

Synthesis of 4-Aminophenyl *N*-Acetyl- β -D-glucosaminide Derivatives and Their Application to the Rate-Assay of *N*-Acetyl- β -D-glucosaminidase

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Four *N*-acetyl- β -D-glucosaminides, 4-amino-2,6-dibromophenyl (1a), 4-amino-2,6-dichlorophenyl (1b), 4-amino-2-chlorophenyl (1c) and 4-aminophenyl *N*-acetyl- β -D-glucosaminides (1d) were synthesized. Substrates 1a–c were hydrolyzed by *N*-acetyl- β -D-glucosaminidase and the released aglycones reacted with *N*-ethyl-*N*-(3-methylphenyl)-*N'*-succinylethylenediamine to produce indoaniline dyes in the presence of bilirubin oxidase under weakly acidic rate-assay conditions (pH 5.0). The K_m values for 1a–c were 1.97, 1.65 and 1.39 mM, respectively. Among these compounds, 1b is considered to be the substrate with most potential for the rate-assay of *N*-acetyl- β -D-glucosaminidase, since it showed the largest V_{max} value and the strongest color generation from colorless to green (λ_{max} 302→718 nm) following enzyme hydrolysis and the coupling reaction. Furthermore, 1b was moderately soluble and stable in aqueous solution and exhibited about a 5.9-fold higher sensitivity to the enzyme than 2-chloro-4-nitrophenyl *N*-acetyl- β -D-glucosaminide.

Key words *N*-acetyl- β -D-glucosaminidase; rate-assay; enzyme activity; 4-aminophenyl *N*-acetyl- β -D-glucosaminide

Urinary *N*-acetyl- β -D-glucosaminidase (NAGase; EC 3.2.1.30) is well known as a sensitive indicator of renal parenchymal damage.¹⁾ The assay of this enzyme is of clinical importance for the diagnosis of renal disease,²⁾ giving early warning of rejection after renal transplantation,³⁾ and monitoring drug nephrotoxicity.⁴⁾

During the last 10 years, some methods for the rate-assay of NAGase have been developed which involve the use of chromogenic substrates.^{5,6)} We also recently reported the synthesis of some convenient and useful substrates having chromophores.^{7–9)} These rate-assay methods require that an aglycone released from the substrate by NAGase is visualized under weakly acidic conditions, because this enzyme shows optimum activity at pH 4–5.¹⁰⁾ Accordingly, the substrate for the rate-assays must have a chromophore with a low pK_a value to fully dissociate under weakly acidic conditions. Unfortunately, these requirements also increase the sensitivity of the substrate to nonenzymatic hydrolysis, possibly from intramolecular nucleophilic attack by the 2-acetamido group at the C-1 position.¹¹⁾ To solve this problem, a method using *N*-acetylglucosamine oxidase and peroxidase as coupled enzymes, to measure *N*-acetyl-D-glucosamine released by NAGase, has been developed,¹²⁾ but is subject to interference from biological substances.

Additionally, a kinetic colorimetric method for measuring γ -glutamyltransferase activity has been established which involves the formation of dye by an oxidative condensation reaction.^{13,14)} This is based on the following reaction: γ -glutamyltransferase transfers the γ -glutamyl group from the donor substrate, L- γ -glutamyl-3,5-dibromo-4-hydroxyanilide, to glycylglycine, and releases 3,5-dibromo-4-hydroxyaniline. The latter reacts with 2,5-dimethylphenol or *N*-ethyl-*N*-(3-methylphenyl)-*N'*-succinylethylenediamine in the presence of ascorbate oxidase or monophenol monooxygenase to form a quinone imine dye. It is expected that the disadvantage of methods using chromogenic substrates can be overcome when this kinetic colorimetric method is applied to the rate-assay of

NAGase.

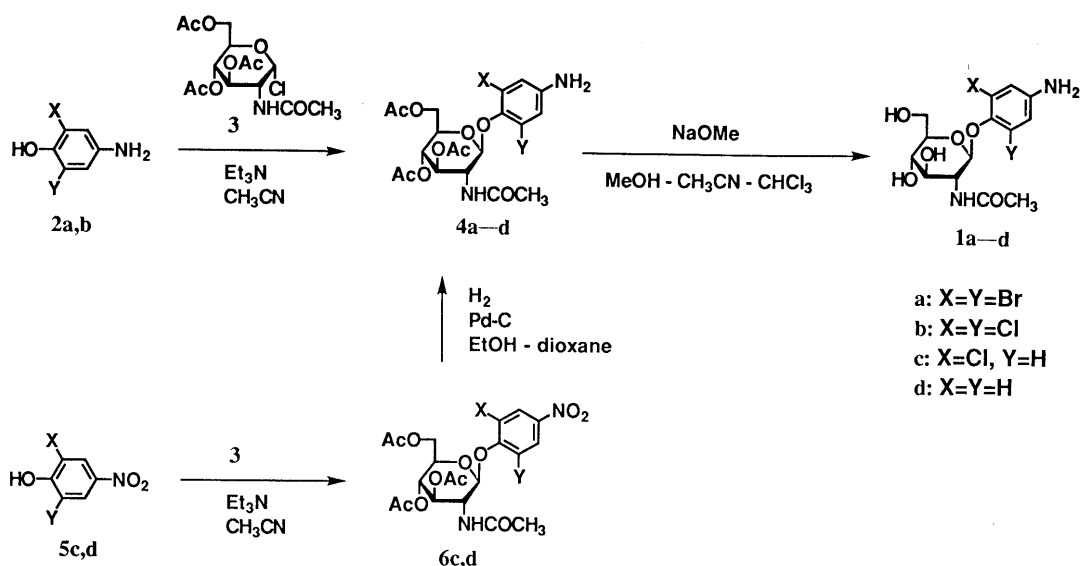
In an effort to develop a convenient and useful substrate, we have synthesized four *N*-acetyl- β -D-glucosaminides of 4-aminophenol derivatives and attempted to apply the above kinetic method, involving the formation of a dye by an oxidative condensation reaction, to the rate-assay of NAGase. In this paper, we describe their synthesis and potential applicability to the rate-assay of NAGase.

