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Carbohydrate Research 342 (2007) 23–30
Carbohydrate RESEARCH

# Substrate specificity of *N*-acetylhexosaminidase from *Aspergillus oryzae* to artificial glycosyl acceptors having various substituents at the reducing ends

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Received 20 June 2006; received in revised form 24 October 2006; accepted 5 November 2006
Available online 10 November 2006

**Abstract**—The substrate specificity of *N*-acetylhexosaminidase (E.C. 3.2.1.51) from *Aspergillus oryzae* was examined using *p*-nitrophenyl 6-*O*-sulfo-*N*-acetyl-β-D-glucosaminide (6-*O*-sulfo-GlcNAc-*O*-*p*NP) as the glycosyl donor and a series of β-D-glucopyranosides and *N*-acetyl-β-D-glucosaminides with variable aglycons at the anomeric positions as the acceptors. When β-D-glucopyranosides with methyl ($CH_3$), allyl ($CH_2CH{=}CH_2$), and phenyl ($C_6H_5$) groups at the reducing end were used as the acceptors, this enzyme transferred the 6-*O*-sulfo-GlcNAc moiety in the donor to the location of O-4 in these glycosyl acceptors with a high regioselectivity, producing the corresponding 6-*O*-sulfo-*N*-acetylglucosaminyl β-D-glucopyranosides. However, β-D-glucopyranose lacking aglycon was a poor substrate for transglycosylation. This *A. oryzae* enzyme could also accept various *N*-acetyl-β-D-glucosaminides carrying hydroxyl (OH), methyl ($CH_3$), propyl ($CH_2CH_2CH_3$), allyl ($CH_2CH{=}CH_2$) and *p*-nitrophenyl (*p*NP; $C_6H_4$–$NO_2$) groups at their aglycons, yielding 6-*O*-sulfo-*N*-acetylglucosaminyl-β(1→4)-disaccharide products.


*Keywords:* Substrate specificity; *N*-Acetylhexosaminidase; 6-*O*-Sulfo-*N*-acetyl-β-D-glucosaminide

## 1. Introduction

Recent studies in glycobiology have revealed that cell-surface oligosaccharides bound to glycoproteins and glycolipids play crucial roles in biological events.[1] O-Sulfated sugars found in glycosaminoglycans and proteoglycans as well as sulfo sialyl $Le^x$ have currently become the focus of studies and gained increasing interest because they serve as bio-informational molecules involved in cell adhesion, inflammation, immunological responses, cell differentiation, and malignant transformation.[2] They also work as cell-surface determinants for various pathogenic bacteria, viruses, bacterial toxins and carbohydrate-binding proteins such as lectins. Among these various O-sulfated sugars, the 6-*O*-sulfo-*N*-acetylglucosamino (6-*O*-sulfo-GlcNAc) structure is a core element in GlyCAM-1 and glycosaminoglycans. Therefore, a lot of syntheses of O-sulfated oligosaccharides involving 6-*O*-sulfo-GlcNAc have been reported.[3] Although purely chemical syntheses for such biologically significant molecules give products with defined structures, multiple protection and deprotection processes are required to construct these functional oligosaccharides. In contrast, enzymatic methods have been

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doi:10.1016/j.carres.2006.11.004

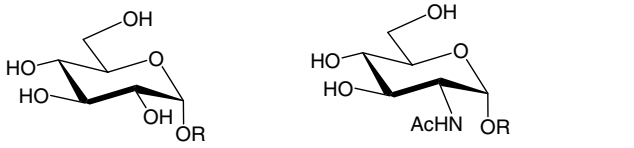
recently focused because of their efficiency and 'eco-friendly' processes.[4] For example, the synthesis of 6-*O*-sulfo-chitooligosaccharides has been reported, in which sulfotransferases and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) were applied.[4b]

Recently, we have proposed two alternative enzymatic methods. Molluscan sulfatases were conveniently used for the regioselective de-O-sulfation of multiply O-sulfated D-glycopyranosides, and the high synthetic potential was disclosed in its application to the library assembly of O-sulfated sugars.[5] Another enzyme, *N*-acetylhexosaminidase (NAHase, E.C. 3.2.1.51) from *Aspergillus oryzae*, was also useful for obtaining *O*-sulfo disaccharides in one step by regioselective transglycosylation.[6] In this paper, we describe the substrate specificity of this enzyme to a series of α-D-glucopyranosides and *N*-acetyl-α-D-glucosaminides having variable substituents at their aglycons, and also examined the utility of transglycosylation. The *p*-nitrophenyl (*p*NP) or allyl O-glycosides synthesized here are convenient for further chemical manipulations, which can be applied to carbohydrate chips and glycomaterials after immobilization into sensor substrates and polymerization.[7]

## 2. Results and discussion

NAHase-catalyzed transglycosylation is effective for oligosaccharide synthesis.[8] The enzyme has been used for the synthesis of 6′-O-acetylated chitobiose and related compounds. Recently, the substrate specificity of the enzyme was also reported using modified substrates.[8d] However, the utility of the enzyme for O-sulfated oligosaccharide synthesis has not yet been fully explored.

In our previous communication, we reported the synthetic utility of NAHase.[6] There, the *A. oryzae* enzyme transferred a 6-*O*-sulfo-GlcNAc moiety in the donor to O-4 of GlcNAcα-*O*-allyl, producing a 92% yield of the corresponding 6-*O*-sulfo-*N*-acetylglucosaminyl disaccharide. In this report, we examined in further detail the substrate specificity of this enzyme using various artificial glycosyl acceptors Glc (**2**–**5**) and GlcNAc (**6**–**10**) series in Scheme 1.




