A New Chemiluminogenic Substrate for *N*-Acetyl-β-D-glucosaminidase, 4′-(6′-Diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide

Kazumi SASAMOTO*,[a] and Yosuke OHKURA[b]

Dojindo Laboratories,[a] Tabaru 2025-5, Mashiki-machi, Kamimashiki-gun, Kumamoto 861-22, Japan and Faculty of Pharmaceutical Sciences, Kyushu University 62,[b] Maidashi, Higashi-ku, Fukuoka 812, Japan. *Received July 13, 1990*

A new type of chemiluminogenic substrate for *N*-acetyl-β-D-glucosaminidase was synthesized. The substrate was obtained by incorporation of an enzyme-removable *N*-acetyl-D-glucosaminide group to the cyclic hydrazide moiety of an arylic hydrazide which contains a highly fluorescent benzofuran framework. Following enzyme-mediated hydrolysis, the substrate released the hydrazide that was subsequently subjected to chemiluminescent oxidation with hydrogen peroxide and a hemin catalyst in an alkaline buffer to generate light. The detection limit of the enzyme using this substrate was 0.6 I.U./l.

Keywords chemiluminogenic substrate; *N*-acetyl-β-D-glucosaminidase; 4′-(6′-diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide; chemiluminescence detection; enzyme assay

Since the enzymatic triggering of chemiluminescence was first realized by Schaap and co-workers[1] using an adamantyl dioxetane as a substrate, it has been gaining much attention for its extreme value in enzyme immunoassays.[2] Alkaline phosphatase or β-galactosidase are being utilized as enzyme labels which trigger the chemiluminescent decomposition of dioxetane in aqueous buffers without the addition of other co-reagents. In a previous paper,[3] we demonstrated through the synthesis of *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide that the enzyme-triggered chemiluminescence could also be achieved by substitution on the heterocyclic ring of luminol with an enzyme-removable group. Thus, the incorporation of an *N*-acetyl-D-glucosaminide group into the cyclic hydrazide moiety of luminol renders the compound nonluminescent; whereas enzymatic hydrolysis with *N*-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30) releases luminol, which is then detected chemiluminescently using hydrogen peroxide and a catalyst. The fact that the detection sensitivity of the enzyme by this method was insufficient under a detection limit of 5 I.U./l, which is approximately 10 times less sensitive compared to conventional spectrophotometric methods, led us to attempt to improve the chemiluminescent efficiency of the cyclic hydrazide. Although the observed efficiency of chemiluminescence is a result of: (1) the selectivity of the chemical reaction, (2) the efficiency of cross-over to the excited state of the light emitter (presumably the corresponding phthalate ion in the luminol system), and (3) the efficiency of fluorescence of the phthalate, the third factor is most important in various organic hydrazides.[4]

In order to exploit a substrate for sensitive detection of NAGase, we synthesized a new chemiluminogenic substrate, 4′-(6′-diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide (**1**, Chart 1) that covalently incorporates an arycyclic hydrazide having a highly fluorescent benzofuran skeleton, and herein report the synthesis and its chemiluminescent response to the enzyme.

Experimental

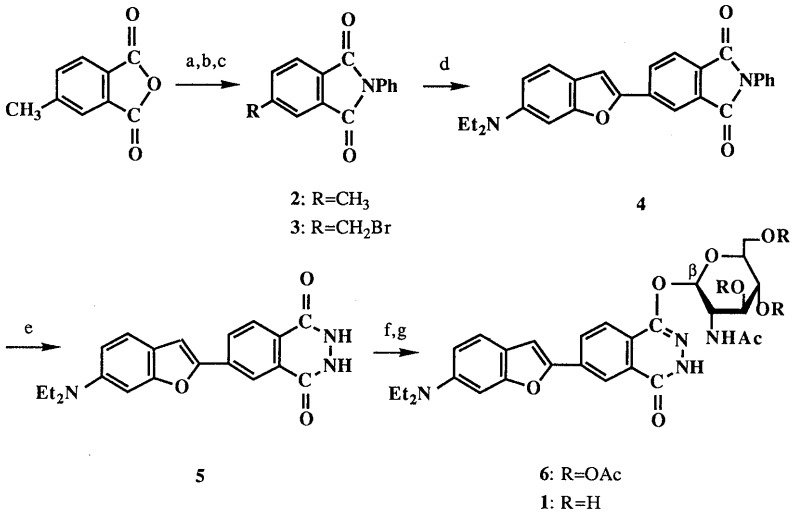
Apparatus Proton nuclear magnetic resonance (^{1}H-NMR) spectra and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were taken with a Bruker AC-200 spectrometer at 200 and 50 MHz, respectively, using tetramethylsilane as an internal standard. The splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet; br, broad. Infrared (IR) spectra were measured in Nujol mull with a Hitachi 260-10 spectrometer. Chemiluminescence measurements were performed on a photon-counting computer-controlled Berthold Lumat LB-9501 luminometer (Wildbad, FRG); chemiluminescence reactions were carried out in 75 × 12 mm clear-polystyrene round-bottom tubes supplied by Sarstedt (Numbrecht, FRG). A Shimadzu LC-6A high-performance liquid chromatograph equipped with a Shimadzu SPD-6AV ultraviolet spectrometer operated at 254 nm was used. The column was a Nucleosil C18 (250 × 4.0 mm i.d., particle size 10 μm; Gasukuro Kogyo Inc., Tokyo, Japan). The column temperature was 40 °C and the flow rate of the mobile phase (aqueous 60% acetonitrile) was 1.0 ml/min. Uncorrected melting points were obtained on a Yamato MP-21 melting point apparatus. Fluorescence spectra were taken on a Hitachi 650-60 spectrometer in 10 × 10 mm quartz cells.

