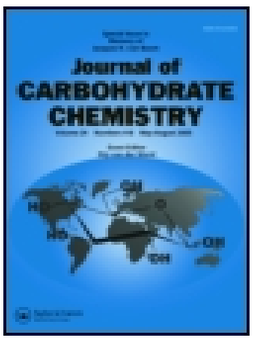

Journal of Carbohydrate Chemistry

ISSN: 0732-8303 (Print) 1532-2327 (Online) Journal homepage: http://www.tandfonline.com/loi/lcar20

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To cite this article: Su-Yan Wang, Pedro Laborda, Ai-Min Lu, Meng Wang, Xu-Chu Duan, Li Liu & Josef Voglmeir (2017): Chemo-enzymatic approach to access diastereopure α-substituted GlcNAc derivatives, Journal of Carbohydrate Chemistry, DOI: 10.1080/07328303.2017.1321116

To link to this article: http://dx.doi.org/10.1080/07328303.2017.1321116

JOURNAL OF CARBOHYDRATE CHEMISTRY
2017, VOL. 0, NO. 0, 1–12
https://doi.org/10.1080/07328303.2017.1321116

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Chemo-enzymatic approach to access diastereopure α-substituted GlcNAc derivatives

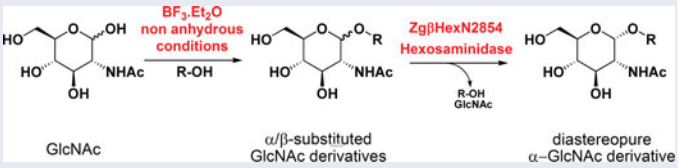
Su-Yan Wang[a], Pedro Laborda[a], Ai-Min Lu[b], Meng Wang[a], Xu-Chu Duan[a], Li Liu [a,c], and Josef Voglmeir [a]

[a]Glycomics and Glycan Bioengineering Research Center (GGBRC), College of Food Science and Technology, Nanjing Agricultural University, Nanjing, People's Republic of China; [b]College of Sciences, Nanjing Agricultural University, Nanjing, People's Republic of China; [c]Qlyco Ltd., Nanjing, People's Republic of China

ABSTRACT

The formation of diastereopure α-substituted GlcNAc derivatives in a simple and straightforward way is a challenging task. Herein, we report the chemical synthesis of diastereomeric α/β-substituted GlcNAc derivatives under non-anhydrous atmosphere using unprotected GlcNAc, followed by a selective enzymatic hydrolysis step using a bacterial *N*-acetyl-hexosaminidase to provide only α-substituted GlcNAc. This enzyme proved to selectively hydrolyze the β-anomeric GlcNAc derivatives, while the α-anomeric GlcNAc derivatives remained unreacted. The released GlcNAc (and therefore the α/β ratios) could be quantified using a coupled enzymatic assay consisting of GlcNAc 2-epimerase and *N*-acetyl- mannosamine dehydrogenase in a simple and accurate way.

GRAPHICAL ABSTRACT



ARTICLE HISTORY
Received 20 February 2017
Accepted 14 April 2017

KEYWORDS
Anomeric substitution; GlcNAc derivatives; *N*-acetyl-hexosaminidase; *O*-substituted GlcNAc quantification

Introduction

Among *O*-linked glycosides, beta-linked *N*-acetylglucosaminyl is a commonly found motif in posttranslational modifications of proteins.[1,2] However, little is known about the role of α-linked GlcNAc modification of proteins or other aglycons in nature. Even though some examples have been found in lower eukaryotes,[3,4] no experimental data on the occurrence of this moiety in higher eukaryotes were found so far. A recent discovery on human polypeptide-GalNAc transferases suggests that

CONTACT Li Liu liu.lichen@qlyco.com; Josef Voglmeir josef.voglmeir@njau.edu.cn College of Food Science and Technology, Nanjing Agricultural University, No.1 Weigang, Nanjing 210095, China.
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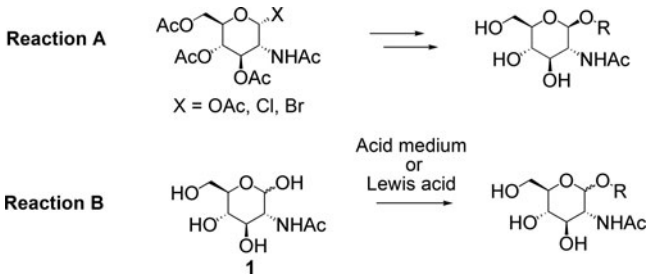


Scheme 1. Reaction A: traditional glycosylation reaction using protected GlcNAc as the starting material; Reaction B: glycosylation reactions using unprotected GlcNAc as the starting material.

