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# Enzymatic synthesis of *N*-glycoprotein linkage region disaccharide mimetics using β-*N*-acetylhexosaminidases from *Aspergillus oryzae* and *Vigna radiata*

Thiruneelakantan Lakshmanan and Duraikkannu Loganathan*

*Department of Chemistry, Indian Institute of Technology Madras, Chennai 600036, India*

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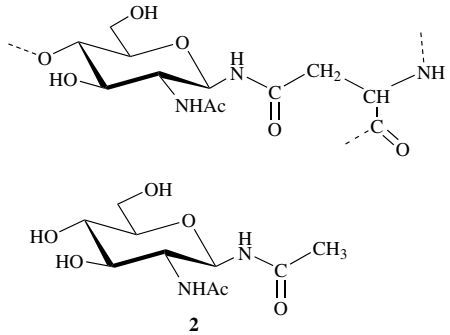
Dedicated to Prof. Girish Kumar Trivedi on his 65th birthday

**Abstract**—Glycosidases are valuable catalysts for the synthesis of a wide array of di- and oligosaccharides. Herein we report on the use of β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose, the simple model of the *N*-glycoprotein linkage region, as a novel acceptor for disaccharide synthesis catalyzed by β-*N*-acetylhexosaminidases from *Aspergillus oryzae* and mung beans (*Vigna radiata*) under transglycosylation as well as reversed hydrolysis. The exclusive formation of the disaccharide model, β-D-GlcNAc-(1→4)-D-GlcNAcβNHAc, by transglycosylation and the corresponding (1→6) analog under reversed hydrolysis both in reasonable yields demonstrates the versatility of β-*N*-acetylhexosaminidase from *Aspergillus oryzae*. The efficacy of the enzyme from *Vigna radiata* for synthesis has been demonstrated for the first time. This is also the first report on the use of derivatized sugars as co-reactants in glycosidase catalyzed reversed hydrolysis mode of synthesis. The excellent (1→6) selectivity of mung beans β-*N*-acetylhexosaminidase under transglycosylation and that from *Aspergillus oryzae* under reversed hydrolysis would prove to be very useful.


## 1. Introduction

Oligosaccharide components of glycoproteins play vital roles in many biological processes as both recognition determinants as well as modulators of the intrinsic properties including folding of proteins.[1] The linkage region constituents, GlcNAc and Asn, are conserved in the *N*-glycoproteins of all the eukaryotes.[2] As the motion of the Asn-GlcNAc linkage (Fig. 1) can significantly alter the presentation of the sugar on the cell surface, elucidation of the structure and conformation of the linkage region of glycoproteins is of fundamental importance for a better understanding of the inter- and intramolecular carbohydrate–protein interactions. In view of the structural complexity and heterogeneity of the glycoprotein glycans, investigation using model compounds would be a valuable approach in this regard. Our recent X-ray crystallographic investigation of β-1-*N*-acetamido derivatives of several monosaccharides as models and analogs of *N*-glycoproteins has yielded very interesting results that support the notion that the linkage region




**2**

**Figure 1.** *N*-Glycoprotein linkage region and the model **2**.

constituents may play a structural role meeting three essential requirements: rigidity, planarity, and linearity.[3] In an effort to extend the study to disaccharide mimetics of the linkage region, we have chosen to explore the glycosidase-base synthetic methodology.

Glycosidases have proven to be very attractive catalysts for the production of oligosaccharides.[4] Glycosylation catalyzed by a variety of glycosidases has been achieved

* Corresponding author. Tel.: +91 44 22578264; fax: +91 44 22570509; e-mail: loganath@iitm.ac.in