Materials NAGase from jack beans was purchased from Sigma (St. Louis, MO, U.S.A.) as a suspension of 0.8 mg of protein per ml in 2.5 M $(NH_4)_2SO_4$ solution (50 I.U./ml) and diluted with water prior to use. Other chemicals were of reagent grade. Stock solutions of substrate **1** and 4-(6-diethylaminobenzofuranyl)phthalhydrazide (**5**) were prepared in dimethylsulfoxide at 20 mM. Column chromatography was performed with silica gel (Kieselgel 60, E. Merck, Darmstadt, FRG).

Synthesis of Substrate 1 (Chart 1) 4-Methyl-*N*-phenylphthalimide (2) To a stirred solution of 4-methylphthalic anhydride (39.64 g, 0.244 mol) in 500 ml of ether was added aniline (22.3 ml, 0.245 mol), and the stirring was continued for 1 h at room temperature. A formed precipitate was collected and then heated with sodium acetate (8.35 g, 0.102 mol) in 90 ml of acetic anhydride at 90 °C for 30 min, during which time an initial suspension became clear and subsequently solidified. The solid was collected and recrystallized from benzene–hexane to give 40.0 g (69%) of compound **2** as a colorless crystalline solid, mp 163—164 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.54 (s, 3H, CH_3), 7.39—7.51 (m, 5H, Ph), 7.57 (dd, J=8.2, 1.1 Hz, 1H, ArH), 7.75 (s, 1H, ArH), 7.83 (d, J=7.6 Hz, 1H, ArH). IR ν_{max} cm^{-1}: 1788 (phthalimide), 1713 (phthalimide), 1622 (aromatic).

4-Bromomethyl-*N*-phenylphthalimide (3) A solution containing compound **2** (10.0 g, 42.15 mmol), *N*-bromosuccinimide (8.50 g, 47.76 mmol), and benzoyl peroxide (250 mg, 1.03 mmol) in 200 ml of carbon tetrachloride was refluxed for 16 h. A formed precipitate was filtered off and the filtrate was concentrated *in vacuo* to give a slight yellow solid, which was purified by column chromatography (benzene). After recrystallization from benzene–petrolium ether, bromide **3** was obtained as an inseparable mixture with compound **2**; the purity was determined to be 89 mol% by ^{1}H-NMR analysis, yield 3.88 g (29%), mp 150—154 °C. ^{1}H-NMR ($CDCl_3$) δ: 4.59 (s, 2H, CH_2Br), 7.36–7.56 (m, 5H, Ph), 7.80 (dd, J=7.7, 1.3 Hz, 1H, ArH), 7.92 (s, 1H, ArH), 7.97 (d, J=5.1 Hz, 1H, ArH). IR ν_{max} cm^{-1}: 1791 (phthalimide), 1723 (phthalimide), 1628 (aromatic), 1609 (aromatic).

4-(6-Diethylaminobenzofuranyl)-*N*-phenylphthalimide (4) A mixture containing 4-diethylaminosalicylaldehyde (3.36 g, 17.39 mmol), bromide **3** (6.0 g, 17.37 mmol based on 89 mol% NMR purity), and K_2CO_3 (11.0 g, 79.59 mmol) in 120 ml of *N*,*N*-dimethylformamide was heated at 130 °C (bath temperature) for 1 h. The resulting brown-colored solution was diluted with ether and washed with aqueous $NaHCO_3$. After drying ($MgSO_4$) followed by concentration *in vacuo*, the crude product was obtained as a yellowish solid which was purified by column chro-



reagents: (a) aniline, Et_2O; (b) Ac_2O, AcONa; (c) NBS, CCl_4; (d) 4-diethylaminosalicylaldehyde, K_2CO_3, DMF; (e) hydrazine, EtOH: (f) 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine, NaH, DMF; (g) $NaOCH_3$, MeOH

Chart 1. Synthetic Route to 4′-(6′-Diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide (**1**)

matography (benzene) followed by recrystallization from benzene–petrolium ether, affording 1.06 g (15%) of compound **4** as red needles, mp 180–181 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (t, J=7.0 Hz, 6H, CH_3), 3.44 (q, J=7.0 Hz, 4H, CH_2), 6.72 (dd, J=8.8, 2.3 Hz, 1H, ArH), 6.78 (br s, 1H, ArH), 7.15 (s, 1H, ArH), 7.26 (s, 1H, furanyl H), 7.41—7.53 (m, 5H, Ph), 7.94(d, J=8.1 Hz, 1H, ArH), 8.13 (dd, J=7.8, 1.5 Hz, 1H, ArH), 8.30 (d, J=7.7 Hz, 1H, ArH). IR ν_{max} cm^{-1}: 1762 (phthalimide), 1710 (phthalimide), 1635 (aromatic), 1619 (aromatic).