**2**, R=H (α/β=3/1)
**3**, R=$CH_3$ (methyl)
**4**, R=$CH_2CH{=}CH_2$ (allyl)
**5**, R=$C_6H_5$ (phenyl)

**6**, R=H (α/β=3/1)
**7**, R=$CH_3$ (methyl)
**8**, R=$CH_2CH_2CH_3$ (propyl)
**9**, R=$CH_2CH{=}CH_2$ (allyl)
**10**, R=$C_6H_5$-*p*$NO_2$ (*p*-nitrophenyl)

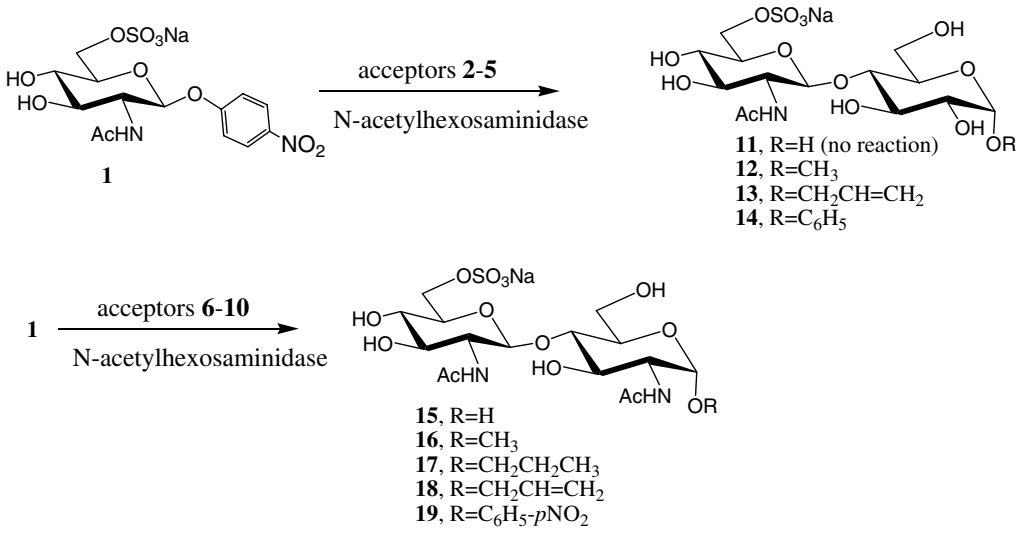
**Scheme 1.** Structures of glycosyl acceptors having various substituents at the aglycon moiety.

In our initial approach, we examined a series of α-D-glucopyranosyl acceptors with hydroxyl (**2**), methyl (**3**), allyl (**4**), and phenyl (**5**) groups at their aglycons. They were subjected to the enzyme reaction with *A. oryzae* NAHase in 50 mM phosphate buffer (pH 6.0) (Scheme 2), in which donor **1** at ca. 0.25 M was applied to the acceptors at ca. 1.3 M in each case. Every enzyme reaction was monitored by HPLC (ODS C-18, MeOH/$H_2O$ = 5:95 containing 0.1% TFA) at regular intervals, and the reaction mixture was stopped when disaccharide products were maximal. After 120–144 h, the amounts of disaccharide products reached saturation. Each product was purified with a column of ODS (C-18) or Bio-Gel P-2, and their structures were determined by HMQC and HMBC 2D NMR analysis.

As a typical example, the HMBC analysis of disaccharide product **14** showed to have the β-(1→4)-linkage from a long-range correlation between H-1′ ($\delta$ 4.63 ppm, $J$ 8.6 Hz) in a GlcNAc residue and C-4 ($\delta$ 82.5 ppm) in a Glc moiety. Further, the C-4 signal appearing at $\delta$ 82.5 ppm in its $^{13}C$ NMR spectrum of compound **14** had clearly shifted downfield as compared to that of other ring carbons [C-2 (73.5), C-3 (73.6), C-5 (74.6), and C-6 (62.6)] in the Glc moiety. Apparently, disaccharide product **14** has the β-(1→4)-linkage, which is similar to the results for 6-*O*-sulfo-GlcNAcβ(1→4)-Glcα-*O*Me **12**.[6] FAB-MS spectrum also supported the structure to be 6-*O*-sulfo-disaccharide product (584 $[M+Na]^+$). In a manner similar to **14**, transfer products using acceptor sugars **3** and **4** were determined to be **12** and **13**, respectively, having the β-(1→4)-linkage (Scheme 2).

The results summarized in Table 1 (entries 2–4) show that this *A. oryzae* enzyme transferred the 6-*O*-sulfo-GlcNAc moiety in donor **1** to the location of O-4 in glycosyl acceptors **3**–**5**, producing 6-*O*-sulfo-*N*-acetylglucosaminyl glucopyranosides (**12**–**14**). None of the transferred compound **11** was produced when the anomeric free glycosyl acceptor **2** was applied (Table 1, entry 1). The evidence reveals that this enzyme catalyzed the regioselective formation of the β-(1→4)-linked sulfo disaccharides when glycosyl acceptors **3**–**5** were used.

Similar results were also observed when using α-D-*N*-acetylglucosaminyl acceptors with hydroxyl (**6**), methyl (**7**), propyl (**8**), allyl (**9**), and *p*-nitrophenyl (**10**) groups (Table 1, entries 5–9). The NAHase catalyzed the transglycosylation reaction irrespective of aglycon structures even in free GlcNAc **6**. Every enzyme transferred the 6-*O*-sulfo-GlcNAc moiety in donor **1** to O-4 of those acceptors **6**–**10** with a high specificity, giving 6′-*O*-sulfo chitobioside **15**–**19** as summarized in Table 1. These transfer yields were higher (38–93% for **6**–**10**) as compared to those of glucopyranosyl acceptors **2**–**5** (0–36%) even when the aglycon structures were identical. For example, when using Glc-α-*O*-$CH_2CH{=}CH_2$ **4** as




**Scheme 2.** Enzymatic reactions with 6-*O*-sulfo-GlcNAc-*O*-*p*NP **1** as the donor and various glycosyl acceptors **2**–**10** were performed in 50 mM phosphate buffer (pH 6.0) at 35 °C.

