Glycosides Having Chromophores as Substrates for Sensitive Enzyme Analysis. IV.[1] Synthesis of *N*-Acetyl-β-D-glucosaminides of Fluorescein Derivatives and Their Application to the Rate-Assay of *N*-Acetyl-β-D-glucosaminidase

Kouichi KASAI,* Riichiro UCHIDA, and Nobuyuki YAMAJI

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278, Japan.
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Three novel *N*-acetyl-β-D-glucosaminides, 2′,7′-dichlorofluorescein mono(*N*-acetyl-β-D-glucosaminide) (6a), fluorescein mono(*N*-acetyl-β-D-glucosaminide) (6b) and 2′,7′-dichlorofluorescein di(*N*-acetyl-β-D-glucosaminide) (7a), were synthesized by the introduction of *N*-acetyl-β-D-glucosaminyl group into fluorescein derivatives followed by the removal of the protecting group. Compounds 6a, 6b and 7a were hydrolyzed by *N*-acetyl-β-D-glucosaminidase to give products showing high absorbance at a long absorption wavelength (500, 465 and 485 nm) under weakly acidic rate-assay conditions (pH 5.0). The K_m values for 6a and 7a were 0.56 and 0.86 mM, respectively. Among these compounds, 7a is considered to be the most potential chromogenic substrate for the rate-assay of *N*-acetyl-β-D-glucosaminidase, since it gives a clear color generation from colorless to orange color (λ_{max} 280→485 nm) by enzyme hydrolysis and has a higher water solubility of more than 30 mM.

Keywords *N*-acetyl-β-D-glucosaminidase; chromogenic substrate; rate-assay; enzyme activity; 2′,7′-dichlorofluorescein di(*N*-acetyl-β-D-glucosaminide); fluorescein

Urinary *N*-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30) is well known as a sensitive indicator for renal parenchymal damage.[2] The assay of this enzyme is of clinical importance in the diagnosis of renal disease,[3] early warning of rejection after renal transplantation,[4] and monitoring of drug nephrotoxicity.[5] Though the NAGase activity can be determined by the use of several synthetic substrates, these substrates are limited to use by an indispensable use of end-point assay and/or an insufficient water solubility.[6] Since the NAGase reaction proceeds under weakly acidic conditions (optimum pH: 4—5),[7] it is urgent that a suitable substrate whose aglycone dissociates in this pH region is found for rate-assay studies.

We recently reported the synthesis of resorufinyl- and resazurinyl-*N*-acetyl-β-D-glucosaminides as potential chromogenic substrates for the rate-assay of NAGase, but they had some disadvantages due to low solubility in water and considerable spectral overlap with the substrate blank at the measuring wavelength.[1]

In an effort to develop a convenient and useful substrate

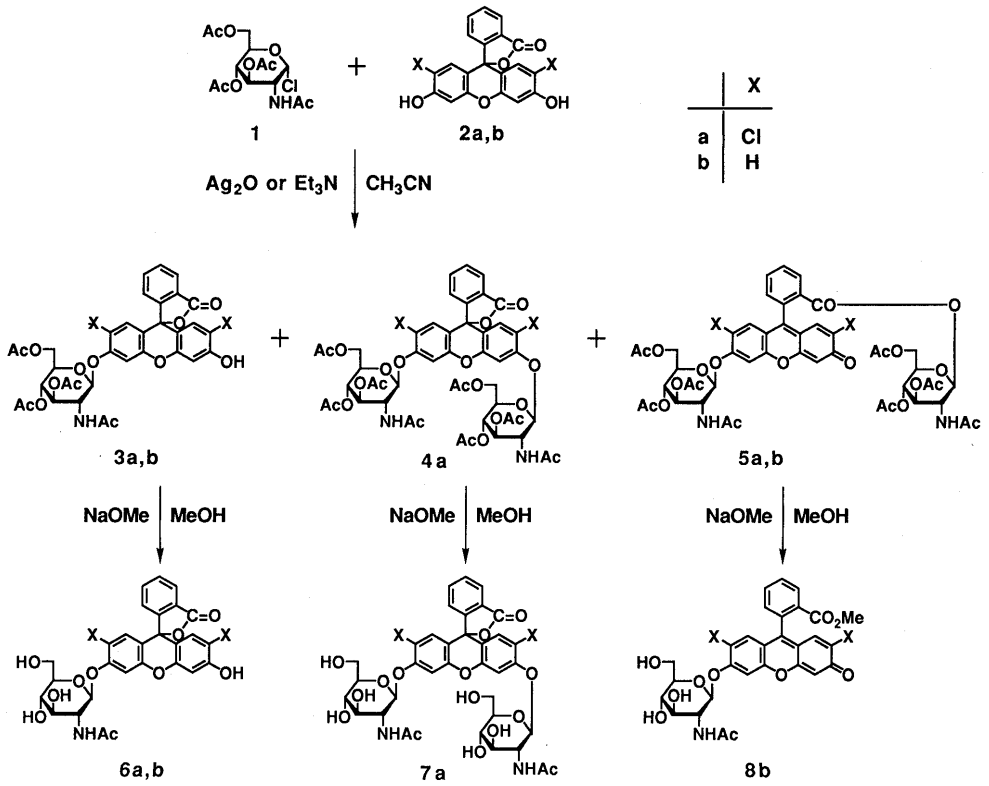

Chart 1

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for rate-assay studies of NAGase, we have selected fluorescein and its dichloro derivative for chromogenic substances in order to visualize its color under weakly acidic conditions and synthesized novel *N*-acetyl-*β*-D-glucosaminides of fluorescein derivatives (Chart 1). In this paper, we describe their synthesis and potential applicability to the rate-assay of NAGase.