4-(6-Diethylaminobenzofuranyl)phthalylhydrazide (5) A suspension of compound **4** (1.0 g, 2.44 mmol) and hydrazine hydrate (5.0 ml, 0.103 mol) in 25 ml of ethanol was refluxed for 30 min, during which time a light-yellow crystalline solid precipitated. The precipitate was collected and washed thoroughly with ethanol. Drying over P_2O_5 *in vacuo* overnight gave hydrazide **5** as a lemon-yellow crystalline solid, yield 0.70 g (82%), mp >269 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.14 (t, J=6.8 Hz, 6H, CH_3), 3.42 (q, J=6.9 Hz, 4H, CH_2), 3.98 (br s, 2H, hydrazido H), 6.74 (dd, J=8.8, 1.5 Hz, 1H, ArH), 6.90 (s, 1H, ArH), 7.45 (d, J=8.6 Hz, 1H, ArH), 7.51 (s, 1H, ArH), 8.08 (d, J=8.4 Hz, 1H, ArH), 8.24 (d, J=8.3 Hz, 1H, ArH), 8.39 (s, 1H, ArH). ^{13}C-NMR (DMSO-d_6) δ: 12.31 (CH_3), 44.10 (CH_2), 93.04 (CH), 104.84 (CH), 109.80 (CH), 117.30, 119.00 (CH), 121.73 (CH), 125.00 (CH), 126.13, 127.07 (CH), 128.58, 133.51, 146.83, 150.62, 155.16, 155.39, 157.22. IR ν_{max} cm^{-1}: 1660, 1641, 1617 (hydrazide).

4′-(6′-Diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide Triacetate (6) To a stirred solution of hydrazide **5** (261 mg, 0.747 mmol) in 10 ml of *N*,*N*-dimethylformamide was added sodium hydride (60% oil dispersed, 30 mg, 0.75 mmol) at room temperature. After 10 min, solid 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine[6] (273 mg, 0.746 mmol) was added and the stirring was continued for 12 h at room temperature. The reaction was worked up by partitioning the reaction mixture between ethyl acetate and water, and the organic layer was washed (aqueous $NaHCO_3$), dried ($MgSO_4$), and concentrated *in vacuo* to leave a brown syrup. Column chromatography (5% methanol in chloroform) afforded compound **6** as a major product along with a more polar by-product (bis(2-acetamide-3,4,6-triacetyl-D-glucopyranosyl) substituted product) which appeared similar to compound **6** in thin layer chromatography (*Rf* values for compound **6** and this by-product are 0.30 and 0.26, respectively; 10% methanol in chloroform, Merck Silica gel 60) and also gave a similar NMR spectrum, yield 60 mg (12%), mp 183—185 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.24 (t, J=6.8 Hz, 6H, CH_3), 2.03 (s, 9H, OAc), 2.12 (s, 3H, NHAc), 3.44 (q, J=7.0 Hz, 4H, CH_2), 3.98 (br s, 1H, hydrazido H), 4.25 (m, 2H, pyranosyl H), 4.73 (m, 1H, pyranosyl H), 5.25 (t, J=7.0 Hz, 2H, pyranosyl H), 5.85 (d, J=8.6 Hz, 1H, anomeric H), 6.53 (d, J=10.0 Hz, 1H, pyranosyl H), 6.74 (d, J=10.4 Hz, 1H, ArH), 7.02 (s, 1H, furanyl H), 7.28 (d, J=10.8 Hz, 1H, NHAc), 7.34 (d, J=8.6 Hz, 1H, ArH), 7.93 (m, 2H, ArH), 8.53 (s, 1H, ArH). IR ν_{max} cm^{-1}: 3300, 1750 (OAc), 1675 (hydrazide).

4′-(6′-Diethylaminobenzofuranyl)phthalylhydrazido-*N*-acetyl-β-D-glucosaminide (1) To a stirred solution of compound **6** (60 mg, 0.088 mmol) in 8 ml of methanol was added 1 M sodium methoxide in methanol (50 μl) and the solution was stored in refrigeration overnight. Deprotected product **1** precipitated out of the solution, which was collected, washed with cold methanol, and dried *in vacuo* over P_2O_5 overnight, yield 27 mg (55%), mp >200 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.14 (t, J=6.9 Hz, 6H, CH_3), 1.74 (s, 3H, NHAc), 3.34 (m, 8H, OH and pyranosyl H), 3.42 (q, J=7.0 Hz, 4H, CH_2), 5.16 (t, J=5.3 Hz, 1H, pyranosyl H), 5.56 (d, J=8.7 Hz, 1H, anomeric H), 6.75 (dd, J=8.7, 1.5 Hz, 1H, ArH), 6.89 (s, 1H, ArH), 7.46 (d, J=8.7 Hz, 1H, ArH), 7.59 (s, 1H, furanyl H), 7.85 (d, J=7.3 Hz, 1H, NHAc), 7.89 (d, J=8.5 Hz, 1H, ArH), 8.36 (dd, J=8.4, 1.8 Hz, 1H, ArH), 8.50 (d, J=1.7 Hz, 1H, ArH). ^{13}C-NMR (DMSO-d_6) δ: 12.32 (CH_3), 44.10 (CH_2), 54.59 (CH_2), 61.00 (CH), 69.86 (CH), 74.00 (CH), 77.56 (CH), 92.93 (CH), 96.06 (CH), 105.08 (CH), 110.00 (CH), 117.21, 119.57 (CH), 121.87 (CH), 122.37 (CH), 124.13, 128.52 (CH), 129.45, 133.78, 146.99, 148.35, 150.16, 157.16, 157.34 (CO), 158.60 (CO), 169.41 (CONH). IR ν_{max} cm^{-1}: 3300 (OH), 1650 (hydrazide). *Anal.* Calcd for $C_{28}H_{32}N_4O_8$: C, 60.86; H, 5.84; N, 10.14. Found: C, 59.60; H, 5.87; N, 10.94.

