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COMMUNICATION

A dimethoxytriazine type glycosyl donor enables a facile chemo-enzymatic route toward α-linked *N*-acetylglucosaminyl-galactose disaccharide unit from gastric mucin†

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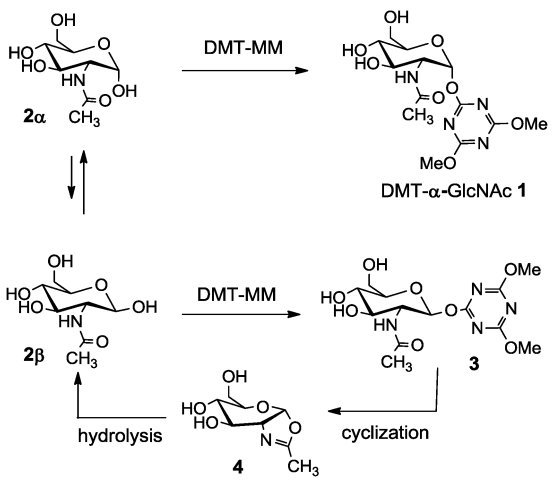
An efficient chemo-enzymatic process for construction of the α-linked disaccharide unit (GlcNAcα1-4Gal) found in gastric mucin has been developed. The process consists of a one-step preparation of a novel triazine type glycosyl donor in water and the subsequent transglycosylation to a galactose derivative catalysed by α-*N*-acetylglucosaminidase.

Oligosaccharides including an α-linked *N*-acetylglucosamine (GlcNAc) unit play biologically important roles in nature. The α-*N*-acetylglucosaminyl galactose (GlcNAcα1-4Gal), the terminal disaccharide unit of gastric mucin, is closely related to the infection of *Helicobacter pylori* in gastric mucosa.[1] Since compounds having the GlcNAcα1-4Gal unit would be expected to be potential inhibitors of the growth of *Helicobacter pylori*, their efficient and practical synthesis in a stereospecific manner is strongly demanded.

In general, stereoselective α-glycosylation (1,2-*cis*-glycosidic bond formation) of *N*-acetylglucosamine is difficult because undesired β-anomers (1,2-*trans*-isomers) are produced predominantly as a result of neighbouring group participation of the 2-acetamido group in the GlcNAc unit. Much effort has so far been devoted to the selective construction of an α-*N*-acetylglucosaminide structure.[2] A 2,3-cyclic carbamate-type protecting group showed a complete α-selectivity in the *N*-acetylglucosaminylation of a galactose derivative.[3] The use of glycosyl donors possessing a 2-azide group also gave α-*N*-acetylglucosaminide selectively.[4–6] However, these chemical processes require a number of steps including protection and deprotection of the hydroxy and amino groups. In this communication, we report a two-step chemo-enzymatic route for preparation of α-*N*-acetylglucosaminyl galactose derivatives by the combined use of a novel dimethoxytriazine type glycosyl donor and an α-*N*-acetylglucosaminidase (α-GlcNAcase).

Recently, we have developed novel glycosyl donors having a 4,6-dimethoxy-1,3,5-triazin-2-yl group on the anomeric carbons with β-configurations (DMT-β-glycosides) for enzymatic glycosylations.[7] The DMT-β-glycosides could be directly prepared in water by treating the corresponding unprotected sugars with a water-soluble agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride[8] (DMT-MM), without protection of the sugar hydroxy groups. In the course of these studies, we found that the reaction of GlcNAc and DMT-MM afforded an adduct **1** (DMT-α-GlcNAc) with the opposite anomeric configuration (Scheme 1). We postulated that if **1** could be recognised by an α-*N*-acetylglucosaminidase that hydrolyses α-glycosidic bonds with retention of configuration, a stereospecific enzymatic synthesis of 1,2-*cis*-glycosides would be possible by using **1** as a glycosyl donor substrate.

A nucleophilic substitution reaction took place between the hemiacetal of GlcNAc and DMT-MM in the presence of 2,6-lutidine in water, giving rise to the corresponding adduct **1** in 84% yield accompanying a small amount of 1,6-disubstituted



Scheme 1 Reaction mechanism for selective formation of **1**.

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DMT derivative (less than 1%). These results showed that the α-hemiacetal **2**α reacted with DMT-MM at the 2-position of the triazine ring almost selectively without protecting other sugar hydroxy groups.