Experimental

Reagents and Materials All chemicals were of reagent grade unless otherwise noted. 2-Chloro-4-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide (CNP-NAG) was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan) as a Meiassei NAG-R kit. NAGase (from bovine kidney) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and diluted with distilled water.

Apparatus All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H-NMR spectra were taken at 199.5 MHz with a JEOL JNM-FX200 NMR spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used for the signal patterns: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. Optical rotations were determined with a JASCO DIP-360 digital polarimeter at 25 °C. Infrared (IR) spectra were taken with a JASCO FT/IR-7300 spectrometer. Ultraviolet (UV) spectra were recorded with a Hitachi 557 spectrometer. High performance liquid chromatography (HPLC) was performed on an Inertsil ODS-2 column (4.6 mm i.d. × 150 mm) using a Waters model 600 multisolvent delivery system and a Waters model 490 UV detector (flow rate: 1 ml/min, detection: 254 nm, temperature: ambient). Column chromatography was performed on Merck Kiesel gel 60 (SiO_2, 230—400 mesh) and YMC-gel ODS-AQ (120—S50, from Yamamura Chemical Laboratories Co., Ltd., Japan).

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-2′,7′-dichloro-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (3a) 2′,7′-Dichlorofluorescein (**2a**) (3.3 g, 8.2 mmol) and triethylamine (Et_3N) (11.4 ml, 82 mmol) were added to a solution of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine[8] (**1**) (3.0 g, 8.2 mmol) in CH_3CN (500 ml) and the whole was stirred at 60 °C for 16 h. The mixture was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with CH_3CN–$CHCl_3$ (3 : 1, v/v) gave 3.04 g (50.7%) of **3a** as an orange colored powder, mp 146—156 °C, $[\alpha]_D^{25}$ −24.5° (*c* = 0.5, MeOH). IR(KBr): 3420, 1750, 1650, 1560, 1540, 1490, 1420, 1370 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 205 (53100), 228 (50900), 282 (10700), 374 (2300), 457 (6000), 485 (4700). ^{1}H-NMR (DMSO-d_6) δ: 1.74, 1.76 (each 3/2H, s, NAc), 1.95, 2.02, 2.06 (each 3H, s, OAc), 4.02—4.34 (4H, m, Glc H), 4.95 (1H, t, *J* = 9.8 Hz, Glc H-4), 5.21, 5.23 (each 1/2H, t, *J* = 9.8 Hz, Glc H-3), 5.47 (1H, d, *J* = 8.3 Hz, Glc H-1), 6.66, 6.68, 6.77, 6.79 (each 1/2H, s, xanthene H), 6.88 (1H, s, xanthene H), 7.28—7.41 (2H, m, xanthene H, arom. H), 7.72—7.89 (2H, m, arom. H), 7.93—8.06 (2H, m, arom. H, NH). *Anal.* Calcd for $C_{34}H_{29}Cl_2NO_{13}\cdot H_2O$: C, 54.56; H, 4.17; N, 1.87. Found: C, 54.26; H, 3.92; N, 1.83. t_R (eluent, CH_3CN : H_2O containing 0.1% H_3PO_4 = 3 : 2, v/v): 16.9, 24.5 min.

3′,6′-Bis[(3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)oxy]-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (4a) Compound **2a** (2.2 g, 5.48 mmol) and Et_3N (76 ml, 549 mmol) were added to a solution of **1** (20 g, 54.8 mmol) in CH_3CN (200 ml) and the whole was stirred at 60 °C for 16 h. The mixture was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with CH_3CN–$CHCl_3$ (2 : 1, v/v) gave 3.31 g (56.9%) of **4a** as a colorless powder, mp 206— 207 °C, $[\alpha]_D^{25}$ −14.4° (*c* = 0.5, MeOH). IR (KBr): 3400, 1750, 1690, 1660, 1560, 1540, 1480, 1410, 1370 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 207 (57900), 228 (60900), 282 (8600). ^{1}H-NMR (DMSO-d_6) δ: 1.75, 1.77 (each 3H, s, NAc), 1.96, 2.03, 2.12 (each 6H, s, OAc), 4.02—4.28 (8H, m, Glc H), 4.98 (2H, t, *J* = 9.5 Hz, Glc H-4), 5.25, 5.27 (each 1H, t, *J* = 9.5 Hz, Glc H-3), 5.48 (2H, d, *J* = 8.8 Hz, Glc H-1), 6.85, 7.28 (each 2H, s, xanthene H), 7.31—7.37 (1H, m, arom. H), 7.72—7.82 (2H, m, arom. H), 7.96 (2H, d, *J* = 7.6 Hz, NH), 8.01—8.05 (1H, m, arom. H). *Anal.* Calcd for $C_{48}H_{48}Cl_2N_2O_{21}\cdot H_2O$: C, 53.49; H, 4.68; N, 2.60. Found: C, 53.60; H, 4.44; N, 2.50. t_R (eluent, CH_3CN : H_2O containing 0.1% H_3PO_4 = 3 : 2, v/v): 14.0 min.