Results and Discussion

Synthesis We initially chose 4-amino-2,6-dibromophenol (2a) as the aglycone of the synthetic substrate, since *N*-substituted 4-amino-2,6-dibromophenol derivative has been used as the donor substrate for measuring γ -glutamyltransferase activity. The synthesis of 4-amino-2,6-dibromophenyl *N*-acetyl- β -D-glucosaminide (1a) was carried out by the direct introduction of a protected glycosyl donor into the aminophenol (Chart 1). Thus, the glycosidation of 2a with 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine (3)¹⁵⁾ as a glycosyl donor in the presence of triethylamine (Et₃N) afforded the corresponding tetraacetyl- β -D-glucosaminide (4a) in 67% yield; no production of the *N*-glycosides was observed. The removal of the protecting groups of 4a with NaOMe in MeOH–CH₃CN–CHCl₃ gave the desired glucosaminide (1a) in 62% yield.

Then, we devised syntheses of three other 4-aminophenyl *N*-acetyl- β -D-glucosaminide derivatives (1b–d) in order to investigate the effects of the halogeno substituents at the 2,6-positions in the aminophenol moiety upon the enzyme reaction. The synthesis of the 4-amino-2,6-dichlorophenyl derivative (1b) was successfully carried out in the same manner as that of 1a using 4-amino-2,6-dichlorophenol (2b) instead of 2a. As 4-amino-2-chlorophenol was not commercially available, the synthesis of the 4-amino-2-chlorophenyl derivative (1c) was attempted by another route which involved reduction of the nitro to the corresponding amino function. Thus, the glycosidation of 2-chloro-4-nitrophenol (5c) with 3 in the presence of

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Et_3N gave the corresponding tetraacetyl- β -D-glucosaminide (**6c**) in 61% yield. The 2-chloro-4-nitrophenyl glycoside (**6c**) was reduced to the 4-amino-2-chlorophenyl glycoside (**4c**) by means of catalytic hydrogenation over palladium-charcoal in 74% yield, although this reduction was accompanied by formation of a small amount of the dehalogenated 4-aminophenyl derivative (**4d**). *O*-Deacetylation of **4c** with NaOMe in $\text{MeOH}-\text{CH}_3\text{CN}-\text{CHCl}_3$ afforded the desired *N*-acetyl- β -D-glucosaminide (**1c**) in 86% yield. To synthesize the last derivative (**1d**), we tried the glycosidation of unsubstituted 4-aminophenol (**2d**) under the same conditions as used for the synthesis of **1a**, but this gave the *N*-glycoside as the major product. This indicates that the reactivity of hydroxyl groups in 4-aminophenol derivatives is decreased by the absence of electron-withdrawing halogen groups at the *o*-position. Therefore, the synthesis of the 4-aminophenyl derivative (**1d**) was accomplished in the same manner as that of **1c** using 4-nitrophenol (**5d**) as a starting material.

The structures of these *N*-acetyl- β -D-glucosaminides (**1a-d**) were confirmed from the results of elemental analyses and the spectral data shown in the experimental section. The proton nuclear magnetic resonance (^1H -NMR) spectra of **1a-d** in dimethylsulfoxide (DMSO)- d_6 showed signals at δ 4.68–4.89 assigned to anomeric protons having large coupling constants ($J_{1,2}=8.3\text{ Hz}$), indicating the presence of the *trans*- β -glycosidic bond.

Properties as NAGase Substrates The four *N*-acetyl- β -D-glucosaminides (**1a-d**) were examined with respect to their suitability for the rate-assay of NAGase. The solubilities of these glucosaminides in 0.05 M citrate buffer (pH 5.0, which is the optimum pH of NAGase)¹⁰ were determined by HPLC analysis. It was found that they had moderate solubility: 2.6 mM for **1a**, 4.5 mM for **1b**, 16 mM for **1c**, and 450 mM for **1d**.

The principle of the NAGase assay using these synthetic substrates (**1a-d**) is illustrated in Chart 2. These substrates are hydrolyzed by NAGase to release the corresponding aminophenols (**2a-d**) and the released aglycones immediately undergo oxidative condensation with *N*-ethyl-

N-(3-methylphenyl)-*N'*-succinylethylenediamine (EMSE) to produce indoaniline dyes in the presence of bilirubin oxidase (BLOD) under the weakly acidic rate-assay conditions (pH 5.0). Since the NAGase hydrolysis reaction is a rate-determining step in the reaction sequence, the NAGase assay is based on the direct spectrophotometric determination of the indoaniline dye and the NAGase activity is measured by calculating the rate of dye formation in terms of the increase in absorbance per minute.

