Novel Chromogenic Substrates for the Rate-Assay of *N*-Acetyl- β -D-glucosaminidase: Resorufinyl- and Resazurinyl-*N*-acetyl- β -D-glucosaminides

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Two novel *N*-acetyl- β -D-glucosaminides, resorufinyl- (1) and resazurinyl-*N*-acetyl- β -D-glucosaminides (2), were synthesized by a coupling reaction of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine with resorufin derivatives followed by deprotection of the sugar moiety. Compounds 1 and 2 were hydrolyzed by *N*-acetyl- β -D-glucosaminidase to give products showing high absorbance at a long absorption wavelength (575 and 610 nm) under rate-assay conditions of a weakly acidic pH. Though these two compounds are considered to be potential chromogenic substrates, 1 is the more favorable substrate for the rate-assay of *N*-acetyl- β -D-glucosaminidase.

Keywords *N*-Acetyl- β -D-glucosaminidase, chromogenic substrate, rate-assay, resorufinyl-*N*-acetyl- β -D-glucosaminide, resazurinyl-*N*-acetyl- β -D-glucosaminide

N-Acetyl- β -D-glucosaminidase (NAGase; EC 3.2.1.30), a lysosomal enzyme, is located predominantly in proximal renal tubules.¹ In recent years, its clinical significance as an indicator for the diagnosis and follow-up of the clinical course of various renal diseases and renal lesion has been emphasized.^{2,3}

NAGase activity has been determined colorimetrically using several chromogenic substrates, such as *p*-nitrophenyl-⁴, *m*-cresolsulfonphthaleinyl-⁵ and 2-methoxy-4-(2'-nitrovinyl)phenyl-*N*-acetyl- β -D-glucosaminides.⁶ However, the procedures for using these substrates involve endo-point assays which require the addition of an alkaline reagent in order to visualize the situation at the termination of the enzyme reaction.

Although 2-chloro-4-nitrophenyl-*N*-acetyl- β -D-glucosaminide (CNP-NAG)⁷ has been used as a chromogenic substrate for the rate-assay (which has the advantages of procedure simplicity), this is not a satisfactory substrate. Although the 2-chloro-4-nitrophenol released by NAGase fully dissociates at over pH 7 ($\epsilon=16000$ at 400 nm), the absorbance under measur-

ing pH is very low ($\epsilon=3580$ at pH 4.8). Therefore, the sensitivity using CNP-NAG is not only low, but also is affected even by minor changes in the pH of the test solution. Since the NAGase reaction proceeds under weakly acidic conditions (optimum pH: 4–5)⁸, it is urgent need to find suitable substrates for rate-assay studies.

We have been studying the synthesis of glucosidic substrates for an enzyme assay⁹, and have reported that resorufinyl- and resazurinyl- β -D-glucopyranosides were hydrolyzed by β -D-glucosidase to give violet products showing high absorbance.¹⁰

In an effort to develop a convenient and useful substrate for rate-assay studies of NAGase, we selected resorufin and resazurin for chromogenic substances and synthesized two novel *N*-acetyl- β -D-glucosaminides [2-(acetyl-amino)-2-deoxy- β -D-glucopyranosides] of resorufin (7-hydroxy-3*H*-phenoxazin-3-one) derivatives: resorufinyl- (1) and resazurinyl-*N*-acetyl- β -D-glucosaminides (2) (Chart 1). We herein report their synthesis and potential applicabilities to the rate-assay of NAGase.

Chart 1 Synthetic scheme of 1 and 2.

Experimental

Apparatus

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^1H -NMR) spectra were taken at 199.5 MHz with a JEOL JNM-FX200 NMR spectrometer with tetramethylsilane used as an internal standard. The infrared (IR) spectra were taken with a JASCO A-202 spectrometer. Ultraviolet (UV) spectra were recorded with a Hitachi 557 spectrometer. Column chromatography was performed on Merck Kieselgel 60 (SiO_2 , 230–400 mesh).

Reagents

NAGase (from Bovine Kidney) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and diluted with distilled water. CNP-NAG was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan) as *Meiassei NAG-R kit*. The other chemicals were of reagent grade.