6-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-9-[2-[[(3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)oxy]carbonyl]phenyl]-2,7-dichloro-3*H*-xanthen-3-one (5a) Compound **2a** (5.4 g, 13.7 mmol) and silver oxide (3.0 g, 13.7 mmol) were added to a solution of **1** (5.0 g, 13.7 mmol) in CH_3CN (500 ml) and the whole was stirred at 40 °C for 16 h. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–CH_3CN (1 : 10, v/v) gave 2.19 g (15.1%) of **5a** as an orange colored powder, mp 153—156 °C, $[\alpha]_D^{25}$ +13.4° (*c* = 0.5, MeOH). IR (KBr): 3420, 1730, 1660, 1620, 1580, 1510, 1370, 1240 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 204 (57800), 234 (54000), 365 (11700), 438 (15800), 463 (26400), 493 (18700). ^{1}H-NMR (DMSO-d_6) δ: 1.63, 1.67 (each 3/2H, s, NAc), 1.75 (3H, s, NAc), 1.90—2.09 (18H, cluster of s, OAc), 3.82—4.53 (8H, m, Glc H), 4.83, 4.85 (each 1/2H, t, *J* = 9.5 Hz, Glc H-4), 4.97 (1H, t, *J* = 9.5 Hz, Glc H-4), 5.10, 5.13 (each 1/2H, t, *J* = 9.5 Hz, Glc H-3), 5.24, 5.26 (each 1/2H, t, *J* = 9.5 Hz, Glc H-3), 5.64 (1H, d, *J* = 8.3 Hz, Glc H-1), 5.68, 5.72 (each 1/2H, d, *J* = 8.5 Hz, Glc H-1), 6.46 (1H, s, xanthene H), 6.82, 6.91, 6.94, 7.03 (each 1/2H, s, xanthene H), 7.53—7.60 (1H, m, arom. H), 7.66 (1H, br s, xanthene H), 7.82—8.12 (4H, m, arom. H × 2, NH × 2), 8.18—8.27 (1H, m, arom. H). *Anal.* Calcd for $C_{48}H_{48}Cl_2N_2O_{21}\cdot H_2O$: C, 53.49; H, 4.68; N, 2.60. Found: C, 53.47; H, 4.64; N, 2.55. t_R (eluent, CH_3CN : H_2O containing 0.1% H_3PO_4 = 3 : 2, v/v): 5.6, 17.5 min.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (3b) and 6-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)oxy]-9-[2-[[(3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]carbonyl]phenyl]-3*H*-xanthen-3-one (5b) Fluorescein (**2b**) (1.0 g, 3.01 mmol) and Et_3N (42 ml, 301 mmol) were added to a solution of **1** (11 g, 30.1 mmol) in CH_3CN (400 ml) and the whole was stirred at 40 °C for 48 h. The mixture was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 16, v/v) gave 1.15 g (57.8%) of **3b** as a yellow colored powder, mp 155—156 °C, $[\alpha]_D^{25}$ −7.7° (*c* = 0.5, MeOH). IR (KBr): 3400, 1750, 1610, 1500, 1470, 1430, 1230 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 205 (43300), 224 (54400), 275 (6200). ^{1}H-NMR (DMSO-d_6) δ: 1.73, 1.74 (each 3/2H, s, NAc), 1.91, 1.97 (each 3H, s, OAc), 1.99, 2.01 (each 3/2H, s, OAc), 3.95—4.18 (4H, m, Glc H), 4.88 (1H, t, *J* = 9.8 Hz, Glc H-4), 5.19, 5.20 (each 1/2H, t, *J* = 9.8 Hz, Glc H-3), 5.39 (1H, d, *J* = 8.6 Hz, Glc H-1), 6.54—6.70 (5H, m, xanthene H), 7.00—7.05 (1H, m, xanthen H), 7.19—7.26 (1H, m, arom. H), 7.64—7.76 (2H, m, arom. H), 7.94—7.98 (2H, m, arom. H, NH). *Anal.* Calcd for $C_{34}H_{31}NO_{13}\cdot H_2O$: C, 60.09; H, 4.89; N, 2.06. Found: C, 60.25; H, 4.71; N, 2.11. t_R (eluent, CH_3CN : H_2O containing 0.1% H_3PO_4 = 3 : 2, v/v): 5.3, 6.3 min.