α-linked GlcNAc to serine or threonine may also be a naturally occurring motif in humans.[5] In addition, several studies reported that mucosal glycans contain terminal α-GlcNAc moieties,[6,7] which were shown to be of interest as therapeutic agents to cure *Helicobacter pylori* infections.[8]

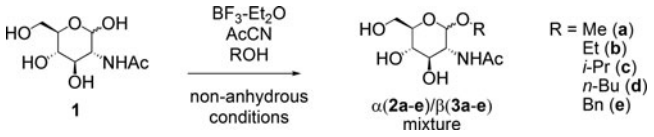
Most reported synthetic schemes to obtain anomerically modified GlcNAc derivatives are based on the complete protection of non-anomeric hydroxyl groups.[9–16] Thus, a suitable leaving group, usually a halogen or an acetyl group, is fixed at the anomeric center of the pyranose ring (Sch. 1, reaction A). After the successful glycosylation and removal of the protecting groups, the resulting anomerically substituted GlcNAc commonly consists mainly of the β-anomer. To increase the proportion of the α-anomer, several efforts have been made to improve the reactivity of the hydroxyl group at the anomeric center of unprotected GlcNAc (**1**) (Sch. 1, reaction B).[11,17–20] Typically, unprotected glycosylation reactions are carried out in strongly acidic media or in the presence of a Lewis acid at elevated temperatures thus allowing the synthesis of the GlcNAc derivative as a diastereomeric α/β-mixture.

Considerable progress in the development of regioselective glycosylation strategies has been achieved in recent years. However, the direct synthesis of anomerically substituted GlcNAc derivatives from unprotected GlcNAc remains a challenging task, due to self-condensation reactions and the requirement of strict anhydrous reaction conditions.[21] In this work, α/β-substituted GlcNAc derivatives are synthesized under non-anhydrous conditions, and β-substituted GlcNAc derivatives were selectively hydrolyzed allowing the facile isolation of the α-substituted GlcNAc derivatives. The released GlcNAc could be subsequently used to quantify the α/β ratios of anomerically substituted GlcNAc.

Results and discussion

Synthesis of anomerically substituted GlcNAc derivatives

Several synthetic routes to anomerically substituted GlcNAc derivatives using unprotected glycosyl donors have been reported to date. Matsumura and co-workers reported the glycosylation of **1** with methanol in the presence of 2% HCl;[19]



Scheme 2. Glycosylation of unprotected GlcNAc (**1**) under non-anhydrous conditions at room temperature. $BF_3{\cdot}Et_2O$ was used as the activator; methanol (**a**), ethanol (**b**), 2-propanol (**c**), *n*-butanol (**d**), and benzyl alcohol (**e**) were used as the glycosyl acceptors; acetonitrile (AcCN) was used as solvent. GlcNAc derivatives were obtained as an α(**2a–e**)/β(**3a–e**) mixture.

Ferreires et al. reported the ultrasound-assisted glycosylation of **1** with *n*-octanol under anhydrous conditions obtaining predominantly the α-anomer with good regioselectivity.[22] Wu and co-workers used $BF_3{\cdot}Et_2O$ and allyl alcohol obtaining 71% of the α-anomer.[23] Roy et al. glycosylated **1** with 2-chloroethanol in the presence of acetic acid.[24] Mahal et al. used 2-azidoethanol and azidotriethylene glycol as acceptors for the direct glycosylation of **1** in the presence of anhydrous Dowex 50 resin to provide only the α anomer.[25] Gao et al. reported glycosylation of **1** with methanol in presence of Amerlite IR-120 (H).[26] Roy et al. synthesized α-propargyl GlcNAc employing H_2SO_4-silica as a catalyst at 65°C.[27] Babic et al. obtained **1** using Fisher glycosylation with benzyl alcohol in the presence of *p*-toluenesulfonic acid monohydrate and benzene.[28] Shaikh and co-workers applied ultrasound-assisted glycosylation between **1** and propargyl alcohol or 2-azidoethanol in the presence of H_2SO_4-silica at 40°C.[13] Another approach by Polanki et al. described the synthesis of α-glycosylation products in high proportion using solvent-free conditions and bismuth nitrate as a catalyst.[18]

