Complete NMR assignment of a bisecting hybrid-type oligosaccharide transferred by *Mucor hiemalis endo*-β-*N*-acetylglucosaminidase

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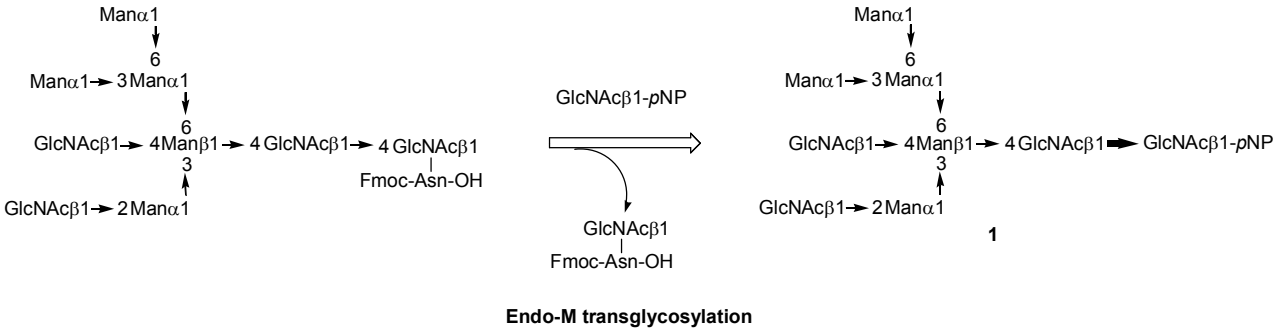
Abstract- This study describes the complete nuclear magnetic resonance (NMR) spectral assignment of a bisecting hybrid-type oligosaccharide **1**, transferred by *Mucor hiemalis endo*-β-*N*-acetylglucosaminidase (Endo-M). Through ^{1}H- and ^{13}C-NMR, DQF-COSY, HSQC, HMBC, TOCSY, and NOESY experiments, we determine the structure of the glycoside linkage formed by the Endo-M transglycosylation, i.e., the connection between GlcNAc and GlcNAc in oligosaccharide **1**.

Keywords: NMR spectral assignment; Bisecting hybrid-type oligosaccharide; Endo-β-*N*-acetylglucosaminidase; Endo-M; Transglycosylation product

With advances in technology, the sensitivity and resolution of NMR spectroscopy have increased. NMR spectroscopy has now become a useful tool for determining the structure of complicated oligosaccharides. Accurate NMR information about oligosaccharide structures helps to elucidate the biological roles of the oligosaccharides that play crucial functions in cell–cell communications, viral infections, and other molecular recognition events.[1]

