Glycosides Having Chromophores as Substrates for Sensitive Enzyme Analysis. V.[1] Synthesis of 6′-*O*-Substituted 2′,7′-Dichlorofluorescein *N*-Acetyl-*β*-D-glucosaminides as Substrates for the Rate-Assay of *N*-Acetyl-*β*-D-glucosaminidase

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Sixteen novel 6′-*O*-substituted 2′,7′-dichlorofluorescein *N*-acetyl-*β*-D-glucosaminides (2a—p) were synthesized from 2′,7′-dichlorofluorescein 2,3,4,6-tetraacetyl-*β*-D-glucosaminide (10), prepared through 3 steps from 2′,7′-dichlorofluorescein (3). These *N*-acetyl-*β*-D-glucosaminides were examined to evaluate their solubility under the weakly acidic rate-assay conditions (pH 5.0) and their kinetic parameters with *N*-acetyl-*β*-D-glucosaminidase. Among these compounds, 6′-*O*-carboxymethyl- (2a), 6′-*O*-(*N*,*N*-dimethylaminoethyl)- (2i), and 6′-*O*-sulfoxyethyl-2′,7′-dichlorofluorescein *N*-acetyl-*β*-D-glucosaminides (2p), are considered to be potential chromogenic substrates for the rate-assay of *N*-acetyl-*β*-D-glucosaminidase, since they showed good solubility of more than 10 mM, moderate K_m values of 1.46—2.04 mM, and higher sensitivity to the enzyme than 2-chloro-4-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide.

Keywords *N*-acetyl-*β*-D-glucosaminidase; chromogenic substrate; rate-assay; enzyme activity; 6′-*O*-substituted 2′,7′-dichlorofluorescein *N*-acetyl-*β*-D-glucosaminide

Glycosides bearing chromophores are very important as substrates for the sensitive enzyme analysis of glycosyl hydrolase activities. For example, *N*-acetyl-*β*-D-glucosaminidase (NAGase, EC 3.2.1.30), a very significant enzyme in the diagnosis of renal disease,[2-5] has been assayed by utilizing several chromogenic substrates.[6-10] We also recently reported the synthesis of some convenient and useful substrates for the rate-assay of NAGase.[1,11] Among these substrates, 2′,7′-dichlorofluorescein di(*N*-acetyl-*β*-D-glucosaminide) (**1**) was considered to have the best potential as a chromogenic substrate, since it gives a clear color generation from colorless to orange (λ_{max} 280 nm→485 nm) upon hydrolysis by the enzyme and has a water solubility of more than 30 mM. These advantages are ascribed to the fixation of **1** in the lactoid form by the substitutents at both hydroxyl groups of the fluorescein moiety. Unfortunately the released chromophore is itself susceptible to hydrolysis by NAGase, because **1** includes two glucosamine residues. Therefore the assay using **1** may result in poor reliability and reproducibility under conditions of high enzyme concentration and long reaction time. To overcome this defect, we decided to replace one of the glucosamine residues with another substituent. Moreover, we aimed to introduce polar substituents into the synthetic substrates to retain the high solubility in aqueous buffer solution.

From this point of view, we have synthesized sixteen novel *N*-acetyl-*β*-D-glucosaminides of 6′-*O*-substituted 2′,7′-dichlorofluorescein derivatives (Chart 1). Here we describe their synthesis and properties as candidate substrates for the rate-assay of NAGase.

Results and Discussion

Synthesis At first, we chose 3′-*O*-carboxymethyl-2′,7′-dichlorofluorescein (**5**) as the chromophore of the synthetic substrate with a view to ensuring good solubility in aqueous solution. Two routes to the synthesis of the desired *N*-acetyl-*β*-D-glucosaminide (**2a**) were examined. The first route was the direct introduction of a glycosyl donor into the chromophore, which involved the preparation of 3′-*O*-substituted fluorescein (**5**) (Chart 2).

The chromophore (**5**) was synthesized by the reaction of the disodium salt of 2′,7′-dichlorofluorescein (**3**) with a two-fold molar quantity of ethyl bromoacetate followed by alkaline hydrolysis, in 75% yield. The glycosidation of **5** with 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-*α*-D-glucosamine (**6**)[12] as a glycosyl donor in the presence of silver oxide (Ag_2O) afforded the corresponding tetraacetyl-*β*-D-glucos-