Both solvents and the catalysts have been demonstrated to be key elements in the chemical glycosylation of unprotected glycosyl donors.[22] In order to avoid self-condensation of unprotected GlcNAc, tetrahydrofuran, 1,4-dioxane, dichloromethane, and acetonitrile, in which **1** can be slightly dissolved, were evaluated in preliminary experiments as solvents; glycosylation of **1** under non-anhydrous conditions could only be detected when acetonitrile was employed (Sch. 2).

The Lewis acids $FeCl_3$, $ZnCl_2$, and $BF_3{\cdot}Et_2O$ were evaluated as glycosylation activators, with only the latter yielding glycosylation products. This result is in agreement with the results reported by Ferrieres et al, in which $ZnCl_2$ did not yield any glycosylation products either.[22] The highest yields for the glycosylation of **1** were observed in the presence of five equivalents of $BF_3{\cdot}Et_2O$. Interestingly, the observed yields for the glycosylation reaction under non-anhydrous conditions (Table 1) are similar or higher than the yield reported for the glycosylation of **1** with *n*-octanol under strict anhydrous conditions (30%).[22] Furthermore, the reported procedure required elevated temperatures (60°C) and ultrasound to achieve the glycosylation of GlcNAc in a dry atmosphere, whereas the glycosylation reaction described herein was performed at room temperature.

Substituted GlcNAc derivatives (**2a–e** and **3a–e**) were synthetized using methanol (**a**), ethanol (**b**), 2-propanol (**c**), *n*-butanol (**d**), and benzyl alcohol (**e**) as glycosyl

Table 1. Yields and α/β-ratios detected for the anomeric substitution reaction when methanol (**a**), ethanol (**b**), 2-propanol (**c**), *n*-butanol (**d**), and benzyl alcohol (**e**) were used as the glycosyl acceptors. *) Low isolated yields of entries 4 and 5 were due to the poor separation of the β-anomer from the α-anomer using silica gel chromatography, allowing that only non-overlapping elution fractions were further used.

Entry	Acceptor	Yield (%)	α/β ratio (colorimetric assay)	α/β ratio (NMR)	Yield (%) α-anomer
1	**a**	74	51:49	52:48	32%
2	**b**	68	80:20	80:20	53%
3	**c**	58	82:18	81:19	44%
4	**d**	18	87:13	88:12	9%*)
5	**e**	25	63:37	58:42	15%*)

acceptors for the nucleophilic substitution. Entries 1–4 in Table 1 show that yields were reduced with the increase in length of the glycosyl acceptor (i.e., entry 1, 74%).

When glycosyl acceptors with bulkier aliphatic chains (*n*-butanol and benzyl alcohol) were used, the yields decreased to below 30%. In all cases, a mixture of the α-anomer (**2a–e**) and the β-anomer (**3a–e**) was obtained. Although the equatorial location of the β-anomer is thermodynamically more favored than the axial location of the α-anomer (Table 1, entries 1–4), the proportion of α-anomer increases with the length of the glycosyl acceptor. Using *n*-butanol as the glycosyl acceptor, only 13% was obtained with a β-configuration, whereas that proportion increased to 49% when using methanol as glycosyl acceptor. The obtained results could be explained by considering the possible approaches of the glycosyl acceptor to the anomeric carbocation intermediate (Fig. 1). With bulkier substrates, the equatorial approach forming the β-anomer is hindered by the steric effect of the amidoacetyl group linked to the C-2 of the pyranose ring and thus, the α-anomer is kinetically favored. This is in agreement with the reported α/β ratio for the glycosylation of **1** with *n*-octanol (90:10).[22] Interestingly, the glycosyl acceptor benzyl alcohol led to an α/β ratio of 63:37, suggesting that the planar aromatic ring did not have relevant steric effects on the equatorial approach.