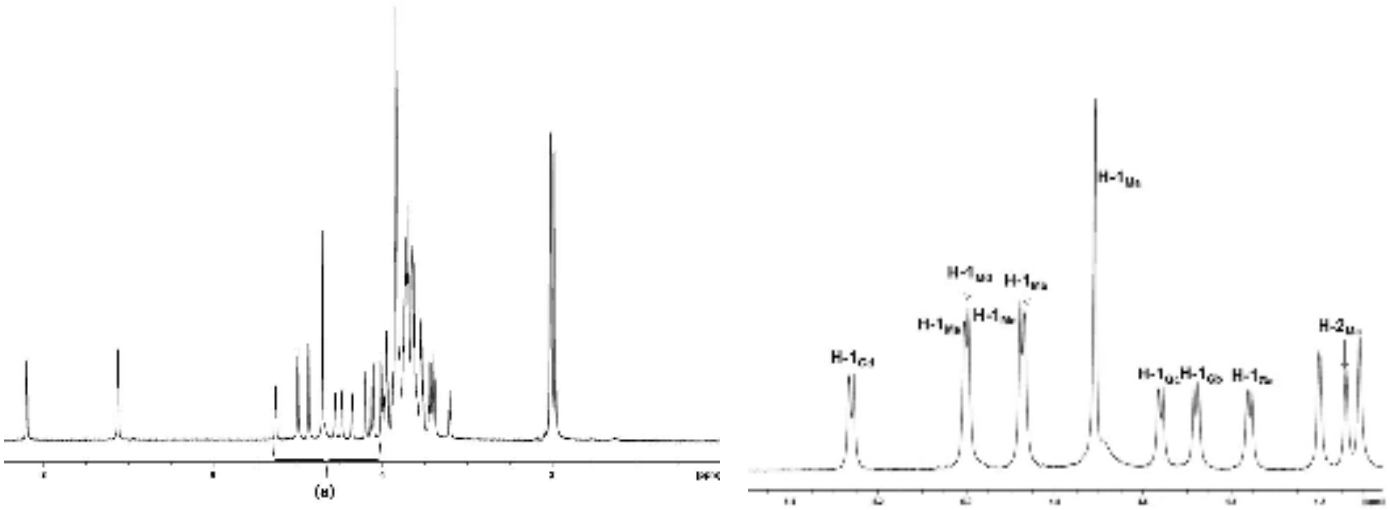
Mucor hiemalis Endo-M hydrolyzes the glycoside linkage in the *N*,*N*'-diacetylchitobiose moiety (GlcNAcβ1→4GlcNAc) of both complex-type and high-mannose-type oligosaccharides of the *N*-linked sugar chains from glycoproteins.[2] In addition to its hydrolysis activity, the enzyme has transglycosylation activity that transfers complex-type or high-mannose-type oligosaccharides to suitable sugar acceptors.[3] Furthermore, recent research has revealed that Endo-M N175Q mutant has dramatically enhanced the transglycosylation activity with sugar oxazoline donors.[4] The discovery of the mutant has improved the usability of Endo-M in the syntheses of various complex carbohydrates. At present, Endo-M is one of the most promising tools for the reconstruction and remodeling of oligosaccharides from glycoproteins.

Our former study showed for the first time that Endo-M has a transglycosylation activity that enables it to transfer a bisecting hybrid-type oligosaccharide from an ovalbumin glycopeptide to the oligosaccharide acceptor *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, as shown in Scheme 1.[5] As a part of our series of continued research, we report the NMR assignment of the transglycosylated product **1** in this study. To our knowledge, this is the first report on the NMR spectral assignment of a hybrid-type oligosaccharide. We believe that the glycoside linkage formed by the Endo-M transglycosylation, i.e., the connection between GlcNAc and GlcNAc in the oligosaccharides, is β1→4 because the enzyme shows hydrolytic characteristics toward the disaccharide unit of the GlcNAc–GlcNAc unit in the transglycosylated products. However, there was no clear evidence to show that the Endo-M transglycosylation provides a β1→4 linkage. Therefore, to determine the structure of the glycoside linkage formed by the Endo-M enzyme, the complete NMR spectral assignment for the bisecting hybrid-type oligosaccharide **1**, which was the Endo-M transglycosylated product, was conducted through ^{1}H- and ^{13}C-NMR, DQF-COSY, HSQC, HMBC, TOCSY, and NOESY experiments.



Scheme 1. Transglycosylation reaction of a bisecting hybrid-type oligosaccharide to GlcNAcβ-*p*NP using Endo-M

Figure 1 presents the ^{1}H-NMR spectrum of **1**. The eight signals of the anomeric protons (H-1) of **1** were observed as shown in (i) of Figure 1. The doublet signals (J = 8.0–8.4 Hz) resonating at 4.36, 4.48, 4.56, and 5.26 ppm are all β-anomeric protons (H-1_{Ga}, H-1_{Gb}, H-1_{Gc}, and H-1_{Gd}) of the four *N*-acetyl glucopyranoside residues (GlcNAc: Ga, Gb, Gc, and Gd). The singlet signals resonating at 4.87, 4.88, 4.99, and 5.00 ppm are the four anomeric protons (H-1_{Mb}, H-1_{Mc}, H-1_{Md}, and H-1_{Me}) in the five mannopyranoside residues (Man: Ma, Mb, Mc, Md, and Me). The signals of these anomeric protons were partially assigned by the support of the HSQC spectrum as mentioned later.



