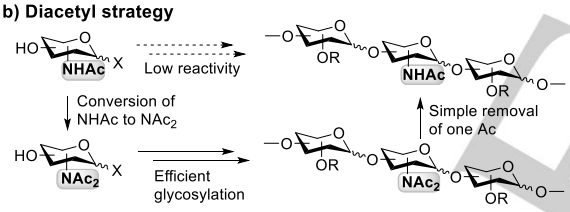


Figure 2. (a) Reactivity in glycosylations and conversion process to NHAc for each amino sugar structure and (b) diacetyl strategy.

The outline of the synthesis for α-gal **5** and H antigen **8** is shown in Figure 3. The synthesis of α-gal has been previously reported by several groups including us.[13g, 22] In this study, α-gal was obtained via glycosylation between **3** and **4** followed by glycosylation of **1** or **2** (Figure 3). In our previous α-gal synthesis, the low reactivity of **1** caused moderate yield in the 2nd glycosylation. In the present study, the yield of the glycosylation was improved by the diacetyl strategy. To demonstrate the effectiveness of this approach, we investigated a one-pot and one-flow glycosylation, in which the enhancement of the reactivity by diacetylation was essential for the efficient construction of α-gal **5**.

The diacetyl strategy was also applied to the synthesis of H antigen **8**. After the first synthesis of B blood type antigen by Lemieux et al., many groups have synthesized ABO blood glycans.[23] In this study, we synthesized H antigen by glycosylation of glucosamine **1** or **2** with galactose **6** and fucose **7** (Figure 3). NAc_2 protection enhanced the reactivity in both glycosylation reactions at proximal and distal positions to GlcNAc.

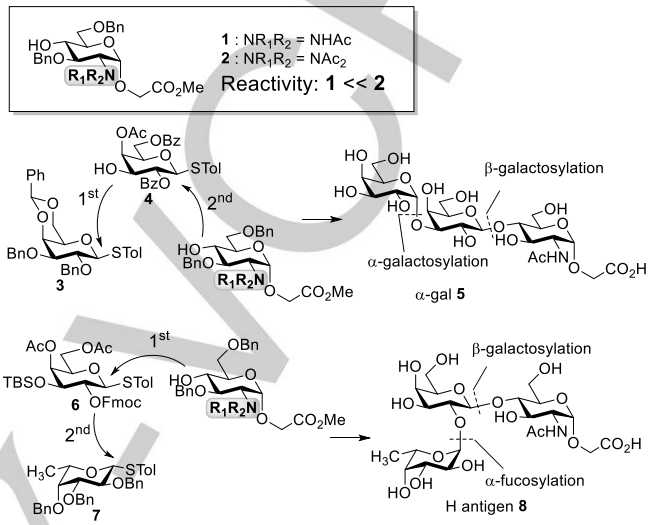
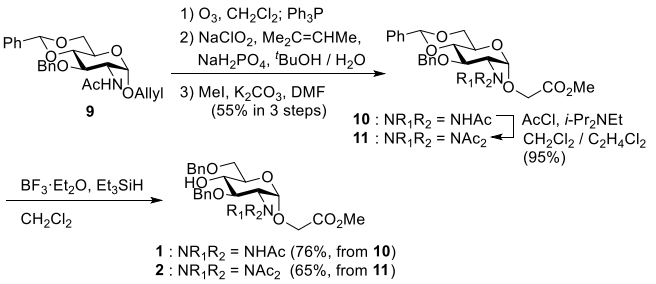


Figure 3. Synthesis of α-gal **5** and H antigen **8** using the diacetyl strategy.

Results and Discussion

We first synthesized glucosaminyl acceptors **1** having NHAc and **2** having NAc_2 (Scheme 1). Synthesis was started from **9**.[24] Ozonolysis of the allyl group in **9** followed by Pinnick oxidation and methyl esterification afforded **10** in 55% yield. Then, selective cleavage of the benzylidene group gave **1** in 76% yield. Meanwhile, NAc_2 protection of **10** using AcCl proceeded in excellent yield (95%). Obtained **11** was then converted to glucosaminyl acceptor **2**.



Scheme 1. Syntheses of acceptors **1** and **2**.