Before trying coupled oxidative condensation with EMSE and BLOD, we confirmed by HPLC analysis that the synthesized *N*-acetyl- β -D-glucosaminides (**1a-d**) release the corresponding aminophenols (**2a-d**) following NAGase hydrolysis. The enzyme reaction was carried out in 0.05 M citrate buffer (pH 5.0) at 37 °C. When each *N*-acetyl- β -D-glucosaminide (**1a-d**) was incubated with NAGase in the presence of EMSE and BLOD, the spectral changes in the reaction mixture were measured (Fig. 1). These substrates were hydrolyzed fully by NAGase under the conditions shown in Fig. 1, and the observed spectra of the reaction mixture shifted from colorless (λ_{max} 290–302 nm) to longer wavelengths (λ_{max} 580–720 nm). It was found that the absorbance of the dye formed using **1d** was very small under the rate-assay conditions of NAGase. The indoaniline dyes formed using dihalogeno derivatives (**1a, b**) as substrates, on the other hand, absorbed at high visible wavelengths and had large molar absorptivities. It was consequently suggested that the NAGase reaction could be directly monitored colorimetrically at the optimum pH range without terminating the reaction, except in the case of **1d**.

The time courses of the NAGase reactions of the three glucosaminides (**1a-c**), at pH 5.0 for 5 min in the presence of EMSE and BLOD, are shown in Fig. 2, where the reactions both with and without NAGase were monitored continuously, at 720 nm for **1a, b**, or at 705 nm for **1c**. The absorbance of the dye formed increased linearly for more than 5 min from the start of the reaction. By measuring the change in absorbance during the period from 2 to

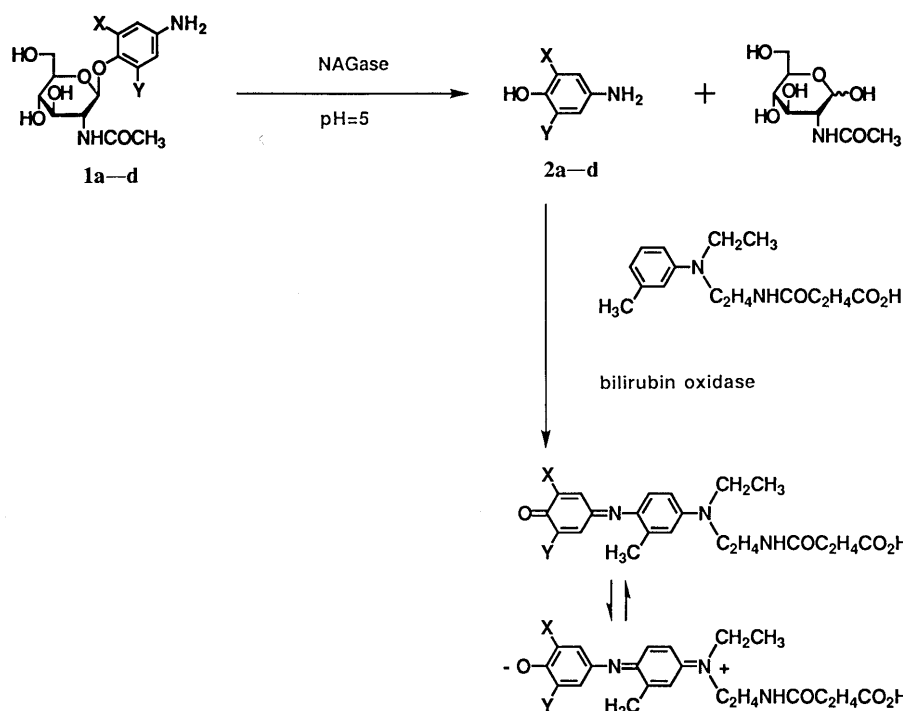


Chart 2

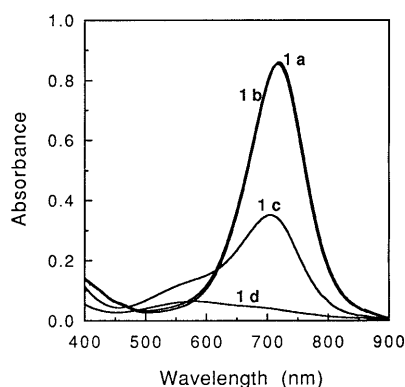


Fig. 1. Absorption Spectra of **1a-d** (0.020 mM) upon Incubation with NAGase (28.1 I.U./l) at 37 °C for 30 min in Citrate Buffer (50 mM, pH 5.0) in the Presence of EMSE (0.986 mg/ml) and BLOD (0.195 U/ml)

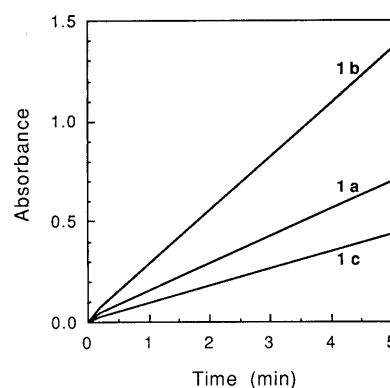


Fig. 2. Continuous Time Courses of the Reactions of **1a** (1.46 mM, Observed at 720 nm), **1b** (2.93 mM, 720 nm) and **1c** (5.85 mM, 705 nm) with NAGase (3.51 I.U./l) at 37 °C over 0 to 5 min in Citrate Buffer (50 mM, pH 5.0) in the Presence of EMSE (0.986 mg/ml) and BLOD (0.195 U/ml)

4 min, the NAGase activity could be determined without terminating the reaction. Of these substrates, the absorbance increase of **1b** per minute at 720 nm was largest under the conditions shown in Fig. 2.