(i) (ii)

Figure 1. (i) ^{1}H NMR spectrum of **1** in D_2O. (ii) is the expansion of (a).

Although one anomeric proton signal (H-1_{Ma}) in the mannopyranoside residue Man was obscure, the TOCSY experiment revealed that the H-1_{Ma} signal determined at 4.69 ppm overlapped with the DOH signal and correlated to the proton signal at 4.14 ppm, i.e., H-2_{Ma}. Thus, the chemical shifts of all nine anomeric protons in **1** were confirmed. In addition, based on the above observation of the proton signals for the H-$1_{Ga–d}$, H-2_{Ma}, and H-$1_{Mb–e}$, the chemical shift assignments of all the other protons on each of the sugar rings in **1** were attempted through TOCSY experiments in which the mixing time was varied. Figure 2 shows a series of TOCSY spectra with the mixing time ranging from 20 ms to 150 ms. In the TOCSY spectrum with a mixing time of 20 ms, 1- or 2-step TOCSY transfers were observed. In the experiments using longer mixing times, the number of steps of the TOCSY transfer gradually increased. The TOCSY spectrum with a mixing time of 150 ms showed 5-step TOCSY transfers. These observations enabled us to assign all the protons on each of the sugar rings in **1**. Table 1 presents the ^{1}H chemical shifts of all the H-1–H-6 protons of Ga–d and Ma–e, respectively. The DQF-COSY spectrum of **1** also supports the TOCSY analysis (data not shown).