In this reaction, α-adduct **1** formed almost exclusively and no β-adduct **3** was obtained. The initial ratio of GlcNAc (**2**α/**2**β) was 6/4, which has not been reflected in the ratio of the resulting adducts. The exclusive formation of the α-adduct can be explained as follows. In addition to the nucleophilic attack of the α-hemiacetal **2**α to DMT-MM, β-hemiacetal **2**β also reacts with DMT-MM to give the corresponding β-adduct **3**. It is assumed that the resulting **3** is very reactive and easily cyclises to the oxazoline derivative **4**.

The resulting oxazoline **4** is much less stable and immediately hydrolysed to **2**β which is in equilibrium with **2**α in water.[9] Since an excess amount of DMT-MM (2 equiv.) was used, α-adduct **1** can accumulate through the re-generation of **2**β.

A typical procedure for preparation of DMT-α-GlcNAc **1** is as follows: a mixture of GlcNAc (221 mg, 1.0 mmol), DMT-MM (553 mg, 2.0 mmol) and 2,6-lutidine (230 μl, 2.0 mmol) in water (6.25 ml) was stirred at room temperature for 24 h. After confirming the disappearance of GlcNAc by TLC, the solvent evaporated and the residue was purified by silica gel column chromatography, giving rise to **1** (288 mg, 84%). The ^{1}H NMR spectrum of **1** showed a doublet peak at 6.4 ppm derived from the anomeric proton with a coupling constant of 3.3 Hz, indicating that the anomeric configuration of the product is α-type.[10]

In order to know whether the resulting DMT-α-GlcNAc can be recognised by the corresponding α-glycosidase, we demonstrated hydrolysis experiments of **1** by using α-*N*-acetylglucosaminidase B1 (α-GlcNAcase B1) cloned from intestinal bacteria, *Bacteroides thetaiotaomicron* VPI5482.[11] The hydrolysis rate of the conventional *p*-nitrophenyl α-*N*-acetylglucosaminide substrate (*p*NP-α-GlcNAc) was also measured as a reference. In the absence of the enzyme, the concentration of **1** in PBS buffer slightly decreased at 37 °C, indicating that the substrate was very stable in the buffer. When α-GlcNAcase enzyme was added to the solution, the substrate was hydrolysed to GlcNAc quantitatively within 3 h liberating 2-hydroxy-4,6-dimethoxy-1,3,5-triazine (closed circle in Fig. 1). These results clearly showed that the substrate **1** can be well recognised by the catalytic site of the α-GlcNAcase to form an enzyme–substrate complex. Interestingly, the most popular substrate for glycosidases, *p*NP-α-GlcNAc, was not hydrolysed under the same conditions (open circle in Fig. 1).

Based on these results, we tried a chemo-enzymatic α-*N*-acetylglucosaminylation by using DMT-α-GlcNAc **1** as glycosyl donor and various galactose derivatives as glycosyl acceptors catalysed by α-GlcNAcase B1 (Table 1). The reaction was carried out by pre-incubating a mixture of **1** and a glycosyl acceptor in PBS buffer (pH 7.4) at 37 °C for 10 min. To this mixture was added a PBS buffer solution of α-GlcNAcase B1 and the resulting product was purified by a preparative HPLC.

When *p*-methoxyphenyl β-galactopyranoside (**5a**) was used as an acceptor, the corresponding disaccharide **6a** was obtained in 52% yield (Table 1, entry 1). A mixture of **1** (50 μmol, final conc.: 50 mM), **5a** (50 μmol, final conc.: 50 mM), and α-GlcNAcase B1 (100 μg, 36.4 μl, PBS solution) in 100 mM sodium phosphate buffer (pH 6.5) was incubated at 37 °C. The reaction mixtures were analysed by HPLC (column: Inertsil ODS-3, eluent: 5% CH_3CN to 15% CH_3CN gradient). The product **6a** was purified by HPLC (column: COSMOSIL, eluent: 8 vol% CH_3CN), and freeze-dried.