Further elution with MeOH–CH_3CN (1 : 10, v/v) gave 0.59 g (19.7%) of **5b** as a yellow colored powder, mp 150—151 °C, $[\alpha]_D^{25}$ −25.5° (*c* = 0.5, MeOH). IR (KBr): 3400, 1750, 1600, 1540, 1520, 1480, 1380 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 204 (41000), 230 (42700), 257 (16000), 264 (16000), 272 (15500), 307 (7500), 358 (9500), 434 (19300), 456 (23700), 485 (16200). ^{1}H-NMR (DMSO-d_6) δ: 1.66, 1.76 (each 3H, s, NAc), 1.88, 1.91, 1.94, 1.95, 2.01, 2.03 (each 3H, s, OAc), 3.82—4.29 (6H, m, Glc H), 4.42—4.52 (2H, m, Glc H), 4.85, 4.93 (each 1H, t, *J* = 9.8 Hz, Glc H-4), 5.13, 5.26 (each 1H, t, *J* = 9.8 Hz, Glc H-3), 5.57 (1H, d, *J* = 8.8 Hz, Glc H-1), 5.69 (1H, d, *J* = 9.0 Hz, Glc H-1), 6.22 (1H, d, *J* = 2.0 Hz, xanthene H), 6.38 (1H, dd, *J* = 9.5, 2.0 Hz, xanthene H), 6.74 (1H, d, *J* = 9.5 Hz, xanthene H), 6.87 (2H, br s, xanthene H), 7.31 (1H, br s, xanthene H), 7.48—7.58 (1H, m, arom. H), 7.76—7.99 (3H, m, arom. H × 2, NH), 8.04 (1H, d, *J* = 9.0 Hz, NH), 8.13—8.22 (1H, m, arom. H). *Anal.* Calcd for $C_{48}H_{50}N_2O_{21}\cdot 5/2H_2O$: C, 55.65; H, 5.35; N, 2.70. Found: C, 55.59; H, 5.10; N, 2.62. t_R (eluent, CH_3CN : H_2O containing 0.1% H_3PO_4 = 3 : 2, v/v): 3.3, 4.7 min.

3′-[[2-(Acetylamino)-2-deoxy-*β*-D-glucopyranosyl]oxy]-2′,7′-dichloro-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one (6a) A solution of **3a** (0.4 g, 0.55 mmol) in absolute MeOH (30 ml) was mixed with 28% sodium methylate (NaOMe) methanol solution (0.21 ml, 2.20 mmol) with stirring and the mixture was stirred for 30 min at room temperature. Then, 1 M phosphate buffer (pH 7, 10 ml) was added to the reaction mixture and half of the solvent was evaporated *in vacuo*. The residual solution was chromatographed on ODS gel. Elution with CH_3CN–H_2O (1 : 4, v/v) gave 0.17 g (51.3%) of **6a** as an orange colored powder, mp 104—124 °C (dec.), $[\alpha]_D^{25}$ −17.5° (*c* = 0.5, MeOH). IR (KBr): 3420, 1750, 1650, 1560, 1510, 1490, 1420 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 207 (36300), 229 (39500), 282 (9500), 369 (3200), 435 (6300), 458 (9600), 488 (9000). ^{1}H-NMR (DMSO-d_6) δ: 1.77, 1.79 (each 3/2H, s, NAc), 3.30—3.58 (4H, m, Glc H), 3.76—3.84 (2H, m, Glc H), 4.55—4.70 (1H, m, OH), 5.01, 5.07 (each 1H, br d, *J* = 5.1 Hz, OH), 5.15 (1H, d, *J* = 8.3 Hz, Glc H-1), 6.67, 6.74, 6.98, 7.29 (each 1H, s, xanthene H), 7.31—7.37 (1H, m, arom. H), 7.70—7.86 (3H, m, arom. H × 2, NH), 8.00—8.04 (1H, m, arom. H). *Anal.* Calcd for $C_{28}H_{23}Cl_2NO_{10}\cdot H_2O$: C, 54.03; H, 4.05; N, 2.25. Found: C, 54.26; H, 3.92; N, 2.10. t_R (eluent,

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MeOH : H_2O containing 0.1% H_3PO_4 = 1 : 1, v/v): 18.0, 19.3 min.

3′,6′-Bis[[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (7a) *O*-Deacetylation of **4a** (1.5 g, 1.42 mmol) with NaOMe was carried out as described for **6a**. Elution with CH_3CN–H_2O (3 : 7, v/v) gave 0.77 g (69.3%) of **7a** as a colorless powder, mp 163—164 °C, $[\alpha]_D^{25}$ −18.6° (c = 0.5, MeOH). IR (KBr): 3400, 1760, 1660, 1640, 1560, 1480, 1410, 1380 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 207 (57900), 228 (60900), 282 (8600). ^{1}H-NMR (DMSO-d_6) δ: 1.77, 1.79 (each 3H, s, NAc), 3.12—3.60 (8H, m, Glc H), 3.73—3.81 (4H, m, Glc H), 4.55—4.68 (2H, m, OH), 5.00 (2H, d, J = 5.1 Hz, OH), 5.07 (2H, d, J = 5.1 Hz, OH), 5.20 (2H, d, J = 8.6 Hz, Glc H-1), 6.79 (2H, s, xanthene H), 7.26, 7.27 (each 1H, s, xanthene H), 7.33—7.37 (1H, m, arom. H), 7.73—7.87 (4H, m, arom. H × 2, NH × 2), 8.02—8.08 (1H, m, arom. H). *Anal.* Calcd for $C_{36}H_{36}Cl_2NO_{15} \cdot 3/2H_2O$: C, 51.81; H, 4.71; N, 3.36. Found: C, 51.95; H, 4.43; N, 3.41. t_R (eluent, MeOH : H_2O containing 0.1% H_3PO_4 = 1 : 1, v/v): 6.5 min.