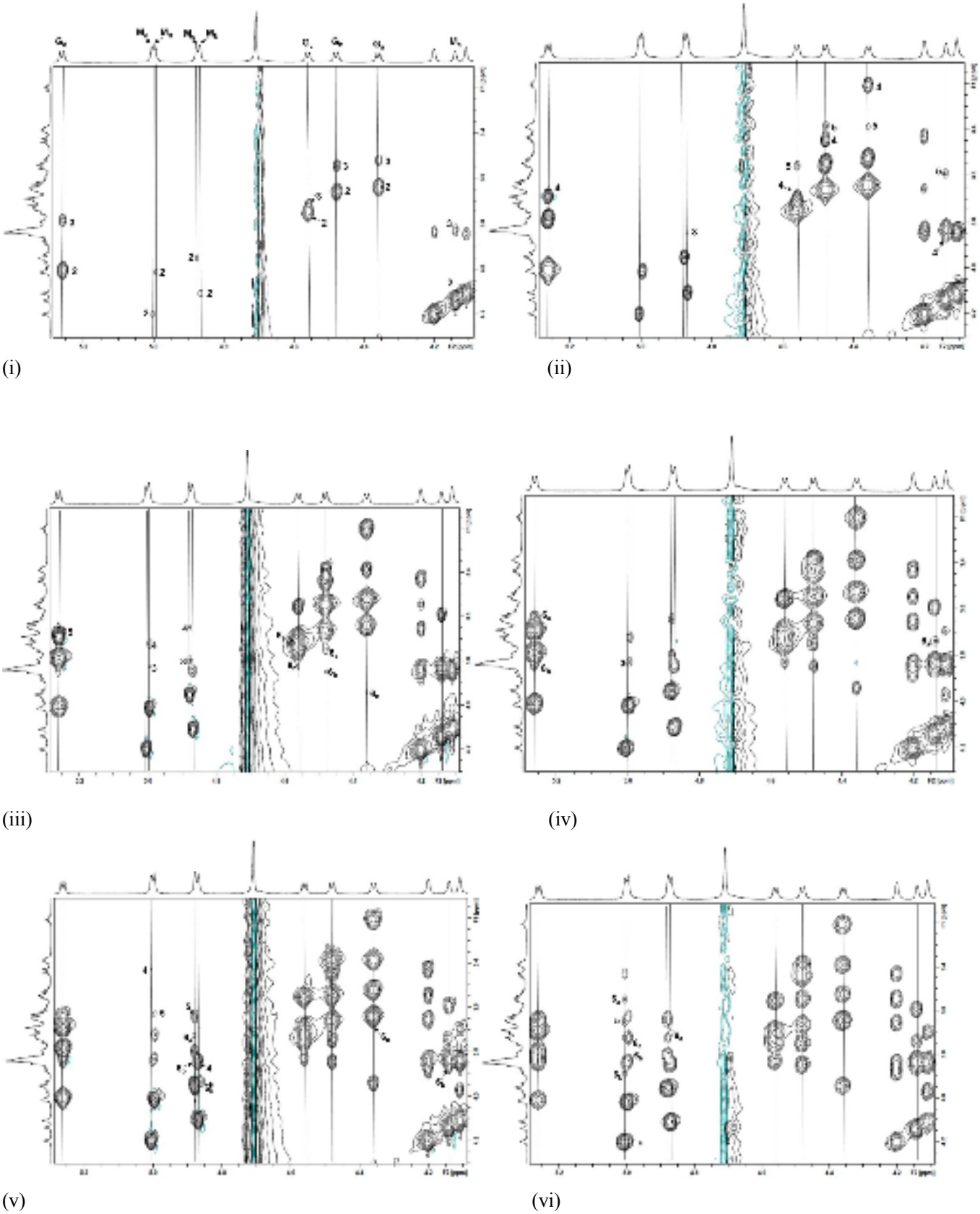
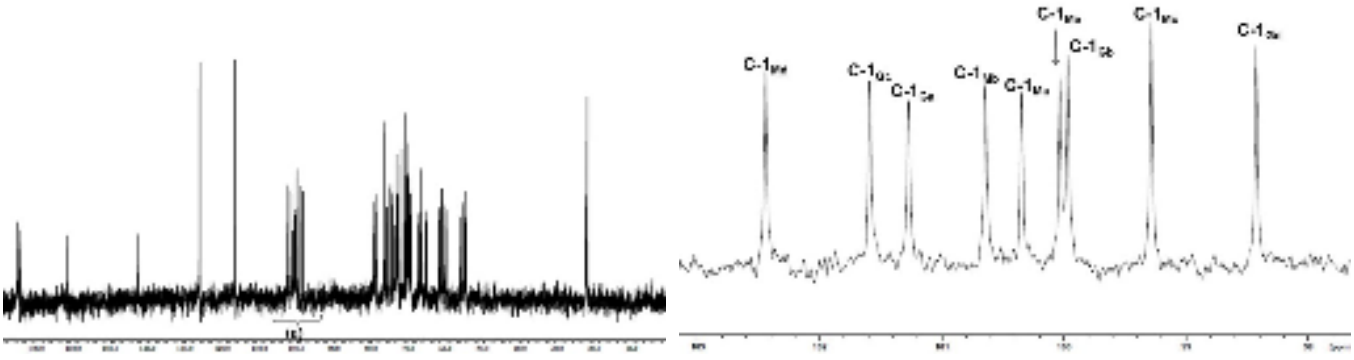


Figure 2. TOCSY spectra of **1**. Mixing time: (i) 20 ms; (ii) 40 ms; (iii) 60 ms; (iv) 80 ms; (v) 100 ms; (vi) 150 ms.