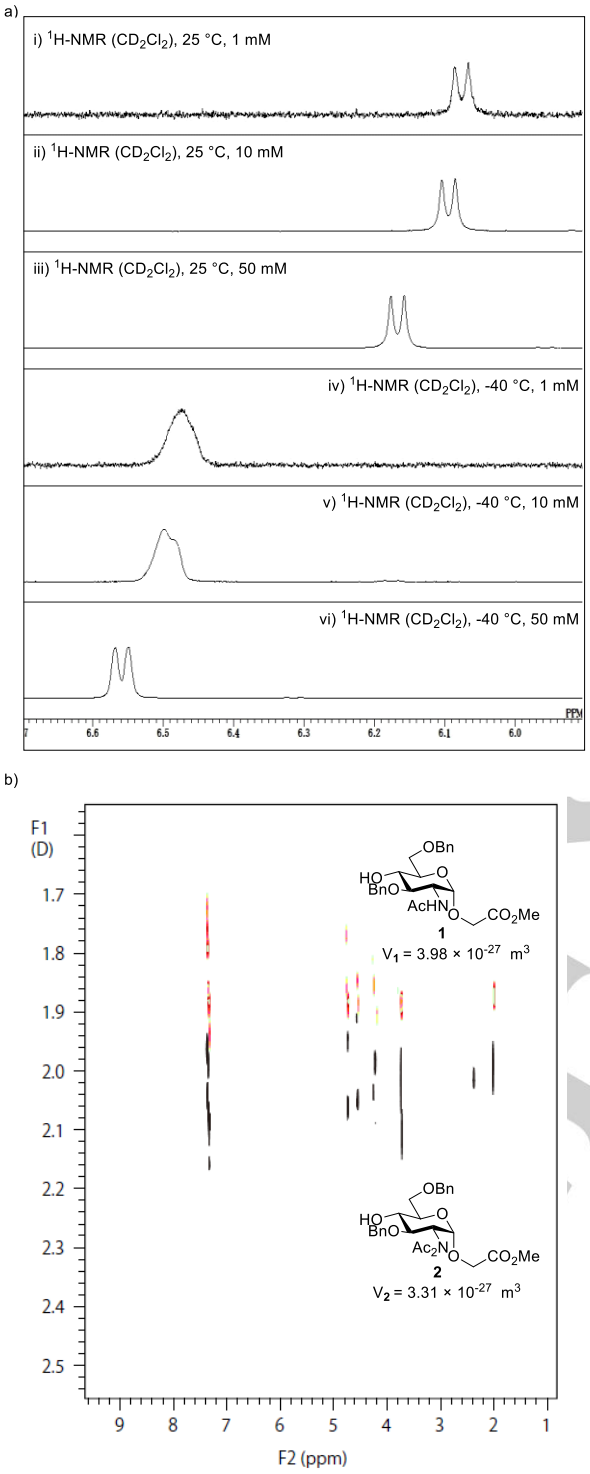


Figure 4. (a) Concentration and temperature dependence of the ^{1}H-NMR spectrum of acetamide acceptor **1**, where downfield shifts of the amide proton are observed upon increasing the concentration and decreasing the temperature. (b) DOSY spectrum of *N*-acetylated **1** (red) and *N*,*N*-diacetylated **2** (black). The effective volume (V) of **1** and **2** estimated by Stokes–Einstein equation was 3.98×10^{-27} m^3 (V_1) and 3.31×10^{-27} m^3 (V_2), respectively.

As described above, formation of intermolecular hydrogen bonds between acceptors is known to decrease their reactivity.[20b] We confirmed the presence of a hydrogen bond involving NHAc in **1** by NMR measurements (Figure 4). When the ^{1}H-NMR spectrum of **1** was measured in CD_2Cl_2 at the concentration of 1, 10, and 50 mM at 25 °C and −40 °C, respectively, the peak of the amide proton was downfield shifted upon increasing the concentration at each temperature (Figure 4a-(i–iii) and (iv–vi)). This downfield shift was also observed as the temperature decreased. These results suggested the existence of intermolecular hydrogen bonding. Vasella et al. and Crich et al. previously reported hydrogen bonding of GlcNAc derivatives. They observed concentration and temperature dependent chemical shift of amide proton of GlcNAc derivatives and concluded that GlcNAc derivatives are likely to take dynamic situation, incorporating both inter- and intramolecular hydrogen bonding.[20b, 25]

We also measured the diffusion-ordered two-dimensional nuclear magnetic resonance spectra (DOSY)[26] of **1** and **2** to gain further information on the hydrogen bonding formed by **1** (Figure 4b). In the DOSY spectrum, the NMR signals are separated according to the self-diffusion coefficient of the molecules. The DOSY spectra of **1** (red, Figure 4b) and **2** (black, Figure 4b) were measured using 50 mM solutions in CD_2Cl_2 at −40 °C. The effective volumes (V) of **1** and **2** were estimated by Stokes–Einstein equation[27] using the self-diffusion coefficient obtained from the DOSY spectra as 3.98×10^{-27} m^3 (V_1) and 3.31×10^{-27} m^3 (V_2), respectively. V_1 was 1.2 times larger than V_2, indicating that **1** formed a supramolecular structure through intermolecular hydrogen bonding, whereas **2** did not aggregate. This difference between V_1 and V_2 was small but significant, considering that the formation of a trimeric cluster of *N*-methylacetamide was predicted under neat conditions by X-ray and neutron scattering with density-functional theory calculations.[28] Our results suggest that the hydrogen-bonding network formed by acetamide **1** is dynamically rearranged at NMR time scale (~10^{-4} sec). This is the first observation of the supramolecular structure construction of GlcNAc by DOSY NMR spectroscopy.

For the efficient synthesis of α-gal, we investigated sequential one-pot and one-flow glycosylations,[29] using thioglycosides as glycosyl donors because they have sufficient stability upon storage and can be smoothly activated during glycosylation to realize high productivity.

One-pot glycosylation reactions can be divided into three types: 1) reactivity-based one-pot glycosylation,[30] 2) orthogonal leaving group-based one-pot glycosylation,[31] and 3) pre-activation-based one-pot glycosylation.[32] Here, we investigated the reactivity-based one-pot glycosylation by applying the so-called armed–disarmed strategy, using a reactive (armed) thioglycoside protected with electron-donating groups and a less reactive (disarmed) thioglycoside protected with electron-withdrawing groups.