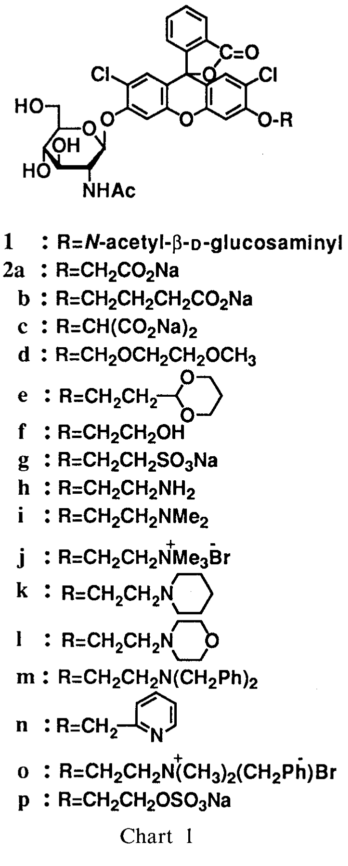

Chart 1

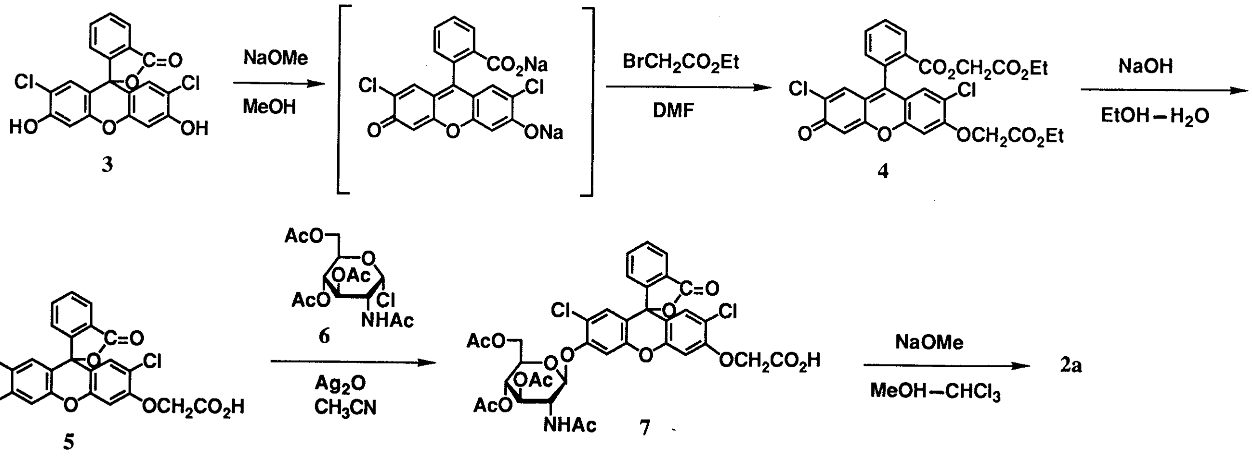

Chart 2

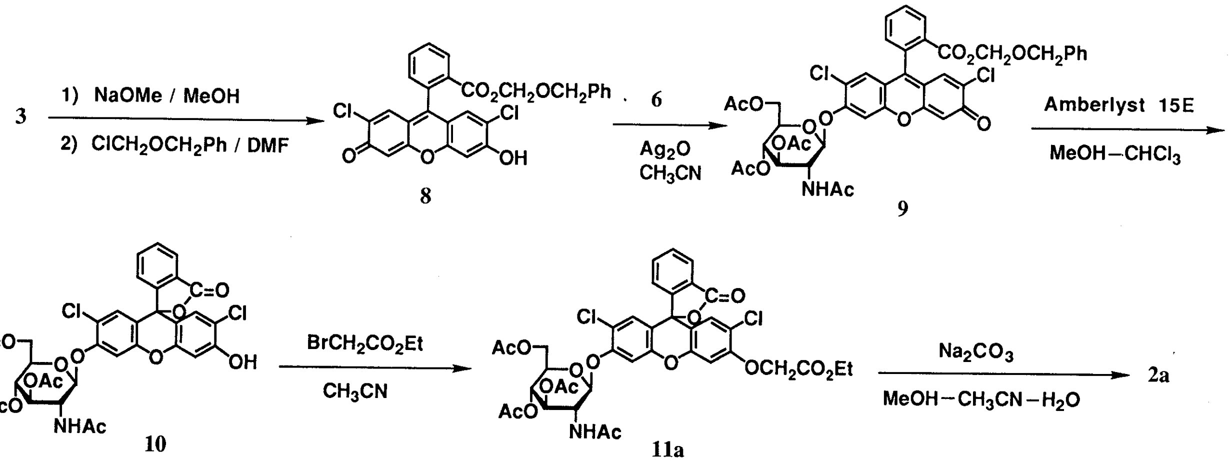

Chart 3

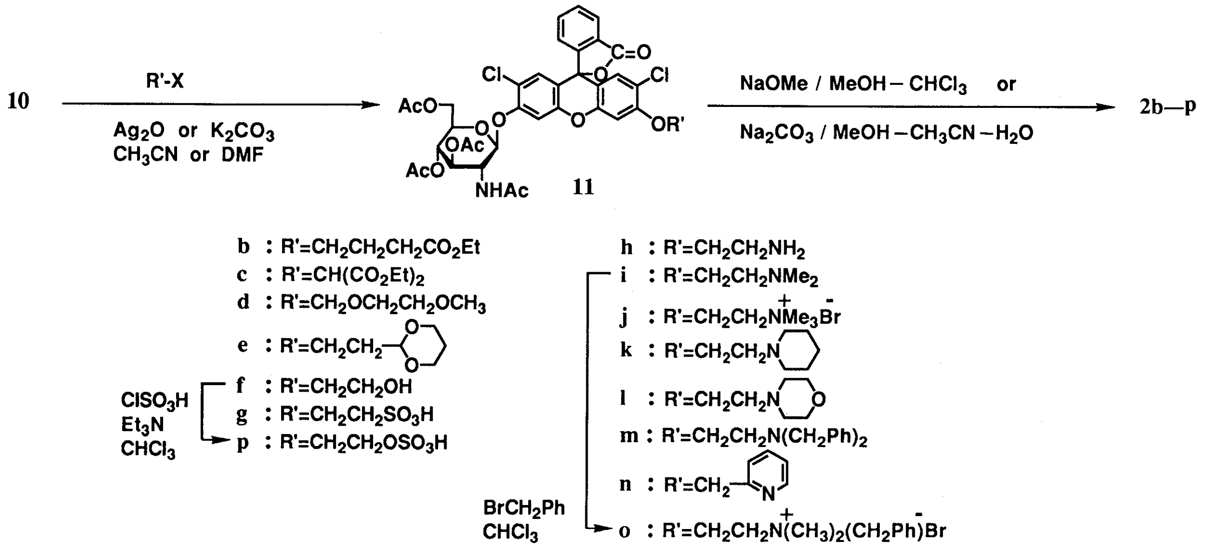

Chart 4

aminide (**7**) in 46% yield as a mixture of diastereoisomers. *O*-Deacetylation of **7** with sodium methoxide (NaOMe) in MeOH–$CHCl_3$ gave the desired *N*-acetyl-β-D-glucosaminide (**2a**) in 67% yield.

The second route was a stepwise method which constructs the chromophore from 2′,7′-dichlorofluorescein 2,3,4,6-tetraacetyl-β-D-glucosaminide (**10**) at a later step (Chart 3).

The synthesis of **10** reported in the previous paper[1] was modified to include the protection of **3** by a benzyloxymethyl group, which could be readily removed under mild acidic conditions. The reaction of the disodium salt of **3** with an equimolar amount of benzyl chloromethyl ether gave the

Table I. Physical Properties and Spectral Data for **11**

Compd. No.	Yield (%)	$[\alpha]_D^{25}$ ° (c, MeOH)	IR (KBr) cm^{-1}	UV λ_{max}^{MeOH} nm (ε)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
11a	74.4	−28.1	3300, 1752, 1696, 1627, 1608,	228 (63500),	$C_{38}H_{35}Cl_2NO_{15} \cdot 1/3H_2O$	55.48	4.37	1.70
		(0.366)	1565, 1485, 1415, 1373	282 (10000)		(55.42	4.26	1.69)
11b	47.7	−26.9	3269, 1749, 1676, 1627, 1608,	228 (66600),	$C_{40}H_{39}Cl_2NO_{15} \cdot 1/3H_2O$	56.48	4.70	1.65
		(0.372)	1566, 1488, 1415, 1372	282 (11300)		(56.43	4.56	1.62)
11c	78.0	−26.4	3278, 1750, 1683, 1626, 1607,	228 (59500),	$C_{41}H_{39}Cl_2NO_{17} \cdot H_2O$	54.31	4.56	1.54
		(0.370)	1569, 1484, 1413, 1371	282 (9000)		(54.17	4.36	1.53)
11d	71.7	−26.7	3370, 1752, 1695, 1627, 1609,	227 (62900),	$C_{38}H_{37}Cl_2NO_{15} \cdot 1/3H_2O$	55.35	4.60	1.70
		(0.393)	1566, 1486, 1415, 1373	281 (9500)		(55.30	4.47	1.66)
11e	53.9	−24.0	3371, 1751, 1685, 1626, 1608,	228 (62700),	$C_{40}H_{39}Cl_2NO_{15} \cdot 2/3H_2O$	56.08	4.75	1.64
		(0.394)	1564, 1488, 1413, 1374	282 (10500)		(55.91	4.57	1.69)
11f	58.7	−29.6	3366, 1751, 1675, 1626, 1607,	228 (59700),	$C_{36}H_{33}Cl_2NO_{14} \cdot H_2O$	54.56	4.45	1.77
		(0.362)	1564, 1487, 1415, 1373	282 (10000)		(54.49	4.27	1.92)
11g	34.5	−24.2	3368, 1751, 1670, 1627, 1608,	228 (59200),	$C_{36}H_{33}Cl_2NO_{16}S \cdot 2H_2O$	49.44	4.26	1.60
		(0.403)	1560, 1488, 1416, 1374	282 (9700)		(49.26	4.18	1.69)
11h	24.3	−22.6	3305, 1751, 1675, 1626, 1607,	228 (54700),	$C_{36}H_{34}Cl_2N_2O_{13} \cdot 2H_2O$	53.41	4.73	3.46
		(0.372)	1565, 1487, 1414, 1372	282 (9500)		(53.58	4.58	3.57)
11i	49.7	−23.8	3370, 1751, 1685, 1627, 1607,	228 (60100),	$C_{38}H_{38}Cl_2N_2O_{13} \cdot H_2O$	55.68	4.92	3.42
		(0.360)	1565, 1488, 1415, 1373	282 (10600)		(55.64	4.66	3.43)
11j	55.9	−24.8	3395, 1748, 1663, 1627, 1607,	227 (53000),	$C_{39}H_{41}Cl_2N_2O_{13} \cdot Br \cdot 2H_2O$	50.23	4.86	3.00
		(0.370)	1566, 1486, 1414, 1372	281 (8400)		(50.06	4.65	3.03)
11k	79.8	−31.6	3422, 1754, 1697, 1626, 1608,	228 (62400),	$C_{41}H_{42}Cl_2N_2O_{13} \cdot 3/2H_2O$	56.69	5.22	3.22
		(0.375)	1566, 1486, 1415, 1373	282 (10200)		(56.80	5.03	3.22)
11l	62.3	−26.7	3370, 1750, 1685, 1626, 1607,	228 (61500),	$C_{40}H_{40}Cl_2N_2O_{14} \cdot H_2O$	55.76	4.91	3.25
		(0.388)	1560, 1488, 1414, 1373	282 (10100)		(55.78	4.71	3.29)
11m	73.8	−15.9[a)]	3376, 1752, 1690, 1625, 1607,	228 (64400),	$C_{50}H_{46}Cl_2N_2O_{13} \cdot 1/3H_2O$	62.57	4.90	2.92
		(0.386)	1564, 1487, 1414, 1371	282 (10700)		(62.57	4.80	2.92)
11n	72.0	−15.9[a)]	3290, 1751, 1677, 1627, 1608,	228 (63500),	$C_{40}H_{34}Cl_2N_2O_{13} \cdot 2/3H_2O$	57.63	4.27	3.36
		(0.370)	1567, 1486, 1414, 1372	282 (10300)		(57.57	4.12	3.35)
11o	65.8	−17.1	3393, 1751, 1671, 1627, 1607,	227 (56000),	$C_{45}H_{45}Cl_2N_2O_{13} \cdot Br \cdot H_2O$	54.56	4.78	2.83
		(0.373)	1566, 1487, 1415, 1372	282 (8800)		(54.47	4.68	2.91)
11p	74.7	−24.4	3300, 1750, 1670, 1608, 1627,	228 (55600),	$C_{36}H_{33}Cl_2NO_{17}S \cdot 3H_2O$	47.59	4.33	1.54
		(0.401)	1560, 1488, 1417, 1374	282 (9000)		(47.38	4.23	1.66)

[a)] Measured in DMF.

benzyloxymethyl ester (**8**) in 82% yield. The glycosidation of **8** with **6** in the presence of Ag_2O afforded the corresponding tetraacetyl-β-D-glucosaminide (**9**) in 65% yield as a mixture of diastereoisomers, whose benzyloxymethyl group was selectively removed by using Amberlyst 15E, an anion exchange resin containing SO_3H groups, to give **10** in 91% yield. The introduction of an ethoxycarbonylmethyl group into the 6′-*O*-position of **10** was carried out to give the protected glucosaminide (**11a**) in 74% yield. The deprotection of **11a** with sodium carbonate (Na_2CO_3) in MeOH–CH_3CN–H_2O (2:2:1) gave the desired glucosaminide (**2a**) in 88% yield.