**Table 1.** *N*-Acetylhexosaminidase-catalyzed transglycosylation using various glycosyl acceptors **2**–**10**[a]

| Entry | Acceptor | Time (h) | Enzyme amount[b] (U/mL) | Specificity | Products | Isolated yields[d] (%) |
|---|---|---|---|---|---|---|
| 1 | **2** | 120 | 27 | —[c] | —[c] | —[c] |
| 2 | **3** | 120 | 23 | O-4 | **12** | 17 |
| 3 | **4** | 120 | 19 | O-4 | **13** | 34 |
| 4 | **5** | 144 | 27 | O-4 | **14** | 36 |
| 5 | **6** | 120 | 24 | O-4 | **15** | 38 |
| 6 | **7** | 144 | 27 | O-4 | **16** | 51 |
| 7 | **8** | 144 | 17 | O-4 | **17** | 87 |
| 8 | **9** | 120 | 28 | O-4 | **18** | 92 |
| 9 | **10** | 120 | 24 | O-4 | **19** | 93 |

[a] *N*-Acetylhexosaminidase from *A. oryzae* was partially purified using commercially available β-D-galactosidase (Sigma) according to the method described in Section 3 and was used in this study. A typical procedure was performed with various acceptors **2**–**10** in the following way: ca. 1.3 M of the acceptor and 0.25 M of donor **1** in 50 mM phosphate buffer (pH 6.0) was incubated at 35 °C in the presence of 17–28 U of the enzyme. The reaction mixture was boiled for 5 min to stop the enzyme reaction, then the mixture was purified with an ODS column (TSK-gel Octadecyl-4PW, ∅ 10 × 45 cm) eluted with $H_2O$ to give disaccharide products. For further information, see Section 3.

[b] One unit of the enzyme hydrolyzed 1 μmol of *p*NP GlcNAc to *p*NP and GlcNAc per min.

[c] Not determined.

[d] Based on the donor added.

the acceptor, the transfer yield of product **13** was low (34%), while in case of GlcNAc-α-*O*-$CH_2CH{=}CH_2$ **9** as the acceptor, the yield of **18** reached 92%. This means that the substituents at C-2 (*N*-acetyl vs 2-OH) largely affected the yields, and the enzyme apparently preferred the GlcNAc series substrates. This *A. oryzae* enzyme also accepted *p*NP α-D-*N*-acetylglucopyranoside **10**, producing a high yield of 6′-O-sulfated disaccharide product **19** with the β-(1→4)-linkage (>93%).

In conclusion, we have described that the NAHase enzyme from *A. oryzae* catalyzed '6-*O*-sulfo-*N*-acetylglucosaminyl-transfer' reactions to a series of Glc and GlcNAc glycosyl acceptors with various substituents at the anomeric positions. The enzyme produces only β-(1→4)-linked disaccharides in a regioselective manner. Apparently, the GlcNAc acceptors having allyl and *p*NP groups as the anomeric substituents are excellent substrates, and the resulting disaccharides can be further applied to assemble glycomaterials and glycosyl chips or arrays after chemical manipulations. For instance, the *p*NP group can be readily reduced to the *p*-aminophenyl (*p*AP) group by conventional hydrogenation,[7a] and then converted to thiolate compounds with appropriate linkers.[9] The allyl group can also be transformed to the corresponding disaccharides carrying an ω-amino group at the aglycon moiety by photo-irradiation.[10] These compounds are useful for the analysis of the specific carbohydrate-protein interaction with surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) techniques.[7a,11]

## 3. Experimental

### 3.1. General methods

β-D-Galactosidase from *A. oryzae* was purchased from Sigma Chemicals. D-GlcNAcα-*O*-$CH_2CH_2CH_3$ was prepared using a similar method described previously.[7a] $SO_3NMe_3$, Glc, GlcNAc, Glcα-*O*-$CH_3$, GlcNAcα-*O*-$CH_3$, Glcα-*O*-$C_6H_5$, GlcNAcα-*O*-*p*NP and Galβ-*O*-*o*NP were purchased from Sigma Chemicals; Glcα-*O*-$CH_2CH{=}CH_2$ and GlcNAcα-*O*-$CH_2CH{=}CH_2$ from GLYCON Biochemicals GmbH (Germany) and purified with a column of ODS C-18 before use; and GlcNAcβ-*O*-*p*NP from Yaizu Suisan Kagaku Co., Ltd (Yaizu, Japan).

**3.1.1. Enzyme assay for β-D-galactosidase and β-NAHase activities.** A mixture of Galβ-*O*-*o*NP (2 mM) in 50 mM sodium phosphate buffer (pH 6.0, 0.9 mL) and β-D-galactosidase was incubated for 5 min at 40 °C. To stop the enzyme reaction, 1 M $Na_2CO_3$ (0.5 mL) was added to the reaction mixture, and the amount of liberated *p*-nitrophenol was determined by measuring each absorbance at 420 and 405 nm using a microplate reader (Biolumin 960, Amersham Pharmacia Biotech). One unit of enzyme was defined as the amount releasing 1 μmol *p*-nitrophenol per min. In a similar manner, the β-NAHase activity was also determined using GlcNAcβ-*O*-*p*NP.

**3.1.2. Analytical methods.** HPLC analysis was carried out using a TSKgel ODS-80TsQA column (4.6 × 250 mm, TOSOH Co.) with a Shimadzu 64D-series liquid chromatograph, and monitored at 210 or 300 nm. The column was eluted with 5% MeOH containing 0.1% TFA at 40 °C, and the flow rate was 1.0 mL/min. Reducing saccharides were analyzed on a column of TSKgel G-Oligo-PW (7.8 × 300 mm) at a flow rate of 1 mL/min at 40 °C. Elution of the column was performed with $H_2O$. $^1H$ and $^{13}C$ NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer at 25 °C. Chemical shifts were expressed as δ relative to sodium 3-(trimethylsilyl) propionate as an external standard. Determination of the amount of protein was carried out using a Bio-Rad protein assay kit. Optical rotations were measured with JASCO DIP-1000 digital polarimeter at an ambient temperature, using a 10-cm micro-cell. FAB mass spectra (FAB-MS) were recorded using a JEOL DX 303 mass spectrometer.