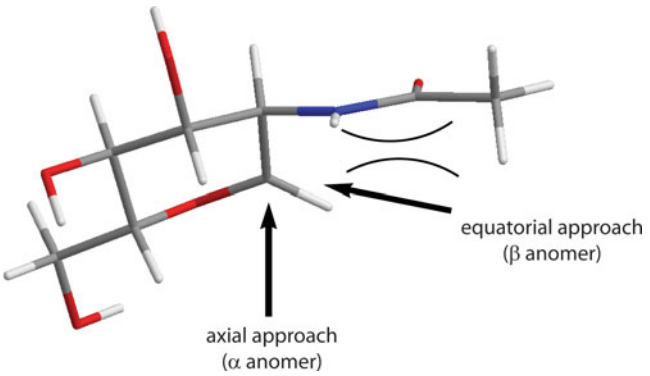


Figure 1. Possible approaches of the glycosyl acceptor to the anomeric carbocation intermediate. The approach that allows the formation of the β-anomer is hindered by the steric effect produced by the acetyl group.

Enzymatic hydrolysis of β-substituted GlcNAc derivatives

Despite most of the GlcNAc glycosylation strategies provide α and β mixtures, the isolation of the α-anomer remains to be a challenging task. In this field, Polanki and coworkers separated the α- and β-anomers after acetylation of the glycosylated GlcNAc derivative by automated flash chromatography equipped with a UV detector and ELSD using a column containing $AgNO_3$-impregnated silica gel.[18] Matsumura et al. reported the purification of the α-GlcNAc derivatives using an anion exchange resin.[19] In this work, the β-anomer was selectively hydrolyzed enzymatically, allowing the purification of the α-anomer by simple silica gel chromatography.

For the selective hydrolysis of the β-substituted portion of GlcNAc derivatives, a bacterial β-*N*-acetyl-hexosaminidase (E.C. 3.2.1.52) derived from *Zobellia galactanivorans* (ZgβHexN2854) was used. This enzyme has been recently employed by our group for the release of GlcNAc from chitinous mushrooms extracts.[29] Using *p*-nitrophenyl-β-GlcNAc (**3f**, Fig. 2A) as a substrate, the enzymatic activity could be easily observed by measuring the absorbance of *p*-nitrophenol, a yellowish chromogenic side product of the reaction (Fig. 2A). Hydrolytic reactions were performed at 37°C and pH 8.0 using 0.02 U/mL of recombinant ZgβHexN2854. This enzyme showed high hydrolytic activity towards **3f**, while *p*-nitrophenyl-α-GlcNAc (**2f**) could not be hydrolyzed by the enzyme (Fig. 2B).

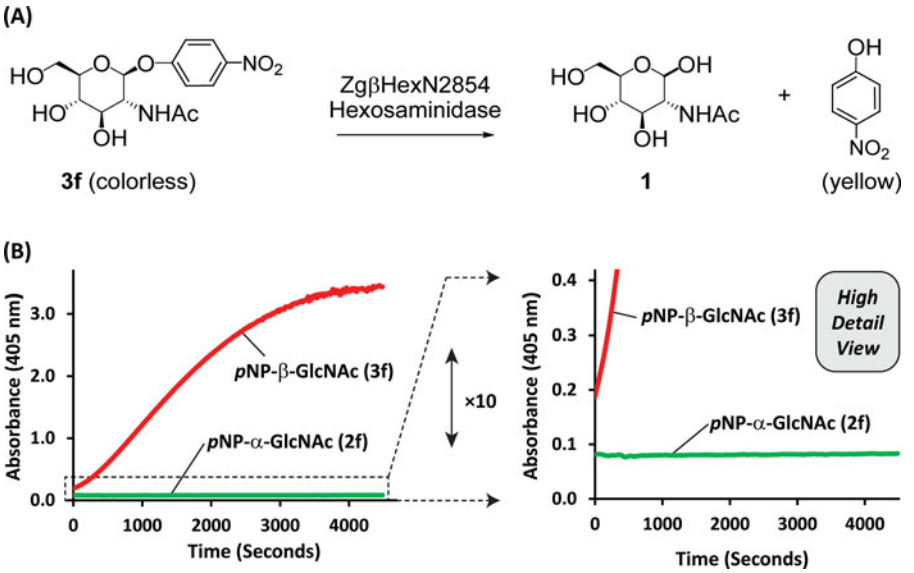
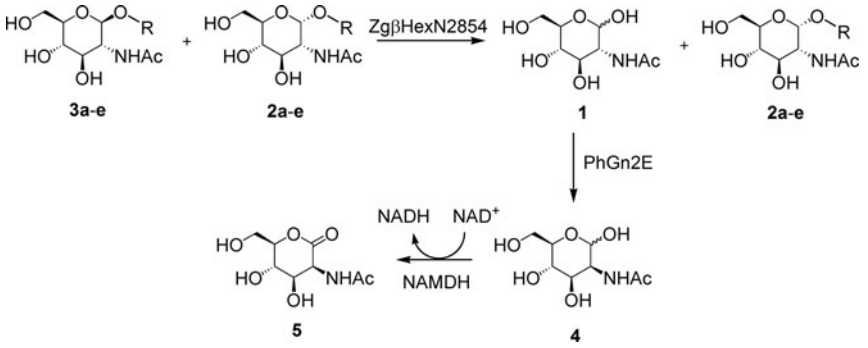