We started the investigation on the one-pot glycosylation procedure by evaluating the condition for each glycosylation. The glycosylation of armed thioglycoside **3** (Scheme S1) with disarmed thioglycoside **4**[13h] was carried out by using NIS and TfOH at −20 °C to give **12** in 80% with perfect α-selectivity

(Table 1, entry 1). However, when scaling up, the yield decreased to 57% mainly due to overreaction of **12**.

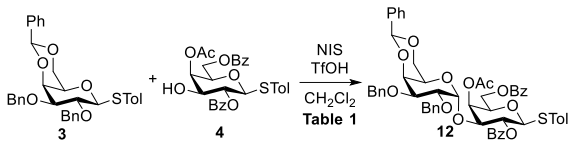


Table 1. The glycosylation between **3** and **4**

Entry	Method	Conditions	Time (min)	Products
1[a]	Batch	NIS (1.5 eq), TfOH (0.5 eq)	5	80%[c], 57%[d]
2[b]	Microflow	NIS (2.1 eq), TfOH (0.8 eq)	1	94%

[a] 1 eq of **3** was used. [b] 1.5 eq of **3** was used. [c] 20 mg of **4** was used. [d] 120 mg of **4** was used.

To overcome the scale-up limitation, we employed a microflow system. Microflow synthesis is an innovative reaction control system that enables efficient mixing, rapid heat transfer, and precise residence time control, as well as good scalability.[33] Scaled-up syntheses can be achieved under the same conditions by continuous flow. These excellent features have been utilized for glycosylation reactions. Thus, a microflow glycosylation was first reported by Seeberger et al.[34] We also achieved efficient and stereoselective α-sialylation,[35] β-mannosylation,[36] and *N*-glycosylation[37] under microflow conditions. The microflow system is obviously advantageous in glycosylations using armed–disarmed strategy, since it allows efficient mixing and accurate temperature control with the concomitant enhancement of the selectivity in thioglycoside activation. In addition, precise residence time control under fluidic conditions prevents overreaction. Importantly, flow reactions can overcome scalability and reproducibility limitations.

We applied the microflow system to the glycosylation between **3** and **4** (Table 1, entry 2). The reaction was completed within 1 min when using a slight excess amount of **3** and reagents. A solution containing **3**, **4**, and NIS was mixed with TfOH in a T-shape mixer at −20 ºC. After 1 min reaction, the reaction mixture was quenched by adding to a solution of Et_3N in CH_2Cl_2. As expected, overreaction of **12** was suppressed, and target disaccharide **12** was obtained in excellent yield (94%). Thus, the scalable synthesis of **12** was achieved under microflow conditions.

Having thiodisaccharide **12** in hand, we then investigated the second glycosylation using glucosaminyl acceptors **1** or **2** to evaluate the effectiveness of the diacetyl strategy (Table 2). Glycosylation between **1** and **12** showed the low reactivity of **1**; Elevating the reaction temperature to 0 ºC was required, and the yield of trisaccharide **13** was 68% (Table 2, entry 1). In contrast, acetylation of the NHAc group in **2** improved the reactivity of the acceptor. Accordingly, the reaction of *N*,*N*-diacetylated acceptor **2** with **12** proceeded at −40 °C to give trisaccharide **14** in excellent yield (91%; Table 2, entry 2). These results indicate that the formation of hydrogen bonding by NHAc in **1** decreases its reactivity and demonstrate the effectiveness of the diacetyl strategy in this glycosylation.

One-pot and one-flow glycosylations were then investigated (Table 3). When the *N*-acetylated glucosaminyl acceptor **1** was used for one-pot glycosylation, the yield of **13** was only 25% due to the low reactivity of **1** in the 2nd glycosylation step (Table 3, entry 1). The reactivity was dramatically enhanced by NAc_2 protection: the 2nd glycosylation using **2** was completed in 5 min at −40 ºC to give **14** in 86% (Table 3, entry 2). In the one-pot glycosylation using the armed–disarmed strategy, the low reactivity in the 2nd glycosylation is inevitable because the disarmed donor should not be activated in the first step. Therefore, highly reactive acceptors are preferred in the 2nd glycosylation for an efficient one-pot glycosylation, which is achieved in this study by diacetyl protection.

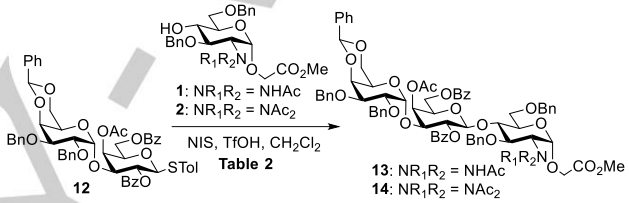


Table 2. Investigation of the reactivity in the [2+1] glycosylation using *N*-acetylated acceptor **1** or *N*,*N*-diacetylated acceptor **2** for the synthesis of protected α-gal **13** or **14**