Enzymatic Reaction A 10 μl of NAGase of a given concentration was added to a pre-incubated mixture (37 °C) of 0.5 ml of 0.1 M phosphate buffer (pH 6.5) and 0.5 ml of aqueous 1% (w/v) cetyltrimethylammonium bromide (CTMAB) containing the substrate (compound **1**, final concentration, 0.1 mM), and the mixture was incubated at 37 °C for 10 min in a tube. The reaction was then terminated by subsequent addition of 0.5 ml of 1 M KOH and 10 μl of 0.1 μM hemin (H_2O). The tube containing this mixture was placed in a luminometer and the chemiluminescent reaction was initiated by adding 10 μl of 0.05% H_2O_2. The resulting light emission was measured in the luminometer as a 10-min integral.

Results and Discussion

Synthesis The synthetic approach to substrate **1** involves the preparation of the arylic hydrazide **5** and its modification in the hydrazide portion with the enzyme-removable *N*-acetyl-D-glucosaminide group, as outlined in Chart 1. Since the red shifts at the emission maxima of the dialkylamino-substituted compounds relative to the corresponding unsubstituted ones were reported in 6-substituted benzofurans,[5b] we first aimed at the synthesis of the arylic hydrazide having a diethylamino group at 6-position. As a precursor to the corresponding hydrazide, an *N*-phenylphthalimide group was employed in the preparation of hydrazide **5** since chromatographic purification is not often applicable to hydrazide compounds because of their slight solubilities in most organic solvents; in addition, *N*-phenylphthalimides can be easily purified by recrystallization. Starting with 4-methylphthalic anhydride, *N*-phenylphthalimide (**2**) was readily obtained in good yield,

and was then subjected to benzylic bromination with *N*-bromosuccinimide to give bromide **3**. Construction of a benzofuran framework was effected by reacting bromide **3** with an equivalent of 4-diethylaminosalicylaldehyde in the presence of a base,[5] affording compound **4** in moderate yield. The conversion of compound **4** to hydrazide **5** proceeded smoothly and in quantitative yield with hydrazine in refluxing ethanol. Incorporation of an *N*-acetyl-D-glucosaminide group to the heterocyclic moiety of hydrazide **5** was achieved as described in the previous paper.[3] The reaction of hydrazide **5** with 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine,[6] readily obtained from commercially available *N*-acetyl-D-glucosamine, in the presence of sodium hydride in *N*,*N*-dimethylformamide yielded compound **6** as a major product. The structure of the coupling product (**6**) was corroborated by the ^{1}H-NMR spectrum data which appeared to be an overlap of the two independent spectra of glucosamine acetate and the benzofuran unit. The anomeric proton resonates at δ 5.85 as a doublet with a coupling constant of 8.6 Hz which is consistent for the vicinal diaxial coupling, thereby suggesting that the coupled product obtained was an α-anomer. The poor coupling yield (12%) is probably due to the heterogenity of the reaction. The coupling reaction also produced a minor by-product, which was not fully characterized because of its poor response to the enzyme. Subsequent removal of the acetyl groups in the sugar moiety performed in a usual manner with sodium methoxide in methanol gave substrate **1** as a highly fluorescent product. At this stage, it was difficult to fully assign the ring protons of the glucosamine unit in ^{1}H-NMR because of overlapping and broadening signals, except for the anomeric proton which appeared as a doublet (J=8.7 Hz) at δ 5.56.

The fluorescence spectra of compounds **1** and **5** are shown in Fig. 1, which indicates that the emission maximum of substrate **1** (λ_{em}=556 nm, excited at 420 nm) is red-shifted from that of hydrazide **5** (λ_{em}=540 nm, excited at 415 nm) and that, although we were unable to obtain it, hydrazide **5** is expected to exhibit similar chemiluminesence spectra upon an oxidative reaction with hydrogen peroxide and a catalyst. The reduction of the fluorescence intensity of substrate **1** is obviously due to the flexible sugar group.