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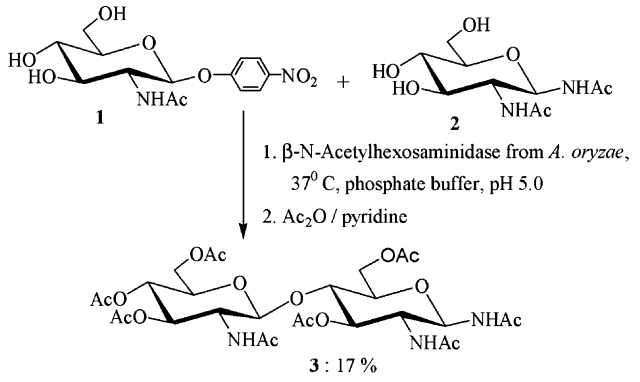
under both kinetically controlled (transglycosylation) as well as equilibrium controlled (reversed hydrolysis) conditions. Only free saccharides or *O*-/*S*-glycosides have so far been employed as acceptors for β-*N*-acetylhexosaminidase catalyzed oligosaccharide synthesis. The yield and the regioselectivity of the disaccharide products formed have been shown to be dependent on the source of the enzyme, the reaction time, and the nature of the acceptor and donor used. Compared to β-galactosidases, the number of commercially available β-*N*-acetylhexosaminidases is rather limited and these are relatively expensive. Enzymatic transglycosylation involving glycosyl amide as an acceptor is a valuable approach first reported from our laboratory initially using β-galactosidase from *Bacillus circulans*[5] and subsequently shown to be successful with almonds β-glucosidase and cloned *Pichia etchellsii* β-glucosidase II.[6] Herein, we demonstrate for the first time (a) disaccharide synthesis using β-1-*N*-acetamido derivative of GlcNAc **2**, the simple model of the *N*-glycoprotein linkage region (Fig. 1), as an acceptor under both transglycosylation and reversed hydrolysis catalyzed by the more often employed commercial β-*N*-acetylhexosaminidase from *Aspergillus oryzae* and (b) the synthetic utility of the partially purified enzyme from germinating mung bean, *Vigna radiata* (L.) R. Wilczek, a novel source for transglycosylation.

## 2. Results and discussion

Since the enzyme catalysis involves the competition of water (>50 M) and the acceptor sugar nucleophile (~100 mM) in trapping the enzyme-held oxocarbenium ion intermediate leading to hydrolysis and synthesis, respectively, use of organic co-solvents should contribute significantly to improving the disaccharide yield. Contrary to the expected increase due to lowering of water activity, use of glycosidases in aqueous organic media on many occasions have led to a decrease in transglycosylation yield.[7] However, a systematic study on *B. circulans* β-galactosidase catalyzed transglycosylation using β-1-*N*-acetamido-D-glycopyranose as an acceptor did result in improved yield of glycosylation in aqueous organic media.[5] In an effort to choose the optimum medium for the β-*N*-acetylhexosaminidase catalyzed disaccharide synthesis, the effect of organic co-solvents on the stability of the title enzymes was first examined. The various aqueous organic media employed contained one of the following solvents: acetone, dioxane, dimethylformamide, dimethylsulfoxide, and acetonitrile [30% (v/v) in the aqueous buffer]. It was observed that both the enzymes lost all activity in less than one minute of incubation at 37 °C in these aqueous organic media. Hence, further experiments were performed in aqueous buffer medium.

### 2.1. Reactions catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae*

Herein, disaccharide synthesis was first explored under transglycosylation conditions employing *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **1** as the donor and β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose **2** as acceptor sugar (Scheme 1) with a donor to acceptor ratio of 1:10. After the disappearance of **1** in 4 h and subsequent inactivation of the enzyme, the solution was lyophilized and the solid obtained was peracetylated by treatment with acetic anhydride and pyridine. Flash column chromatographic purification of the peracetylated product on silica gel furnished essentially a single disaccharide in 17% yield. This disaccharide was unambiguously characterized as the (1→4)-linked regioisomer **3** based on high resolution ESI-MS data and 500 MHz NMR data obtained from two-dimensional homonuclear proton DQF-COSY and heteronuclear $^{1}H$–$^{13}C$ HMQC, and HMBC.