**3.1.3. Preparation of a GlcNAc–Cellulofine adsorbent.[12]** To partially purify β-NAHase from *A. oryzae*, the GlcNAc–Cellulofine adsorbent for column packings was prepared as follows. A mixture of GlcNAcβ-*p*NP (600 mg) and a catalytic amount of palladium-on-charcoal (150 mg) was stirred in MeOH (150 mL) at an ambient temperature under a hydrogen atmosphere for 1 h. After filtration and removal of the solvent under diminished pressure, the resultant syrup was dissolved in 27 mL of 0.2 M acetate buffer (pH 6.0). To this solution, 21 g of suction-dried formyl-Cellulofine, prepared according to the method of Itoh et al.,[13] was added, and the mixture was pre-incubated for 1.5 h at 40 °C with gentle shaking. To the suspended solution, 150 mg of $NaCNBH_3$ was added, and the mixture was incubated overnight at 40 °C. After washing the GlcNAc-conjugated Cellulofine with 400 mL of distilled water, the remaining formyl groups were blocked by treatment with 43 mL of 0.2 M Tris–HCl (pH 7.4) and 150 mg of $NaCNBH_3$ at an ambient temperature for 2 h. The gel thus obtained was finally filtered and washed again with 400 mL of distilled water to give GlcNAc-conjugated Cellulofine used for affinity chromatography.

**3.1.4. Partial purification of β-NAHase from *A. oryzae*.** Commercially available β-galactosidase (Sigma) contains β-NAHase. β-NAHase was partially purified as follows: the crude β-NAHase contaminated with β-galactosidase from *A. oryzae* (80 U) was dissolved in 60 mM sodium phosphate buffer (80 mM, pH 6.0). The solution was loaded onto the GlcNAc–Cellulofine column (∅ 2.5 × 15 cm) equilibrated with the same buffer as described above. The column was developed with 0.1 M AcOH (700 mL) containing 0.1 M NaCl and 1% GlcNAc, and the β-NAHase was eluted. The fractions containing β-NAHase activity without β-galactosidase activity were collected and concentrated to a small volume (2 mL) by Amico Diaflo Unit with a PM10 membrane. The activity was determined to be ca. 36 U/mL.

### 3.2. β-D-6-*O*-Sulfo-GlcNAc-*O*-*p*NP, sodium salt (1)

To a solution of *p*NP GlcNAc (5.0 g, 14.6 mmol) in dry DMF (50 mL) was added dropwise sulfur trioxide–trimethylamine complex (12.2 g, 87.6 mmol) in DMF (10 mL). The reaction mixture was stirred at an ambient temperature for 9 h, diluted with MeOH (150 mL) and evaporated in vacuo. The residue was then purified on a column of ODS-C18 and treated with an ion-exchange resin (Dowex $Na^+$) to yield **1** (3.8 g, 59 %). $[\alpha]_D^{27}$ −32.8 (*c* 2.3, water). $^1H$ NMR ($D_2O$, 500 MHz): δ 8.16 (d, 2H, $J_{o\text{-Ph},m\text{-Ph}}$ 9.2 Hz, *m*-Ph), 7.14 (d, 2H, $J_{o\text{-Ph},m\text{-Ph}}$ 9.2 Hz, *o*-Ph), 5.31 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 4.43 (dd, 1H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 11.6 Hz, H-6b), 4.28 (dd, 1H, $J_{5,6a}$ 5.8, $J_{6a,6b}$ 11.6 Hz, H-6a), 4.08 (dd, 1H, $J_{1,2}$ 8.5, $J_{2,3}$ 10 Hz, H-2), 3.97 (ddd, 1H, $J_{4,5}$ 10, $J_{5,6a}$ 5.8, $J_{5,6b}$ 1.9 Hz, H-5), 3.75 (t, 1H, $J_{2,3}$ 10, $J_{3,4}$ 10 Hz, H-3), 3.65 (t, 1H, $J_{3,4}$ 10, $J_{4,5}$ 10 Hz, H-4), 2.06 (s, 3H, C$H_3$CONH–); $^{13}C$ NMR ($D_2O$, 500 MHz): δ 177.8 ($CH_3$*C*ONH–), 164.4 (C–Ph), 145.3 (*p*-Ph), 128.9 (*m*-Ph), 119.3 (*o*-Ph), 101.4

(C-1), 76.9 (C-5), 76.1 (C-3), 72.2 (C-4), 69.7 (C-6), 58.0 (C-2), 25.0 (*C*$H_3$CONH–). Anal. Calcd for $C_{14}H_{17}N_2NaO_{11}S$: C, 37.84; H, 3.86; N, 6.30; S, 7.22. Found C, 38.06; H, 3.80; N, 6.30; S, 7.43. FAB-mass: *m*/*z* 467 $[M+Na]^+$ (matrix: glycerol). These NMR data accorded well with the previous reports.[14]