Figure 2. A: ZgβHexN2854-catalyzed hydrolysis of *p*-nitrophenyl-β-GlcNAc (**3f**). ZgβHexN2854 showed no activity towards *p*-nitrophenyl-α-GlcNAc (**2f**). B: UV response at 405 nm of ZgβHexN2854-catalyzed hydrolysis reaction when **2f** or **3f** was used as the substrates. Hydrolytic reactions were performed at 37°C and pH 8.0 (50 mM Na_3PO_4/citrate buffer).



Scheme 3. ZgβHexN2854-catalyzed hydrolysis of **3a**–**e** to obtain **1**. **2a**–**e** remained unreacted in the presence of ZgβHexN2854. PhGn2E catalyzed the epimerization reaction of **1** to ManNAc (**4**). Subsequently, **4** was oxidized to *N*-acetyl-D-mannosamino-lactone (**5**) using *N*-acetyl-D-mannosamine dehydrogenase (NAMDH). The NAMDH-catalyzed oxidation of **4** to **5** involved the reduction of NAD^+ to NADH, which was subsequently detected photometrically at 340 nm.

These results encouraged us to carry out the selective hydrolysis of β-GlcNAc derivatives of the obtained α(**2a–e**)/β(**3a–e**) mixtures (Sch. 3, upper part). ZgβHexN2854 could successfully release the anomerically-linked substituents of **3a**, **3b**, **3c**, **3d** within 12 h, while 24 h were needed for the complete hydrolysis of compound **3e** (Fig. S1). As expected, α-GlcNAc derivatives (**2a**, **2b**, **2c**, **2d**, and **2e**) remained unreacted during the incubation with ZgβHexN2854, and could be easily purified by silica gel chromatography (Figs. S2–S6).

Detection of β-linked GlcNAc

In this work, an enzyme coupled plate reader-based photometric assay for the accurate measurement of β-linked GlcNAc was developed. Thus, free GlcNAc released by the ZgβHexN2854 was interconverted into ManNAc (**4**) using a bacterial GlcNAc-2-epimerase (PhGn2E).[30] Then, the obtained ManNAc was selectively oxidized by a bacterial *N*-acetyl-D-mannosamine dehydrogenase (NAMDH) in the presence of NAD^+, affording *N*-acetyl-D-mannosamino-lactone (**5**) and NADH (Sch. 3, lower part).[31] Although several colorimetric assays for the detection of reducing sugars have been reported so far,[32] the developed colorimetric assay allows the specific quantification of β-GlcNAc.

Both the epimerization and oxidation reactions were carried out in microwell plate format. A linear correlation between the NADH-based absorbance at 340 nm and the concentration of GlcNAc could be established in the range of 0.25 mM and 4 mM of GlcNAc (Fig. 3) after a 75 min reaction time. The observed concentration of GlcNAc in the hydrolysis reaction revealed the concentration of β-GlcNAc derivatives and, consequently, the α/β ratios. A proton NMR-based calculation of the ratios in the α/β mixtures confirmed the reliability of the coupled photometric assay (^{1}H NMR spectra are shown in Figs. S7–S11).