Entry	Acceptor	Temp (°C)	Products
1	**1**	0	68%
2	**2**	−40	91%

Although the one-pot construction of α-gal **14** was achieved, reproducibility and scalability still remained unsolved because the 1st glycosylation was difficult to control. As mentioned above (Table 1), the 1st glycosylation requires precise control of reaction temperature and time to suppress the overreaction of disaccharide **12**. To overcome these issues, we employed a microflow system (Table 3, entry 3; Figure 5). Thus, a solution containing **3**, **4**, and NIS was mixed with TfOH in a T-shape mixer at −20 ºC. After 1 min reaction, *N*,*N*-diacetylated acceptor **2** and TfOH were successively added at −40 ºC. The resulting mixture was collected in the flask and stirred for additional 5 min at −40 ºC. Target trisaccharide **14** was then obtained in 82% yield, which is comparable to the maximum yield of the one-pot reaction under batch conditions (Table 3, entries 2 and 3). Therefore, it is demonstrated that the one-flow reaction under microflow conditions can overcome the issues of scalability and reproducibility in the one-pot procedure. Although an orthogonal leaving group-based one-flow glycosylation has been reported,[38] to the best of our knowledge, this is the first report on a one-flow glycosylation using the armed–disarmed strategy.

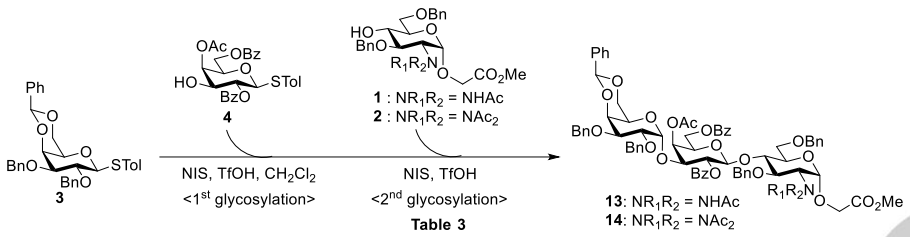


Table 3

Table 3. Investigation of one-pot and one-flow glycosylation using *N*-acetylated acceptor **1** or *N*,*N*-diacetylated acceptor **2**

Entry	Acceptor	Method	Temp (°C)	Time	Products
1	**1**	One-pot	−20	3 h	25%
2	**2**	One-pot	−40	5 min	86%[a]
3	**2**	One-flow	−40	5 min	82%

[a] Low reproducibility

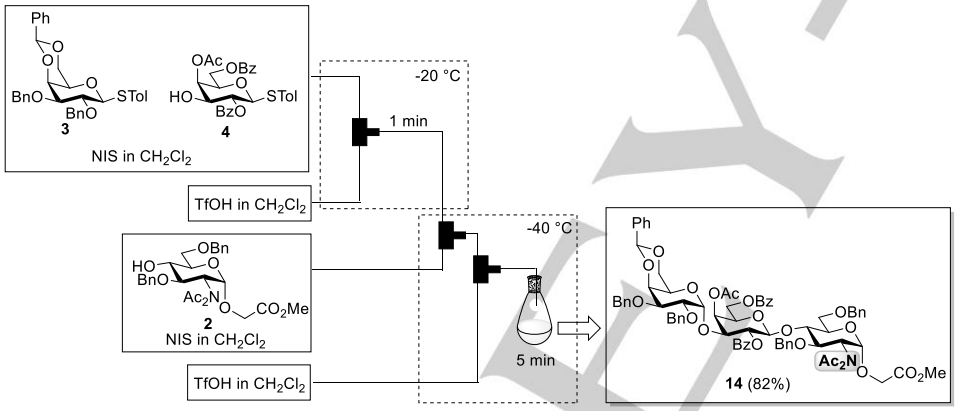
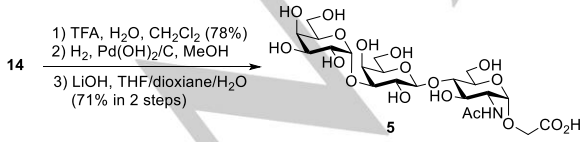


Figure 5. One-flow synthesis of protected α-gal **14** under microfluidic conditions.

Global deprotection of obtained **14** gave α-gal **5** (Scheme 2). After cleavage of the benzylidene group, hydrogenation followed by hydrolysis under basic conditions afforded α-gal **5**. It is noteworthy that the removal of one acetyl group from NAc_2 proceeded smoothly under basic conditions. Since basic treatment is necessary for removal of acyl protection on hydroxy groups, no extra steps were required for the conversion of NAc_2 to NHAc.



Scheme 2. Deprotection of protected α-gal **14**.

We then verify the effectiveness of the diacetyl strategy in the synthesis of H antigen **5**. We employed stepwise construction of glycan chain: fucose was introduced to the 2-position of galactose after reliable β-selective galactosylation using neighboring group participation. 9-Fluorenylmethyloxycarbonyl (Fmoc) group[39] was hence used for the protection of the 2-hydroxy group on the galactose residue, since the β-directing neighboring group participation of Fmoc has been previously reported. After the synthesis of galactosyl donors **15** and **6** (Scheme S2), we investigated the glycosylation using *N*-acetylated **1** and *N*,*N*-diacetylated **2** (Table 4) for comparative purposes. As expected, **2** showed higher reactivity than **1**. When **1** was used as an acceptor for the glycosylation of **15**, elevation of the reaction temperature to 0 ºC was required, and the yield of disaccharide **16** was only 7% (Table 4, entry 1). Unexpectedly, only the undesired α isomer was obtained in this case even though the 2-position of **15** was Fmoc-protected. In contrast, the reactivity was improved when using *N*,*N*-diacetylated acceptor **2**. The reaction proceeded at −40 °C to give disaccharide **17** in 35% yield with an α/β selectivity of 1/1.7 (Table 4, entry 2). To improve the β-selectivity, the glycosyl donor was replaced with **6** because 4,6-*O*-benzylidene-protected galactose was reported to block the β-face leading to an increase of the α-selectivity.[40] Thus, when **6** was used as a galactosyl donor, NAc_2 protection improved the reactivity. Glycosylation of **1** showed low reactivity and required a temperature increase to 0 °C to obtain **18** in 24% yield (Table 4, entry 3). Conversely, glycosylation using **2** proceeded smoothly at −20 °C to give disaccharide **19** in 76% yield with perfect β-selectivity (Table 4, entry 4).