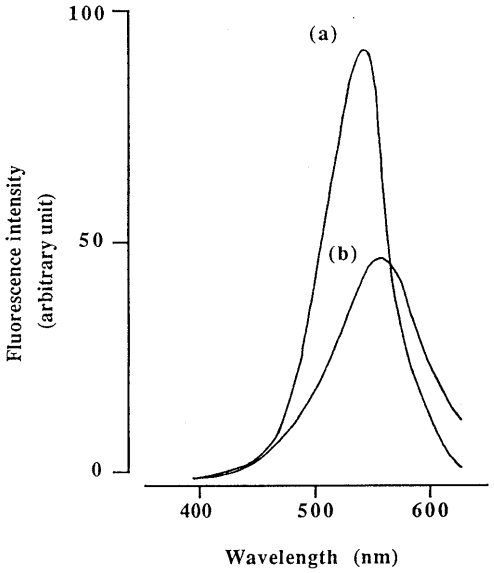


Fig. 1. Fluorescence Spectra of (a) Hydrazide **5** and (b) Substrate **1**
Both were measured in a DMSO solution.

^{1}H-NMR spectra of substrate **1** (Fig. 2a) shows two ABX patterns similar to those of hydrazide **5** (Fig. 2b), except for the H-2 proton that is shifted upfield. Since this shielding of the H-2 proton seems to favor an *O*-glycosylation at the carbonyl oxygen closer to the H-2 proton, the structure of substrate **1** was tentatively assigned as indicated in Chart 1. A nuclear Overhauser effect experiment failed to give any further information supporting the structure.

The acid susceptibility of substrate **1** due to its glycosidic bond was confirmed by high-performance liquid chromatography (HPLC) analysis based on monitoring the release of hydrazide **5** from substrate **1** following an acid treatment. When substrate **1** (5 mM) was incubated in 1 M hydrochloric acid at 37 °C, the complete disappearance of substrate **1** (retention time, 4.0 min) within 30 min resulting in hydrazide **5** (retention time, 6.7 min) was observed by HPLC.

Chemiluminescence Chart 2 illustrates the principle of the chemiluminescent detection of NAGase using compound **1** as the substrate. Substrate **1** can be regarded as a masked chemiluminescer in the sense that it is non-luminescent until the enzyme removes the sugar moiety. The released product (hydrazide **5**) is then subjected to chemiluminescent oxidation in an alkaline buffer with hydrogen peroxide and a catalyst to generate light and phthalate **7**. In this way the enzyme triggers the chemiluminescence of substrate **1** and a linear relationship is expected between the quantity of the enzyme and light production.

In order to attain this, we first investigated chemiluminescent reaction conditions for hydrazide **5**, the results of which are shown in Table I. Chemiluminescence of hydrazide **5** in a partly aqueous dimethylsulfoxide (DMSO) solution was found to be highly dependent on the ratio of DMSO to water; the presence of DMSO greatly enhanced the chemiluminescence. Since a similar enhancement effect by DMSO can be observed in the fluorescence of hydrazide **5**, the great loss of the chemiluminescence with an increasing water ratio can best be rationalized by an assumed