The Michaelis constants (K_m) and maximum velocities (V_{max}) for the three glucosaminides (**1a-c**) were obtained from Lineweaver-Burk plots and are summarized in Table I. All three glucosaminides had acceptable levels of affinity and V_{max} values in the NAGase reaction. The V_{max} value for **1b** was found to be the largest indicating that it is the most favorable substrate for the rate-assay of NAGase.

Since **1b** was found to be the most favorable substrate among the *N*-acetyl- β -D-glucosaminides investigated, the standard curve of the absorbance increase in the reaction mixture of **1b** against the concentration of NAGase under rate-assay conditions was examined (Fig. 3). A good linearity was observed over the range 0.7 to 7 I.U./l of final NAGase concentration. Comparison of the absorbance increase using **1b** with that of 2-chloro-4-nitro-

TABLE I. Properties of 4-Aminophenyl *N*-Acetyl- β -D-glucosaminides (**1**) as NAGase Substrates

Compd. No.	K_m (mM)	V_{max} (mM/min)
1a	1.94	6.9×10^{-3}
1b	1.65	9.2×10^{-3}
1c	1.39	5.7×10^{-3}

phenyl-*N*-acetyl- β -D-glucosaminide (CNP-NAG),⁵⁾ a commonly used substrate in the rate-assay of NAGase, showed the sensitivity of **1b** to be 5.9 times higher than that of CNP-NAG.

Spectrophotometrically, the ideal assay method should show no overlapping of the substrate and product spectra, the product should absorb at long visible wavelengths where absorbance from bilirubin or hemoglobin is neg-

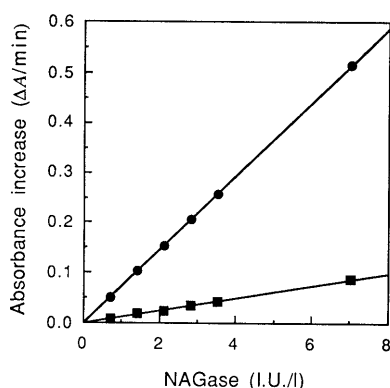


Fig. 3. Standard Curves of **1b** (2.93 mM, ●, Observed at 720 nm) and CNP-NAG (2.21 mM, ■, 400 nm) under the Rate-Assay Conditions at 37°C Absorbance Increase against the Concentration of NAGase

1b: $y = 0.0734x + 0.00014$, $r = 1.000$. CNP-NAG: $y = 0.0123x - 0.00004$, $r = 0.996$. Each point is a mean value ($n = 3$).

ligible and the absorptivity of product should be high. The method we propose using **1b** as substrate in the presence of EMSE and BLOD satisfies these criteria because this method produces a clear color generation from colorless to green ($\lambda_{\max}$ 302→718 nm) following NAGase hydrolysis with an adequate absorbance ($\epsilon = 42000$) at pH 5.0 (which is the optimum pH of NAGase). In addition, the substrate (**1b**) is sufficiently stable and soluble under rate-assay conditions. Therefore, the synthetic substrate (**1b**) is considered to be an excellent substrate for the rate-assay of NAGase.

On the basis of the above results, we are now investigating the application of the substrates described in this paper to the assay of NAGase in biological samples such as human urine and serum.

Experimental

Reagents and Materials All chemicals were of reagent grade unless otherwise noted. CNP-NAG was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan) as a Meiassei NAG-R kit. NAGase (from bovine kidney) and BLOD (from *Myrothecium verrucaria*) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and diluted with distilled water. EMSE was a gift from Kyowa Medex Co., Ltd. (Tokyo, Japan).

Apparatus All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. $^1\text{H-NMR}$ spectra were recorded at 199.5 MHz with a JEOL JNM-FX200 NMR spectrometer using tetramethylsilane as an internal standard. Optical rotations were determined with a JASCO DIP-360 digital polarimeter at 25°C. Infrared (IR) spectra were recorded with a JASCO FT/IR-7300 spectrometer. Ultraviolet (UV) spectra were recorded with a Hitachi 557 spectrometer or a Hitachi U-3300 spectrophotometer attached to a Hitachi AS-3000 intelligent auto-sampler. High-performance liquid chromatography (HPLC) was performed on an Inertsil ODS-2 column (4.6 mm i.d. $\times$ 150 mm) using a Waters model 600 multisolute delivery system and a Waters model 490 UV detector (eluent, MeOH–H₂O containing 5 mM sodium 1-heptanesulfonate, 1:4, v/v; flow rate, 1.0 ml/min; detection, UV at 254 nm; temperature, ambient). Column chromatography was performed on Merck Kiesel gel 60 (SiO₂, 230–400 mesh) and YMC-gel ODS-AQ (120–S50, from Yamamura Chemical Laboratories Co., Ltd., Japan).