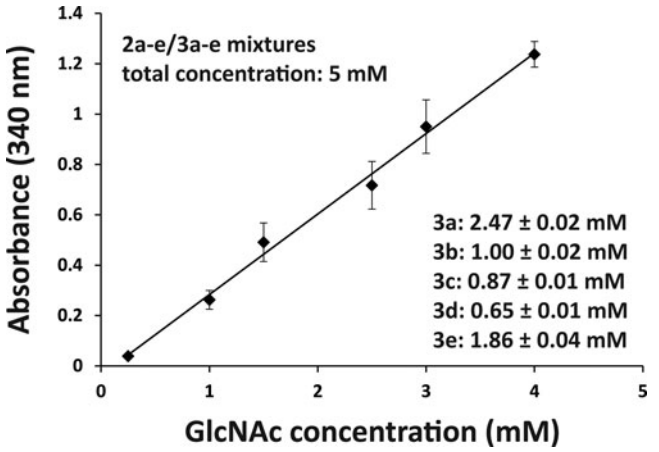
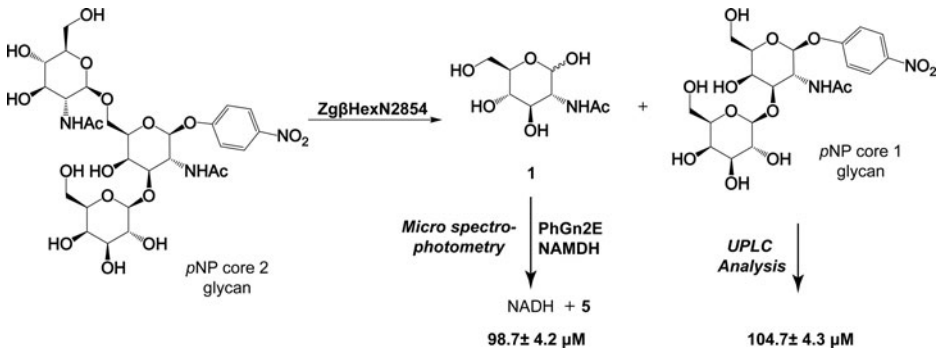


Figure 3. Linear regression of the developed colorimetric assay for the quantitative detection of GlcNAc. GlcNAc obtained from the ZgβHexN2854-catalyzed hydrolysis of **3a–e** was detected. Reaction conditions: 5 μL of the enzymatic hydrolysis were incubated with NAD^+ (2 mM), Na_3PO_4-citrate buffer (50 mM, pH 8.0), PhGn2E (5.8 μM), and NAMDH (3.4 μM, final volume: 50 μL) at 37°C for 75 min. Absorbance was measured at 340 nm in 15 s intervals.



Scheme 4. Quantification of the β-linked GlcNAc in a core 2 glycan structure. After the ZgβHexN2854-catalyzed hydrolysis of the *p*NP core 2 glycan, the released GlcNAc (**1**) was quantified micro spectrophotometrically using a coupled epimerization-oxidation assay. The *p*NP core 1 glycan by-product was quantified using UPLC-based analysis.

To further demonstrate the versatility of the applied method, the enzyme-coupled photometric assay was also applied to quantify a more complex core 2 glycan structure bearing β-linked GlcNAc (*p*NP-α-GalNAc[β1,3Gal]β1,6GlcNAc, Sch. 4). The GlcNAc concentration obtained from the assay (98.7 ± 4.2 μM) was in good agreement with concentration obtained from the UPLC-based assay which detected the (aquimolar) reaction by-product *p*NP-α-GalNAcβ1,3Gal (104.7 ± 4.3 μM).

Conclusion

In summary, an alternative approach for the synthesis to anomerically substituted GlcNAc derivatives using unprotected GlcNAc as the starting material has been developed. The herein described synthesis method allows the anomeric modification of GlcNAc under non-anhydrous conditions, and allowed the formation

of α/β mixtures using different alcohols as glycosyl acceptors. A bacterial β-*N*-acetyl hexosaminidase catalyzed the selective hydrolysis of the β-anomers, while α-anomers remained unreacted. Hence, this procedure allowed the easy isolation of α-diastereoisomers by silica-gel column chromatography, circumventing the extensive chromatographic or crystallographic workup generally required for the separation of anomers. Furthermore, the applied enzyme coupled photometric assay could be successfully used to measure the ratios of the chemically obtained α/β mixtures in a fast, easy, and reliable way. This assay might be especially useful in procedures wherein the NMR analysis is unfeasible, such as in high-throughput or sub-mg syntheses.