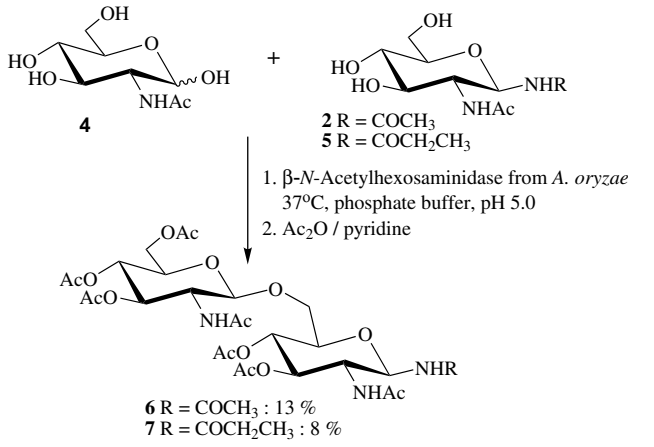



**Scheme 1.** Transglycosylation catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae*.

Aglycon moiety of the acceptor sugar is known to influence the regioselectivity of glycosidase catalyzed transglycosylation.[8] Reaction of *N*-acetyl-D-glucosamine, the free sugar, as the acceptor with **2** as the donor catalyzed by the title enzyme has been reported to result in the formation of a mixture of (1→4)- and (1→6)-linked disaccharides with the isomeric ratio being 9:1 after 40 h and 8:92 after 210 h.[9] Self-condensation of **1** catalyzed by the enzyme from the same source also afforded *p*-nitrophenyl β-chitobioside and the (1→6)-linked isomer as major and minor products, respectively.[10] In contrast, when methyl 2-acetamido-2-deoxy-α-D-glucopyranoside[9] was employed as an acceptor, the transfer was directed exclusively to the 4-position indicating that acceptor sugars carrying acetamido and methyl aglycon moieties appear to share similar binding characteristics.

Reversed hydrolysis is an alternate mode for glycosidase catalyzed oligosaccharide synthesis and only free sugars have been used as reactants in the earlier reported studies.[4] The product saccharides obtained in this manner exist as anomeric mixtures and also often consist of several regioisomers making their separation extremely difficult. We, therefore, explored reversed hydrolysis using **2** or **5** with a fixed aglycon group as one of the reactants, taken in large excess, along with GlcNAc as a co-reactant (Scheme 2). Thus reaction of GlcNAc (1 equiv) with **2** (10 equiv) in phosphate buffer, pH 5.0, at 37 °C for 5 days followed by the usual workup, derivatization and chromatographic purification afforded a single disaccharide in 13% yield. The product obtained was unambiguously characterized as the (1→6)-linked regio-

isomer **6** based on NMR and MS data as mentioned earlier. Encouraged by these results, reversed hydrolysis involving the propionamido derivative, **5**, and GlcNAc as co-reactants was then performed under similar conditions. This reaction also turned out to be successful furnishing the corresponding (1→6)-linked disaccharide, **7**, in 8% yield (Scheme 2).

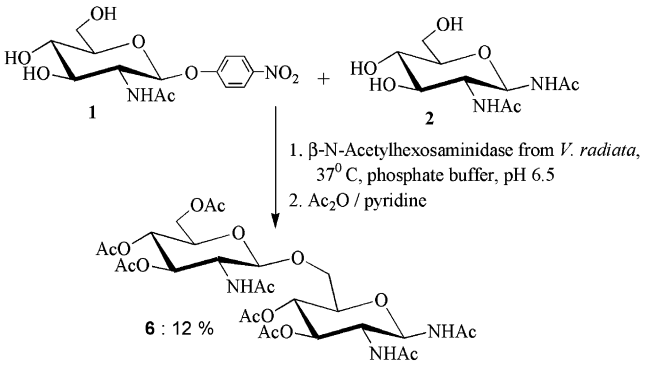



**Scheme 2.** Reversed hydrolysis catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae*.

The (1→6) selectivity of this enzyme under reverse hydrolysis conditions would prove to be valuable for the synthesis of various analogs of the *N*-glycoprotein linkage region complementing its (1→4) selectivity under transglycosylation. It is clear that the (1→4)-linked disaccharide **3** formed by transglycosylation is a kinetically favored product, while the (1→6)-linked disaccharide (**6** or **7**) is obtained when the reaction reaches equilibrium. Any (1→4)-linked disaccharide formed initially under reversed hydrolysis gets either hydrolyzed or transformed to the (1→6)-linked product. Thus the regioselectivity of the disaccharides synthesized is modulated by the mode of synthesis.