4-Amino-2,6-dibromophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (4a) 4-Amino-2,6-dibromophenol (**2a**) (26.7 g, 100 mmol) and triethylamine (Et₃N, 140 ml, 1.0 mol) were added to a solution of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine (**3**)¹⁵ (18.3 g, 50 mmol) in CH₃CN (500 ml) and the mixture was stirred at 40°C for 1 h. After addition of CHCl₃ (2 l) to the reaction mixture, the solution was washed with 0.1 N NaOH (1 l $\times$ 2) and water (2 l), decolorized using activated charcoal powder, dried over MgSO₄, and

evaporated *in vacuo*. The residue was suspended in Et₂O–CHCl₃ (1:1, v/v) and the precipitate collected by filtration was recrystallized from CH₃CN–Et₂O to give 19.85 g (66.6%) of **4a** as colorless needles, mp 207.5–209°C, $[\alpha]_D^{25} - 61.9^\circ$ ($c = 0.391$, MeOH). IR (KBr): 3296, 1751, 1736, 1665, 1602, 1558, 1470, 1375 cm⁻¹. UV $\lambda_{\max}^{\text{EtOH}}$ nm (ϵ): 242 (10600), 309 (2400). $^1\text{H-NMR}$ (DMSO-*d*₆) δ : 1.81 (3H, s, NAc), 1.94 (6H, s, OAc), 1.97 (3H, s, OAc), 3.70–3.80 (1H, m, Glc H), 3.95–4.15 (3H, m, Glc H), 4.88 (1H, t, $J = 9.5$ Hz, Glc H-4), 5.17 (1H, d, $J = 8.1$ Hz, Glc H-1), 5.27 (1H, dd, $J = 10.5, 9.5$ Hz, Glc H-3), 5.33 (2H, br s, NH₂), 6.77 (2H, s, arom. H), 7.93 (1H, d, $J = 9.3$ Hz, NH). Anal. Calcd for C₂₀H₂₄Br₂N₂O₉: C, 40.29; H, 4.06; Br, 26.80; N, 4.70. Found: C, 40.17; H, 4.01; Br, 26.39; N, 4.56.

4-Amino-2,6-dibromophenyl 2-(Acetylamino)-2-deoxy- β -D-glucopyranoside (1a) A solution of **4a** (10.0 g, 16.8 mmol) in MeOH–CH₃CN–CHCl₃ (1:1:1, v/v, 600 ml) was mixed with 28% NaOMe in MeOH (0.65 ml, 3.4 mmol) with stirring. After stirring the mixture for 1 h at room temperature, it was stored in a refrigerator for 2 h. The precipitated crystals, collected by filtration, were recrystallized from H₂O–EtOH to give 4.90 g (62.2%) of **1a** as colorless needles, mp 192–195°C, $[\alpha]_D^{25} - 33.4^\circ$ ($c = 0.400$, MeOH). IR (KBr): 3304, 1635, 1568, 1472, 1387 cm⁻¹. UV $\lambda_{\max}^{\text{EtOH}}$ nm (ϵ): 241 (10300), 308 (2500). $^1\text{H-NMR}$ (DMSO-*d*₆) δ : 1.83 (3H, s, NAc), 2.95–3.20 (2H, m, Glc H), 3.40–3.50 (2H, m, Glc H), 3.60–3.80 (2H, m, Glc H), 3.89 (1H, t, $J = 5.6$ Hz, OH), 4.80–4.95 (2H, m, OH), 4.89 (1H, d, $J = 8.3$ Hz, Glc H-1), 5.28 (2H, br s, NH₂), 6.77 (2H, s, arom. H), 7.70 (1H, d, $J = 8.8$ Hz, NH). Anal. Calcd for C₁₄H₁₈Br₂N₂O₆: C, 35.77; H, 3.86; Br, 33.99; N, 5.96. Found: C, 35.74; H, 3.84; Br, 33.48; N, 5.97. t_R : 7.6 min.

4-Amino-2,6-dichlorophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (4b) 4-Amino-2,6-dichlorophenol (**2b**) (17.8 g, 100 mmol) and Et₃N (140 ml, 1.0 mol) were added to a solution of **3** (18.3 g, 50 mmol) in CH₃CN (500 ml) and the mixture was stirred at 40°C for 2 h. After addition of CHCl₃ (2 l) to the reaction mixture, the solution was washed with 0.1 N NaOH (1 l $\times$ 2) and water (2 l), decolorized using activated charcoal powder, dried over MgSO₄, and evaporated *in vacuo*. The residue was suspended in Et₂O–CHCl₃ (1:1, v/v) and the precipitate collected by filtration was recrystallized from CH₃CN–Et₂O to give 15.86 g (62.3%) of **4b** as colorless needles, mp 231–233°C, $[\alpha]_D^{25} - 58.9^\circ$ ($c = 0.397$, MeOH). IR (KBr): 3290, 1752, 1736, 1667, 1602, 1562, 1480, 1375 cm⁻¹. UV $\lambda_{\max}^{\text{EtOH}}$ nm (ϵ): 241 (10300), 308 (2500). $^1\text{H-NMR}$ (DMSO-*d*₆) δ : 1.80 (3H, s, NAc), 1.94 (6H, s, OAc), 1.97 (3H, s, OAc), 3.72–3.82 (1H, m, Glc H), 3.90–4.15 (3H, m, Glc H), 4.88 (1H, t, $J = 9.5$ Hz, Glc H-4), 5.09 (1H, d, $J = 8.3$ Hz, Glc H-1), 5.25 (1H, dd, $J = 10.3, 9.5$ Hz, Glc H-3), 5.36 (2H, br s, NH₂), 6.57 (2H, s, arom. H), 7.97 (1H, d, $J = 9.3$ Hz, NH). Anal. Calcd for C₂₀H₂₄Cl₂N₂O₉: C, 47.35; H, 4.77; Cl, 13.98; N, 5.52. Found: C, 47.39; H, 4.77; Cl, 13.84; N, 5.59.