### 3.3. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-Glc-*O*-$CH_3$, sodium salt (12)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (54 mg, 0.12 mmol) and Glcα-*O*-$CH_3$ (120 mg, 0.62 mmol) were dissolved in 50 mM sodium phosphate buffer (480 μL, pH 6.0) followed by the addition of 11 U of the partially purified β-NAHase preparation described above. The mixture was incubated at 35 °C for 120 h and was passed through a DEAE Sephadex A-25 column with $H_2O$ as the eluent. Subsequently, the product was eluted with 0.2 M ammonium acetate. The fractions containing the product were combined and concentrated, and the residue was purified on the columns of Dowex 50W-X8 ($H^+$) and Bio-Gel P-2. The fractions containing the title compound were evaporated, and the residue was treated with an ion-exchange resin (Dowex $Na^+$) to give sodium salt **12** in a 17% total yield (9.8 mg) based on the donor added. $[\alpha]_D^{25}$ +27.5 (*c* 0.47, water); FAB-mass: *m*/*z* 522 $[M+Na]^+$ (matrix: 3-nitrobenzyl alcohol); $^1$H NMR ($D_2O$, 500 MHz): δ 4.80 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 4.60 (d, 1H, $J_{1',2'}$ 8.6 Hz, H-1′), 4.39 (dd, 1H, $J_{5',6'b}$ 1.5, $J_{6'a,6'b}$ 11 Hz, H-6′b), 4.22 (dd, 1H, $J_{5',6'a}$ 6.4, $J_{6'a,6'b}$ 11 Hz, H-6′a), 3.81 (dd, 1H, $J_{5,6b}$ 1.4, $J_{6a,6b}$ 12 Hz, H-6b), 3.81 (m, 1H, $J_{4,5}$ 8.6, $J_{5,6a}$ 5.2, $J_{5,6b}$ 1.4 Hz, H-5), 3.78 (t, 1H, $J_{1',2'}$ 8.6, $J_{2',3'}$ 8.6 Hz, H-2′), 3.73 (m, 1H, $J_{4',5'}$ 8.6, $J_{5',6'a}$ 6.4, $J_{5',6'b}$ 1.5 Hz, H-5′), 3.69 (dd, 1H, $J_{1,2}$ 3.7, $J_{2,3}$ 6.8 Hz, H-2), 3.66 (dd, 1H, $J_{5,6a}$ 5.2, $J_{6a,6b}$ 12 Hz, H-6a), 3.76 (dd, 1H, $J_{2,3}$ 6.8, $J_{3,4}$ 8.6 Hz, H-3), 3.60 (t, 1H, $J_{2',3'}$ 8.6, $J_{3',4'}$ 8.6 Hz, H-3′), 3.54 (t, 1H, $J_{3,4}$ 8.6, $J_{4,5}$ 8.6 Hz, H-4), 3.52 (t, 1H, $J_{3',4'}$ 8.6, $J_{4',5'}$ 8.6 Hz, H-4′), 3.41 (s, 3H, C$H_3$O–), 2.08 (s, 3H, C$H_3$CONH–′); $^{13}$C NMR ($D_2O$, 500 MHz): δ 177.3 ($CH_3$*C*ONH–′), 104.3 (C-1′), 101.6 (C-1), 82.9 (C-4), 76.5 (C-5′), 76.1 (C-3′), 74.6 (C-5), 73.6 (C-3), 72.7 (C-2), 72.3 (C-4′), 70.0 (C-6′), 62.9 (C-6), 58.3 (C-2′), 57.9 (*C*$H_3$O–), 24.9 (*C*$H_3$CONH–′).

### 3.4. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-Glc-*O*-$CH_2CH{=}CH_2$, sodium salt (13)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (150 mg, 0.34 mmol) and Glcα-*O*-$CH_2CH{=}CH_2$ (374 mg, 1.70 mmol) were dissolved in 50 mM sodium phosphate buffer (1.3 mL, pH 6.0) followed by the addition of 25 U of the partially purified β-NAHase preparation from *A. oryzae*. The mixture was incubated at 35 °C for 120 h and was loaded onto a TSKgel Octadecyl-4PW column (∅ 10 × 45 cm) equilibrated with $H_2O$ at a flow rate of 1.0 mL/min. The fractions containing **13** were concentrated in vacuo, and the residue was treated with an ion-exchange resin (Dowex $Na^+$) to yield sodium salt **13** (60 mg, 34% total yield based on the donor added). $[\alpha]_D^{25}$ +67.4 (*c* 1.7, water); FAB-mass: *m*/*z* 548 $[M+Na]^+$ (matrix: 3-nitrobenzyl alcohol); $^1$H NMR ($D_2O$, 500 MHz): δ 5.98 (m, 1H, $CH_2{=}CHCH_2O$–), 5.39–5.26 (2H, $CH_2{=}CHCH_2O$–), 4.96 (d, 1H, $J_{1,2}$ 2.1 Hz, H-1), 4.60 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1′), 4.39 (dd, 1H, $J_{5',6'b}$ 2.0, $J_{6'a,6'b}$ 11 Hz, H-6′b), 4.23 (dd, 1H, $J_{5',6'a}$ 6.0, $J_{6'a,6'b}$ 11 Hz, H-6′a), 4.23–4.08 (2H, $CH_2{=}CHCH_2O$–), 3.84 (m, 1H, $J_{4,5}$ 9.2, $J_{5,6a}$ 4.6, $J_{5,6b}$ 1.6 Hz, H-5), 3.80 (dd, 1H, $J_{5,6b}$ 1.6, $J_{6a,6b}$ 12 Hz, H-6b), 3.78 (dd, 1H, $J_{1',2'}$ 8.3, $J_{2',3'}$ 9.2 Hz, H-2′), 3.75 (dd, 1H, $J_{1,2}$ 2.1, $J_{2,3}$ 6.5 Hz, H-2), 3.73 (m, 1H, $J_{4',5'}$ 9.8, $J_{5',6'a}$ 6.0, $J_{5',6'b}$ 2.0 Hz, H-5′), 3.66 (dd, 1H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12 Hz, H-6a), 3.60 (dd, 1H, $J_{2,3}$ 6.5, $J_{3,4}$ 9.2 Hz, H-3), 3.57 (dd, 1H, $J_{2',3'}$ 9.2, $J_{3',4'}$ 9.8 Hz, H-3′), 3.55 (t, 1H, $J_{3,4}$ 9.2, $J_{4,5}$ 9.2 Hz, H-4), 3.52 (t, 1H, $J_{3',4'}$ 9.8, $J_{4',5'}$ 9.8 Hz, H-4′), 2.07 (s, 3H, C$H_3$CONH–′); $^{13}$C NMR ($D_2O$, 500 MHz): δ 177.3 ($CH_3$*C*ONH–′), 136.3 ($CH_2{=}$*C*$HCH_2O$–), 121.0 (*C*$H_2{=}CHCH_2O$–), 104.3 (C-1′), 99.7 (C-1), 82.9 (C-4), 76.5 (C-5′), 76.2 (C-3′), 74.6 (C-5), 73.6 (C-3), 73.0 (C-2), 72.4 (C-4), 71.3 ($CH_2{=}$C*H*$CH_2O$–), 70.0 (C-6′), 62.9 (C-6), 58.3 (C-2′), 25.0 (*C*$H_3$CONH–′).