### 2.2. Reactions catalyzed by β-*N*-acetylhexosaminidase from *V. radiata*

As mentioned earlier, the number of commercially available β-*N*-acetylhexosaminidases is rather limited. β-*N*-Acetylhexosaminidase from the mung beans, *V. radiata*, was chosen for the present work for evaluating its synthetic utility. The enzyme was partially purified from mung bean sprouts obtained after 5 days of germination of seeds. The purification using ammonium sulfate precipitation followed by a DEAE-Sephadex column separation, resulted in a modest but definite 4-fold increase in the enzyme specific activity. Transglycosylation was carried out using this enzyme employing β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose as acceptor **2**, and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **1** as the donor sugar (Scheme 3). The single disaccharide product obtained in 12% yield after the usual workup, derivatization and chromatographic purification was fully characterized as the (1→6)-linked disaccharide **6**. Interestingly, the (1→6) regioselectivity of the mung bean enzyme complements the (1→4) selectivity of disaccharide observed in transglycosylation catalyzed by *A. oryzae* enzyme. This is the second report on enzymatic synthesis catalyzed by *N*-acetylhexosaminidase from a plant source, the other being from jack beans.[11] Attempted reaction of a ten fold excess of **2** with GlcNAc in phosphate buffer, pH 6.5 at 37 °C under reversed hydrolysis conditions in the presence of β-*N*-acetylhexosaminidase from *V. radiata* did not prove to be successful.




**Scheme 3.** Transglycosylation catalyzed by β-*N*-acetylhexosaminidase from *V. radiata*.

## 3. Conclusion

We have demonstrated disaccharide synthesis using β-1-*N*-amido derivatives of GlcNAc as novel acceptors, under transglycosylation as well as reversed hydrolysis, catalyzed by β-*N*-acetylhexosaminidases from two different sources. The versatility and value of β-*N*-acetylhexosaminidase from *A. oryzae* for the synthesis of linkage region disaccharide mimetics is evident from the exclusive formation of the model, β-D-GlcNAc-(1→4)-D-GlcNAcβ NHAc **3**, by transglycosylation and the corresponding (1→6) analog under reversed hydrolysis both in reasonable yields. The successful synthesis of (1→6)-linked disaccharide **7** from the propionamido acceptor under reversed hydrolysis catalyzed by the same enzyme is also noteworthy.

The efficacy of β-*N*-acetylhexosaminidase from *V. radiata* for synthesis has been demonstrated for the first time adding a new and cheaper source of this enzyme for further exploitation. The excellent (1→6) selectivity of this enzyme under transglycosylation and that of β-*N*-acetylhexosaminidase from *A. oryzae* under reversed hydrolysis would prove to be very useful for preparing several disaccharide analogs of lipid A, the hydrophobic anchor of lipopolysaccharide (LPS), present on the outer membranes of most Gram-negative bacteria.

## 4. Experimental

### 4.1. General

All sugars were purchased from the Sigma–Aldrich, USA or from Pfanstiehl Laboratories Inc., USA, and used as such without further purification.

β-*N*-Acetylhexosaminidase from *A. oryzae* was purchased from Sigma and used as such. Mung bean seeds were purchased from a local shop. Amido sugars **2** and **5** were prepared from 2-deoxy-2-acetamido-β-D-glucopyranosylamie[12] as reported earlier.[3] DEAE-Sepharose and bovine serum albumin were bought from Sigma, USA. Thin layer chromatograms were performed on 25 mm E. Merck silica gel plates (60F-254) or on micro slides coated with silica gel (200–300 mesh) of 0.1 mm thickness. Detection was done by spraying the plates with 10% sulfuric acid in ethanol and heating on a hot plate. Column chromatography was performed using silica gel under gravity (60–120 mesh) or under flash conditions (230–400 mesh).

Melting points were determined on a Toshniwal melting point apparatus and are uncorrected. Optical rotation was measured at 25 °C on a JASCO-DIP 200 digital polarimeter. Ultraviolet spectral absorbance was measured for solutions contained in 1 cm quartz cuvettes using a Hitachi 22A spectrophotometer. Infrared spectra were recorded on a Shimadzu R-470 spectrophotometer with a spectral resolution of 4 $cm^{-1}$ in the region of 4000–2000 $cm^{-1}$ and 1.5 $cm^{-1}$ in the region 2000–400 $cm^{-1}$. NMR spectra were recorded on a JEOL GSX-400, Varian DRX-500, BRUKER AMX 600 or BRUKER AVANCE DRX-400 spectrometer using tetramethylsilane as an internal standard in $CDCl_3$. ESI-MS spectra were measured on a Micromass Q-Tof or Applied Biosystems QSTAR mass spectrometer.