Synthesis of 1

Resorufin (3.5 g, 16.4 mmol) and Ag_2O (3.8 g, 16.4 mmol) were added to a solution of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine¹¹ (2.0 g, 5.5 mmol) in CH_3CN (250 ml) and the entire mixture was stirred at 40°C for 10 h. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with $\text{CH}_3\text{CN}-\text{CHCl}_3$ (2:3, v/v) gave 1.22 g (41.2% yield) of 7-[(3,4,6-tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranosyl)oxy]-3H-phenoxazin-3-one as orange colored needles ($\text{CHCl}_3-\text{Et}_2\text{O}$), mp $208.0-210.0^\circ\text{C}$ (dec.). Anal. Calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_{11}$: C, 57.56; H, 4.83; N, 5.16. Found: C, 57.52; H, 4.73; N, 4.93. IR (KBr): 3480, 3300, 1745, 1660, 1605, 1565, 1510 cm^{-1} . ^1H -NMR ($\text{DMSO}-d_6$) δ : 1.79 (3H, s), 1.96 (3H, s), 2.01 (3H, s), 2.03 (3H, s), 4.00–4.30 (4H, m), 4.95 (1H, br t, $J=9.8\text{ Hz}$), 5.25 (1H, br t, $J=9.8\text{ Hz}$), 5.56 (1H, d, $J=8.8\text{ Hz}$), 6.25 (1H, d, $J=1.7\text{ Hz}$), 6.80 (1H, dd, $J=10.0, 1.7\text{ Hz}$), 7.07 (1H, dd, $J=8.8, 2.4\text{ Hz}$), 7.20 (1H, d, $J=2.4\text{ Hz}$), 7.53 (1H, d, $J=10.0\text{ Hz}$), 7.79 (1H, d, $J=8.8\text{ Hz}$), 8.04 (1H, d, $J=8.8\text{ Hz}$, NH). UV λ_{max} (MeOH)nm (ϵ): 450 (19200), 375 (11600), 248 (15700).

Subsequently, anhydrous K_2CO_3 (28 mg, 0.2 mmol) was added to a solution of the acetylglucosaminide (434 mg, 0.8 mmol) in absolute MeOH and CH_3CN with stirring. After stirring the reaction mixture for 1 h at room temperature, it was stored in a refrigerator for 2 h. The precipitated crystals were collected by filtration and washed with cold methanol. The obtained crystals were recrystallized from methanol to give 303 mg (91.0% yield) of 7-[[2-(acetylamino)-2-deoxy- β -D-glucopyranosyl]oxy]-3H-phenoxazin-3-one (1) as orange colored powder, mp $195.0-198.0^\circ\text{C}$ (dec.). Anal. Calcd for $\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}_8$: C, 57.69; H, 4.84; N, 6.73. Found: C, 57.57; H, 4.63; N, 6.93. IR (KBr): 3380, 3250, 1640, 1600, $1560, 1505\text{ cm}^{-1}$. ^1H -NMR ($\text{DMSO}-d_6$) δ : 1.82 (3H, s),

3.10–3.80 (6H, m), 4.55 (1H, br t, $J=5.6\text{ Hz}$), 5.03 (2H, br t, $J=5.9\text{ Hz}$), 5.20 (1H, d, $J=8.3\text{ Hz}$), 6.26 (1H, d, $J=2.0\text{ Hz}$), 6.79 (1H, dd, $J=10.0, 2.0\text{ Hz}$), 7.02 (1H, dd, $J=8.8, 2.4\text{ Hz}$), 7.08 (1H, d, $J=2.4\text{ Hz}$), 7.52 (1H, d, $J=10.0\text{ Hz}$), 7.76 (2H, d, $J=8.8\text{ Hz}$). UV λ_{max} (MeOH)nm (ϵ): 450 (18500), 382 (11500), 249 (15500).

Synthesis of 2

Resazurin (2.8 g, 12.1 mmol) and Ag_2O (2.8 g, 12.2 mmol) were added to a solution of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine (1.5 g, 4.1 mmol) in CH_3CN (200 ml) and the entire mixture was stirred at 40°C for 8 h. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with $\text{CH}_3\text{CN}-\text{CHCl}_3$ (2:3, v/v) gave 840 mg (36.7% yield) of 7-[(3,4,6-tri-O-acetyl-2-(acetylamino)-2-deoxy- β -D-glucopyranosyl)oxy]-3H-phenoxazin-3-one 10-oxide as reddish needles ($\text{CHCl}_3-\text{Et}_2\text{O}$), mp $220.0-221.0^\circ\text{C}$ (dec.). Anal. Calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_{12}$: C, 55.92; H, 4.69; N, 5.02. Found: C, 55.88; H, 4.65; N, 4.91. IR (KBr): 3480, 3320, 1740, 1660, 1630, 1540 cm^{-1} . ^1H -NMR (CDCl_3) δ : 1.97 (3H, s), 2.07 (3H, s), 2.08 (3H, s), 2.10 (3H, s), 3.95–4.05 (1H, m), 4.15–4.35 (3H, m), 5.14 (1H, br t, $J=9.8\text{ Hz}$), 5.49 (1H, br t, $J=10.0\text{ Hz}$), 5.52 (1H, d, $J=8.3\text{ Hz}$), 6.15 (1H, d, $J=8.1\text{ Hz}$, NH), 6.22 (1H, d, $J=2.0\text{ Hz}$), 6.74 (1H, dd, $J=10.0, 2.0\text{ Hz}$), 7.01 (1H, dd, $J=10.0, 2.0\text{ Hz}$), 7.03 (1H, d, $J=2.0\text{ Hz}$), 8.01 (1H, d, $J=10.0\text{ Hz}$), 8.13 (1H, d, $J=10.0\text{ Hz}$). UV λ_{max} (MeOH)nm (ϵ): 520 (14600), 489 (14600), 375 (8900), 358 (10300), 348 (10300), 270 (9500).