3′-[[2-(Acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (6b) *O*-Deacetylation of **3b** (0.56 g, 0.85 mmol) with NaOMe was carried out as described for **6a**. Elution with CH_3CN–H_2O (1 : 9, v/v) gave 0.32 g (70.6%) of **6b** as a light orange colored powder, mp 203—205 °C (dec.), $[\alpha]_D^{25}$ +43.3° (c = 0.5, MeOH). IR (KBr): 3400, 1750, 1640, 1600, 1510, 1470, 1380 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 225 (42000), 275 (8800), 308 (2000), 360 (2200), 452 (7200), 480 (6200). ^{1}H-NMR (DMSO-d_6) δ: 1.77 (3H, s, NAc), 3.20—3.80 (6H, m, Glc H), 4.56 (1H, br s, OH), 4.95—5.05 (2H, m, OH), 5.12, 5.18 (each 1/2H, d, J = 8.5 Hz, Glc H-1), 6.19 (1H, d, J = 2.0 Hz, xanthene H), 6.31 (1H, dd, J = 9.5, 2.0 Hz, xanthene H), 6.78—6.90 (3H, m, xanthene H), 7.05—7.15 (2H, m, xanthene H, arom. H), 7.49—7.54 (2H, m, arom. H), 7.77 (1H, d, J = 9.0 Hz, NH), 8.02—8.06 (1H, m, arom. H). *Anal.* Calcd for $C_{28}H_{25}NO_{10} \cdot H_2O$: C, 60.76; H, 4.92; N, 2.53. Found: C, 60.90; H, 4.72; N, 2.56. t_R (eluent, MeOH : H_2O containing 0.1% H_3PO_4 = 1 : 1, v/v): 4.6 min.

6-[[2-(Acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-9-[2-[(methoxy)carbonyl]phenyl]-3*H*-xanthen-3-one (8b) *O*-Deacetylation of **5b** (0.24 g, 0.24 mmol) with NaOMe was carried out as described for **6a**. Elution with CH_3CN–H_2O (3 : 7, v/v) gave 0.12 g (89.6%) of **8b** as an orange colored powder, mp 156—157 °C, $[\alpha]_D^{25}$ +31.1° (c = 0.5, MeOH). IR (KBr): 3420, 1720, 1640, 1600, 1510, 1470 cm^{-1}. UV λ_{max}^{EtOH} nm (ε): 205 (37300), 231 (42600), 257 (16800), 264 (16800), 268 (17000), 308 (8100), 360 (9700), 434 (20400), 456 (26300), 485 (19100). ^{1}H-NMR (DMSO-d_6) δ: 1.80 (3H, s, NAc), 3.23—3.77 (6H, m, Glc H), 3.69 (3H, s, OCH_3), 4.52 (1H, br s, OH), 5.02 (2H, br s, OH), 5.22 (1H, d, J = 8.6 Hz, Glc H-1), 6.23 (1H, d, J = 2.0 Hz, xanthene H), 6.38 (1H, dd, J = 9.7, 2.0 Hz, xanthene H), 6.80 (1H, br d, J = 9.7 Hz, xanthene H), 6.85 (2H, br s, xanthene H), 7.20 (1H, br s, xanthene H), 7.45—7.50 (1H, m, arom. H), 7.73—7.91 (3H, m, arom. H × 2, NH), 8.18—8.25 (1H, m, arom. H). *Anal.* Calcd for $C_{29}H_{27}NO_{10} \cdot 1/2H_2O$: C, 62.36; H, 5.05; N, 2.51. Found: C, 62.56; H, 5.01; N, 2.47. t_R (eluent, MeOH : H_2O containing 0.1% H_3PO_4 = 1 : 1, v/v): 3.4 min.

Michaelis Constants and Maximum Velocitites A solution of NAGase (274.9 I.U./l, 0.05 ml) was added to the solution of **6a** or **7a** (0.05—3 mM, 2.95 ml) in the citrate buffer (50 mM, pH 5.0) and the mixture was incubated at 37 °C. After 1 min, the increase in absorbance at 500 nm (for **6a**) or 485 nm (for **7a**) against H_2O was measured continuously for 3 min. For the substrate blank, H_2O was added instead of the 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 (30—600 I.U./l, 0.05 ml) was added to the solution of **7a** (1.73 mM, 2.95 ml) or CNP-NAG (1.73 mM 2.95 ml) in the citrate buffer (50 mM, pH 5.0) and the mixture was incubated at 37 °C. After 1 min, the increase in absorbance at 485 nm (for **7a**) or 400 nm (for CNP-NAG) against H_2O was measured continuously for 3 min. For the substrate blank, H_2O was added instead of the NAGase solution.