### 4.2. Reactions catalyzed by β-*N*-acetylhexosaminidase from *A. oryzae*

**4.2.1. Enzyme assay.**[13] A sample of enzyme solution (10 μL) in 0.05 M citrate buffer (pH 5.0) was added to a solution (90 μL) of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**, 5 mM) in the same buffer. The reaction mixture was incubated at 30 °C for 10 min. The reaction was then quenched by the addition of 3.9 mL of 0.1 M sodium carbonate solution. The absorbance of the released *p*-nitrophenol was measured at 420 nm. The solid protein had an activity of 1 U/mg. One unit of the enzyme is defined as the amount of enzyme that releases 1 μL of *p*-nitrophenol/min under the conditions stated above.

**4.2.2. Transglycosylation reaction**

**4.2.2.1. Synthesis of β-1-*N*-acetamido-2-acetamido-2-deoxy-3,6-di-*O*-acetyl-4-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-D-glucopyranose 3.** To a mixture of β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose **2** (acceptor, 760 mg, 2.9 mmol) in phosphate buffer pH 5.0 (3 mL) and β-*N*-acetylhexosaminidase (1 U, 1 mg, 100 μL of the same buffer) both pre-incubated at 37 °C, was added a solution of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **1** (donor, 100 mg, 0.29 mmol) in phosphate buffer pH 5.0 (11 mL) also maintained at the same temperature, over a period of 30 min. The progress of the reaction was monitored by the amount of released *p*-nitrophenol by UV ($\lambda$ = 420 nm). After 4 h, when the entire donor had reacted, the reaction was quenched by cooling in ice followed by heating in a boiling water bath for 10 min. The released *p*-nitrophenol was removed by extraction with ether. The aqueous solution was freeze dried and the residue was peracetylated by stirring it overnight at room temperature with acetic anhydride and pyridine (2 mL each). Pyridine was removed by successive co-evaporation using toluene and later using dichloromethane and the resulting solid obtained was dissolved in chloroform and impregnated onto Celite and purified by flash column chromatography on silica gel. Initial elution with $CHCl_3$–acetone = 95:5 followed by 83:17 gave the peracetate of the hydrolysis product (GlcNAc) of the donor sugar and that of the excess of the acceptor sugar in that order. The product disaccharide was eluted using $CHCl_3$–acetone = 77:23 to obtain 33.5 mg of **3**. Yield, 17%; colorless syrup; $[\alpha]_D$ = +12.1 (*c* 0.78, $CHCl_3$); $^1H$ NMR (500 MHz, $CDCl_3$): $\delta$ 7.13 (d, 1H, *J* = 9.2 Hz, NH-1), 5.43 (t, 1H, *J* = 9.8 Hz, H-3′), 5.20 (t, 1H, *J* = 9.6 Hz, H-3), 5.01 (t, 1H, *J* = 9.8 Hz, H-4′), 4.80 (t, 1H, *J* = 9.3 Hz, H-1), 4.45 (m, 1H, H-6′b), 4.42 (d, 1H, *J* = 8.9 Hz, H-1′), 4.20 (m, 1H, H-6b), 4.13–4.11 (m, 2H, H-2, H-6′a), 4.10 (t, 1H, *J* = 8.9 Hz, H-2′), 4.02 (dd, 1H, *J* = 2.0 and 12.4 Hz, H-6a), 3.79 (t,1H, *J* = 9.6 Hz, H-4), 3.68 (m, 1H, H-5′), 3.61 (m, 1H, H-5), 2.15–1.92 (7s, 24H, $COCH_3$); $^{13}C$ NMR (120 MHz, $CDCl_3$): $\delta$ 172.5, 171.8, 171.5, 171.4, 171.2, 170.4, 170.3, 169.7 (C=O), 100.2 (C-1′), 80.6 (C-1), 74.5 (C-4), 74.1 (C-5), 72.7 (C-4′), 71.4 (C-2′), 71.8 (C-5′), 70.3 (C-3′), 68.3 (C-3), 65.1 (C-2), 63.3 (C-6), 61.9 (C-6′), 20.6, 21.0, 22.6, 23.2, 24.9, 26.0, 29.2 and 29.4 ppm ($CO\mathit{CH}_3$). ESI-MS: Calcd for $C_{28}H_{42}N_3O_{16}$ [$M+H^+$]: 676.2565. Found 676.2553.