4-Amino-2,6-dichlorophenyl 2-(Acetylamino)-2-deoxy- β -D-glucopyranoside (1b) A solution of **4b** (10.0 g, 19.7 mmol) in MeOH–CH₃CN–CHCl₃ (1:1:1, v/v, 600 ml) was mixed with 28% NaOMe in MeOH (0.76 ml, 4.0 mmol) with stirring. After stirring the mixture for 1 h at room temperature, it was stored in a refrigerator for 2 h. The precipitated crystals, collected by filtration, were recrystallized from H₂O–EtOH to give 6.25 g (83.2%) of **1b** as colorless needles, mp 205–208°C, $[\alpha]_D^{25} - 37.4^\circ$ ($c = 0.380$, MeOH). IR (KBr): 3295, 1634, 1571, 1480, 1391 cm⁻¹. UV $\lambda_{\max}^{\text{EtOH}}$ nm (ϵ): 245 (9300), 308 (2300). $^1\text{H-NMR}$ (DMSO-*d*₆) δ : 1.82 (3H, s, NAc), 2.95–3.20 (2H, m, Glc H), 3.35–3.50 (2H, m, Glc H), 3.60–3.75 (2H, m, Glc H), 4.00 (1H, t, $J = 5.6$ Hz, OH), 4.81 (1H, d, $J = 8.3$ Hz, Glc H-1), 4.84 (1H, d, $J = 5.6$ Hz, OH), 4.90 (1H, d, $J = 5.1$ Hz, OH), 5.30 (2H, br s, NH₂), 6.55 (2H, s, arom. H), 7.73 (1H, d, $J = 9.0$ Hz, NH). Anal. Calcd for C₁₄H₁₈Cl₂N₂O₆: C, 44.11; H, 4.76; Cl, 18.60; N, 7.35. Found: C, 44.15; H, 4.75; Cl, 18.44; N, 7.36. t_R : 5.3 min.

2-Chloro-4-nitrophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (6c) 2-Chloro-4-nitrophenol (**5c**) (17.4 g, 100 mmol) and Et₃N (140 ml, 1.0 mol) were added to a solution of **3** (18.3 g, 50 mmol) in CH₃CN (500 ml) and the mixture was stirred at 40°C for 2 h. After addition of CHCl₃ (2 l) to the reaction mixture, the solution was washed with 0.1 N NaOH (1 l $\times$ 2) and water (2 l), dried over MgSO₄, and evaporated *in vacuo*. The residue was suspended in Et₂O–CHCl₃ (1:1, v/v) and the precipitate collected by filtration was recrystallized from CH₃CN–Et₂O to give 15.21 g (60.5%) of **6c** as colorless needles, mp 206–207°C, $[\alpha]_D^{25} - 65.0^\circ$ ($c = 0.404$, MeOH). IR (KBr): 3301, 1749, 1667, 1586, 1542, 1522, 1376, 1347 cm⁻¹. UV $\lambda_{\max}^{\text{EtOH}}$ nm (ϵ): 208 (16100), 283 (9000). $^1\text{H-NMR}$ (DMSO-*d*₆) δ : 1.78 (3H, s, NAc), 1.96 (3H, s, OAc), 2.01 (3H, s, OAc), 2.02 (3H, s, OAc), 4.05–4.20 (4H, m, Glc H), 4.98 (1H, t, $J = 9.3$ Hz, Glc H-4), 5.24 (1H, dd, $J = 10.3, 9.3$ Hz, Glc

H-3), 5.57 (1H, d, $J=8.3$ Hz, Glc H-1), 7.53 (1H, d, $J=9.3$ Hz, H-6 of arom.), 8.00 (1H, d, $J=8.8$ Hz, NH), 8.22 (1H, dd, $J=9.3$, 2.7 Hz, H-5 of arom.), 8.32 (1H, d, $J=2.7$ Hz, H-3 of arom.). *Anal.* Calcd for $C_{20}H_{23}ClN_2O_{11}$: C, 47.77; H, 4.61; Cl, 7.05; N, 5.57. Found: C, 47.84; H, 4.63; Cl, 7.19; N, 5.43.