Figure 3 shows the ^{13}C-NMR spectra of **1**. All 54 carbon signals from C-1 to C-6 on each sugar ring (Ga–d and Ma–e) of **1** were separately observed. Then, the assignment of the ^{13}C chemical shift of all the C-1–C-6 of **1** was performed through an HSQC experiment (Figure 4). All the C-1–C-6 carbon signals on each sugar ring (Ga–d and Ma–e) of **1** were determined by the correlations between the proton and carbon signals. Furthermore, the J_{CH} values of the five Man residues were measured to explore a β-mannopyranosyl linkage.[6] The $J_{C\text{-}1,\,H\text{-}1}$ value of the Ma residue was 156.6 Hz, indicating a

β-mannnopyranosyl linkage, while the other Mb-e residues had $J_{C\text{-}1,\ H\text{-}1}$ values of 170.9–172.4 Hz, indicating an α-mannopyranosyl linkage. The ^{13}C chemical shift data from C1 to C6 on each of the sugar residues in **1** and the J_{CH} values of the five Man residues are given in parentheses in Table 1.



(i) (ii)

Figure 3. ^{13}C NMR spectrum of **1**. (ii) is the expansion of (a).

The connection of the nine sugar residues (Ga–d and Ma–e) of **1** was determined by an HMBC experiment. The inter-ring HMBC cross peaks with the eight anomeric protons (H-1) of **1** were successfully observed, as shown in Figure 5. The observed HMBC correlations are as follows: a: H-1_{Ga}–C-4_{Ma}; b: H-1_{Gb}–C-2_{Me}; c: H-1_{Gc}–C-4_{Gd}; d: H-1_{Ma}–C-4_{Gc}; e: H-1_{Mc}–C-6_{Mb}; f: H-1_{Mb}–C-6_{Ma}; g: H-1_{Me}–C-3_{Ma}; and h: H-1_{Md}–C-3_{Mb}. The following HMBC correlations were also observed: C-1_{Gc}–H-4_{Gd}, C-1_{Ma} –H-4_{Gc}, C-1_{Me}–H-3_{Ma}, C-1_{Gb}–H-2_{Me}, C-1_{Ga}–H-4_{Ma}, C-1_{Mb}–H-6_{Ma}, C-1_{Mc}–H-3_{Mb}, and C-1_{Md}–H-4_{Mb} (data not shown). The connection of these sugar residues based on the HMBC correlations is described in Scheme 2. The HMBC experiment clarified that Endo-M transglycosylation formed the GlcNAc–GlcNAc unit of **1** and that the linkage was β1→4. The assignment of the ^{1}H and ^{13}C chemical shifts of the four acetoamide groups of the GlcNAc units in **1** was also determined through the HMBC experiment. Thus, the complete NMR assignment of the bisecting hybrid-type oligosaccharide **1** was achieved.

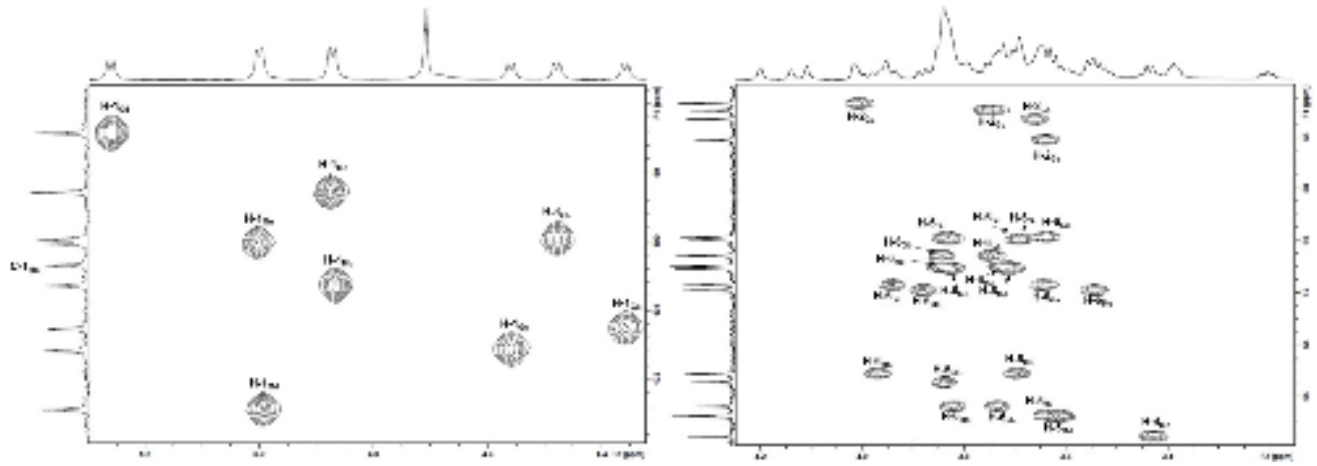
For the purpose of additional NMR study, an NOESY experiment was conducted to provide 3D conformational information about **1**, which is useful for understanding the protein binding structure. The NOESY spectrum of **1** showed 29 inter-ring NOE signals (data not shown). These NOE correlations are shown in Scheme 3.