We selected the latter method for the synthesis of a wide variety of 6′-*O*-substituted 2′,7′-dichlorofluorescein *N*-acetyl-β-D-glucosaminides (**2**), because compound **10** is the common intermediate. The introduction of various kinds of substituents into the 6′-*O*-position of **10** was attempted. Consequently, thirteen acetylated glucosaminides (**11b**—**n**) were synthesized (Chart 4). Moreover, benzylation of the glucosaminide (**11i**) having a dimethylamino group with benzyl bromide gave the corresponding quaternary ammonium salt (**11o**) in 66% yield and sulfonylation of the glucosaminide (**11f**) having a hydroxyl group with chlorosulfonic acid ($ClSO_3H$) gave the corresponding sulfate (**11p**) in 75% yield. Then the desired *N*-acetyl-β-D-glucosaminides (**2b**—**p**) were obtained by the deprotection reactions of **11b, c** with Na_2CO_3 and **11d**—**p** with NaOMe.

The structures of these 2,3,4,6-tetraacetyl-β-D-glucosaminides (**11**) and *N*-acetyl-β-D-glucosaminides (**2**) were confirmed by the results of elemental analyses and the spectral data shown in Tables I and II (for **11**) and III and IV (for **2**), respectively. The proton nuclear magnetic resonance (^{1}H-NMR) spectra of **11** and **2** showed signals at δ 5.10—5.52 assigned to anomeric protons having large coupling constants ($J_{1,2}=8.1$—8.6 Hz), indicating the presence of the *trans*-β-glycosidic bond.

Each of the glucosaminides (**2**, **7**, **9**, **10** and **11**) is assumed to be a mixture of diastereoisomers from the results of ^{1}H-NMR spectroscopy and HPLC analysis. The isomerism may be attributed to the configuration at the spiro carbon and the axial dissymmetry at the C_1–C_2 bond of the fluorescein moiety. At present, we are investigating separation of the diastereoisomers and analysis of their absolute configurations.

Properties as NAGase Substrate The sixteen *N*-acetyl-β-D-glucosaminides (**2**) were examined for their suitability for the rate-assay of NAGase by measuring their solubilities and kinetic parameters for NAGase under weakly acidic conditions. The solubilities of these glucosaminides in 0.05 M citrate buffer (pH 5.0, which is the optimum pH of the NAGase)[13)] were determined by HPLC analysis and are summarized in Table V. It was found that four