**4.2.3. Reversed hydrolysis reactions**

**4.2.3.1. Synthesis of β-1-*N*-acetamido-2-acetamido-2-deoxy-3,6-di-*O*-acetyl-6-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-D-glucopyranose 6.** A solution of 2-acetamido-2-deoxy-β-D-glucosamine **4** (100 mg, 0.45 mmol) in phosphate buffer pH 5.0 (1 mL) was mixed with β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose **2** (1.17 g, 4.5 mmol) in 3 mL of the same buffer. To this solution incubated at 37 °C, was added the enzyme (3 U, 3 mg) in the same buffer solution (0.5 mL) pre-incubated at 37 °C. After 5 days, the reaction was arrested, worked-up, peracetylated and the resultant solid purified by flash column chromatography as described above. The product disaccharide was eluted using $CHCl_3$–acetone = 75:25 to obtain 39 mg of the title compound. Yield, 13%; colorless syrup; $[\alpha]_D$ = +4.7 (*c* 0.65, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 7.52 (d, 1H, *J* = 8.8 Hz, NH-1), 6.14 (d, 1H, *J* = 8.3 Hz, NH-2), 5.05 (t, 1H, *J* = 9.5 Hz, H-4′), 5.00 (t, 1H, H-3′), 4.98 (t, 1H, *J* = 9.7 Hz, H-3), 4.87 (t, 1H, *J* = 9.7 Hz, H-4), 4.77 (dd, 1H, *J* = 8.8 and 9.7 Hz, H-1), 4.65 (d, 1H, *J* = 8.6 Hz, H-1′), 4.25–4.15 (m, 3H, H-6′a, H-6′b, H-2′), 4.05 (m, 1H, H-2), 3.68 (m, 1H, H-5), 3.65 (m, 1H, H-6a,), 3.64 (dd, 1H, H-6b), 3.61 (m, 1H, H-5′) and 2.08–1.99 ppm (8s, 24H, $COCH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ 172.8, 172.4, 172.0, 171.0, 170.8, 170.7, 169.5, 169.3 (C=O), 102.0 (C-1′), 81.4 (C-1), 77.3 (C-5), 73.0 (C-2), 72.7 (C-3), 71.7 (C-4), 68.7

(C-4′), 68.1 (C-3′), 67.8 (C-4′), 67.1 (C-6), 65.1 (C-2′), 63.3 (C-6′), 20.6, 20.8, 22.7, 23.1, 23.4, 24.9, 28.9 ($CO\mathit{CH}_3$) ppm. ESI-MS: Calcd for $C_{28}H_{42}N_3O_{16}$ [M+H$^+$]: 676.2565. Found 676.2558.

**4.2.3.2. Synthesis of β-1-*N*-propionamido-2-acetamido-2-deoxy-3,6-di-*O*-acetyl-6-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-D-glucopyranose 7.** A solution of 2-acetamido-2-deoxy-β-D-glucosamine **4** (100 mg, 0.45 mmol) in phosphate buffer pH 5.0 (1 mL) was mixed with β-1-*N*-propionamido-2-acetamido-2-deoxy-D-glucopyranose **5** (1.25 g, 4.5 mmol) in 5 mL of the same buffer. To this solution incubated at 37 °C, was added the enzyme, (3 U, 3 mg) in the same buffer (0.5 mL) pre-incubated at 37 °C. After 7 days, the reaction was arrested, worked-up, peracetylated and the resultant solid purified by flash column chromatography as described above. The product disaccharide was eluted using $CHCl_3$–acetone = 78:22 to obtain 25 mg of the title compound. Yield, 8%; colorless syrup; $[\alpha]_D = -1.3$ (*c* 0.75, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 7.57 (d, $J$ = 8.6 Hz, 1H, NH-1), 6.21 (d, $J$ = 8.5 Hz, 1H, NH-2), 5.08–5.00 (m, 3H, H-3′, H-4′, H-3), 4.92 (t, $J$ = 9.8 Hz, 1H, H-4), 4.80 (dd, $J$ = 8.6 and 9.7 Hz, 1H, H-1), 4.65 (d, $J$ = 8.6, 1H, H-1′), 4.23–4.08 (m, 3H, H-6′a, H-6′b, H-2′), 4.03 (m, 1H, H-2), 3.67–3.73 (m, 3H, H-6a, H-6b, H-5′), 3.59 (m, 1H, H-5), 2.28 (q, $J$ = 6.5 $OCH_2$), 2.12–1.98 (7s, 21H, $COCH_3$) and 0.89 ppm (t, $J$ = 6.5 Hz, $COCH_2CH_3$); ESI-MS: Calcd for $C_{29}H_{44}N_3O_{16}$ [M+H$^+$]: 690.2722. Found 690.2728.