Thus, this study described the complete NMR spectral assignment of a bisecting hybrid-type oligosaccharide **1** transferred by *Mucor hiemalis* Endo-M. Through ^{1}H- and ^{13}C-NMR, DQF-COSY, HSQC, HMBC, TOCSY, and NOESY experiments, it was confirmed that the glycoside linkage between GlcNAc and GlcNAc in **1**, which was formed by the Endo-M transglycosylation, was β1→4.

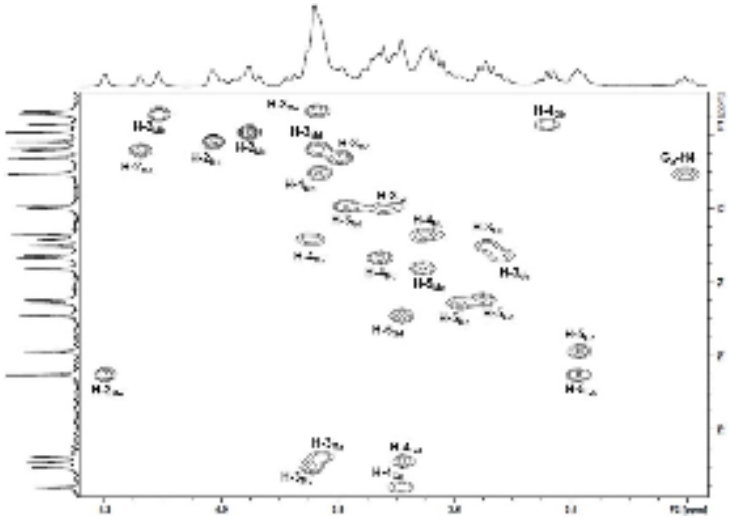
Experimental

The sample concentration was approximately 6.5 mM in D_2O (Cambridge Isotope Laboratories, Inc., 99.9% minimum in *D*). All spectra (^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC, HMBC, J_{CH}, and NOESY) were recorded at 298 K and at either 800 MHz (^{1}H) or 200 MHz (^{13}C). A standard Bruker Avance 800 US^2 spectrometer equipped with a TCI CryoProbe with a Z-field gradient was used to record the proton spectra. All NMR spectra were measured by using pulse sequences and standard procedures offered by Bruker. All chemical shifts were referenced to the lock D_2O. The measuring conditions for the 2D spectra were as follows: data size 2048 (F_2)/512 (F_1) and a relaxation delay of 1.5 s. The TOCSY experiments were

performed with mixing times of 20, 40, 60, 80, 100, and 150 ms. The NOESY experiment was performed with a mixing time of 500 ms.



(i) (ii)



(iii)

Figure 4. HSQC spectrum of **1**.