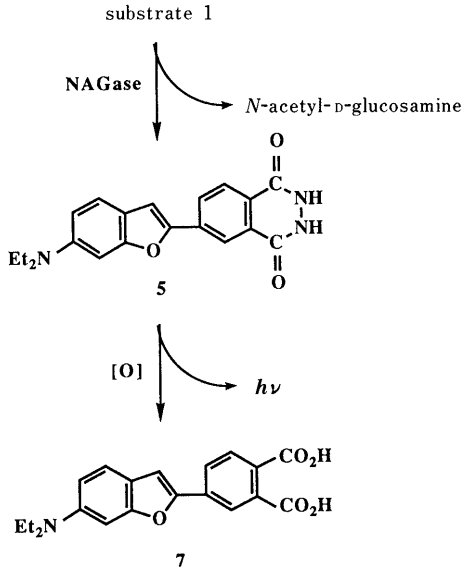


Chart 2. Principle of NAGase Detection Using Substrate **1**

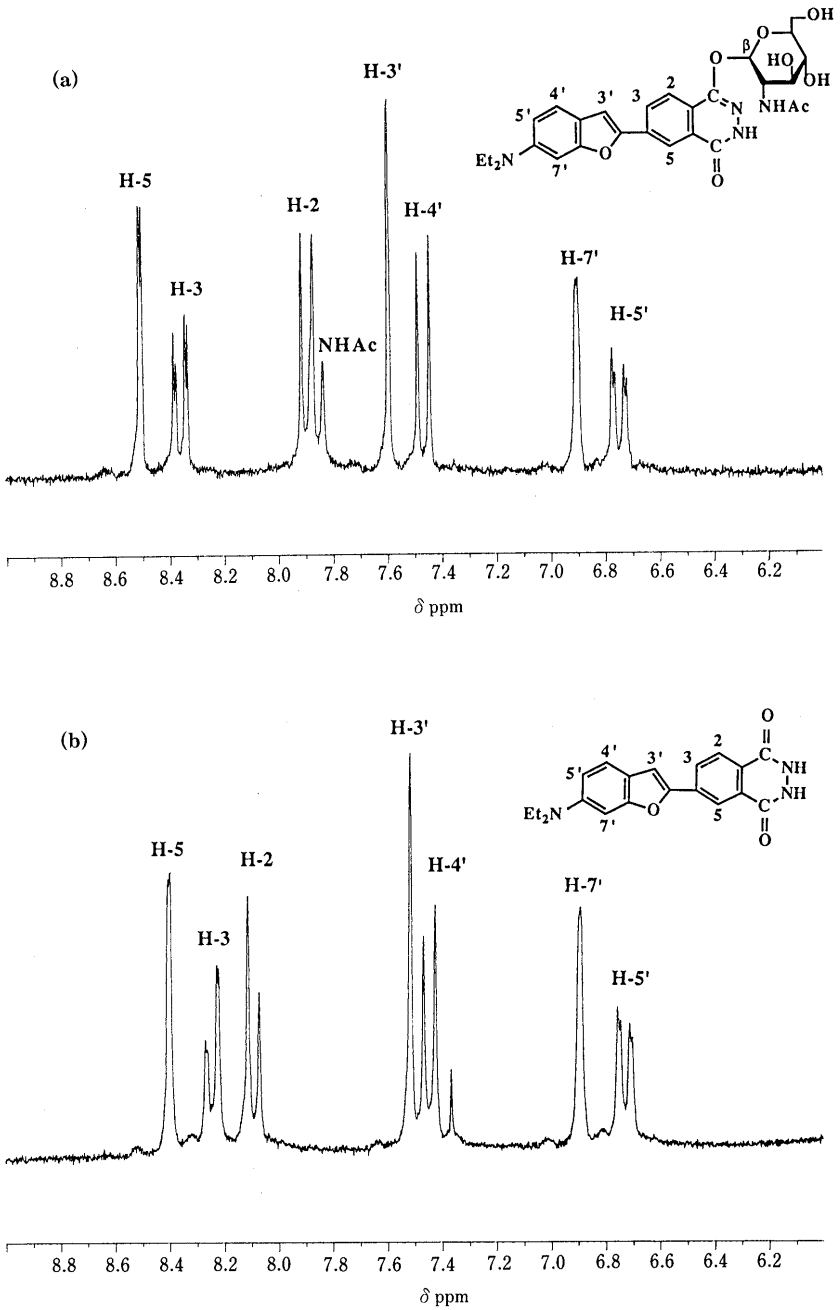


Fig. 2. ^{1}H-NMR Spectra of (a) Substrate **1** and (b) Hydrazide **5**

TABLE I. Chemiluminescence Intensities of Hydrazide **5** under Various Conditions

Reaction medium[a]		Chemiluminescence intensity[b]
DMSO + buffer	(3 : 1, v/v)	381
DMSO + buffer	(1 : 1, v/v)	80
DMSO + buffer	(1 : 3, v/v)	5.6
Buffer		1
Buffer + SDS	(1 : 1, v/v)	2.3
Buffer + Tween	(1 : 1, v/v)	0.4
Buffer + CTMAB	(1 : 1, v/v)	195

a) Buffer = 0.1 M boric acid–0.2 M KOH (1 : 1 (v/v), pH 12.6); SDS = aqueous 1% (w/v) sodium dodecyl sulfate; Tween = aqueous 1% (w/v) Tween 20; CTMAB = aqueous 1% (w/v) cetyltrimethylammonium bromide. *b*) Chemiluminescence reactions were initiated by adding 10 μl of hydrogen peroxide (0.05%) to a mixture containing 10 μl of hydrazide **5** (20 mM, DMSO) and 10 μl of hemin (0.1 μM) in 2.0 ml of the reaction media; the chemiluminescence intensities (arbitrary unit) were measured as a 10-min integral.

quenching of the fluorescence of the emitting species (presumably the corresponding phthalate (**7**)) by water. The solvation of the emitting species by an aprotic solvent such as DMSO prevents the fluorescence from being quenched, thereby enhancing the chemiluminescence. Hydrazide **5** is far more susceptible to aqueous quenching than luminol, whose chemiluminescence is, by contrast, insensitive to the ratio of DMSO under these conditions.

White and Bursey[7] reported the chemiluminescent behaviors of di- and tri-methoxysubstituted luminol analogs which were expected to chemiluminesce more efficiently than the parent luminol because of higher fluorescent quantum yields of the corresponding phthalates. These analogs, however, were found to be less efficient chemiluminescers than luminol in water, although they were more efficient in the DMSO system; they explained this as a hydrogen-

bonding effect, which is caused to a greater extent in these analogs than in luminol. Hydrazide **5** appears to be subject to this hydrogen bonding effect caused by the bulk of water, presumably because there are more sites for hydrogen bonding than in luminol. Compared to luminol in identical aqueous conditions (50% (v/v) DMSO), hydrazide **5** emits 4 times as much light.

The addition of CTMAB, a cationic surfactant, also enhances the chemiluminescence of hydrazide **5** by twice the magnitude. No significant increase in light production was observed either with non-ionic or anionic surfactants. The micellar-enhanced chemiluminescence by an alkylammonium salt surfactant in the luminol system has been well discussed based on a microenvironmental effect[8]; a microenvironment provides an effective surface for the chemiluminescent reaction, which leads to an acceleration of the reaction[9]; it also solubilizes the light emitter, thus increasing the fluorescent quantum yield.[10] Taking into account the fact that chemiluminescence enhancement is also observed by DMSO, the effect of the cationic surfactant can be better interpreted as an increase of the fluorescent efficiency of the phthalate, rather than that of the reaction rate.