TABLE II. ^{1}H-NMR Spectral Data (199.5 MHz) for **11**

Compd. No.	δ (ppm, in $CDCl_3$)
11a	1.32 (3H, t, *J*=7.1 Hz, CH_3), 1.93, 1.95 (3H, s, NAc), 2.06, 2.07, 2.15 (each 3H, s, OAc), 3.85—4.00 (2H, m, Glc H), 4.15—4.30 (2H, m, Glc H), 4.30 (2H, q, *J*=7.1 Hz, $C\underline{H}_2CH_3$), 4.73 (2H, s, $OC\underline{H}_2CO_2$), 5.13, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.28, 5.51 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.42, 5.61 (1H, dd, *J*=10.3, 9.3 Hz, Glc H-3), 5.68, 5.75 (each 1/2H, d, *J*=8.1 Hz, NH), 6.65—6.80 (3H, m, Xa), 7.10—7.20 (2H, m, Xa and Ar), 7.60—7.75 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11b	1.26 (3H, t, *J*=7.1 Hz, CH_3), 1.92, 1.94 (each 3/2H, s, NAc), 2.06, 2.07, 2.16 (each 3H, s, OAc), 2.10—2.25 (2H, m, $C\underline{H}_2CH_2CO_2$), 2.56 (2H, t, *J*=7.1 Hz, $CH_2C\underline{H}_2CO_2$), 3.85—4.05 (2H, m, Glc H), 4.13 (2H, t, *J*=6.1 Hz, $OC\underline{H}_2CH_2$), 4.15 (2H, q, *J*=7.1 Hz, $C\underline{H}_2CH_3$), 4.25—4.35 (2H, m, Glc H), 5.13, 5.16 (each 1/2H, t, *J*=9.0 Hz, Glc H-4), 5.28, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.42, 5.62 (each 1/2H, dd, *J*=10.3, 9.0 Hz, Glc H-3), 5.87, 5.93 (each 1/2H, d, *J*=8.6 Hz, NH), 6.70—6.80 (3H, m, Xa), 7.10—7.20 (2H, m, Xa and Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11c	1.32, 1.33 (each 3H, t, *J*=7.1 Hz, CH_3), 1.92, 1.94 (each 3/2H, s, NAc), 2.06, 2.07 (each 3H, s, OAc), 2.17, 2.24 (each 3/2H, s, OAc), 3.80—4.05 (2H, m, Glc H), 4.25—4.45 (6H, m, $C\underline{H}_2CH_3 \times 2$ and Glc H × 2), 5.11, 5.14 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.18 (1H, s, $OC\underline{H}(CO_2)_2$), 5.29, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.41, 5.62 (each 1/2H, dd, *J*=10.1, 9.3 Hz, Glc H-3), 5.75 (1H, br d, *J*=7.8 Hz, NH), 6.70—6.85 (2H, m, Xa), 7.10—7.30 (3H, m, Xa × 2 and Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11d	1.91, 1.93 (each 3/2H, s, NAc), 2.06, 2.07, 2.17 (each 3H, s, OAc), 3.37, 3.38 (each 3/2H, s, OCH_3), 3.55—3.60, 3.84—3.90 (each 2H, m, $OC\underline{H}_2C\underline{H}_2O$), 3.90—4.05 (2H, m, Glc H), 4.20—4.35 (2H, m, Glc H), 5.12, 5.15 (each 1/2H, t, *J*=9.0 Hz, Glc H-4), 5.38 (2H, s, OCH_2O), 5.29, 5.53 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.43, 5.63 (each 1/2H, dd, *J*=10.3, 9.0 Hz, Glc H-3), 5.90, 5.97 (each 1/2H, dd, *J*=8.5 Hz, NH), 6.70—6.80 (2H, m, Xa), 7.12, 7.14 (each 1H, s, Xa), 7.15—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11e	1.91, 1.93 (each 3/2H, s, NAc), 2.06, 2.07 (each 3H, s, OAc), 2.16, 2.17 (each 3/2H, s, OAc), 2.10—2.20 (4H, m, $OCH_2C\underline{H}_2$), 3.70—4.10 (6H, m, Glc H × 2 and $OCH_2 \times 2$), 4.18 (2H, t, *J*=6.2 Hz, $OC\underline{H}_2CH_2$), 4.25—4.32 (2H, m, Glc H), 4.82 (1H, t, *J*=5.1 Hz, $CH_2C\underline{H}O_2$), 5.13, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.28, 5.51 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.43, 5.62 (1H, dd, *J*=10.0, 9.3 Hz, Glc H-3), 5.69, 5.77 (1H, br d, *J*=8.3 Hz, NH), 6.70—6.80 (2H, m, Xa), 7.12, 7.14 (each 1H, s, Xa), 7.15—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11f	1.92, 1.94 (each 3/2H, s, NAc), 2.06, 2.07 (each 3H, s, OAc), 2.14, 2.16 (each 3/2H, s, OAc), 3.80—4.10 (4H, m, Glc H), 4.19, 4.28 (each 2H, t, *J*=4.6 Hz, $OC\underline{H}_2CH_2$), 5.12, 5.16 (each 1/2H, t, *J*=9.5 Hz, Glc H-4), 5.28, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.40, 5.61 (each 1/2H, dd, *J*=10.3, 9.5 Hz, Glc H-3), 5.90, 6.05 (each 1/2H, d, *J*=8.6 Hz, NH), 6.72, 6.76, 6.77, 6.78, 6.81, 6.87, 7.07, 7.11 (each 1/2H, s, Xa), 7.10—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11g[a)]	1.75, 1.77 (each 3/2H, s, NAc), 1.95, 2.02, 2.11 (each 3H, s, OAc), 2.95 (2H, br t, *J*=7.6 Hz, CH_2SO_3), 4.10—4.35 (4H, m, Glc H), 4.46 (2H, br t, *J*=7.6 Hz, $OC\underline{H}_2CH_2$), 4.96 (1H, t, *J*=9.3 Hz, Glc H-4), 5.20, 5.21 (each 1/2H, t, *J*=9.3 Hz, Glc H-3), 5.49 (1H, d, *J*=8.3 Hz, Glc H-1), 6.76, 6.78, 6.79, 6.80 (each 1/2H, s, Xa), 7.13, 7.14 (each 1/2H, s, Xa), 7.30—7.40 (1H, m, Ar), 7.41 (1H, s, Xa), 7.70—7.90 (2H, m, Ar), 7.95—8.10 (2H, m, Ar and NH)
11h	1.93, 1.95 (each 3/2H, s, NAc), 2.06 (6H, s, OAc), 2.15 (3H, s, OAc), 3.17 (2H, t, *J*=5.4 Hz, $OCH_2C\underline{H}_2N$), 3.85—4.05 (2H, m, Glc H), 4.09 (2H, t, *J*=5.4 Hz, $OC\underline{H}_2CH_2N$), 4.20—4.35 (3H, m, Glc H), 5.13, 5.16 (each 1/2H, t, *J*=9.5 Hz, Glc H-4), 5.28, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.43, 5.62 (each 1/2H, dd, *J*=10.3, 9.3 Hz, Glc H-3), 5.76 (1H, d, *J*=8.5 Hz, NH), 6.70—6.80 (3H, m, Xa), 7.10—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11i	1.92, 1.94 (each 3/2H, s, NAc), 2.06 (6H, s, OAc), 2.15 (3H, s, OAc), 2.39 (6H, s, NCH_3), 2.85 (2H, t, *J*=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.85—4.05 (1H, m, Glc H), 4.18 (2H, t, *J*=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.25—4.35 (3H, m, Glc H), 5.12, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.29, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.48, 5.62 (each 1/2H, dd, *J*=10.5, 9.3 Hz, Glc H-3), 5.93, 5.99 (each 1/2H, d, *J*=8.1 Hz, NH), 6.70—6.80 (3H, m, Xa), 7.10, 7.12 (each 1/2H, s, Xa), 7.10—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.07 (1H, m, Ar)
11j	1.90, 1.92 (each 3/2H, s, NAc), 2.05 (6H, s, OAc), 2.13 (3H, s, OAc), 3.51, 3.54 (each 9H, s, NCH_3), 3.80—3.95 (1H, m, Glc H), 4.05—4.25 (3H, m, Glc H), 4.29 (2H, t, *J*=5.4 Hz, $OCH_2C\underline{H}_2N$), 4.72 (2H, t, *J*=5.4 Hz, $OC\underline{H}_2CH_2N$), 5.12, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.29, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.48, 5.62 (each 1/2H, dd, *J*=10.3, 9.3 Hz, Glc H-3), 6.06, 6.17 (each 1/2H, d, *J*=8.8 Hz, NH), 6.65—6.80 (3H, m, Xa), 7.05–7.25 (2H, m, Xa and Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.07 (1H, m, Ar)
11k	1.40—1.50 (2H, m, $NCH_2CH_2C\underline{H}_2$), 1.55—1.70 (4H, m, $NCH_2C\underline{H}_2CH_2$), 1.91, 1.94 (each 3/2H, s, NAc), 2.05, 2.06 (3H, s, OAc), 2.15, 2.16 (each 3/2H, s, OAc), 2.58 (4H, t, *J*=5.1 Hz, $NC\underline{H}_2CH_2CH_2$), 2.87 (2H, t, *J*=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.80—4.05 (1H, m, Glc H), 4.21 (2H, t, *J*=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.20—4.35 (3H, m, Glc H), 5.12, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.27, 5.53 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.41, 5.62 (each 1/2H, dd, *J*=10.0, 9.3 Hz, Glc H-3), 5.97, 6.07 (each 1/2H, d, *J*=8.3 Hz, NH), 6.70—6.80 (3H, m, Xa), 7.09, 7.12 (each 1/2H, s, Xa), 7.10—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11l	1.92, 1.94 (each 3/2H, s, NAc), 2.07 (6H, s, OAc), 2.15 (3H, s, OAc), 2.63 (4H, br t, *J*=4.6 Hz, $OCH_2C\underline{H}_2N$), 2.88 (2H, t, *J*=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.72 (4H, br t, *J*=4.6 Hz, $OC\underline{H}_2CH_2N$), 3.85—4.05 (2H, m, Glc H), 4.21 (2H, t, *J*=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.25—4.35 (2H, m, Glc H), 5.13, 5.16 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.29, 5.53 (each 1/2H, d, *J*=8.3 Hz, Glc H-1), 5.43, 5.62 (each 1/2H, dd, *J*=10.3, 9.3 Hz, Glc H-3), 5.90, 5.93 (each 1/2H, d, *J*=8.8 Hz, NH), 6.70—6.80 (3H, m, Xa), 7.10, 7.11 (each 1/2H, s, Xa), 7.10—7.20 (1H, m, Ar), 7.60—7.80 (2H, m, Ar), 8.00—8.05 (1H, m, Ar)
11m	1.90, 1.92 (each 3/2H, s, NAc), 2.05, 2.06 (each 3H, s, OAc), 2.12, 2.13 (each 3/2H, s, OAc), 2.98 (2H, br t, *J*=5.4 Hz, $OCH_2C\underline{H}_2N$), 3.75 (4H, s, $NC\underline{H}_2Ph$), 3.80—4.00 (1H, m, Glc H), 4.08 (2H, br t, *J*=5.4 Hz, $OC\underline{H}_2CH_2N$), 4.20—4.35 (3H, m, Glc H), 5.12, 5.16 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.29, 5.54 (each 1/2H, d, *J*=8.3 Hz, Glc H-1), 5.42, 5.64 (each 1/2H, dd, *J*=10.3, 9.3 Hz, Glc H-3), 6.06, 6.17 (each 1/2H, d, *J*=8.8 Hz, NH), 6.63, 6.64, 6.71, 6.72, 6.73, 6.74, 7.10, 7.11 (each 1/2H, s, Xa), 7.15—7.40 (11H, m, Ar), 7.60—7.75 (2H, m, Ar), 8.00—8.07 (1H, m, Ar)
11n	1.90, 1.93 (each 3/2H, s, NAc), 2.05, 2.07, 2.14 (each 3H, s, OAc), 3.85—4.05 (2H, m, Glc H), 4.15—4.35 (2H, m, Glc H), 5.10, 5.14 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.31 (2H, br s, OCH_2), 5.28, 5.52 (each 1/2H, d, *J*=8.1 Hz, Glc H-1), 5.42, 5.62 (each 1/2H, dd, *J*=10.0, 9.3 Hz, Glc H-3), 5.96, 6.04 (each 1/2H, d, *J*=8.8 Hz, NH), 6.71, 6.74, 6.77, 6.78, 6.83, 6.86, 7.09, 7.12 (each 1/2H, s, Xa), 7.12—7.18 (1H, m, Ar), 7.23—7.30 (1H, m, Py), 7.57 (1H, br d, *J*=7.8 Hz, Py), 7.65—7.80 (3H, m, Py and Ar × 2), 8.00—8.10 (1H, m, Ar), 8.55—8.65 (1H, m, Py)
11o	1.94, 1.97 (each 3/2H, s, NAc), 2.06, 2.07 (each 3H, s, OAc), 2.13, 2.15 (each 3/2H, s, OAc), 3.47 (6H, s, NCH_3), 3.80—4.00 (1H, m, Glc H), 4.20—4.35 (3H, m, Glc H), 4.34 (2H, br t, *J*=5.4 Hz, $OCH_2C\underline{H}_2N$), 4.82 (2H, br t, *J*=5.4 Hz, $OC\underline{H}_2CH_2N$), 5.01 (2H, s, $NC\underline{H}_2Ph$), 5.12, 5.15 (each 1/2H, t, *J*=9.3 Hz, Glc H-4), 5.29, 5.52 (each 1/2H, d, *J*=8.3 Hz, Glc H-1), 5.48, 5.62 (each 1/2H, dd, *J*=10.1, 9.3 Hz, Glc H-3), 5.90, 5.93 (each 1/2H, d, *J*=8.5 Hz, NH), 6.70—6.85 (3H, m, Xa), 7.10, 7.12 (each 1/2H, s, Xa), 7.10—7.50 (6H, m, Ar), 7,60—7.85 (2H, m, Ar), 8.00—8.10 (1H, m, Ar)
11p[a)]	1.75, 1.77 (each 3/2H, s, NAc), 1.96, 2.02, 2.11 (each 3H, s, OAc), 4.05—4.40 (8H, m, Glc H × 4 and $OC\underline{H}_2C\underline{H}_2O$), 4.96 (1H, t, *J*=9.5 Hz, Glc H-4), 5.22, 5.23 (each 1/2H, d, *J*=9.5 Hz, Glc H-3), 5.50 (1H, d, *J*=8.3 Hz, Glc H-1), 6.78, 6.79, 6.80, 6.81 (each 1/2H, s, Xa), 7.14 (1H, br s, Xa), 7.30—7.40 (2H, m, Ar and Xa), 7.70—7.90 (2H, m, Ar), 7.95—8.05 (2H, m, Ar and NH)

Abbreviations: Glc, D-glucopyranosyl; Ar, aromatic proton; Xa, proton of xanthene ring; Py, proton of pyridine ring. *a*) Measured in DMSO-d_6.