Subsequently, anhydrous K_2CO_3 (28 mg, 0.2 mmol) was added to a solution of the acetylglucosaminide (447 mg, 0.8 mmol) in absolute MeOH and CH_3CN with stirring. After stirring the reaction mixture for 1 h at room temperature, it was stored in a refrigerator for 2 h. The precipitated crystals were collected by filtration and washed with cold methanol. The obtained crystals were recrystallized from methanol to give 154 mg (44.5% yield) of 7-[[2-(acetylamino)-2-deoxy- β -D-glucopyranosyl]oxy]-3H-phenoxazin-3-one 10-oxide (2) as reddish powder, mp $150.5-153.0^\circ\text{C}$ (dec.). Anal. Calcd for $\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}_9$: C, 55.56; H, 4.66; N, 6.48. Found: C, 55.29; H, 4.55; N, 6.27. IR (KBr): 3380, 3250, 1650, 1630, 1600, 1545, 1470 cm^{-1} . ^1H -NMR ($\text{DMSO}-d_6$) δ : 1.82 (3H, s), 3.10–3.80 (6H, m), 4.54 (1H, br t, $J=4.9\text{ Hz}$), 5.03 (2H, br t, $J=5.9\text{ Hz}$), 5.23 (1H, d, $J=8.8\text{ Hz}$), 6.15 (1H, d, $J=1.7\text{ Hz}$), 6.67 (1H, dd, $J=10.0, 1.7\text{ Hz}$), 7.04 (1H, dd, $J=9.3, 2.0\text{ Hz}$), 7.17 (1H, d, $J=2.0\text{ Hz}$), 7.77 (1H, d, $J=8.8\text{ Hz}$, NH), 7.98 (1H, d, $J=10.0\text{ Hz}$), 8.07 (1H, d, $J=9.3\text{ Hz}$). UV λ_{max} (MeOH)nm (ϵ): 522 (14400), 490 (14400), 379 (8700), 359 (9400), 349 (9400), 270 (9200).

Enzyme reactions

The pre-incubated substrate solutions (1 ml) of 1 (2.47 mM) or 2 (0.31 mM) in a citrate buffer (0.05 M, pH 5.0) containing β -cyclodextrin (20 mg ml^{-1}) were, in each case, mixed with a NAGase solution (0.05 ml) of a given concentration. The mixture was incubated at

37°C. After 1 min, the increase in the UV absorbance at 575 nm (for 1) or 610 nm (for 2) against H₂O was measured continuously for 2 min interval.

Results and Discussion

Our route to the synthesis of the desired *N*-acetyl- β -D-glucosaminides involved a direct introduction of resorufinyl groups into a glycosyl donor, followed by deprotection of the sugar moiety (Chart 1). By means of the Koenigs-Knorr reaction, resorufinyl- and resazurinyl-2,3,4,6-tetraacetyl- β -D-glucosaminides were obtained from 1-chloro-1-deoxy-2,3,4,6-tetraacetyl- α -D-glucosamine in 41 and 37% yields, respectively. *O*-Deacetylation of these two compounds with K₂CO₃ in MeOH-CH₃CN gave 1 and 2 in 91 and 45% yields, respectively.

The structures of the two *N*-acetyl- β -D-glucosaminides (1 and 2) were established from the results of elemental analyses and the spectral data shown in the experimental section. The proton nuclear magnetic resonance (¹H-NMR) spectra of 1 and 2 in dimethylsulfoxide (DMSO)-*d*₆ showed signals at δ 5.20 and 5.23 assigned to anomeric protons having large coupling constants ($J_{1,2}$ =8.3 and 8.8 Hz), indicating the presence of the β -glycosidic bond in 1 and 2.

The principle of the NAGase assay using these synthetic substrates (1 and 2) is illustrated in Chart 2. The enzyme assay is based on the direct spectrophotometric determination of the chromophore which is released from the substrate by the enzymatic hydrolysis. Thus, the NAGase activity is measured by calculating the rate of chromophore formation in terms of the increase in absorbance per minute.