In order to use CTMAB in the enzyme reaction of NAGase with substrate **1**, the effect of CTMAB over the enzyme activity was independently examined using *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide (PNP-NAG),[11] a commonly used substrate in NAGase assay (Fig. 3). NAGase activity with CTMAB, calculated from the slope in Fig. 3, is 56% higher than without CTMAB of the same enzyme solution.

Since the use of CTMAB was found to be beneficial in the sense that it enhances the chemiluminescence of hydrazide **5** and also activates the enzyme, we next investigated a chemiluminescent response of substrate **1** to an enzyme of different concentrations. Enzymatic reactions were carried out in a 0.1 M phosphate buffer (pH 6.5) in the presence of CTMAB (0.5% (w/v)) at 37 °C for 10 min and subsequently terminated by the addition of an alkali. Figure 4 shows the chemiluminescent intensities obtained from the enzymatic reactions *versus* NAGase activities determined by the PNP-NAG method.

A linear relationship between chemiluminescence and enzyme activity implies that the principle in Chart 2 is operative. The detection limit of 0.6 I.U./l might be satisfactory considering that a mean value of urinary NAGase activity in healthy adults is reportedly in the range of 3.0[12] to 4.6[13] I.U./l. The present method permits sensitivity of the assay of NAGase of less than 1 I.U./l, roughly 10-fold more sensitive compared to the previous method using *o*-aminophthalylhydrazido-*N*-acetyl-*β*-D-glucosaminide,[3] whose detection limit is 5 I.U./l and independent from the addition of CTMAB. However, the background emission is relatively high, which suggests a non-enzymatic hydrolysis of substrate **1**. This hydrolytic instability might be ascribed to a pH sensitivity of the diethylamino group at 6-position. A K_m value of 0.23 mM was calculated from the Lineweaver–Burk plot under these conditions.

In summary, we have synthesized both a new type of arylic hydrazide and, by introducing the enzyme-cleavable group, a new chemiluminogenic substrate for NAGase.

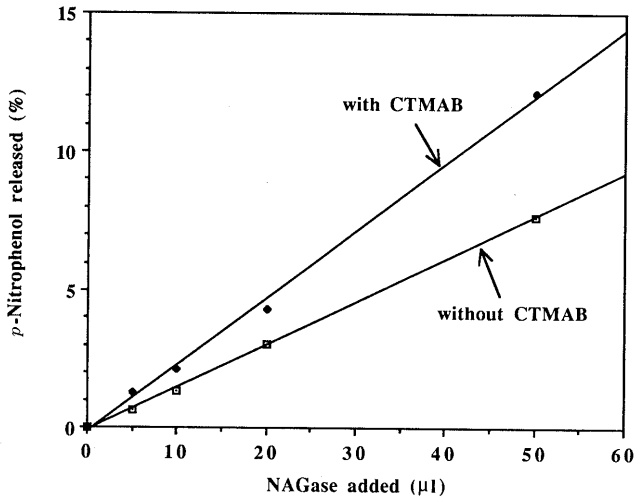


Fig. 3. Effect of the Addition of CTMAB on the NAGase Activity Using *p*-Nitrophenyl-*N*-acetyl-*β*-D-glucosaminide (PNP-NAG)

NAGase (55.6 I.U./ml) of a given amount (0—50 μl) was added to 1.0 ml of a pre-incubated 0.1 M citrate buffer (pH 5.0) containing PNP-NAG (4 mM), and the mixture was incubated at 37 °C for 5 min. Then 2.0 ml of 0.1 M bicarbonate buffer (pH 11.0) was added to terminate the enzymatic reaction and to measure the released *p*-nitrophenol at 400 nm. The same reaction was carried out in the presence of CTMAB (0.5% (w/v)).

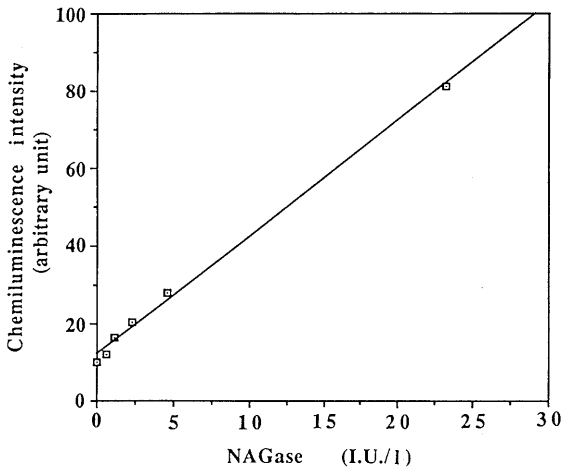


Fig. 4. Chemiluminescent Response of Substrate **1** to NAGase of Different Activities

NAGase solutions were treated according to the procedure described under Enzymatic Reaction.