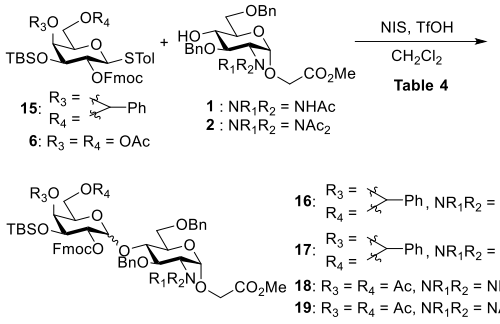


Table 4. Investigation of the reactivity in the glycosylation using *N*-acetylated **1** or *N*,*N*-diacetylated acceptor **2**

Entry	Donor	Acceptor	Temp (°C)	Products
1	**15**	**1**	−40 to 0	7% (α only)
2	**15**	**2**	−40	35% (α:β = 1:1.7)[a]
3	**6**	**1**	−20 to 0	24% (β only)[b]
4	**6**	**2**	−20	76% (β only)

[a] α/β selectivity was estimated by ^{1}HNMR. [b] Product **18** can not be isolated. Thus, yield was estimated by ^{1}HNMR.

We next compared the reactivity of the disaccharide acceptors **20** (*N*-acetylated) and **21** (*N*,*N*-diacetylated) in the [2+1] glycosylation (Table 5)**.** After the syntheses of **20** and **21** by cleavage of the Fmoc group of **18** and **19**, respectively, glycosylations with fucosyl donor **7**[30a] were investigated. In the case of **20**, the glycosylation did not proceed at −30 °C, and trisaccharide **22** was obtained in 45% yield when the reaction temperature was elevated to rt (Table 5, entry 1). In contrast, glycosylation with **21** proceeded smoothly at −30 °C to give **23** in 83% yield (Table 5, entry 2). Both glycosylations proceeded with perfect α-selectivity, and diacetyl protection of NHAc in GlcNAc enhanced the reactivity at proximal and distal positions to GlcNAc in both cases. This remote effect strongly supports the hypothesis that aggregation induced by NHAc hydrogen bond decreases the reactivity. *N*,*N*-diacetyl protection can enhance the reactivity even when the reaction site is far from the NAc_2 group, indicating that the diacetyl strategy can be generally applied to the synthesis of glycans containing GlcNAc.

Finally, global deprotection of **23** gave H antigen **8** (Scheme 2). After removal of the TBS group with TASF, hydrolysis of the acetyl group followed by hydrogenation afforded **8**. In this case, NAc_2 was also smoothly converted to NHAc during hydrolysis.

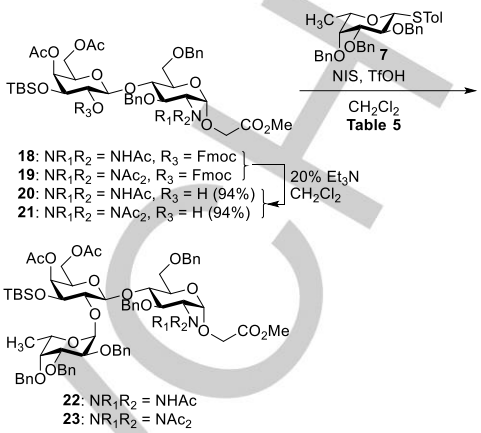
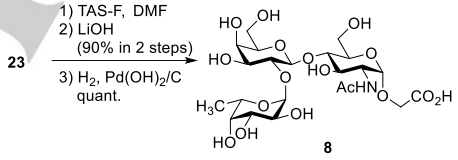


Table 5. Investigation of the reactivity using *N*-acetylated disaccharide **20** and *N*,*N*-diacetylated disaccharide **21**

Entry	Acceptor	Conditions	Products
1	**20**	−30 °C to rt	45%
2	**21**	−30 °C	83%



Scheme 3. Deprotection of protected H antigen **23**.