Identification of Enzyme Reaction Products by HPLC When **6a** or **7a** was used as the substrates, a 0.02 ml of the final reaction mixture obtained under the conditions described in Figs. 1 and 2 was injected into the HPLC system using MeOH–H_2O containing 0.1% H_3PO_4 (3 : 2, v/v) as an eluent. In this case, the retention time was 17.0 min for **2a**, 5.7 min for **6a**, 2.8 min for **7a**. When **6b** was tested, a 0.02 ml of the final reaction mixture obtained under the conditions described in Fig. 1 was injected into the HPLC system using MeOH–H_2O containing 0.1% H_3PO_4 (1 : 1, v/v) as an eluent. In this case, the retention time was 13.1 min for **2b**, 4.6 min for **6b**.

Results and Discussion

Synthesis At first, we selected 2′,7′-dichlorofluorescein (**2a**) as the aglycone of the synthetic substrate in view of the color generation under weakly acidic conditions. Thus the glycosidation of **2a** with 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine (**1**) as a glycosyl donor was examined (Table I). Treatment of **2a** with equimolecular amounts of glycosyl chloride **1** in the presence of silver oxide (Ag_2O) as a catalyst afforded the corresponding monoether **3a**, diether **4a** and ester ether **5a** in 18, 12 and 15% yields, respectively (entry 1). For the more effective syntheses of **3a** and **4a**, which are easily converted into NAGase substrates, we further searched for a suitable catalyst of this reaction. Consequently, the glycosidation using Et_3N instead of Ag_2O as a catalyst gave the monoether **3a** as a major product in a 51% yield (entry 2). When the reaction was carried out in the presence of Et_3N with ten-fold molar quantity of glycosyl chloride **1**, the diether **4a** was obtained as a major product in a 57% yield (entry 3).

O-Deacetylation of the monoether **3a** and diether **4a** with NaOMe in MeOH gave the desired *N*-acetyl-β-D-glucosaminides (**6a** and **7a**) in 51 and 69% yields, respectively. However, the reaction of the ester ether **5a** gave a complex mixture of products which were difficult to purify.

Next, in order to clarify the influence of Cl groups on the glycosidation and the enzyme reaction, fluorescein (**2b**) is used as the aglycone. The glycosidation of **2b** with excess **1** using Et_3N afforded the corresponding monoether **3b** and ester ether **5b** in 38 and 20% yields, respectively, but in contrast with the result of entry 3, the corresponding diether compound could not be isolated (entry 4). This indicates that the reactivity of hydroxyl groups in fluorescein derivatives is increased by the presence of electron-withdrawing Cl groups. The given monoether **3b** afforded the desired *N*-acetyl-β-D-glucosaminide (**6a**) in a 71% yield by *O*-deacetylation. On the other hand, the deprotection of the ester ether **5b** with NaOMe in MeOH gave the corresponding methyl ester **8b** in a 90% yield by ester exchange reaction.

The structures of these *N*-acetyl-β-D-glucosaminides (**6a**, **6b**, **7a** and **8b**) were confirmed from the results of elemental analyses and the spectral data shown in the experimental section. The ^{1}H-NMR spectra of **6a**, **6b**, **7a** and **8b** in dimethylsulfoxide-d_6 (DMSO-d_6) showed signals at δ 5.12—5.22 assigned to anomeric protons having large coupling constants ($J_{1,2}$ = 8.3—8.6 Hz), indicating the presence of the *trans*-β-glycosidic bond.

Each monoether compound (**3a**, **3b**, **6a** and **6b**) and ester ether compound (**5a**, **5b** and **8b**) is assumed to be a mixture of diastereoisomers from the results of ^{1}H-NMR

TABLE I. Glycosidation of **2a** and **2b**

Entry	Aglycone	**1** (mol eq)	Catalyst[a]	Temp. (°C)	Time (h)	Yields (%)		
						3a, b	**4a, b**	**5a, b**
1	**2a**	1	Ag_2O	40	16	18	12	15
2	**2a**	1	Et_3N	40	16	51	1	3
3	**2a**	10	Et_3N	60	16	25	57	6
4	**2b**	10	Et_3N	40	48	38	0	20

a) Carried out 10 eq of **1** in CH_3CN.

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spectroscopy and HPLC analysis. These isomerisms may be attributed to the configuration at the spiro carbon and the axial dissymmetry in the C_1–C_2 bond of fluorescein moiety. At present, we are currently investigating for separation of diastereoisomers and analysis of their absolute configurations.

Enzyme Reaction Since the solubility of **8b** was not sufficient for the enzyme reaction, the other three *N*-acetyl-β-D-glucosaminides of fluorescein derivatives (**6a**, **6b** and **7a**) were examined for their suitability for the rate-assay of NAGase. Though fluorometric measurement is sensitive, it requires special equipment and is subject to interference from biological substances such as peptides and fluorescent compounds. So, we selected colorimetric measurement which is widely used in clinical diagnosis.