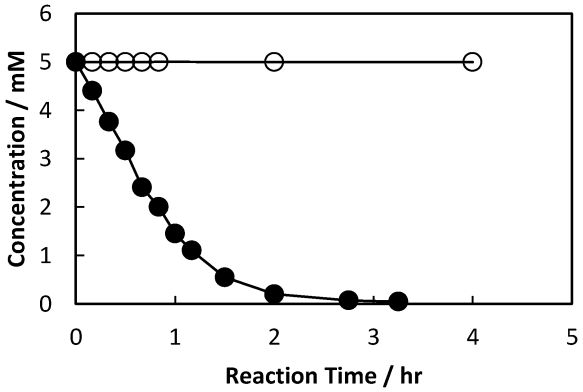
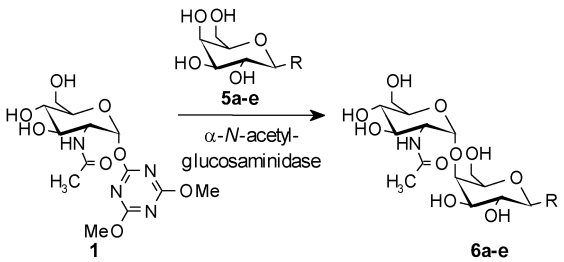
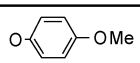
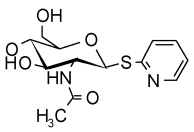
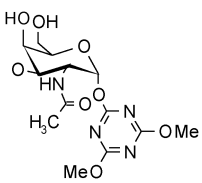


Fig. 1 Time course of the enzymatic hydrolysis of **1** (closed circle) and *p*NP-α-GlcNAc(open circle) by α-*N*-acetylglucosaminidase B1 from *Bacteroides thetaiotamicron* VPI5482(10 μg ml^{-1}) in PBS buffer at 37 °C. The decrease of **1** and *p*NP-α-GlcNAc was evaluated by normal phase HPLC and UV-VIS spectrometer, respectively.

Table 1 α-GlcNAcase-catalysed transglycosylation reaction by using **1**



Entry	Acceptor	R	Yield[a] (%)
1	**5a**		52
2	**5b**	OMe	17[b]
3	**5c**		29[b]
4	**5d**		59
5	**5e**		51

[a] Determined by HPLC. [b] Isolated yield.

The ^{1}H-NMR spectrum of the resulting disaccharide showed a doublet signal at around δ 4.9 ppm ascribable to the H-1′ proton with a coupling constant value of 3.6 Hz. The ^{13}C-NMR showed a signal due to the C4 at around 77.7 ppm. All of these data clearly indicated that the glycosylation proceeded in a regio- and stereo-selective manner, affording

an α-1,4 glycosidic bond.[12] Methyl β-D-galactopyranoside (**5b**) and isopropyl β-D-1-thiogalactopyranoside (**5c**) were also α-glycosylated at the 4-positions, affording the corresponding disaccharide derivatives **6b** and **6c**, respectively (entries 2 and 3).

We also demonstrated the transglycosylation reaction by using *N*-acetyllactosamine derivative **5d** and galacto-*N*-biose derivative **5e** as glycosyl acceptors. The glycosyl acceptor **5d** could be directly prepared from the corresponding unprotected sugars according to the method described in the previous papers.[13] The glycosyl acceptor **5e** was prepared in a similar manner as **1** by treating galacto-*N*-biose with DMT-MM in water. The transglycosylation reaction of the GlcNAc moiety with **5d** and **5e** proceeded in a regio- and stereo-selective manner, giving rise to the corresponding trisaccharide derivatives having a GlcNAcα-1,4Galβ-1,4GlcNAc structure and a GlcNAcα-1,4Galβ-1,3GalNAc structure, respectively (entries 4 and 5). These structures can be found in the oligosaccharides of mucin type glycoproteins.

The mechanism of the present enzymatic transglycosylation has not been made clear, however, it is assumed that either the glycosidic oxygen atom or the nitrogen atom (1 or 3 position) of the triazine ring of **1** is protonated with the carboxylic acid of an acidic amino acid located at the catalytic center of α-GlcNAcase, affording a glycosyl-enzyme intermediate with β-configuration, liberating 2-hydroxy-4,6-dimethoxy-1,3,5-triazine. The resulting intermediate is then attacked by the 4-OH of the galactose unit in the glycosyl acceptors to give the transglycosylated products with double inversion of configuration.