Conclusions

In summary, we have demonstrated the effectiveness of diacetyl strategy in the synthesis of α-gal **5** and H antigen **8**. The formation of hydrogen bond through NHAc in GlcNAc was clearly shown by NMR analysis. Although the effective volume of **1** was 1.2 times larger than that of **2** according to the DOSY spectra, this small difference indicates that hydrogen bonds formed by NHAc were dynamically rearranged, which significantly reduced the reaction rate of glycosylations. Conversion of NHAc to NAc_2 dramatically improved the reactivity in all cases. The diacetyl strategy enabled the effective one-pot and one-flow glycosylation using armed and disarmed donors. Even though the glycosylation position was far from NHAc, diacetyl protection enhanced the reactivity. In the synthesis of α-gal and H antigen, glycans were elongated from the nonreducing and reducing ends, respectively. Since the reactivity in glycosylations tends to decrease as the size of fragments increases, the donor reactivity decrease in the former and the

acceptor reactivity decrease in the latter with the glycan elongation. The reactivity of acceptors by the diacetyl strategy proved to be enhanced in both cases. These results clearly indicate that the diacetyl strategy can be a powerful tool for the synthesis of GlcNAc-containing glycans. We also showed that the reactivity was affected by the supramolecular structure based on hydrogen bonding and can be controlled by slight structure modification such as amide acetylation. Application of this concept not only to other glycans but also to a wide range of organic compounds is further important work.

Experimental Section

Glycosylation between 3 and 4, Compound 12

Batch conditions: To a suspension of donor **3** (20 mg, 37.1 μmol), acceptor **4** (13.3 mg, 24.7 μmol), *N*-iodosuccinamide (8.36 mg, 37.2 μmol), and activated MS4A (ca. 20 mg) in CH_2Cl_2 (0.5 mL) was slowly added a solution of TfOH (1.07 μL, 12.39 μmol) in CH_2Cl_2 (0.3 mL) at -20 ºC under Ar atmosphere. After being stirred for 5 mins at -20 ºC, the reaction was quenched with Et_3N and sat. $Na_2S_2O_3$ aq., and filtered using membrane filter (Fluoro pore®). The filtrate was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography (CH_2Cl_2/EtOAc = 60/1) to give **12** (19.2 mg, 80%) as a white powder.

Microflow conditions: A microreactor system consisted of syringe (HAMILTON CO., RENO. NEVADA, size 5 mL), syringe pump (HARVARD 11 plus single syringe pump), T-Shaped micro mixer, and tubes (inner diameter Φ = 80 mm). A solution of donor **3** (56.85 mg, 0.11 mmol), accepor **4** (31.46 mg, 58.62 μmol), and NIS (27.7 mg, 0.12 mmol) in CH_2Cl_2 (1.45 mL) in the syring A was pumped at the flow rate of 0.3 mL/min. A solution of TfOH (4.16 μL, 46.84 μmol) in CH_2Cl_2 (1.45 mL) in the syringe B was pumped at the flow rate of 0.3 mL/min. These solutions were mixed at -20 ºC. The reaction mixture was collected for 60 s in a flask which was filled with a solution of Et_3N in CH_2Cl_2 at -20 ºC. Then, sat. $Na_2S_2O_3$ aq. was added, and the reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$ aq., brine, dried over Na_2SO_4, filtrated, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (CH_2Cl_2/EtOAc = 60/1) to give **12** (10.6 mg, 94%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07-6.90 (m, 29H), 5.62 (d, *J* = 3.0 Hz, 1H), 5.52 (m, 1H), 5.18 (s, 1H), 5.09 (d, *J* = 3.3 Hz, 1H), 4.79 (d, *J* = 10.1 Hz, 1H), 4.73-4.54 (m, 4H), 4.48-4.38 (m, 2H), 4.04-3.99 (m, 2H), 3.95 (dd, *J* = 10.2, 3.3 Hz, 1H), 3.82 (d, *J* = 11.5 Hz, 1H), 3.74 (dd, *J* = 10.2, 3.4 Hz, 1H), 3.57 (d, *J* = 3.3 Hz, 1H), 3.39 (d, *J* = 12.0 Hz, 2H), 1.90 (s, 3H), 1.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.2, 166.0 (2x), 164.8, 138.8, 138.6, 138.1, 137.7, 133.5, 133.2, 129.8, 129.6, 128.8, 128.6, 128.4, 128.3, 128.1 (2x), 127.6, 127.5, 126.2, 100.8 (2x), 96.0, 87.5, 76.1, 75.2, 74.4, 74.3, 73.9 (2x), 72.1, 68.8, 65.8, 62.9, 21.1, 20.4. HRMS (ESI-LTQ-Orbitrap) m/z $[M+Na]^+$ calcd for $C_{56}H_{54}O_{13}S$ 989.3177, found 989.3189.