Table III. Physical Properties and Spectral Data for **2**

Compd. No.	Yield (%)	$[\alpha]_D^{25}$ ° (c, MeOH)	IR (KBr) cm^{-1}	UV λ_{max}^{MeOH} nm (ε)	Formula	Analysis (%) Calcd (Found) C	H	Cl	N	t_R (min) (Eluent)[a]
2a	67.2[b]	−9.8	3283, 1758, 1660,	229 (56700),	$C_{30}H_{24}Cl_2NO_{12}$	49.40	4.01	9.72	1.92	4.5
	87.9[c]	(0.408)	1625, 1608, 1566	282 (9500)	$\cdot Na \cdot 5/2H_2O$	(49.31	3.81	9.61	1.98)	(C)
2b	80.2	−12.5	3282, 1764, 1660,	229 (59300),	$C_{32}H_{28}Cl_2NO_{12}$	51.35	4.31	9.47	1.87	6.5
		(0.390)	1626, 1605, 1565	282 (9900)	$\cdot Na \cdot 2H_2O$	(51.10	4.16	9.35	1.91)	(C)
2c	44.2	−13.6	3297, 1756, 1660,	229 (59000),	$C_{31}H_{23}Cl_2NO_{14}$	47.35	3.46	9.02	1.78	4.5
		(0.393)	1625, 1608, 1565	282 (9800)	$\cdot 2Na \cdot 2H_2O$	(47.74	3.42	9.14	1.82)	(C)
2d	79.3	−14.0	3297, 1768, 1663,	227 (59400),	$C_{32}H_{31}Cl_2NO_{12}$	53.42	4.76	9.85	1.95	3.7
		(0.395)	1625, 1608, 1565	282 (9000)	$\cdot 3/2H_2O$	(53.16	4.45	9.97	1.88)	(E)
2e	70.1	−12.0	3307, 1767, 1663,	229 (59500),	$C_{34}H_{33}Cl_2NO_{12}$	55.44	4.79	9.63	1.90	4.5
		(0.415)	1627, 1608, 1563	282 (10100)	$\cdot H_2O$	(55.58	4.60	9.62	1.89)	(E)
2f	87.3	−13.3	3297, 1760, 1662,	228 (58500),	$C_{30}H_{27}Cl_2NO_{11}$	54.07	4.39	10.64	2.10	3.9
		(0.395)	1625, 1607, 1564	282 (9800)	$\cdot H_2O$	(54.16	4.20	10.80	2.15)	(C)
2g	90.1	−10.6	3306, 1759, 1655,	228 (53300),	$C_{30}H_{26}Cl_2NO_{13}S$	46.76	3.92	9.20	1.82	6.3
		(0.393)	1625,1608, 1565	282 (8800)	$\cdot Na \cdot 2H_2O$	(46.72	3.90	9.28	1.94)	(E)
2h	47.0	−12.5	3304, 1770, 1664,	228 (57000),	$C_{30}H_{28}Cl_2N_2O_{10}$	52.72	4.72	10.37	4.10	2.1
		(0.385)	1627, 1607, 1564	282 (9200)	$\cdot 2H_2O$	(52.58	4.58	10.43	4.21)	(A)
2i	64.1	−14.5	3297, 1767, 1661,	228 (59500),	$C_{32}H_{32}Cl_2N_2O_{10}$	54.02	5.10	9.97	3.94	2.8
		(0.397)	1625, 1608, 1565	282 (10000)	$\cdot 2H_2O$	(53.84	4.84	10.18	3.91)	(A)
2j	67.1	−9.6	3285, 1761, 1662,	227 (50600),	$C_{33}H_{35}Cl_2N_2O_{10}$	49.15	4.87		3.47	2.9
		(0.371)	1625, 1606, 1565	281 (8600)	$\cdot Br \cdot 2H_2O$	(49.28	4.63	—[d]	3.48)	(A)
2k	50.8	−12.0	3304, 1770, 1664,	228 (61000),	$C_{35}H_{36}Cl_2N_2O_{10}$	55.93	5.36	9.43	3.73	2.6
		(0.388)	1627, 1607, 1564	282 (10200)	$\cdot 2H_2O$	(55.76	5.16	9.46	3.62)	(B)
2l	69.1	−11.9	3308, 1767, 1664,	229 (60300),	$C_{34}H_{34}Cl_2N_2O_{11}$	54.85	5.01	9.52	3.76	5.0
		(0.395)	1627, 1607, 1564	282 (10100)	$\cdot 3/2H_2O$	(54.55	4.72	9.59	3.82)	(A)
2m	62.7	−7.9	3314, 1770, 1664,	228 (64600),	$C_{44}H_{40}Cl_2N_2O_{10}$	62.49	5.01	8.38	3.31	6.0
		(0.376)	1626, 1607, 1564	282 (10700)	$\cdot H_2O$	(62.29	4.74	8.41	3.33)	(C)
2n	78.8	−12.7[e]	3292, 1766, 1663,	229 (61500),	$C_{34}H_{28}Cl_2N_2O_{10}$	55.82	4.41	9.69	3.83	6.4
		(0.382)	1628, 1607, 1567	282 (10200)	$\cdot 2H_2O$	(55.67	4.20	9.73	3.89)	(D)
2o	71.0	−7.5	3292, 1763, 1665,	228 (57000),	$C_{39}H_{39}Cl_2N_2O_{10}$	53.07	4.91		3.17	5.6
		(0.397)	1627, 1607, 1565	282 (9100)	$\cdot Br \cdot 2H_2O$	(53.25	4.64	—[d]	3.13)	(B)
2p	70.9	−10.7	3320, 1760, 1663,	228 (55800),	$C_{30}H_{26}Cl_2NO_{14}S$	44.79	4.01	8.81	1.74	6.5
		(0.402)	1625, 1607, 1564	282 (9200)	$\cdot Na \cdot 3H_2O$	(44.85	3.83	8.91	1.72)	(F)

a) Eluent A, CH_3CN–H_2O containing 0.1% H_3PO_4 = 25 : 75, v/v; B, CH_3CN–H_2O containing 0.1% H_3PO_4 = 30 : 70, v/v; C, CH_3CN–H_2O containing 0.1% H_3PO_4 = 35 : 65, v/v; D, CH_3CN–H_2O containing 0.1% H_3PO_4 = 40 : 60, v/v; E, CH_3CN–H_2O containing 0.1% H_3PO_4 = 45 : 55, v/v; F, CH_3CN–H_2O containing 0.1% H_3PO_4 = 50 : 50, v/v. *b*) From **3**. *c*) From **11a**. *d*) Not measured. *e*) Measured in DMF.

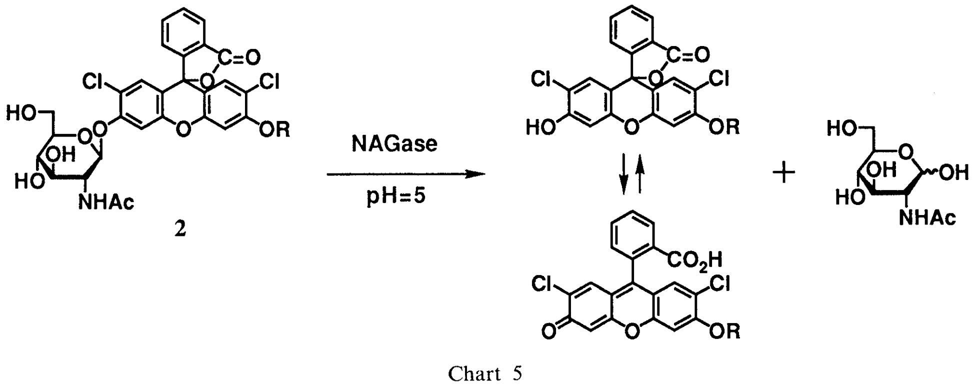

Chart 5

glucosaminides (**2a**, **i**, **j**, **p**) had good solubility (more than 10 mM).

Since the solubilities of eight glucosaminides (**2b**—**f**, **h**, **m**, **n**) were not sufficient for the enzyme reaction, only the other eight glucosaminides (**2a**, **g**, **i**—**l**, **o**, **p**) were subjected to evaluation of their kinetic parameters for NAGase. The enzyme reaction was carried out in 0.05 M citrate buffer (pH 5.0) at 37 °C. All eight substrates released 3′-*O*-substituted fluorescein chromophores ($\lambda_{max}^{pH\,5.0}$ 486—488 nm) and the absorbance at 490 nm was directly measured during the period from 2 to 5 min after the start of the enzyme reaction. The principle of the NAGase assay using these synthetic substrates (**2**) is illustrated in Chart 5. The Michaelis constants (K_m) and maximum velocities (V_{max}) for the glucosaminides (**2a**, **g**, **i**—**l**, **o**, **p**) were obtained from Lineweaver–Burk plots and are summarized in Table V. All eight glucosaminides had suitable levels of affinity and V_{max} values in the NAGase reaction. In particular, 6′-*O*-carboxymethyl- (**2a**), 6′-*O*-(*N*,*N*-dimethylaminoethyl)- (**2i**), and 6′-*O*-sulfoxyethyl-2′,7′-dichlorofluorescein *N*-acetyl-*β*-D-glucosaminides (**2p**) lacked the disadvantages of **1** while retaining the advantages.