### 3.5. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-Glc-*O*-$C_6H_5$, sodium salt (14)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (50 mg, 0.11 mmol) and Glcα-*O*-$C_6H_5$ (144 mg, 0.56 mmol) were dissolved in 50 mM sodium phosphate buffer (450 μL, pH 6.0) followed by the addition of 12 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 144 h and was loaded onto a TSKgel Octadecyl-4PW column (∅ 2.5 × 46 cm) equilibrated with $H_2O$ at a flow rate of 1.0 mL/min. Fractions containing the product were evaporated, and the residue was then treated with an ion-exchange resin (Dowex $Na^+$) to afford sodium salt **14** (23 mg, 36% total yield based on the donor added). $[\alpha]_D^{27}$ +99.0 (*c* 0.37, water); FAB-mass: *m*/*z* 584 $[M+Na]^+$ (matrix: glycerol); $^1$H NMR ($D_2O$, 500 MHz): δ 7.41 (t, 2H, *m*-Ph), 7.19 (d, 2H, *o*-Ph), 7.15 (t, 1H, *p*-Ph), 5.66 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 4.63 (d, 1H, $J_{1',2'}$ 8.6 Hz, H-1′), 4.41 (dd, 1H, $J_{5',6'b}$ 2.0, $J_{6'a,6'b}$ 11 Hz, H-6′b), 4.25 (dd, 1H, $J_{5',6'a}$ 6.4, $J_{6'a,6'b}$ 11 Hz, H-6′a), 4.07 (t, 1H, $J_{1',2'}$ 8.6, $J_{2',3'}$ 8.6 Hz, H-2′), 3.83 (m, 1H, $J_{4,5}$ 9.5, $J_{5,6a}$ 4.6, $J_{5,6b}$ 1.2 Hz, H-5), 3.80 (dd, 1H, $J_{5,6b}$ 1.2, $J_{6a,6b}$ 12 Hz, H-6b), 3.77 (dd, 1H, $J_{1,2}$ 3.7, $J_{2,3}$ 7.0 Hz, H-2), 3.73 (m, 1H, $J_{4',5'}$ 9.5, $J_{5',6'a}$ 6.4, $J_{5',6'b}$ 2.0 Hz, H-5′), 3.66 (dd, 1H, $J_{2,3}$ 7.0, $J_{3,4}$ 9.5 Hz, H-3), 3.63 (dd, 1H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 12 Hz, H-6a), 3.60 (dd, 1H, $J_{2',3'}$ 8.6, $J_{3',4'}$ 9.5 Hz, H-3′), 3.58 (t, 1H, $J_{3,4}$ 9.5, $J_{4,5}$ 9.5 Hz, H-4), 3.54 (t, 1H, $J_{3',4'}$ 9.5,

$J_{4',5'}$ 9.5 Hz, H-4′), 2.04 (s, 3H, C$H_3$CONH–′); $^{13}$C NMR ($D_2O$, 500 MHz): δ 177.3 ($CH_3$*C*ONH–′), 158.7 (C–Ph), 132.7 (*m*-Ph), 126.0 (*p*-Ph), 120.0 (*o*-Ph), 104.4 (C-1′), 99.3 (C-1), 82.5 (C-4), 76.5 (C-5′), 76.1 (C-3′), 74.6 (C-5), 73.6 (C-3), 73.5 (C-2), 72.3 (C-4′), 70.0 (C-6′), 62.6 (C-6), 58.3 (C-2′), 24.9 (*C*$H_3$CONH–′).

### 3.6. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-GlcNAc, sodium salt (15)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (25 mg, 0.06 mmol) and GlcNAc (65 mg, 0.29 mmol) were dissolved in 50 mM sodium phosphate buffer (252 μL, pH 6.0) followed by the addition of 6 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 120 h and was loaded onto a Bio-Gel P-2 column equilibrated with $H_2O$. The fractions containing the product were evaporated, and the residue was treated with an ion-exchange resin (Dowex $Na^+$) to give sodium salt **15** (5.0 mg, 17% yield based on the donor added). $[\alpha]_D^{25}$ +11.7 (*c* 1.3, water); FAB-mass: *m*/*z* 548 $[M+Na]^+$ (matrix: 3-nitrobenzyl alcohol); $^1$H NMR ($D_2O$, 400 MHz): δ 5.17 (d, 1H, $J_{1,2}$ 3.2 Hz, H-1α), 4.67 (d, 1H, $J_{1,2}$ 8.4 Hz, H-1β), 4.61 (d, 1H, $J_{1',2'}$ 8.4 Hz, H-1′), 2.03 (s, 3H, C$H_3$CONH–′), 2.01 (s, 3H, C$H_3$CONH–); $^{13}$C NMR ($D_2O$, 600 MHz): δ 103.2 (C-1′), 96.5 (C-1β), 92.0 (C-1α), 82.0 (C-4α), 81.5 (C-4β).

### 3.7. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-GlcNAc-*O*-$CH_3$, sodium salt (16)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (25 mg, 0.06 mmol) and GlcNAcα-*O*-$CH_3$ (66 mg, 0.28 mmol) were dissolved in 50 mM sodium phosphate buffer (225 μL, pH 6.0) followed by the addition of 6 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 144 h and was loaded onto a TSKgel Octadecyl-4PW column (2.5 × 46 cm) equilibrated with $H_2O$ at a flow rate of 1.0 mL/min. Fractions containing the title compound were concentrated in vacuo, and the residue was treated with Dowex $Na^+$ resin to give sodium salt **16** in a 53% total yield (16 mg) based on the donor added. $[\alpha]_D^{27}$ +62.3 (*c* 0.47, water); FAB-mass: *m*/*z* 563 $[M+Na]^+$ (matrix: glycerol); $^1$H NMR ($D_2O$, 500 MHz): δ 4.77 (d, 1H, $J_{1,2}$ 3.1 Hz, H-1), 4.63 (d, 1H, $J_{1',2'}$ 8.6 Hz, H-1′), 4.37 (dd, 1H, $J_{5',6'b}$ 1.8, $J_{6'a,6'b}$ 11.3 Hz, H-6′b), 4.23 (dd, 1H, $J_{5',6'a}$ 5.8, $J_{6'a,6'b}$ 11.3 Hz, H-6′a), 3.91 (dd, 1H, $J_{1,2}$ 3.1, $J_{2,3}$ 10.7 Hz, H-2), 3.87 (t, 1H, $J_{2,3}$ 10.7, $J_{3,4}$ 10.7 Hz, H-3), 3.82 (dd, 1H, $J_{5,6b}$ 1.8, $J_{6a,6b}$ 11.6 Hz, H-6b), 3.78 (dd, 1H, $J_{1',2'}$ 8.6, $J_{2',3'}$ 9.2 Hz, H-2′), 3.68 (dd, 1H, $J_{5,6a}$ 5.2, $J_{6a,6b}$ 11.6 Hz, H-6a), 3.62 (t, 1H, $J_{3,4}$ 10.7, $J_{4,5}$ 10.7 Hz, H-4), 3.59 (t, 1H, $J_{2',3'}$ 9.2, $J_{3',4'}$ 9.2 Hz, H-3′), 3.54 (t, 1H, $J_{3',4'}$ 9.2, $J_{4',5'}$ 9.2 Hz, H-4′), 3.37 (s, 3H, C$H_3$O–), 2.08 (s, 3H, C$H_3$CONH–), 2.04 (s, 3H, C$H_3$CONH–′); $^{13}$C NMR ($D_2O$, 500 MHz): δ 177.4 ($CH_3$*C*ONH–), 177.3 ($CH_3$*C*ONH–′), 104.3 (C-1′), 100.4 (C-1), 83.0 (C-4), 76.5 (C-5′), 76.1 (C-3′), 72.8 (C-5), 72.4 (C-3), 72.2 (C-4′), 69.8 (C-6′), 62.9 (C-6), 58.3 (C-2′), 58.0 (C-2), 56.0 (*C*$H_3$O–), 24.9 (*C*$H_3$CONH–), 24.7 (*C*$H_3$CONH–′).