Glycosylation between 12 and 2, Compound 14

To a suspension of disaccharide donor **12** (37.5 mg, 38.7 μmol), acceptor **2** (20.0 mg, 38.7 μmol), *N*-iodosuccinamide (11.5 mg, 46.5 μmol), and activated MS4A (ca. 40 mg) in CH_2Cl_2 (1 mL) was slowly added a solution of TfOH (1.4 μL, 15.48 μmol) in CH_2Cl_2 (1 mL) at -40 ºC under Ar atmosphere. After being stirred for 20 mins at -40 ºC, the reaction was quenched with Et_3N and sat. $Na_2S_2O_3$ aq., and filtration using membrane filter (Fluoro pore®). The filtrate was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography (Toluene/EtOAc = 5/1 to 4/1) to give **14** (48.2 mg, 91%) as a white powder.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.05-7.17 (m, 35H), 5.48 (d, *J* = 3.4 Hz, 1H), 5.41 (dd, J = 9.9, 8.0 Hz, 1H), 5.17 (s, 1H), 5.10 (d, J = 10.9 Hz, 1H), 5.00 (d, *J* = 3.4 Hz, 1H), 4.95 (d, *J* = 3.7 Hz, 1H), 4.74 (m, 2H), 4.69 (d, *J* = 9.0 Hz, 1H), 4.66-4.59 (m, 4H), 4.56 (d, *J* = 12.3, 1H), 4.36 (dd, *J* = 11.2, 3.8 Hz, 1H), 4.32 (d, *J* = 12.2 Hz, 1H), 4.14-4.00 (m, 5H), 3.92 (dd, *J* = 10.0, 3.5 Hz, 1H), 3.82 (d, *J* = 12.5 Hz, 1H), 3.73-3.67 (m, 3H), 3.64 (s, 3H), 3.62-3.61 (m, 2H), 3.56 (d, *J* = 3.2 Hz, 1H), 3.42 (dd, *J* = 11.1, 1.8 Hz, 1H), 3.37 (dd, *J* = 12.5, 1.6 Hz, 1H), 3.26 (s, 1H), 2.28 (s, 6H), 1.75, (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ =175.6, 170.1, 169.6, 166.0, 164.4, 139.2, 138.8, 138.7, 137.9, 137.7, 133.7, 133.3, 129.9, 129.7, 128.9, 128.8, 128.7, 128.6, 128.4, 128.2, 128.0, 127.7, 127.6, 127.3, 127.2, 126.3, 100.9, 99.9, 98.1, 96.6, 76.1, 76.0, 74.5, 74.5, 74.4, 74.0, 73.8, 73.3, 72.2, 71.8, 71.1, 71.0, 68.9, 67.6, 65.5, 63.7, 62.9, 61.8, 59.3, 51.9, 26.8, 20.4. HRMS (ESI-LTQ-Orbitrap) m/z $[M+Na]^+$ calcd for $C_{76}H_{79}NO_{22}$, 1380.4986, found 1380.4985.

One-flow glycosylation using 3, 4, and 2, Compound 14

A microreactor system consisted of syringe (HAMILTON CO., RENO. NEVADA, size 5 mL), syringe pump (HARVARD 11 plus single syringe pump), T-Shaped micro mixer, and tubes (inner diameter Φ = 80 mm). Preparation of the solutions were carried out at rt under Ar atmosphere. A solution of **3** (78.41 mg, 0.15 mmol), **4** (43.39 mg, 80.9 μmol), and NIS (38.2 mg, 0.17 mmol) in CH_2Cl_2 (2.0 mL) in the syringe A was pumped at the flow rate of 0.3 mL/min. A solution of TfOH (5.74 μL, 64.64 μmol) in CH_2Cl_2 (2.0 mL) in the syringe B was pumped at the flow rate of 0.3 mL/min. These solutions were mixed at -20 ºC. After 1 min reaction, a solution of **2** (34.2 mg, 66.3 μmol) and NIS (32.7 mg, 0.145 mmol) in CH_2Cl_2 (2.0 mL) in the syringe C was pumped at the flow rate of 0.3 mL/min, and mixed at -40 ºC. A solution of TfOH (5.74 μL, 64.6 μmol) in CH_2Cl_2 (2.0 mL) in the syringe D was pumped at the flow rate of 0.3 mL/min, and mixed at -40 ºC. The reaction mixture was collected for 60 s in a flask containing MS4A. After being stirred for 5 min at -40 ºC under Ar atmosphere, the reaction mixture was diluted with CH_2Cl_2 (5 mL), quenched with Et_3N and sat. $Na_2S_2O_3$ aq. at -40 ºC, and extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$ aq., brine, dried over Na_2SO_4, filtrated, and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography (Toluene/EtOAc = 5/1→4/1) to give **14** (10.7 mg, 82%).

Glycosylation between 6 and 2, Compound 19

To a suspension of donor **6**, acceptor **2**, *N*-iodosuccinimide (8.25 mg, 36.65 μmol), and MS4A powder (ca. 32 mg) in CH_2Cl_2 (610 μL) was added trifluoromethanesulfonic acid (1.1 μL, 12.21 μmol) at -20 ºC under Ar atmosphere. After being stirred for 20 mins at -20 ºC, the reaction mixture was quenched with sat. $NaHCO_3$ aq. and 10% $Na_2S_2O_3$ aq. and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by preparative layer chromatography (hexane/EtOAc = 1/1) to give **19** (25.4 mg, 76%) as a colorless oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.79 (dd, *J* = 7.6, 2.6 Hz, 2H), 7.62 (dd, *J* = 7.3, 2.6 Hz, 2H), 7.43 (t, *J* = 7.3 Hz, 2H), 7.36-7.19 (m, 12H), 5.06 (d, *J* = 3.7 Hz, 1H), 5.01 (d, *J* = 11.0 Hz, 1H), 4.98 (d, *J* = 3.9 Hz, 1H), 4.74 (dd, *J* = 9.4, 8.1 Hz, 1H), 4.66 (d, *J* = 12.4 Hz, 1H), 4.63-4.56 (m, 3H), 4.48 (dd, *J* = 10.3, 6.2 Hz, 1H), 4.41 (dd, *J* = 11.3, 3.7 Hz, 1H), 4.35 (d, *J* = 8.1 Hz, 1H), 4.29 (d, *J* = 12.1 Hz, 1H), 4.24 (t, *J* = 6.2 Hz, 1H), 4.16 (d, *J* = 16.5 Hz, 1H), 4.11 (d, *J* = 16.5 Hz, 1H), 3.96 (t, *J* = 9.3 Hz, 1H), 3.88 (dd, *J* = 11.3, 6.6 Hz, 1H), 3.76-3.69 (m, 3H), 3.69 (s, 3H), 3.44-3.36 (m, 3H), 2.30 (s, 6H), 2.03 (s, 3H), 1.97 (s, 3H), 0.77 (s, 9H), 0.02 (s, 3H), -0.06 (s, 3H). ^{13}C-NMR (500MHz, $CDCl_3$): δ = 175.49, 170.33, 169.95, 169.45, 154.12, 143.45, 143.40, 141.53, 141.38, 139.18, 137.90, 128.47, 127.93, 127.89, 127.85, 127.78, 127.34, 127.14, 127.12, 127.02, 124.82, 124.79, 120.07, 120.05, 100.08, 98.24, 77.90, 77.20, 76.61, 76.04, 73.22, 71.11, 70.91, 70.64, 69.45, 69.22, 67.39, 63.85, 61.46, 59.06, 51.84, 46.81, 26.72, 25.30, 20.62, 17.67, -4.93, -5.32. HRMS (ESI-LTQ-Orbitrap) m/z $[M+Na]^+$ calcd for $C_{58}H_{71}NO_{18}SiNa$, 1120.4333, found 1120.4320.