Table IV. ^{1}H-NMR Spectral Data (199.5 MHz) for **2**

Compd. No.	δ (ppm, in DMSO-d_6)
2a	1.77, 1.79 (each 3/2H, s, NAc), 3.20—3.60 (4H, m, Glc H), 3.75—3.90 (2H, m, Glc H), 4.37 (2H, s, OCH_2CO_2), 4.74 (2H, br s, OH), 5.10, 5.12 (each 1/2H, d, J=8.6 Hz, Glc H-1), 5.45 (1H, br s, OH), 6.69, 6.70, 6.74, 6.75 (each 1/2H, s, Xa), 6.82, 7.31 (each 1H, s, Xa), 7.35—7.40 (1H, m, Ar), 7.70—7.85 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2b	1.76, 1.78 (each 3/2H, s, NAc), 1.85—2.10 (4H, m, $C\underline{H}_2C\underline{H}_2CO_2$), 3.40—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.20 (2H, t, J=6.4 Hz, $OC\underline{H}_2CH_2$), 4.86 (1H, br s, OH), 5.12, 5.14 (each 1/2H, d, J=8.3 Hz, Glc H-1), 5.87 (2H, br s, OH), 6.74, 6.76, 7.12, 7.27 (each 1H, s, Xa), 7.30—7.40 (1H, m, Ar), 7.70—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2c	1.78, 1.80 (each 3/2H, s, NAc), 3.20—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.66 (1H, br s, OH), 4.82 (1H, s, $OCH(CO_2)_2$), 5.04 (2H, br s, OH), 5.13, 5.15 (each 1/2H, d, J=8.3 Hz, Glc H-1), 6.67, 6.73, 6.94, 7.28 (each 1H, s, Xa), 7.25—7.35 (1H, m, Ar), 7.65—7.85 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2d$^{a)}$	1.89, 1.91 (each 3/2H, s, NAc), 3.30 (3H, s, OCH_3), 3.53—3.58, 3.84—3.89 (each 2H, m, $OC\underline{H}_2C\underline{H}_2O$), 3.65—4.05 (7H, m, Glc H × 6 and OH), 4.40 (1H, br s, OH), 4.64 (1H, br s, OH), 5.38, 5.43 (each 1/2H, d, J=8.6 Hz, Glc H-1), 5.48 (2H, s, OCH_2O), 6.83, 6.84, 6.85, 6.86 (each 1/2H, s, Xa), 7.24, 7.30 (each 1H, s, Xa), 7.30—7.40 (2H, m, Ar and NH), 7.70—7.90 (2H, m, Ar), 8.00—8.05 (1H, m, Ar)
2e$^{a)}$	1.88, 1.90 (each 3/2H, s, NAc), 1.95—2.15 (4H, m, $OCH_2C\underline{H}_2$), 3.50—4.05 (10H, m, Glc H × 6 and OCH_2 × 2), 4.08 (1H, br d, J=4.2 Hz, OH), 4.28 (2H, t, J=6.6 Hz, $OC\underline{H}_2CH_2$), 4.37 (1H, br d, J=3.7 Hz, OH), 4.53—4.60 (1H, m, OH), 4.83 (1H, t, J=5.1 Hz, $CH_2C\underline{H}O_2$), 5.38, 5.43 (each 1.2H, d, J=8.5 Hz, Glc H-1), 6.82—6.85 (2H, m, Xa), 7.06, 7.28 (each 1H, s, Xa), 7.30—7.40 (2H, m, Ar and NH), 7.70—7.90 (2H, m, Ar), 8.00—8.05 (1H, m, Ar)
2f	1.79, 1.81 (each 3/2H, s, NAc), 3.20—3.60 (4H, m, Glc H), 3.65—3.90 (4H, m, $OC\underline{H}_2CH_2$ and Glc H), 4.22 (2H, t, J=4.6 Hz, $OC\underline{H}_2CH_2$), 4.68, 4.87 (each 1H, br s, OH), 5.04 (2H, br s, OH), 5.14, 5.15 (each 1/2H, d, J=8.5 Hz, Glc H-1), 6.77, 6.78 (each 1H, s, Xa), 7.20—7.40 (3H, m, Xa × 2 and Ar), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.10 (1H, m, Ar)
2g	1.77, 1.79 (each 3/2H, s, NAc), 2.96 (2H, br t, J=7.4 Hz, CH_2SO_3), 3.30—3.60 (4H, m, Glc H), 3.70—3.90 (2H, m, Glc H), 4.45 (2H, br t, J=7.4 Hz, $OC\underline{H}_2CH_2$), 4.62 (1H, br t, J=5.5 Hz, OH), 4.98 (1H, br d, J=5.4 Hz, OH), 5.03 (2H, br d, J=5.1 Hz, OH), 5.12, 5.15 (each 1/2H, d, J=8.6 Hz, Glc H-1), 6.76, 6.77 (each 1H, s, Xa), 7.15, 7.16 (each 1/2H, s, Xa), 7.25—7.40 (2H, m, Xa and Ar), 7.65—7.85 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2h	1.79, 1.80 (each 3/2H, s, NAc), 2.92 (2H, t, J=5.4 Hz, $OCH_2C\underline{H}_2N$), 3.20—3.60 (4H, m, Glc H), 3.70—3.90 (2H, m, Glc H), 4.25 (2H, t, J=5.4 Hz, $OC\underline{H}_2CH_2N$), 4.60—4.75 (1H, m, OH), 4.95—5.10 (2H, m, OH), 5.14, 5.15 (each 1/2H, d, J=8.6 Hz, Glc H-1), 6.76, 6.78 (each 1H, s, Xa), 7.20—7.35 (3H, m, Xa × 2 and Ar), 7.70—7.90 (3H, m, Ar × 2 and NH), 8.00—8.10 (1H, m, Ar)
2i	1.78, 1.79 (each 3/2H, s, NAc), 2.25 (6H, s, NCH_3), 2.70 (2H, t, J=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.30—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.23 (2H, t, J=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.60—4.70 (1H, m, OH), 5.01, 5.08 (each 1H, br d, J=5.1 Hz, OH), 5.14, 5.15 (each 1/2H, d, J=8.6 Hz, Glc H-1), 6.76, 6.78 (each 1H, s, Xa), 7.20—7.35 (3H, m, Xa × 2 and Ar), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2j	1.78, 1.80 (each 3/2H, s, NAc), 3.22 (9H, s, NCH_3), 3.30—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 3.89 (2H, t, J=5.4 Hz, $OCH_2C\underline{H}_2N$), 4.68 (2H, t, J=5.4 Hz, $OC\underline{H}_2CH_2N$), 4.60—4.75 (1H, m, OH), 4.95—5.10 (2H, m, OH), 5.16, 5.17 (each 1/2H, d, J=8.5 Hz, Glc H-1), 6.80—6.85 (2H, m, Xa), 7.20—7.35 (3H, m, Xa × 2 and Ar), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2k	1.30—1.55 (6H, m, $NCH_2CH_2C\underline{H}_2$ and $NCH_2C\underline{H}_2CH_2$ × 2), 1.78, 1.80 (each 3/2H, s, NAc), 2.40—2.55 (4H, m, $NC\underline{H}_2CH_2CH_2$), 2.72 (2H, t, J=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.35—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.28 (2H, t, J=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.68 (1H, br s, OH), 5.03 (1H, br s, OH), 5.08 (1H, br s, OH), 5.13, 5.14 (each 1/2H, d, J=8.5 Hz, Glc H·1), 6.76, 6.77, 6.78, 6.79 (each 1/2H, s, Xa), 7.22, 7.25 (each 1H, s, Xa), 7.27—7.37 (1H, m, Ar), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.10 (1H, m, Ar)
2l	1.78, 1.80 (each 3/2H, s, NAc), 2.52 (4H, br t, J=4.6 Hz, $OCH_2C\underline{H}_2N$), 2.77 (2H, t, J=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.20—3.50 (4H, m, Glc H), 3.57 (4H, br t, J=4.6 Hz, $OC\underline{H}_2CH_2N$), 3.65—3.85 (2H, m, Glc H), 4.30 (2H, t, J=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.68 (1H, m, OH), 4.95—5.10 (2H, m, OH), 5.14, 5.15 (each 1/2H, d, J=8.5 Hz, Glc H-1), 6.76, 6.78 (each 1H, s, Xa), 7.20—7.35 (3H, m, Xa × 2 and Ar), 7.70—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2m	1.79, 1.81 (each 3/2H, s, NAc), 2.87 (2H, br t, J=5.6 Hz, $OCH_2C\underline{H}_2N$), 3.05—3.60 (4H, m, Glc H), 3.71 (4H, s, $NC\underline{H}_2Ph$), 3.73—3.90 (2H, m, Glc H), 4.30 (2H, br t, J=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.60—4.75 (1H, m, OH), 4.95—5.10 (2H, m, OH), 5.14, 5.15 (each 1/2H, d, J=8.6 Hz, Glc H-1), 6.75, 6.77 (each 1H, s, Xa), 7.10, 7.11 (each 1/2H, s, Xa), 7.20—7.40 (12H, m, Xa and Ar × 11), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)
2n	1.78, 1.79 (each 3/2H, s, NAc), 3.30—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.60—4.70 (1H, m, OH), 5.00—5.10 (2H, m, OH), 5.14, 5.15 (each 1/2H, d, J=8.6 Hz, Glc H-1), 5.41 (2H, s, OCH_2), 6.76, 6.79, 6.82, 6.83 (each 1/2H, s, Xa), 7.25—7.40 (4H, m, Xa × 2 and Py and Ar), 7.56 (1H, br d, J=7.6 Hz, Py), 7.65—7.95 (4H, m, Py and Ar × 2 and NH), 8.00—8.05 (1H, m, Ar), 8.61 (1H, br d, J=4.4 Hz, Py)
2o	1.78, 1.80 (each 3/2H, s, NAc), 3.12 (6H, s, NCH_3), 3.20—4.00 (8H, m, $OCH_2C\underline{H}_2N$ and Glc H), 4.60—4.70 (1H, m, OH), 4.74 (2H, s, $NC\underline{H}_2Ph$), 4.78 (2H, t, J=5.6 Hz, $OC\underline{H}_2CH_2N$), 4.95—5.10 (2H, m, OH), 5.17, 5.18 (each 1/2H, d, J=8.3 Hz, Glc H-1), 6.81, 6.84, 6.85, 6.88 (each 1/2H, s, Xa), 7.25, 7.36 (each 1H, s, Xa), 7.25—7.40(1H, m, Ar), 7.50—7.65 (5H, m, Ph), 7.70—7.90 (3H, m, Ar × 2 and NH), 8.00—8.10 (1H, m, Ar)
2p	1.78, 1.79 (each 3/2H, s, NAc), 3.35—3.60 (4H, m, Glc H), 3.70—3.85 (2H, m, Glc H), 4.09, 4.37 (each 2H, br t, J=5.1 Hz, $OC\underline{H}_2C\underline{H}_2O$), 4.60—4.70 (1H, m, OH), 5.00, 5.05 (each 1H, d, J=5.1 Hz, OH), 5.14, 5.15 (each 1/2H, d, J=8.3 Hz, Glc H-1), 6.76, 6.77 (each 1H, s, Xa), 7.21, 7.22 (each 1/2H, s, Xa), 7.25—7.40 (2H, m, Xa and Ar), 7.65—7.90 (3H, m, Ar × 2 and NH), 8.00—8.05 (1H, m, Ar)

Abbreviations: Glc, D-glucopyranosyl; Ar, aromatic proton; Xa, proton of xanthene ring; Py, proton of pyridine ring. *a*) Measured in acetone-d_6.