### 3.8. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-GlcNAc-*O*-$CH_2CH_2CH_3$, sodium salt (17)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (30 mg, 0.07 mmol) and GlcNAcα-*O*-$CH_2CH_2CH_3$ (88 mg, 0.33 mmol) were dissolved in 50 mM sodium phosphate buffer (0.3 mL, pH 6.0) followed by the addition of 5 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 144 h and was loaded onto a TSKgel Octadecyl-4PW column (1.7 × 25 cm) equilibrated with $H_2O$ at a flow rate of 1.0 mL/min. Fractions containing the product were processed in the same way as described above to afford compound **17** as sodium salt in a 87% total yield (33.4 mg) based on the donor added. $[\alpha]_D^{25}$ +23.1 (*c* 0.07, water); FAB-mass: *m*/*z* 591 $[M+Na]^+$ (matrix: 3-nitrobenzyl alcohol); $^1$H NMR ($D_2O$, 500 MHz): δ 4.89 (d, 1H, $J_{1,2}$ 2.8 Hz, H-1), 4.63 (d, 1H, $J_{1',2'}$ 8.6 Hz, H-1′), 4.37 (dd, 1H, $J_{5',6'b}$ 2.0, $J_{6'a,6'b}$ 10.7 Hz, H-6′b), 4.24 (dd, 1H, $J_{5',6'a}$ 5.5, $J_{6'a,6'b}$ 10.7 Hz, H-6′a), 3.92 (t, 1H, $J_{2,3}$ 10.7, $J_{3,4}$ 10.7 Hz, H-3), 3.90 (dd, 1H, $J_{1,2}$ 2.8, $J_{2,3}$ 10.7 Hz, H-2), 3.81–3.68 (m, 2H, H-6a, H-6b), 3.78 (dd, 1H, $J_{1',2'}$ 8.6, $J_{2',3'}$ 10 Hz, H-2′), 3.76 (m, 1H, H-5), 3.72 (m, 1H, $J_{4',5'}$ 10, $J_{5',6'a}$ 5.5, $J_{5',6'b}$ 2.0 Hz, H-5′), 3.64 (t, 1H, $J_{3,4}$ 10.7, $J_{4,5}$ 10.7 Hz, H-4), 3.59 (t, 1H, $J_{2',3'}$ 10, $J_{3',4'}$ 10 Hz, H-3′), 3.54 (t, 1H, $J_{3',4'}$ 10, $J_{4',5'}$ 10 Hz, H-4′), 3.43 (2H, $CH_3CH_2CH_2O$–), 2.08 (s, 3H, C$H_3$CONH–), 2.04 (s, 3H, C$H_3$CONH–′), 1.59 (2H, $CH_3CH_2CH_2O$–), 0.912 (t, 3H, C$H_3CH_2CH_2O$–); $^{13}$C NMR ($D_2O$, 500 MHz): δ 177.3 ($CH_3$*C*ONH–), 177.2 ($CH_3$*C*ONH–′), 104.3 (C-1′), 99.0 (C-1), 83.1 (C-4), 76.5 (C-5′), 76.1 (C-3′), 72.9 (C-5), 72.8 ($CH_3CH_2$*C*$H_2O$–), 72.3 (C-3), 72.2 (C-4′), 69.8 (C-6′), 62.9 (C-6), 58.3 (C-2′), 56.2 (C-2), 25.0 (*C*$H_3$CONH–), 24.8 ($CH_3$*C*$H_2CH_2O$–), 24.6 (*C*$H_3$CONH–′), 12.7 (*C*$H_3CH_2CH_2O$–).

### 3.9. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-GlcNAc-*O*-$CH_2CH{=}CH_2$, sodium salt (18)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (100 mg, 0.23 mmol) and GlcNAcα-*O*-$CH_2CH{=}CH_2$ (300 mg, 1.15 mmol) were dissolved in 50 mM sodium phosphate buffer (0.9 mL, pH 6.0) followed by the addition of 25 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 120 h and was loaded onto a TSKgel Octadecyl-4PW column (1.7 × 25 cm) equilibrated with $H_2O$ at a flow rate of 1.0 mL/min. Fractions containing compound **18** were evaporated, and the residue was processed in the same way as described above to afford