4-Amino-2-chlorophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (4c) A stirred solution of **6c** (10.0 g, 19.9 mmol) in 1,4-dioxane (400 ml) and EtOH (200 ml) was hydrogenated immediately after the addition of 10% Pd/C (1.0 g), at atmospheric pressure for 6 h at room temperature. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica-gel. Elution with $CH_3CN-CHCl_3$ (1:3, v/v) gave 6.94 g (73.8%) of **4c** as colorless needles, mp 202–204 °C (CH_3CN-Et_2O), $[\alpha]_D^{25} -57.3^\circ$ ($c=0.377$, MeOH). IR (KBr): 3274, 1746, 1653, 1505, 1372 cm^{-1} . UV λ_{max}^{EtOH} nm (ϵ): 240 (10300), 302 (2100). 1H -NMR (DMSO- d_6) δ : 1.78 (3H, s, NAc), 1.93 (3H, s, OAc), 1.98 (3H, s, OAc), 2.02 (3H, s, OAc), 3.90–4.25 (4H, m, Glc H), 4.89 (1H, t, $J=9.5$ Hz, Glc H-4), 4.98 (2H, brs, NH_2), 5.00 (1H, d, $J=8.3$ Hz, Glc H-1), 5.18 (1H, dd, $J=10.3$, 9.5 Hz, Glc H-3), 6.46 (1H, dd, $J=8.8$, 2.7 Hz, H-5 of arom.), 6.60 (1H, d, $J=2.7$ Hz, H-3 of arom.), 6.97 (1H, d, $J=8.8$ Hz, H-6 of arom.), 7.92 (1H, d, $J=9.3$ Hz, NH). *Anal.* Calcd for $C_{20}H_{25}ClN_2O_9$: C, 50.80; H, 5.33; Cl, 7.50; N, 5.92. Found: C, 50.75; H, 5.33; Cl, 7.40; N, 5.81.

4-Amino-2-chlorophenyl 2-(Acetylamino)-2-deoxy- β -D-glucopyranoside (1c) A solution of **4c** (5.0 g, 10.6 mmol) in MeOH- $CH_3CN-CHCl_3$ (1:1:1, v/v, 300 ml) was mixed with 28% NaOMe in MeOH (0.40 ml, 2.1 mmol) with stirring. After stirring the mixture for 1 h at room temperature, it was stored in a refrigerator for 2 h. The precipitated crystals, collected by filtration, were recrystallized from $H_2O-EtOH$ to give 3.15 g (85.9%) of **1c** as colorless needles, mp 209–212 °C, $[\alpha]_D^{25} -48.5^\circ$ ($c=0.378$, MeOH). IR (KBr): 3382, 1638, 1541, 1505, 1374 cm^{-1} . UV λ_{max}^{EtOH} nm (ϵ): 239 (9700), 302 (2200). 1H -NMR (DMSO- d_6) δ : 1.81 (3H, s, NAc), 3.10–3.80 (6H, m, Glc H), 4.47 (1H, t, $J=5.6$ Hz, OH), 4.68 (1H, d, $J=8.3$ Hz, Glc H-1), 4.85–4.95 (4H, m, OH $\times$ 2, NH_2), 6.44 (1H, dd, $J=8.8$, 2.7 Hz, H-5 of arom.), 6.58 (1H, d, $J=2.7$ Hz, H-3 of arom.), 7.02 (1H, d, $J=8.8$ Hz, H-6 of arom.), 7.66 (1H, d, $J=8.8$ Hz, NH). *Anal.* Calcd for $C_{14}H_{19}ClN_2O_6$: C, 48.49; H, 5.52; Cl, 10.22; N, 8.08. Found: C, 48.53; H, 5.54; Cl, 10.06; N, 8.05. t_R : 3.0 min.

4-Nitrophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (6d) 4-Nitrophenol (**5d**) (13.9 g, 100 mmol) and Et_3N (140 ml, 1.0 mol) were added to a solution of **3** (18.3 g, 50 mmol) in CH_3CN (500 ml) and the mixture was stirred at 40 °C for 4 h. After addition of $CHCl_3$ (2 l) to the reaction mixture, the solution was washed with 0.1 N NaOH (1 $\times$ 2) and water (2 l), dried over $MgSO_4$, and evaporated *in vacuo*. The residue was suspended in $Et_2O-CHCl_3$ (1:1, v/v) and the precipitate collected by filtration was recrystallized from CH_3CN-Et_2O to give 10.82 g (46.2%) of **6d** as colorless needles, mp 248.5 °C, $[\alpha]_D^{25} -47.6^\circ$ ($c=0.381$, MeOH). IR (KBr): 3336, 1749, 1664, 1517 cm^{-1} . UV λ_{max}^{EtOH} nm (ϵ): 218 (8200), 291 (10700). 1H -NMR (DMSO- d_6) δ : 1.78 (3H, s, NAc), 1.96 (3H, s, OAc), 2.01 (6H, s, OAc), 4.00–4.25 (4H, m, Glc H), 4.95 (1H, t, $J=9.3$ Hz, Glc H-4), 5.25 (1H, dd, $J=10.3$, 9.3 Hz, Glc H-3), 5.53 (1H, d, $J=8.3$ Hz, Glc H-1), 7.23 (2H, d, $J=9.3$ Hz, H-2,6 of arom.), 8.01 (1H, d, $J=8.8$ Hz, NH), 8.22 (2H, d, $J=9.3$ Hz, H-3,5 of arom.). *Anal.* Calcd for $C_{20}H_{24}N_2O_{11}$: C, 51.28; H, 5.16; N, 5.98. Found: C, 51.23; H, 5.16; N, 5.94.