Since **2a** was considered to be the most favorable substrate among the present glucosaminides, the standard curve of the absorbance increase in the reaction mixture of **2a** against the concentration of NAGase under the rate-assay conditions was examined (Fig. 1). A good linearity was observed in the range from 0.5 to 10 I.U./l final NAGase concentration. When the absorbance increase with **2a** was compared with that in the case of 2-chloro-4-nitrophenyl-*N*-acetyl-β-D-glucosaminide (CNP-NAG),[9] a commonly used substrate in the rate-assay of NAGase, the sensitivity of **2a** was 2.7 times higher than that of CNP-NAG. Furthermore the dissociation of the released chromophore (**3a**) was as high as 83% at pH 5.0 [ε at 490 nm (pH 5.0) = 20100]. Therefore the synthetic substrate (**2a**) is considered to be an excellent chromogenic substrate for the rate-assay of NAGase.

Table V. Properties of *N*-Acetyl-*β*-D-glucosaminides (**2**) as NAGase Substrates

Compd. No.	Solubility (mM)	K_m (mM)	V_{max} (mM/min)
2a	45	1.46	6.4×10^{-3}
2b	0.2	NT	NT
2c	2	NT	NT
2d	0.2	NT	NT
2e	0.02	NT	NT
2f	0.5	NT	NT
2g	6	0.81	3.9×10^{-3}
2h	2	NT	NT
2i	12	2.01	5.3×10^{-3}
2j	120	2.11	3.5×10^{-3}
2k	5	1.70	3.0×10^{-3}
2l	3	1.72	3.4×10^{-3}
2m	0.05	NT	NT
2n	0.01	NT	NT
2o	3	0.55	2.5×10^{-3}
2p	30	2.04	8.2×10^{-3}

NT = not tested.

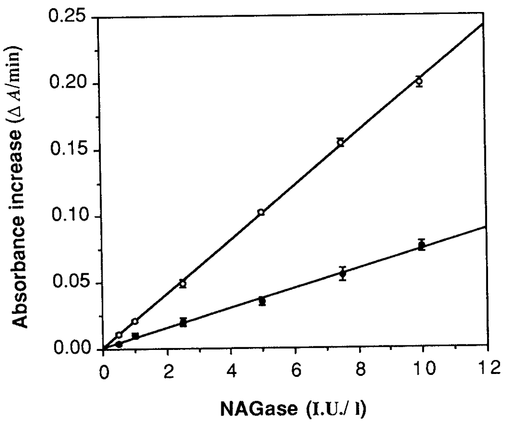

Fig. 1. Standard Curves of **2a** (4.0 mM, ○, Observed at 490 nm) and CNP-NAG (1.7 mM, ●, 400 nm) under the Rate-Assay Conditions at 37 °C against the Concentration of NAGase

2a: $y=0.2004x+0.00078$. $r=0.998$. CNP-NAG: $y=0.0074x+0.00008$. $r=0.988$. Each point and bar show the mean value and the standard deviation ($n=3$).

Based on the above results, we are investigating the application of the substrates described in this paper for 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) 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. 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. 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, 1.0 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).

2′,7′-Dichlorofluorescein Ethoxycarbonylmethyl Ester Ethoxycarbonylmethyl Ether (4) A solution of 2′,7′-dichlorofluorescein (**3**) (10.0 g, 25 mmol) in MeOH (250 ml) was mixed with 28% NaOMe in MeOH (9.75 ml, 50 mmol) and the mixture was stirred for 30 min at room temperature. After removal of the solvent *in vacuo*, the residue was dissolved in *N*,*N*-dimethylformamide (DMF) (750 ml). The solution was mixed with ethyl bromoacetate (5.6 ml, 50 mmol) and the mixture was stirred at 80 °C for 2 h. The residue obtained after evaporation of the solvent *in vacuo* was purified by column chromatography on silica gel. Elution with CH_3CN–$CHCl_3$ (1 : 3, v/v) gave 12.7 g (88.6%) of **4** as an orange powder, mp 165—166 °C. IR (KBr): 1758, 1725, 1634, 1617, 1593 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 371 (10500), 463 (28300), 493 (22600). ^{1}H-NMR ($CDCl_3$) δ: 1.17, 1.34 (each 3H, t, $J=7.1$ Hz, CH_3), 4.21, 4.32 (each 2H, q, $J=7.1$ Hz, $C\underline{H}_2CH_3$), 4.56, 4.65 (2H, ABq, $J=15.9$ Hz, $OC\underline{H}_2CO_2$), 4.82 (2H, s, $OC\underline{H}_2CO_2$), 6.56, 6.90, 6.97, 7.02 (each 1H, s, xanthene H), 7.28—7.35 (1H, m, arom. H), 7.70—7.90 (2H, m, arom. H), 8.35—8.43 (1H, m, arom. H). *Anal.* Calcd for $C_{28}H_{22}Cl_2O_9 \cdot H_2O$: C, 58.65; H, 3.87. Found: C, 58.64; H, 3.87.

2′,7′-Dichlorofluorescein Carboxymethyl Ether (5) A solution of **4** (12.0 g, 20.9 mmol) in EtOH (800 ml) and H_2O (200 ml) was mixed with 2 N NaOH (40 ml) and the mixture was stirred for 2 h at room temperature. After removal of the solvent *in vacuo*, the residue was dissolved in H_2O (500 ml). This solution was acidified with 2 N HCl (40 ml), and the resulting precipitates were collected and purified by column chromatography on silica gel. Elution with MeOH–$CHCl_3$ (1 : 4, v/v) gave 8.55 g (89.1%) of **5** as a yellow powder. IR (KBr): 3201, 1762, 1602, 1584, 1490 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 283 (10200), 460 (6200), 489 (5600). ^{1}H-NMR (DMSO-d_6) δ: 4.59 (2H, s, $OC\underline{H}_2CO_2$), 6.61, 6.70 (each 1H, s, xanthene H), 6.89 (2H, br s, xanthene H), 7.25—7.35 (1H, m, arom. H), 7.70—7.85 (2H, m, arom. H), 8.00—8.05 (1H, m, arom. H). *Anal.* Calcd for $C_{22}H_{12}Cl_2O_7 \cdot 2H_2O$: C, 53.35; H, 3.26. Found: C, 53.22; H, 3.05.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-carboxymethyloxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (7) Compound **5** (5.0 g, 10.9 mmol) and Ag_2O (25.3 g, 109 mmol) were added to a solution of 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-*α*-D-glucosamine (**6**)[12] (40.0 g, 109 mmol) in CH_3CN (2.5 l) and the mixture was stirred at 50 °C for 20 h. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 4, v/v) gave 3.93 g (45.8%) of **5** as a colorless powder, $[\alpha]_D^{25}$ −20.9° ($c=0.378$, MeOH). IR (KBr): 3305, 1751, 1690, 1628, 1607, 1565, 1486, 1413, 1373 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 228 (55500), 282 (9700). ^{1}H-NMR (DMSO-d_6) δ: 1.74, 1.76 (each 3/2H, s, NAc), 1.95, 2.01, 2.08 (each 3H, s, OAc), 4.10—4.30 (4H, m, Glc H), 4.47 (2H, br s, $OC\underline{H}_2CO_2$), 4.97 (1H, t, $J=9.5$ Hz, Glc H-4), 5.21, 5.22 (each 1/2H, t, $J=9.5$ Hz, Glc H-3), 5.46 (1H, d, $J=8.5$ Hz, Glc H-1), 6.72, 6.74, 6.80, 6.81 (each 1/2H, s, xanthene H), 6.83 (1H, s, xanthene H), 7.28—7.40 (2H, m, xanthene H and arom. H), 7.70—7.90 (2H, m, arom. H), 7.98—8.05 (2H, m, arom. H and NH). *Anal.* Calcd for $C_{36}H_{31}Cl_2NO_{15} \cdot 2H_2O$: C, 52.44; H, 4.28; N, 1.70. Found: C, 52.32; H, 4.16; N, 1.72.