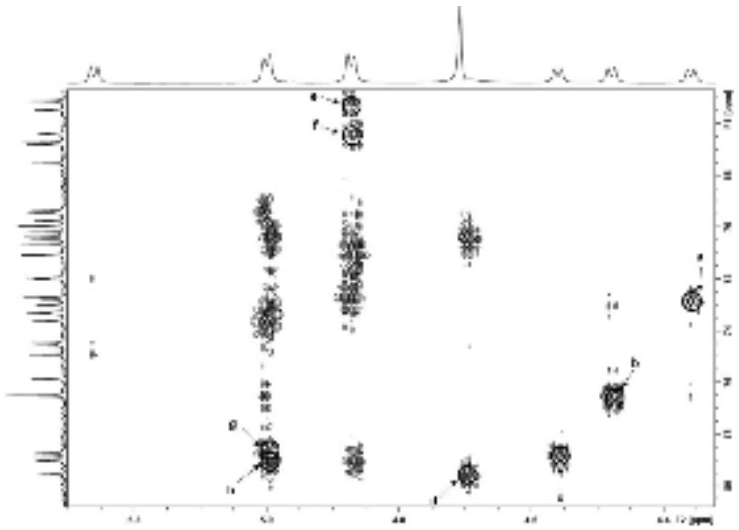


Figure 5. HMBC spectrum of **1**.

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Table 1. The ^{1}H and ^{13}C chemical shift assignments of **1**.

	Sugar unit	H-1 [C-1]	H-2 [C-2]	H-3 [C-3]	H-4 [C-4]	H-5 [C-5]	H-6 [C-6]	Acetoamido group	
								CH_3	C=O
GlcNAc	Ga	4.36 (d, J = 8.2 Hz) [101.3]	3.68 [56.1]	3.52 [73.2]	3.20 (t, J = 9.3 Hz) [71.1]	3.39 [76.5]	3.64, 3.94 [61.7]	2.00 (s) [22.1]	[175.2]
	Gb	4.48 (d, J = 8.2 Hz) [99.9]	3.66 [55.3]	3.54 [73.0]	3.44 (t, J = 9.8 Hz) [69.7]	3.39 [75.9]	3.75, 3.84 [60.6]	2.01 (s) [22.3]	[175.4]
	Gc	4.56 (d, J = 8.0 Hz) [101.6]	3.75 [55.0]	3.72 [72.0]	3.68 [79.6]	3.55 [74.5]	3.69, 3.83 [59.94]	2.03 (s) [22.4]	[175.6]
	Gd	5.26 (d, J = 8.4 Hz) [98.4]	4.0 [54.7]	3.78 [71.9]	3.71 [78.8]	3.69 [74.9]	3.64, 3.83 [59.85]	1.96 (s) [22.1]	[175.6]
Man	Ma	4.69 (s) [100.3, J_{ch} = 156.6 Hz]	4.14 (s) [70.3]	3.82 [78.7]	3.84 [72.8]	3.59 [74.6]	3.73, 3.86 [66.4]	-	-
	Mb	4.87 (s) [100.6, J_{ch} = 170.9 Hz]	4.10 (s) [69.5]	3.80 [79.0]	3.81 [71.1]	3.84 [65.5]	3.70, 3.95 [65.1]	-	-
	Mc	4.88 (s) [99.3, J_{ch} = 170.9 Hz]	3.95 [69.9]	3.80 [70.6]	3.65 [72.7]	3.62 [66.7]	3.72, 3.86 [61.0]	-	-
	Md	4.99 (s) [102.4, J_{ch} = 172.0 Hz]	4.01 [70.2]	3.83 [70.4]	3.72 [73.3]	3.63 [66.8]	3.72, 3.83 [61.1]	-	-
	Me	5.00 (s) [100.0, J_{ch} = 172.4 Hz]	4.20 (s) [76.5]	3.80 [69.4]	3.42 [67.5]	3.65 [73.6]	3.54, 3.88 [61.9]	-	-