### 4.3. Reactions catalyzed by β-*N*-acetylhexosaminidase from *V. radiata*

#### 4.3.1. Extraction and partial purification of enzyme

**4.3.1.1. Extraction.** Mung bean [*V. radiata* (L.) R. Wilczek] seeds (25 g) immersed in distilled water for 2 h at room temperature and sterilized with 0.5% hydrogen peroxide for 2 min was washed thoroughly and allowed to germinate in a dark germination chamber for 5 days at room temperature.[14] The seeds were kept in an atmosphere of 100% humidity by spreading them in folds of cheesecloth. At the end of 5 days, the germinated seeds were homogenized in 50 mM Tris–HCl buffer, pH 7.5 (50 mL) containing 10 mM β-mercaptoethanol and the solid particles from the resultant slurry removed by squeezing the liquid through cheesecloth. The clear liquid thus obtained was centrifuged at 16,000*g* for 40 min. The supernatant (100 mL) was dialyzed against 50 mM Tris–HCl buffer as above and taken to the next step. The dialyzed solution contained 52 mg of protein with a specific (β-*N*-acetylhexosaminidase) activity of 0.13 U/mg. All the following steps were performed at 4 °C.

**4.3.1.2. Ammonium sulfate fractionation.** The supernatant (100 mL) was saturated to 30% by the addition of solid ammonium sulfate, stirred till it dissolved completely and kept aside for 1 h. The precipitate formed was separated by centrifugation (30,000*g* for 20 min) and the pellet reconstituted in Tris buffer, pH 7.5 (5 mL). It was found to have a negligible specific activity (0.007 U/mg). The supernatant was then saturated to 60% ammonium sulfate. The precipitate formed was centrifuged as above. The pellet was reconstituted in 15 mL of the same buffer and assayed for the specific activity. It was dialyzed twice against phosphate buffer pH 6.5. The dialyzed solution containing 22 mg of protein with a specific activity of 0.25 U/mg was further purified by DEAE-Sepharose column as described below.

**4.3.1.3. DEAE-Sepharose chromatography.** The enzyme solution obtained in the above step was applied to a DEAE-Sepharose column (2 × 20 cm), which had been equilibrated with 0.05 M sodium phosphate buffer, pH 6.5 (100 mL). The column was eluted with 200 mL of the same buffer and fractions of 5 mL were collected. β-*N*-Acetylhexosaminidase activity was observed in fractions 5–15. These fractions were mixed and the solution was dialyzed against phosphate buffer pH 6.5 and the resultant enzyme solution containing 8 mg of protein with a specific activity of 0.48 U/mg was used for synthesis.

**4.3.2. Enzyme assay.**[14] The reaction mixture contained 750 μL of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (5 mM) and 250 μL of the diluted enzyme extract (100 μL made up to 1 mL in 50 mM citrate buffer, pH 4.5) incubated at 37 °C for 15 min. The reaction was quenched by the addition of 1.4 mL of 0.2 M sodium carbonate and the intensity of the color developed by the release of the *p*-nitrophenol was measured at 420 nm with the corrections being made for the appropriate blank.

**4.3.3. Transglycosylation reaction.** To a mixture of β-1-*N*-acetamido-2-acetamido-2-deoxy-D-glucopyranose **2** (760 mg, 2.9 mmol, 3 mL) in phosphate buffer pH 6.5 and β-*N*-acetylhexosaminidase (2 U, 10 mL, phosphate buffer, pH 6.5) incubated at 37 °C, was added a solution of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **1** (donor, 100 mg, 0.29 mmol) in the same buffer (11 mL), maintained at 37 °C, over a period of 30 min. The progress of the reaction was monitored by the amount of released *p*-nitrophenol by UV ($\lambda$ = 420 nm). After the entire donor sugar had reacted (8 h) as indicated by the released *p*-nitrophenol, the reaction was quenched by heating in a boiling water bath for 10 min followed by cooling in ice. The released *p*-nitrophenol was removed by extraction with diethyl ether (5 × 20 mL). The aqueous solution was freeze dried and the residue was peracetylated, worked-up and the resultant solid purified by flash column chromatography as described above. The product disaccharides were eluted using $CHCl_3$–acetone = 75:25 to obtain 23.6 mg (12%) of a fraction whose physical and spectral data matched well with those of **6** and hence identified as the (1→6)-linked disaccharide.

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