2′,7′-Dichlorofluorescein Phenylmethyloxymethyl Ester (8) A solution of **3** (40.0 g, 100 mmol) in MeOH (1 l) was mixed with 28% NaOMe methanol solution (38.5 ml, 200 mmol) with stirring and the mixture was stirred for 30 min at room temperature. After removal of the solvent *in vacuo*, the residue was dissolved in DMF (3 l). The solution was mixed with benzyl chloromethyl ether (15.3 ml, 110 mmol) and the mixture was stirred at room temperature for 3 h. The residue obtained after evaporation of the solvent *in vacuo* was dissolved in MeOH (500 ml) and $CHCl_3$ (500 ml). The solution was mixed with Amberlite IRC-50 (40 g) and the mixture was stirred at room temperature for 0.5 h. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 3, v/v) gave 42.8 g (82.1%) of **8** as a reddish-colored powder, mp 118—127 °C. IR (KBr): 3066, 1726, 1635, 1589, 1501, 1451 cm^{-1}. UV $\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε): 502 (47500). ^{1}H-NMR (DMSO-d_6) δ: 4.35 (2H, s, $OC\underline{H}_2Ph$), 5.29 (2H, s, $OC\underline{H}_2O$), 6.31, 6.74 (each 2H, s, xanthene H), 7.10—7.17 (2H, m, Ph), 7.25—7.35 (3H, m, Ph), 7.43—7.50 (1H, m, arom. H), 7.70—7.90 (2H, m, arom. H), 8.10—8.17 (1H, m, arom. H). *Anal.* Calcd for $C_{28}H_{18}Cl_2O_6$: C, 64.51; H, 3.48. Found: C, 64.46; H, 3.52.

6-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-9-[2-[[(phenylmethyloxymethyl)oxy]carbonyl]phenyl]-2,7-dichloro-3*H*-xanthen-3-one (9) Compound **8** (40.0 g, 76.7 mmol) and Ag_2O (35.6 g, 154 mmol) were added to a solution of **6** (56.2 g, 154 mmol) in CH_3CN (2000 ml) and the mixture was stirred at 50 °C for 20 h. 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 : 20, v/v) gave 42.3 g (64.8%) of **9** as an orange powder, $[\alpha]_D^{25}$ $-17.8°$ ($c=0.399$, MeOH). IR (KBr): 3282, 1749, 1678, 1593, 1524 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 367 (10900), 438 (18900), 462 (25100), 491 (18400). ^{1}H-NMR ($CDCl_3$) δ: 1.90, 1.92 (each 3/2H, s, NAc), 2.07 (6H, s, OAc), 2.12, 2.15 (each 3/2H, s, OAc), 4.00—4.15 (1H, m, Glc H), 4.20—4.35 (3H, m, Glc H), 4.53, 4.54 (each 2H, s, $OC\underline{H}_2Ph$), 5.15 (1H, t, $J=9.5$ Hz, Glc H-4), 5.30—5.60 (2H, m, $OC\underline{H}_2O$ and Glc H-3), 5.51, 5.69 (each 1/2H, d, $J=8.1$ Hz, Glc H-1), 6.18, 6.39 (each 1/2H, d, $J=8.8$ Hz, NH), 6.60, 6.61 (each 1/2H, s, xanthene H), 6.94, 7.04 (each 1H, s, xanthene H), 7.20—7.35 (6H, m, Ph and arom. H), 7.41, 7.43 (each 1/2H, s, xanthene H), 7.70—7.90 (2H, m, arom. H), 8.10—8.15 (1H, m, arom. H). *Anal.* Calcd for $C_{42}H_{37}Cl_2NO_{14} \cdot 1/2H_2O$: C, 58.68; H, 4.46; N, 1.63. Found: C, 58.45; H, 4.31; N, 1.59.

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 (10)[1] Amberlyst 15E (80 g) was added to a solution of **9** (40.0 g, 47.0 mmol) in $CHCl_3$ (800 ml) and MeOH (800 ml) and the whole was stirred at room temperature for 5 h. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 20, v/v) gave 31.2 g (90.9%) of **10**.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-ethoxycarbonylmethyloxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (11a) Compound **10** (2.0 g, 2.74 mmol) and Ag_2O (1.27 g, 5.48 mmol) were added to a solution of ethyl bromoacetate (0.6 ml, 5.4 mmol) in CH_3CN (200 ml) and the mixture was stirred at 50 °C for 2 h. 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 : 20, v/v) gave 1.66 g (74.4%) of **11a**. Physical properties and spectral data are summarized in Tables I and II.

Compounds **11b—e** were prepared in a similar manner.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-(2-hydroxyethyl)oxy-2′,7′-dichlorospiro[isobenzofuran-(3*H*),9′-[9*H*]xanthen]-3-one (11f) Compound **10** (2.0 g, 2.74 mmol) and Ag_2O (6.35 g, 27.4 mmol) were added to a solution of 2-bromoethanol (1.94 ml, 27.4 mmol) in DMF (200 ml) and the mixture was stirred at 60 °C for 20 h. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 20, v/v) gave 1.25 g (58.9%) of **11f**. Physical properties and spectral data are summarized in Tables I and II.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-[2-(*N*,*N*-dimethylamino)ethyl]oxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (11i) Compound **10** (2.0 g, 2.74 mmol) and K_2CO_3 (7.58 g, 54.8 mmol) were added to a solution of 2-chloroethyl-*N*,*N*-dimethylammonium chloride (3.95 g, 27.4 mmol) in DMF (200 ml) and the mixture was stirred at 60 °C for 20 h. After removal of the insoluble material by filtration, the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 25, v/v) gave 1.09 g (49.7%) of **11i**. Physical properties and spectral data are summarized in Tables I and II.

Compounds **11g**, **h**, **j—n** were prepared in a similar manner.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-[2-[*N*,*N*,*N*-dimethyl(phenylmethyl)ammonio]ethyl]oxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one Bromide (11o) Benzyl bromide (0.18 ml, 1.5 mmol) was added to a solution of **111** (1.0 g, 1.25 mmol) in $CHCl_3$ (20 ml) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 7, v/v) gave 800 mg (65.8%) of **11o**. Physical properties and spectral data are summarized in Tables I and II.

3′-[(3,4,6-Tri-*O*-acetyl-2-(acetylamino)-2-deoxy-*β*-D-glucopyranosyl)-oxy]-6′-(2-sulfoxyethyl)oxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (11p) Chlorosulfonic acid (0.17 ml, 2.6 mmol) and triethylamine (0.36 ml, 2.6 mmol) were added to a solution of **11f** (1.0 g, 1.29 mmol) in CH_2Cl_2 (20 ml) and the mixture was stirred under ice-cooling for 2 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with MeOH–$CHCl_3$ (1 : 10, v/v) gave 824 mg (74.7%) of **11a**. Physical properties and spectral data are summarized in Tables I and II.

Sodium Salt of 3′-[[2-(Acetylamino)-2-deoxy-*β*-D-glucopyranosyl]-oxy]-6′-carboxymethoxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one (2a) i) A solution of **7** (2.4 g, 3.05 mmol) in MeOH (96 ml) and $CHCl_3$ (48 ml) was mixed with 28% NaOMe in MeOH (1.2 ml, 6.2 mmol) and the mixture was stirred for 30 min at room temperature. The solvent was evaporated *in vacuo* and the residue was chromatographed on octadecyl silica (ODS) gel. Elution with EtOH–H_2O (1 : 4, v/v) gave 1.40 g (67.2%) of **2a** as a colorless powder. Physical properties and spectral data are summarized in Tables III and IV.

Compounds **2g**, **p** were prepared in a similar manner.

ii) Na_2CO_3 (647 mg, 6.1 mmol) was added to a solution of **11a** (1.0 g, 1.22 mmol) in MeOH–CH_3CN–H_2O (2 : 2 : 1, v/v/v, 30 ml) and the mixture was stirred under ice-cooling for 1 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on ODS gel. Elution with CH_3CN–H_2O (1 : 4, v/v) gave 734 mg (87.9%) of **2a**.

Compounds **2b**, **c** were prepared in a similar manner.

3′-[[2-(Acetylamino)-2-deoxy-*β*-D-glucopyranosyl]oxy]-6′-[2-(*N*,*N*-dimethylamino)ethyl]oxy-2′,7′-dichlorospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (2i) A solution of **11i** (500 mg, 0.62 mmol) in MeOH (20 ml) and CH_3CN (10 ml) was mixed with 28% NaOMe in MeOH (0.06 ml, 0.31 mmol) and the mixture was stirred under ice-cooling for 2 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on ODS gel. Elution with CH_3CN–H_2O (1 : 4, v/v) gave 270 mg (64.1%) of **2i** as a colorless powder. Physical properties and spectral data are summarized in Tables III and IV.

Compounds **2d—f**, **h**, **j—o** were prepared in a similar manner.

Solubilities of 2 A suspension of **2** in the 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 solution, Toyo Roshi Kaisha Ltd., Tokyo). The filtrate was appropriately diluted with water and 0.02 ml of the solution was subjected to HPLC analysis using CH_3CN–H_2O containing 0.1% H_3PO_4 as an eluent. Concentration of **2** was determined from the peak area ratio to the standard.

Michaelis Constants and Maximum Velocities A solution of NAGase (274.9 I.U./l, 0.05 ml) was added to a solution of **2** (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 2 min, the increase in absorbance at 490 nm 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 a solution of **2a** (4.00 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 2 min, the increase in absorbance at 490 nm (for **2a**) 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.

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