Glycosylation between 21 and 7, Compound 23

To a suspension of donor **30**, acceptor **21**, *N*-iodosuccinimide (36.5 mg, 162.2 μmol), and MS4A powder (ca. 100 mg) in CH_2Cl_2 (1.06 mL) was added and trifluoromethanesulfonic acid (1.41 μL, 16.0 μmol) at -30 ºC under Ar atmosphere. After being stirred at -30 ºC for 20 mins, the reaction mixture was quenched with sat. $NaHCO_3$ aq. and 10% $Na_2S_2O_3$ aq. and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography (toluene/acetone = 5/1) to give **23** (57.2 mg, 83%) as a light yellow oil.

^{1}H-NMR (500 MHz, $CDCl_3$) : δ = 7.42-7.19 (m, 25H), 5.46 (d, *J* = 3.9 Hz, 1H), 5.04 (d, *J* = 10.6 Hz, 1H), 5.01-4.97 (m, 3H), 4.94 (d, *J* = 3.7 Hz, 1H), 4.74-4.68 (m,4H), 4.64 (d, *J* = 12.0 Hz, 1H), 4.53 (dt, *J* = 10.6, 5.0 Hz, 2H), 4.42-4.35 (m, 3H), 4.26 (d, *J* = 7.9 Hz, 1H), 4.18 (d, *J* = 16.5 Hz, 1H), 4.14-4.06 (m, 3H), 3.99-3.93 (m, 2H), 3.89-3.78 (m, 5H), 3.66 (s, 3H), 3.53 (dd, *J* = 9.3, 3.4 Hz, 1H), 3.43 (dd, *J* = 10.8, 1.5 Hz, 1H), 3.35 (t, *J* = 6.6 Hz, 1H), 2.29 (s, 6H), 2.00 (s, 3H), 1.92 (s, 3H), 1.28 (d, *J* = 6.6 Hz, 3H), 0.79 (s, 9H), 0.06 (s, 3H), 0.01 (s, 3H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ = 175.49, 170.41, 170.16, 169.66, 139.02, 138.97, 138.80, 138.65, 137.90, 128.59, 128.36, 128.22, 128.14, 127.99, 127.97, 127.92, 127.79, 127.68, 127.57, 127.50, 127.31, 127.19, 127.15, 100.52, 98.74, 97.43, 79.99, 77.51, 76.27, 76.04, 75.91, 74.97, 74.26, 73.74, 73.52, 73.42, 73.32, 71.73, 71.27, 70.59, 70.33, 67.77, 66.53, 64.49, 61.95, 59.15, 51.81, 26.66, 25.75, 20.69, 20.65, 17.57, 16.70, -4.07, -4.57. HRMS (ESI-LTQ-Orbitrap) m/z $[M+Na]^+$ calcd for $C_{70}H_{89}NO_{20}SiNa$ 1314.5639, found 1314.5661.

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

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Key Topic: Carbohydrate synthesis

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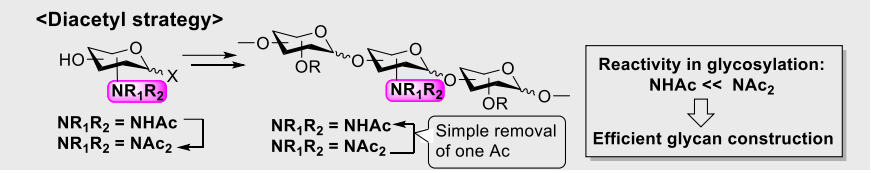

Diacetyl strategy: Acetamide (NHAc) is tentatively converted to diacetylimide (NAc_2) during oligosaccharide construction. Formation of hydrogen bonds through NHAc in GlcNAc was confirmed by NMR measurements, and *N*,*N*-diacetyl protection improved the reactivity in glycosylation to realize the efficient construction of glycans.

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Efficient synthesis of antigenic trisaccharides containing *N*-acetylglucosamine: protection of NHAc as NAc_2