Figure 1 shows the spectral changes of 1 and 2 when incubated with NAGase in 0.05 M citrate buffer (pH 5.0) at 37°C. When we used each 1 and 2 as a substrate, the observed spectra of the reaction mixture shifted to a longer wavelength. The dissociation of the released chromophores, however, was not complete at pH 5.0, since the absorption maxima of these chromophores at pH 7 were 580 nm (ϵ =34000 for 1) and 620 nm (ϵ =39000 for 2).

Figure 2 shows the differential spectra of each 1 and 2 upon incubation with NAGase, as well as NAGase without incubation under the same conditions as in Fig. 1. The absorption maxima of the differential spec-

tra of 1 and 2 were observed at 572 nm (ϵ =8330) and 605 nm (ϵ =13300), respectively. We therefore decided to detect at 575 or 610 nm for the NAGase reaction, even though it might be interfered by a considerable spectral overlap with the reagent blank. It was consequently suggested that the enzyme reaction could be directly monitored at the optimum pH range without terminating the reaction.

The time courses of the enzyme reactions of 1 and 2 at pH 5.0 at 37°C for 5 min are shown in Fig. 3, in which the

Fig. 1 Absorption spectra of 1 (0.025 mM) and 2 (0.031 mM) both with and without NAGase (2750 units l⁻¹) at 37°C for 10 min at pH 5.0: a, 1 without NAGase; b, 1 with NAGase; c, 2 without NAGase; d, 2 with NAGase.

Fig. 2 Differential spectra of b-a and d-c shown in Fig. 1.

Chart 2 Principle of the NAGase assay by 1 and 2.

Fig. 3 Time courses of the reactions of **1** (2.47 mM) and **2** (0.31 mM) with NAGase (275 units l^{-1}) at 37°C for 5 min at pH 5.0 observed at 575 and 610 nm, respectively: a, **1** without NAGase; b, **1** with NAGase; c, **2** without NAGase; d, **2** with NAGase.

Fig. 4 Standard curves of **1** (2.47 mM, O), **2** (0.31 mM, ●) and CNP-NAG (2.33 mM, □) under the rate-assay conditions at 37°C against the concentration of NAGase, observed at 575, 610 and 400 nm, respectively.

reactions with the enzyme as well as without the enzyme were monitored spectrophotometrically at 575 nm for **1** and 610 nm for **2**. The reaction rate was constant for more than 5 min from the start of the reaction. By measuring the change in the absorbance during the period from 1 to 3 min we could determine the NAGase activity. The absorbance increase of **1** per minute at 575 nm in the enzyme reaction was about 2.8-fold larger than that of **2** at 610 nm.

The linearity of this method between the absorbance and the standard NAGase concentration was examined and good linearity was observed in the range from 13.8 to 275 units l^{-1} of the NAGase concentration (Fig. 4). The increase in absorbance of **1** at 575 nm and **2** at 610 nm when incubated with NAGase of different concentrations was compared with that of CNP-NAG at 400 nm under the same conditions; it was observed that the sensitivity of **1** is about 1.4 times higher than that of CNP-NAG; that of **2** is only about half that of CNP-NAG.

We determined the Michaelis constants (K_m) and maximum velocities ($V_{\max}$) for **1** and **2** from Lineweaver-Burk plots. The K_m values were 0.62 mM for **1** and 0.40 mM for **2**. It was found that **1** and **2** had the desired levels of affinity in the NAGase reaction. A comparison of the $V_{\max}$ values for these two substrates showed that the $V_{\max}$ values for **1** was about 2.3 times larger than that for **2**, indicating that **1** is a more favorable substrate than **2**.

When we dissolved both **1** and **2** for preparing substrate solutions, these concentrations were difficult to increase beyond 2.47 mM (for **1**) and 0.31 mM (for **2**), even when using β -cyclodextrin. Therefore, the initial velocities in these substrate concentrations using for NAGase determination were estimated to be 75% (for **1**) and 43% (for **2**) of the $V_{\max}$ values, respectively. Thus, even though the tested condition was unsatisfactory for **2**, that of **1** was sufficient to perform an adequate NAGase rate-assay.

Although the present synthetic substrates (**1** and **2**) have disadvantages due to low solubility in water and the considerable spectral overlap with the reagent blank in the determining wavelength, they are considered to be potential chromogenic substrates for the rate-assay of NAGase. Especially, compound **1** is the first substrate found which enables us to perform an NAGase assay colorimetrically by using the absorption maximum at a longer absorption wavelength, even for a weakly acidic pH.

Based on the present results, we are currently investigating how to create a more suitable substrate for the NAGase assay.

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