When each *N*-acetyl-β-D-glucosaminide (**6a**, **6b** and **7a**) was incubated with NAGase in 0.05 M citrate buffer (pH 5.0) at 37 °C, the spectral changes of the reaction mixture were measured (Figs. 1 and 2) and the hydrolyzed products were determined by HPLC analysis (Chart 2). Compound **6a** was hydrolyzed to dichlorofluorescein (**2a**) and *N*-acetyl-D-glucosamine by NAGase with the spectral shift of 15 nm (λ_{max} 485→500 nm; Fig. 1, A and B). The released chromophore from **6b** was fluorescein (**2b**) and the shift of the absorption maixma was not observed in the reaction mixture (Fig. 1, C and D). This may be ascribed to the slight dissociation of the released chromophore at pH 5.0 due to the lack of two Cl groups. On the other hand, the NAGase reaction of **7a** produced dichlorofluorescein monoether (**6a**) and *N*-acetyl-D-glucosamine in the initial stage by hydrolyzing only one of the *N*-acetyl-β-D-glucosaminyl groups. Therefore, **7a** gives a clear color generation from colorless to orange color (λ_{max} 280→485 nm) by proceeding with the reaction of NAGase hydrolysis (Fig. 2). Though **2a** could not be detected by HPLC analysis of this reaction mixture, **2a** might be produced from **7a** *via* **6a** in higher enzyme concentration and longer reaction time. Consequently, using their spectral changes, except for **6b**, it is possible to determine NAGase activity

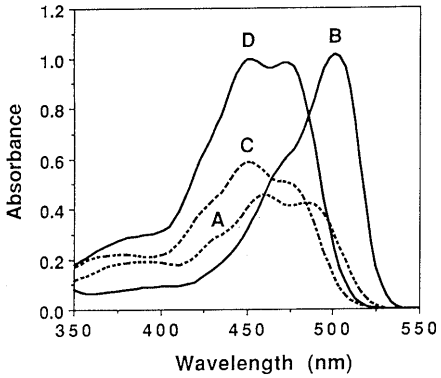

Fig. 1. Absorption Spectra of **6a** (0.025 mM) and **6b** (0.05 mM) upon Incubation with and without NAGase (50 I.U./l) at 37 °C for 30 min in Citrate Buffer (50 mM, pH 5.0)

A, **6a** without NAGase; B, **6a** with NAGase; C, **6b** without NAGase; D, **6b** with NAGase.

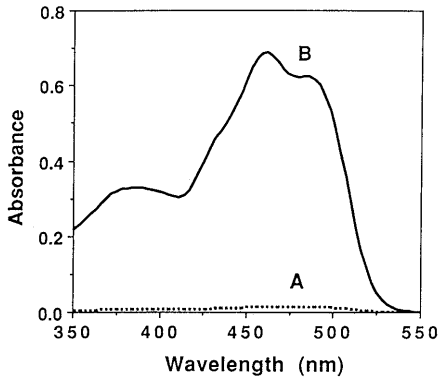

Fig. 2. Absorption Spectra of **7a** (1.7 mM) upon Incubation with and without NAGase (5 I.U./l) at 37 °C for 5 min in Citrate Buffer (50 mM, pH 5.0)

A, without NAGase; B, with NAGase.

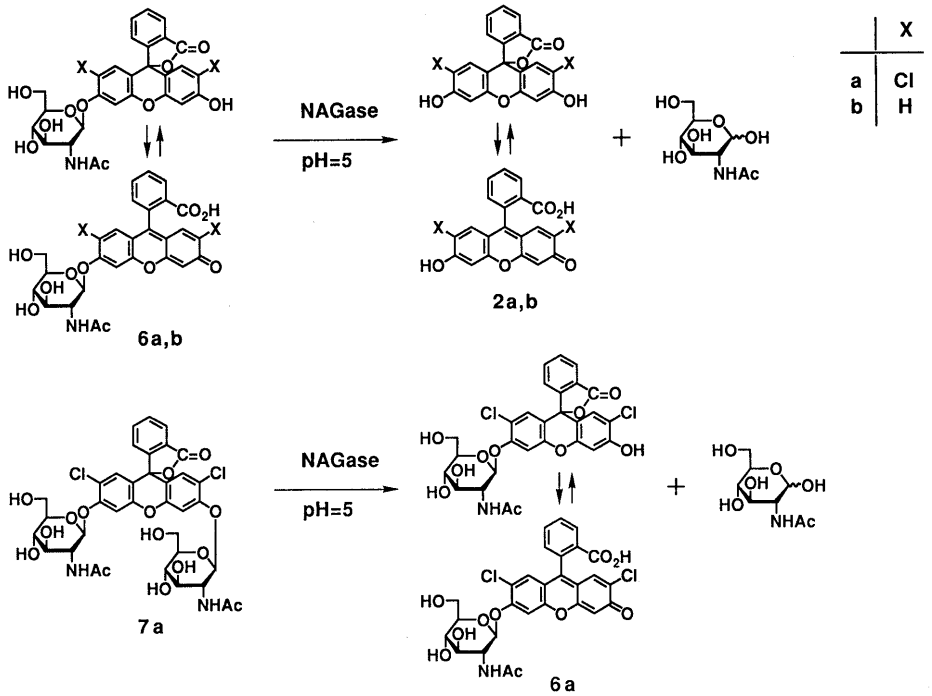

Chart 2

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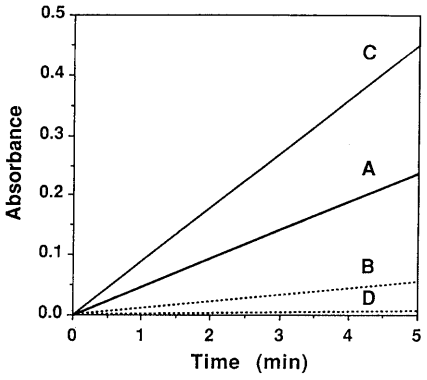