Experimental

Materials

Buffers, salts, and other chemicals used in this study were bought at the highest grade from commercial suppliers without further purification or modification. The expression and purification of PhGn2E was performed as previously reported.[30] ZgβHexN2854 hexosaminidase and NAMDH *N*-acetyl-D-mannosamine dehydrogenase were obtained from Qlyco Ltd, Nanjing. NMR spectra were registered on a Bruker AV-400 instrument using the residual proton signal from the solvent as the internal standard.

Glycosylation reaction

To a solution of **1** (100 mg, 0.45 mmol) in acetonitrile (15 mL) and the corresponding alcohol (20 mmol, methanol (**a**), ethanol (**b**), 2-propanol (**c**), *n*-butanol (**d**), or benzyl alcohol (**e**)), boron trifluoride diethyl etherate (300 μL, 2.3 mmol) was added. The reaction mixture was stirred at room temperature overnight. Then, the solvent was evaporated under reduced pressure. The residue was then subjected to silica-gel column chromatography. The α/β mixture of the *N*-acetyl-D-glucosamine derivative was obtained as a white solid by eluting the compound with an AcOEt/MeOH 9:1 mixture (**2a/3a** 51:49 mixture: 74% yield, **2b/3b** 80:20 mixture: 68% yield, **2c/3c** 82:18 mixture: 58% yield, **2d/3d** 87:13 mixture: 18% yield, **2e/3e** 63:37 mixture: 25% yield).

Enzymatic assay using chromogenic pNP-GlcNAc as substrate

Reaction mixtures (final volume 50 μL) containing *p*NP-α-GlcNAc or *p*NP-β-GlcNAc (1 mM final concentration), and 0.001 U of ZgβHexN28545 hexosaminidase in Na_3PO_4/citrate buffer (50 mM, pH 8.0) were analyzed photo-spectrometrically at 405 nm in 15 s intervals over a time period of 75 min in a multi-well plate reader.

Hydrolysis of β-substituted GlcNAc derivatives

The purified α/β mixtures **2a/3a**, **2b/3b**, **2c/3c**, **2d/3d**, or **2e/3e** were dissolved in Na_3PO_4/citrate buffer (50 mM, pH 8.0) and 0.1 U of ZgβHexN2854 hexosaminidase were added (final volume 5 mL). The reaction mixture was incubated at 37 °C for 12 h in the case of **2a/3a**, **2b/3b**, **2c/3c**, and **2d/3d** and 24 h in the case of **2e/3e**. Then, the solvent was evaporated under reduced pressure. The residue was subjected to silica-gel column chromatography. **2a**, **2b**, **2c**, **2d**, and **2e** were obtained as white solids by eluting them with an AcOEt/MeOH 9:1 mixture (Yields: **2a**: 32%, **2b**: 53%, **2c**: 44%, **2d**: 9%, **2e**: 15%).

NMR signal annotations

2a: ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.76 (d, 1H, $J = 8$ Hz), 5.01 (d, 1H, $J = 5.6$ Hz), 4.74 (d, 1H, $J = 5.6$ Hz), 4.54 (t, 1H, $J = 6$ Hz), 4.52 (d, 1H, $J = 3.6$ Hz), 3.69–3.61 (m, 2H), 3.51–3.39 (m, 2H), 3.33–3.28 (m, 1H), 3.23 (s, 3H), 3.15–3.08 (m, 1H), 1.82 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6): δ .169.9, 98.4, 73.2, 71.4, 71.3, 61.4, 54.8, 54.2, 23.2.

2b: ^{1}H NMR (400 MHz, D_2O): δ 4.83 (d, 1H, $J = 3.6$ Hz), 3.89–3.61 (m, 6H), 3.53–3.33 (m, 2H), 2.00 (s, 3H), 1.16 (t, 3H, $J = 6.8$ Hz).

^{13}C NMR (100 MHz, D_2O): δ 174.3, 96.5, 71.6, 71.0, 69.9, 63.8, 60.4, 53.5, 21.7, 13.9.

2c: ^{1}H NMR (400 MHz, D_2O): δ 4.93 (d, 1H, $J = 3.6$ Hz), 3.91–3.66 (m, 6H), 3.42 (m, 1H), 2.00 (s, 3H), 1.17 (d, 1H, $J = 6$ Hz), 1.09 (d, 1H, $J = 6$ Hz).