sodium salt **18** in a 81% total yield (105 mg) based on the donor added. $[\alpha]_D^{27}$ +66.8 (*c* 2.4, water); FAB-mass: *m*/*z* 589 $[M+Na]^+$ (matrix: glycerol); $^1H$ NMR ($D_2O$, 500 MHz): $\delta$ 5.95 (m, 1H, $CH_2$=C*H*$CH_2O$–), 5.34–5.26 (2H, C$H_2$=$CHCH_2O$–), 4.94 (d, 1H, $J_{1,2}$ 2.1 Hz, H-1), 4.63 (d, 1H, $J_{1',2'}$ 8.5 Hz, H-1′), 4.37 (dd, 1H, $J_{5',6'b}$ 2.0, $J_{6'a,6'b}$ 11 Hz, H-6′b), 4.21 (dd, 1H, $J_{5',6'a}$ 4.6, $J_{6'a,6'b}$ 11 Hz, H-6′a), 4.23–4.02 (2H, $CH_2$=CHC$H_2O$–), 3.93 (t, 1H, $J_{2,3}$ 9.2, $J_{3,4}$ 9.2 Hz, H-3), 3.91 (dd, 1H, $J_{1,2}$ 2.1, $J_{2,3}$ 9.2 Hz, H-2), 3.80 (dd, 1H, $J_{1',2'}$ 8.5, $J_{2',3'}$ 10 Hz, H-2′), 3.78–3.69 (m, 2H, H-6a, H-6b), 3.78 (m, 1H, H-5), 3.71 (m, 1H, $J_{4',5'}$ 10, $J_{5',6'a}$ 4.6, $J_{5',6'b}$ 2.0 Hz, H-5′), 3.59 (t, 1H, $J_{3,4}$ 9.2, $J_{4,5}$ 9.2 Hz, H-4), 3.56 (t, 1H, $J_{2',3'}$ 10, $J_{3',4'}$10 Hz, H-3′), 3.54 (t, 1H, $J_{3',4'}$ 10, $J_{4',5'}$ 10 Hz, H-4′), 2.08 (s, 3H, C$H_3$CONH–), 2.04 (s, 3H, C$H_3$CONH–′); $^{13}C$ NMR ($D_2O$, 500 MHz): $\delta$ 177.4 ($CH_3$*C*ONH–), 177.3 ($CH_3$*C*ONH–′), 136.3 ($CH_2$=*C*H$CH_2O$–), 120.7 (*C*$H_2$=$CHCH_2O$–), 104.3 (C-1′), 98.4 (C-1), 83.1 (C-4), 76.5 (C-5′), 76.1 (C-3′), 73.1 (C-5), 72.3 (C-3), 72.1 (C-4′), 71.3 ($CH_2$=CH*C*$H_2O$–), 69.8 (C-6′), 62.9 (C-6), 58.3 (C-2′), 56.1 (C-2), 24.9 (*C*$H_3$CONH–), 24.6 (*C*$H_3$CONH–′).

### 3.10. β-D-6-*O*-Sulfo-GlcNAc-(1→4)-α-D-GlcNAc-*O*-*p*NP, sodium salt (19)

6-*O*-Sulfo-GlcNAc-*O*-*p*NP (25 mg, 0.06 mmol) and GlcNAcα-*O*-*p*NP (100 mg, 0.29 mmol) were dissolved in 50 mM sodium phosphate buffer (252 μL, pH 6.0) followed by the addition of 6 U of the partially purified β-NAHase preparation. The mixture was incubated at 35 °C for 120 h and was loaded onto a TSKgel Octadecyl-4PW column (2.5 × 46 cm) equilibrated with 10% MeOH at a flow rate of 1.0 mL/min. Fractions containing compound **19** were processed in the same way described above to give sodium salt **19** in a 93% total yield (34 mg) based on the donor added. $[\alpha]_D^{27}$ +99.8 (*c* 0.30, water); FAB-mass: *m*/*z* 670 $[M+Na]^+$ (matrix: 3-nitrobenzyl alcohol); $^1H$ NMR ($D_2O$, 500 MHz): $\delta$ 8.22 (d, 2H, *m*-Ph), 7.25 (d, 2H, *o*-Ph), 5.77 (d, 1H, $J_{1,2}$ 2.8 Hz, H-1), 4.66 (d, 1H, $J_{1',2'}$ 8.3 Hz, H-1′), 4.39 (dd, 1H, $J_{5',6'b}$ 1.9, $J_{6'a,6'b}$ 11 Hz, H-6′b), 4.25 (dd, 1H, $J_{5',6'a}$ 5.8, $J_{6'a,6'b}$ 11 Hz, H-6′a), 4.16–4.15 (m, 2H, H-2, H-3), 3.79 (dd, 1H, $J_{1',2'}$ 8.3, $J_{2',3'}$ 10 Hz, H-2′), 3.77 (m, 1H, H-5), 3.76 (m, 1H, $J_{4',5'}$ 10, $J_{5',6'a}$ 5.8, $J_{5',6'b}$ 1.9 Hz, H-5′), 3.75 (m, 1H, H-4), 3.72 (dd, 1H, $J_{5,6b}$ 1.7, $J_{6a,6b}$ 12 Hz, H-6b), 3.65 (dd, 1H, $J_{5,6a}$ 4.3, $J_{6a,6b}$ 12 Hz, H-6a), 3.60 (t, 1H, $J_{2',3'}$ 10, $J_{3',4'}$ 10 Hz, H-3′), 3.55 (t, 1H, $J_{3',4'}$ 10, $J_{4',5'}$ 10 Hz, H-4′), 2.03 (s, 3H, C$H_3$CONH–), 2.03 (s, 3H, C$H_3$CONH–′); $^{13}C$ NMR ($D_2O$, 500 MHz): $\delta$ 177.7 ($CH_3$*C*ONH–), 177.7 ($CH_3$*C*ONH–′), 164.0 (C–Ph), 145.2 (*p*-Ph), 128.8 (*m*-Ph), 119.4 (*o*-Ph), 104.4 (C-1′), 97.9 (C-1), 82.5 (C-4), 76.6 (C-5′), 76.1 (C-3′), 74.1 (C-5), 72.2 (C-3), 72.2 (C-4′), 69.9 (C-6′), 62.6 (C-6), 58.3 (C-2′), 55.7 (C-2), 24.9 (*C*$H_3$CONH–), 24.6 (*C*$H_3$CONH–′).


## Acknowledgements

A part of this work was financially supported by a Grant-in-Aid for Scientific Research (B) (No. 17380202) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and also by the Special Coordination Fund from the Science and Technology Agency of the Japanese Government.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