Hydrazide **5** is more efficient than luminol, but more susceptible to aqueous quenching due to the hydrogen bonding effect. A number of arylic hydrazides have been synthesized in an effort to increase chemiluminescent efficiency, but only few are known to have a higher chemiluminescent quantum yield than luminol.[14] However, in an aprotic system, substitution of an electron-releasing group such as a methoxy or amino group on the phthalic hydrazide skeleton, for example, makes the parent luminol more efficient chemiluminescently because of an increase of the reaction rate and a higher fluorescence quantum yield of the corresponding phthalate.[4] In designing a highly efficient chemiluminescent compound, improving the fluorescence efficiency of a light emitter is a prerequisite. Most such compounds, however, suffer from substantial aqueous quenching, and sometimes lability to the oxidation or self-quenching of the fluorescence. The chemilumi-

nescence of hydrazide **5** is susceptible to aqueous quenching. However, it can be enhanced by cationic micelle through hydrophobic microenvironmental effects. Attaching a water-soluble substituent without affecting the chemiluminescene could offer an alternative solution to the problem of this aqueous quenching. In addition to chemiluminescent efficiency, the longer chemiluminescence wavelength of hydrazide **5** compared to luminol is another advantage because biological samples have less background emissions at longer wavelengths.

Regarding substrate **1**, because a chemiluminescence assay of enzymes being used currently involves multi-step enzymatic reactions to detect hydrogen peroxide as the final signal,[15] the approach using this substrate is straightforward in the sense that the substrate produces light directly on the action of the enzyme to be detected. Although sensitive detection of this enzyme of less than 0.5 I.U./l could be attainable by the conventional colorimetric[11,16] or fluorometric[17] methods, the present method offers an alternative to these spectrophotometric methods because of its simplicity and comparable sensitivity, and therefore it seems to have potential value in enzyme immunoassays. Furthermore, this kind of approach which involves incorporation of an enzyme-removable group into the hydrazide portion of an arycyclic hydrazide is not confined to a specific enzyme, basically being applicable to other enzymes such as those employed in enzyme immunoassays. In order to attain lower detection limits, however, the background emission caused by non-enzymatic hydrolysis should be minimized. Another factor that limits sensitivity and is inherent to this method is that the detection limit of the enzyme is restricted by the released amount of hydrazide **5**, rather than of the catalyst or hydrogen peroxide, which appears in a smaller amount than hydrazide **5** in this type of chemiluminescence reaction. We are currently working in this direction.

References

1) A. P. Schaap, M. D. Sandison and R. S. Handley, *Tetrahedron Lett.*, **28**, 1159 (1987).
2) I. Bronstein, B. Edwards and J. C. Voyta, *J. Bioluminescence and Chemiluminescence*, **4**, 99 (1989); J. C. Voyta, B. Edwards and I. Bronstein, *Clin. Chem.*, **34**, 1157 (1988); I. Bronstein, J. C. Voyta, G. H. G. Thorpe, L. J. Kricka and G. Armstrong, *ibid.*, **35**, 1441 (1989); I. Bronstein, J. C. Voyta and B. Edwards, *Anal. Biochem.*, **180**, 95 (1989).
3) K. Sasamoto and Y. Ohkura, *Chem. Pharm. Bull.*, **38**, 1323 (1990).
4) E. H. White and D. F. Roswell, *Acc. Chem. Res.*, **3**, 54 (1970) and references cited therein.
5) *a*) O. Dann, G. Bergen, E. Dermant and G. Voltz, *Justus Liebigs Ann. Chem.*, **749**, 68 (1971); *b*) S. Akiyama, H. Akimoto, S. Nakatsuji and K. Nakashima, *Bull. Chem. Soc. Jpn.*, **58**, 2192 (1985).
6) *Org. Synth. Coll. Vol.*, **V**, 1 (1973); D. H. Leabach, *Biological Preparations*, **10**, 118 (1963).
7) E. H. White and M. M. Bursey, *J. Org. Chem.*, **31**, 1912 (1966).
8) M. Yamada and S. Suzuki, *Anal. Lett.*, **17**, 251 (1984).
9) H. Karatani, *Bull. Chem. Soc. Jpn.*, **60**, 2023 (1987).
10) H. Karatani, *Chem. Lett.*, **1986**, 377.
11) E. Horak, S. M. Hopfer and F. W. Sunderman, Jr., *Clin. Chem.*, **27**, 1180 (1981).
12) P. H. Whiting, A. J. Nicholls and G. R. D. Catto, *Clin. Chim. Acta*, **108**, 1 (1980).
13) D. Maruhn, *Clin. Chim. Acta*, **73**, 453 (1976).
14) C. C. Wei and E. H. White, *Tetrahedron. Lett.*, **1971**, 3359; K. D. Gunderman, W. Horstmann and G. Bergmann, *Justus Liebigs Ann. Chem.*, **684**, 127 (1965).
15) S. Takayasu, M. Maeda and T. Tsuji, *J. Immunol. Methods*, **83**, 317 (1985).
16) A. Noto, Y. Ogawa, S. Mori, M. Yoshioka, T. Kitakaze, T. Hori, M. Nakamura and T. Miyake, *Clin. Chem.*, **29**, 1713 (1983).
17) D. H. Leabach and P. G. Walker, *Biochem. J.*, **78**, 151 (1961).