In conclusion, a novel compound, 4,6-dimethoxy-1,3,5-triazin-2-yl α-*N*-acetylglucosaminide (DMT-α-GlcNAc), was found to be an efficient glycosyl donor substrate for α-*N*-acetylglucosaminidase from *Bacteroides thetaiotaomicron* (α-GlcNAcase B1), and could transfer the GlcNAc unit to the 4-OH of various galactose derivatives, affording the core disaccharide (GlcNAcα1-4Gal) unit of the oligosaccharide chain on mucin type glycoproteins. It is noteworthy that this new glycosyl donor can be prepared in water directly from GlcNAc without using any protecting groups. There have been no reports on glycosyl donors that can transfer a GlcNAc unit to an acceptor in an α-selective manner due to the difficulty of their preparation and low reactivity in the catalytic site enzymes. The DMT-α-GlcNAc is the first artificial substrate that can be recognized by α-GlcNAcase B1 efficiently and can be employed as the glycosyl donor for chemo-enzymatic synthesis. The present synthetic process which consists of a one-step preparation of the glycosyl donor and the subsequent effective transglycosylation by α-*N*-acetylglucosaminidase will be a practical method in synthetic organic chemistry for production of the mucin core disaccharide derivatives and will contribute to basic research in glycoscience by supplying key oligosaccharide materials on gram-scale.[14] Further application as well as the elucidation of detailed transglycosylation mechanism of the reaction is now in progress.

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9 The use of a weak base of 2,6-lutidine is crucial for the selective formation of α-adduct **1**. Under more basic conditions, for example, in the presence of triethylamine, the oxazoline **4** was not hydrolysed to **2**β, giving rise to a mixture of **1** and **4**. M. Noguchi, T. Tanaka, H. Gyakushi, A. Kobayashi and S. Shoda, *J. Org. Chem.*, 2009, **74**, 2210.
10 ^{1}H NMR(500 MHz, D_2O): δ 6.38(1H, d, H-1, $J_{1,2}$ = 3.3 Hz), 4.03(1H, dd, H-2, $J_{1,2}$ = 3.3 Hz, $J_{2,3}$ = 10.8 Hz), 3.89(6H, s, –OCH_3), 3.83(1H, dd, H-3, $J_{2,3}$ = 10.6 Hz, $J_{3,4}$ = 9.9 Hz), 3.74–3.66(3H, m, H-5, H-6a, H-6b), 3.51(1H, t, H-4, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz), 1.85(3H, s, –COCH_3). ^{13}C NMR(126 MHz, D_2O): δ 174.7, 173.2, and 171.7(triazine, –COCH$_3$), 94.3(C-1), 74.2(C-5), 70.4(C-3), 69.3(C-4), 60.1(C-6), 55.8(–OCH$_3$), 52.8(C-2), 21.6(–COCH$_3$). ESI-MS: *m*/*z* calcd for $C_{13}H_{20}N_4O_8[M+Na]^+$: 383.1173, found: 383.1174.
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12 ^{1}H NMR (500 MHz, CD_3OD): δ 7.08 (2H, d, Ph, J = 9.1 Hz), 6.86 (2H, d, Ph, J = 9.1 Hz), 4.95 (1H, d, H-1′, J = 3.6 Hz), 4.84 (1H, d, H-1, J = 7.6 Hz), 4.29 (1H, ddd, H-5′, J = 2.5, 4.0, 9.9 Hz), 4.04 (1H, d, H-4, J = 2.8 Hz), 3.97 (1H, dd, H-2′, J = 3.6, 10.9 Hz), 3.83 (1H, dd, H-6a′, J = 2.2, 11.8 Hz), 3.79–3.71 (8H, m, H-2, H-5, H-6a, H-3′, H-6′b, OCH_3), 3.69–3.65 (2H, m, H-3, H-6b), 3.46 (1H, t, H-4′, J = 9.4, 9.5 Hz), 2.02 (3H, s, CH_3). ^{13}C NMR (126 MHz, CD_3OD): δ 173.8 (CO of Ac), 156.7 (Ph), 153.0 (Ph), 119.1 (Ph), 115.5 (Ph), 104.0 (C-1), 100.3 (C-1′), 77.7 (C-4), 76.8 (C-5), 74.3 (C-3), 73.7 (C-5′), 72.6 (C-3′), 72.4 (C-2), 72.0 (C-4′), 62.3 (C-6′), 60.7 (C-6), 56.1 (OCH_3), 55.5 (C-2′), 22.7 (CH_3 of Ac).
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14 In the present chemo-enzymatic process, isolation of the glycosyl donor **1** is indispensable. When the reaction of GlcNAc and DMC was carried out in the presence of α-GlcNAcase, the enzyme was inactivated due to interaction of nucleophilic moieties of the enzyme and DMC.