Fig. 3. Continuous Time Courses of the Reactions of **6a** (0.5 mM) and **7a** (1.7 mM) with and without NAGase (5 I.U./l) at 37 °C from 0 to 5 min in Citrate Buffer (50 mM, pH 5.0) Observed at 520 and 485 nm, Respectively

A, **6a** with NAGase; B, **6a** without NAGase; C, **7a** with NAGase; D, **7a** without NAGase.

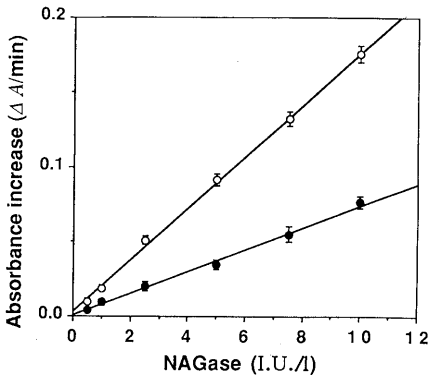

Fig. 4. Standard Curves of **7a** (1.7 mM, ○) and CNP-NAG (1.7 mM, ●) under the Rate-Assay Conditions at 37 °C against the Concentration of NAGase, Observed at 485 and 400 nm, Respectively

7a: $y=0.0173x+0.0033$. $r=0.992$. CNP-NAG: $y=0.0074x+0.000078$. $r=0.988$. Each point and bar shows the mean value and the standard deviation ($n=3$).

TABLE II. Kinetic Parameters of **6a** and **7a**

Compd. No.	K_m (mM)	V_{max} (M/min)
6a	0.56	4.1×10^{-6}
7a	0.86	7.3×10^{-6}

under the rate-assay conditions.

The time courses of the NAGase reactions of **6a** and **7a** at pH 5.0 for 5 min are shown in Fig. 3, in which the reactions with the enzyme as well as without the enzyme were monitored spectrophotometrically at 520 nm for **6a** and 485 nm for **7a.** The absorbance of the reactant of **6a** and NAGase increased linearly, but **6a** was unstable in an aqueous solution because of the considerable spontaneous hydrolysis. Therefore, the true enzyme activity must be calculated from the difference between lines A and B in Fig. 3. In the case of **7a**, even after 5 min the constant increase in absorbance was observed and the absorbance increase of **7a** per minute at 485 nm was 2.4-fold larger than that of **6a** at 520 nm. By measuring the change in absorbance during the period from 1 to 4 min, we could determine the NAGase activity.

The Michaelis constants (K_m) and maximum velocities (V_{max}) for the two glucosaminides (**6a** and **7a**) were obtained through Lineweaver–Burk plots and are summarized in Table II. The K_m values for **6a** and **7a** were 0.56 and 0.86 mM, respectively, which are similar to those found when previous substrates are used.[6b,c,e] Though the K_m value for **7a** was slightly larger than that for **6a**, the V_{max} value for **7a** was larger than that for **6a**, which may be caused by the difference of repulsion forces between affinity sites of the enzyme and the hydrolyzed products.

Since **7a** was considered to be the most favorable substrate among the present *N*-acetyl-β-D-glucosaminides, the standard curve of the absorbance increase of **7a** against the concentration of NAGase under the rate-assay conditions was examined (Fig. 4). A good linearity was observed in the range from 0.5 to 10 I.U./l of the NAGase concentration (final concentration). When the absorbance increase in the reaction mixture of **7a** was compared with that of CNP-NAG,[6d] a commonly used substrate in the rate-assay of NAGase, the sensitivity of **7a** was 2.3 times higher than that of CNP-NAG.

In summary, compound **6a** is not only unstable in an aqueous solution but also had a considerable spectral overlap with the chromophore. Moreover, compound **6b** shows no spectral shift under the rate-assay conditions. However, these defects for the rate-assay of NAGase are greatly improved in **7a**. Since compound **7a** is fixed in the lactoid form and the released chromophore by NAGase tautomerizes to the quinoid form in the reaction mixture (Chart 2), it gives a clear color generation from colorless to orange color (λ_{max} 280→485 nm) by the NAGase hydrolysis with an adequate absorbance at pH 5.0 (which is the optimum pH of the NAGase). In addition, the substrate (**7a**) shows sufficient stability and high solubility of more than 30 mM under rate-assay conditions by having two glucosamine residues. Therefore, **7a** is considered to be an excellent chromogenic substrate for the rate-assay of NAGase due to the above adventages.

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

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