^{13}C NMR (100 MHz, D_2O): δ 174.3, 95.0, 71.7, 70.9, 70.8, 70.0, 60.4, 53.6, 22.2, 21.7, 20.3.

2d: ^{1}H NMR (400 MHz, D_2O): δ 4.83 (d, 1H, $J = 3.6$ Hz), 3.88–3.79 (m, 2H), 3.77–3.64 (m, 4H), 3.47–3.40 (m, 2H), 1.99 (s, 3H), 1.61–1.48 (m, 2H), 1.39–1.26 (m, 2H), 0.86 (t, 3H, $J = 7.6$ Hz).

^{13}C NMR (100 MHz, D_2O): δ 174.3, 96.5, 71.6, 70.8, 69.9, 67.8, 60.3, 53.7, 30.5, 21.7, 18.6, 12.9.

2e: ^{1}H NMR (400 MHz, D_2O): δ 7.43–7.34 (m, 4H), 7.22–7.18 (m, 1H) 4.90 (d, 1H, $J = 3.6$ Hz), 4.73 (d, 1H, $J = 12$ Hz), 4.51 (d, 1H, $J = 11.6$ Hz), 3.85–3.56 (m, 5H), 3.85–3.56 (m, 1H), 3.45 (M, 1H), 1.91 (s, 3H).

^{13}C NMR (100 MHz, D_2O): δ 174.3, 137.0, 128.7, 128.6, 128.4, 95.8, 72.1, 70.8, 70.0, 69.7, 60.5, 53.7, 21.8.

Enzymatic assay using non-chromogenic GlcNAc derivatives as substrate

To an aqueous solution containing the ZgβHexN2854-catalyzed hydrolysis reaction solution (5 μL), 2 mM NAD^+ in 50 mM Na_3PO_4/citrate buffer (pH 8.0), PhGn2E (5.8 μM), and NAMDH (3.4 μM) were added (Final volume: 50 μL). The reaction mixture was analyzed using the same continuous plate reader-based method described by Awad et al.[33] and was recorded for 75 min. The linear relationship

between absorbance values and GlcNAc concentration was established by testing various GlcNAc standard solutions (between 0.25 mM and 4 mM).

The determination of the β-GlcNAc contents of the *p*NP-core 2 glycan was performed using a micro spectrophotometer (Nanodrop 1000, Thermo), which allowed to reduce assay volumes from 50 μL to 10 μL. Approximately, 0.5 mg of the *p*NP-core 2 glycan (Carbosynth Ltd., UK) was dissolved in 707 μL of dH_2O to obtain a 1 mM stock solution. One microliter of this solution was then mixed with 1 μL of a 1 mM *p*NP-β-gal standard solution (used as an inert internal standard for latter HPLC quantification) and 0.02 U of ZgβHexN2854 hexosaminidase in MES buffer (pH 6.8, 50 mM, final volume 6 μL), and incubated overnight at 37°C. To this mixture 1 µL NAD^+ (20 mM), 1 µL Na_3PO_4/citrate buffer (pH 8.0, 1 M), 1 µL of PhGn2E (1.16 µM), and 1 µL NAMDH (0.68 µM) were added. After 75 min of incubation at 37°C, the sample was first analyzed with a micro spectrophotometer at 340 nm and subsequently subject to UPLC analysis using a reversed phase column (Phenomenex Kinetex C18, bead size 1.7 µm, dimensions 150 × 2.10 mm) with ammonium formate (50 mM, pH 4.5, 0.2 mL/min) mixed with increasing concentrations of acetonitrile as eluent (from 15% to 40% in the first 6 min). The eluents (*p*NP-β-gal and *p*NP-α-GalNAcβ1, 3Gal) were detected with an UV wavelength of 300 nm and quantified based on their relative peak areas.

Funding

This work was supported in part by the Natural Science Foundation of China (grant numbers 31471703, A0201300537, and 31671854 to J.V. and L.L.), and the 100 Foreign Talents Plan (grant number JSB2014012 to J.V.). The authors would like to thank F. J. Gimenez-Warren for language editing of this manuscript.

ORCID

Li Liu http://orcid.org/0000-0002-2178-9237
Josef Voglmeir http://orcid.org/0000-0002-4096-4926

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