4-Aminophenyl 3,4,6-Tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranoside (4d) A stirred solution of **6d** (10.0 g, 21.4 mmol) in 1,4-dioxane (400 ml) and EtOH (200 ml) was hydrogenated immediately after the addition of 10% Pd/C (1.0 g), at atmospheric pressure for 3 h at room temperature. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica-gel. Elution with $CH_3CN-CHCl_3$ (1:2, v/v) gave 8.55 g (91.3%) of **4d** as colorless needles, mp 134–142 °C (CH_3CN-Et_2O), $[\alpha]_D^{25} -32.6^\circ$ ($c=0.349$, MeOH). IR (KBr): 3279, 1750, 1655, 1510, 1374 cm^{-1} . UV λ_{max}^{EtOH} nm (ϵ): 235 (9700), 295 (1600). 1H -NMR (DMSO- d_6) δ : 1.79 (3H, s, NAc), 1.93 (3H, s, OAc), 1.98 (3H, s, OAc), 2.01 (3H, s, OAc), 3.80–4.25 (4H, m, Glc H), 4.88 (1H, t, $J=9.5$ Hz, Glc H-4), 4.99 (2H, brs, NH_2), 5.04 (1H, d, $J=8.3$ Hz, Glc H-1), 5.18 (1H, dd, $J=10.3$, 9.5 Hz, Glc H-3), 6.52 (2H, d, $J=8.8$ Hz, H-3,5 of arom.), 6.73 (2H, d, $J=8.8$ Hz, H-2,6 of arom.), 7.96 (1H, d, $J=9.0$ Hz, NH). *Anal.* Calcd for $C_{20}H_{26}N_2O_9$: C, 54.79; H, 5.98; N, 6.39. Found: C, 54.50; H, 5.94; N, 6.26.

4-Aminophenyl 2-(Acetylamino)-2-deoxy- β -D-glucopyranoside (1d) A solution of **4d** (5.0 g, 11.4 mmol) in MeOH- $CH_3CN-CHCl_3$ (1:1:1, v/v, 300 ml) was mixed with 28% NaOMe in MeOH (0.44 ml, 2.3 mmol) with

stirring and the mixture was stirred for 1 h at room temperature. After removal of three quarters of the solvent *in vacuo*, the residual solution was stored in a refrigerator overnight. The precipitated crystals collected by filtration were recrystallized from EtOH to give 2.19 g (61.8%) of **1d** as colorless needles, mp 210–214 °C, $[\alpha]_D^{25} -11.8^\circ$ ($c=0.353$, MeOH). IR (KBr): 3292, 1625, 1554, 1513, 1375 cm^{-1} . UV λ_{max}^{EtOH} nm (ϵ): 235 (9300), 296 (1700). 1H -NMR (DMSO- d_6) δ : 1.82 (3H, s, NAc), 3.10–3.75 (6H, m, Glc H), 4.45 (1H, t, $J=5.6$ Hz, OH), 4.61 (2H, brs, NH_2), 4.71 (1H, d, $J=8.3$ Hz, Glc H-1), 4.83–4.93 (2H, m, OH), 6.47 (2H, d, $J=8.8$ Hz, H-3,5 of arom.), 6.69 (2H, d, $J=8.8$ Hz, H-2,6 of arom.), 7.69 (1H, d, $J=8.8$ Hz, NH). *Anal.* Calcd for $C_{14}H_{20}N_2O_6$: C, 53.84; H, 6.45; N, 8.97. Found: C, 53.57; H, 6.36; N, 8.79. t_R : 2.3 min.

Solubilities of 1 A suspension of **1** in citrate buffer (50 mM, pH 5.0) was stirred for 30 min at room temperature. The mixture was filtered through a disposable syringe filter unit (Dismic-25CS, 0.45 μm for aqueous solutions, Toyo Roshi Kaisha Ltd., Tokyo). The filtrate was appropriately diluted with water and 0.02 ml of the solution was subjected to HPLC analysis. The concentration of **1** was determined from the ratio of the peak area to the standard.

Identification of NAGase Hydrolysis Products by HPLC A solution of NAGase (1152 I.U./l, 0.05 ml) was added to a solution of **1** (0.02 mM, 2.00 ml) in the presence of EMSE (1.0 mg/ml) and BLOD (0.2 U/ml) in citrate buffer (50 mM, pH 5.0) and the mixture was incubated at 37 °C. After 30 min, 0.02 ml of the final reaction mixture was injected into the HPLC system. The retention times of products were 16.1 min for **2a**, 10.4 min for **2b**, 9.6 min for **2c** and 12.0 min for **2d**.

Michaelis Constants and Maximum Velocities A solution of NAGase (144 I.U./l, 0.05 ml) was added to a solution of **1** (0.2–4.0 mM, 2.00 ml) in the presence of EMSE (1.0 mg/ml) and BLOD (0.2 U/ml) in citrate buffer (50 mM, pH 5.0) and the mixture was incubated at 37 °C. After 2 min, the increase in absorbance at 720 nm (for **1a, b**) or 705 nm (for **1c**) against H_2O was measured continuously for 2 min. For the substrate blank, H_2O was added instead of NAGase solution. The K_m and V_{max} values for the substrates were calculated from Lineweaver-Burk plots.

Standard Curves under Rate-Assay Conditions A solution of NAGase (28.8–288 I.U./l, 0.05 ml) was added to a solution of **1b** (3.00 mM, 2.00 ml) or CNP-NAG (2.33 mM, 2.00 ml) in the presence of EMSE (1.0 mg/ml) and BLOD (0.2 U/ml) in citrate buffer (50 mM, pH 5.0) and the mixture was incubated at 37 °C. After 2 min, the increase in absorbance at 720 nm (for **2a**) or 400 nm (for CNP-NAG) against H_2O was measured continuously for 2 min. For the substrate blank, H_2O was added instead of NAGase solution.

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