A Chemiluminogenic Substrate for *N*-Acetyl-β-D-glucosaminidase, *o*-Aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide

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A new type of chemiluminogenic substrate for *N*-acetyl-β-D-glucosaminidase, *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide, was prepared by incorporating an enzyme-removable substituent, *N*-acetyl-D-glucosaminide group, into the hydrazide moiety of luminol. This substrate releases luminol upon enzymatic hydrolysis. The enzyme was detected chemiluminescently using this substrate.

Keywords chemiluminogenic substrate; *N*-acetyl-β-D-glucosaminidase; *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide; luminol; chemiluminescence detection; enzyme assay

Luminol is one of the most important chemiluminescent reagents and has been widely used for biological assays.[1] Derivatization has been attempted in the search for an analogue which has a chemiluminescent quantum yield higher than the parent structure. From the finding that the modification of the hydrazide moiety of luminol renders the molecule nonluminescent,[2] we anticipated that, by incorporating an enzyme-removable substituent into the hydrazide moiety, luminol could be utilized as a substrate for chemiluminescence detection of enzyme. The chemiluminogenic substrates were first reported by Schaap *et al.* for the thermally stable dioxetane derivatives in which enzyme acted directly on the substrates to generate light.[3] Of these compounds, 3-(2′-spiroadamantane)-4-methoxy-4-(3′′-phosphoryloxy)phenyl-1,2-dioxetane and 3-(2′-spiroadamantane)-4-methoxy-4-(3′′-β-D-galactopyranoyloxy)-phenyl-1,2-dioxetane have recently been applied to enzyme immunoassays to detect alkaline phosphatase and β-D-galactosidase, respectively.[4] However, deprotonation of the phenolic hydroxy group is required to trigger chemiluminescence, thereby limiting the use to assay conditions above pH 9.

In the course of our studies to develop substrates for the rate-assay of *N*-acetyl-β-D-glucosaminidase (NAGase),[5] we selected *N*-acetyl-D-glucosaminide group as a masking group for luminol. We herein report the synthesis of this chemiluminogenic substrate, *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide (LUM-NAG; Chart 1), and its potential applicability to enzyme assay.

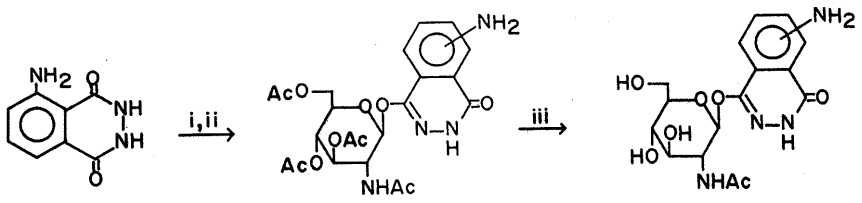
Experimental

Apparatus Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken in dimethylsulfoxide-d_6 with a Hitachi R-24B spectrometer at 60 MHz using tetramethylsilane as an internal standard. The splitting patterns were designated as follows: s, singlet; d, doublet; m, multiplet; br, broad. Infrared (IR) spectra were recorded in Nujol mull with a Hitachi 260-10 spectrometer. Chemiluminescent measurements were done on an Analytical Luminescence Laboratory MONOLIGHT 2010 luminometer (San Diego, CA, U.S.A.). A Shimadzu LC-4A high-performance liquid chromatograph (HPLC) equipped with a Shimadzu SPD-2AS ultraviolet (UV) spectrophotometer was used. The spectrophotometer was operated at 280 nm. 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. The mobile phase was aqueous 50% (v/v) acetonitrile and the flow rate was 1.0 ml/min. Uncorrected melting points were obtained on a Yamato MP-21 melting point apparatus.

Materials All chemicals were of reagent grade. NAGase from jack beans was purchased from Sigma (St. Louis, MO, U.S.A.) as a suspension of 0.8 mg of protein (62 I.U./mg) per ml in 2.5 M $(NH_4)_2SO_4$ solution at pH 7 (50 I.U./ml) and diluted with 0.1 M phosphate buffer (pH 6.3) prior to use. Peroxidase (POD, type II from horseradish) was purchased from Sigma as a crystalline solid (200 I.U./mg solid); a solution of 1 mg/dl in water was freshly prepared before use. A 40 mM stock solution of LUM-NAG was prepared in dimethylsulfoxide. α,β,γ,δ-Tetrakis(4-carboxyphenyl)porphine (TCPP) was obtained from Dojindo Laboratories (Kumamoto, Japan). Fe(III)–TCPP complex was obtained by refluxing TCPP with an equivalent mole of $FeCl_3 \cdot 6H_2O$ in acetic acid (6 mM) overnight; the stock solution was prepared by dissolving it in water at the concentration of 2.0×10^{-6} M.

Synthesis of LUM-NAG (Chart 1) To a stirred dimethylformamide (100 ml) solution of luminol (2.22 g, 12.53 mmol) was added sodium hydride (60% in oil, 0.5 g, 12.5 mmol) at room temperature. After 5 min, 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine[6] (4.50 g, 12.30 mmol) was added in portions and the stirring was continued overnight at room temperature. The reaction mixture was poured onto 1 M $NaHCO_3$ and the product was extracted with ethyl acetate. The ethyl acetate layer was washed with saturated brine, dried over $MgSO_4$ and concentrated to give the crude product, which was chromatographed on silica gel (5% methanol in chloroform) to afford the coupled product, *o*-aminophthalylhydrazido-2,3,4,6-tetraacetyl-β-D-glucosaminide, as a colorless crystalline solid in 10% yield after recrystallization from ethanol. ^{1}H-NMR δ, ppm; 1.75 (s, 3H, NHAc), 1.98 (s, 9H, OAc), 3.67—5.33 (m, 6H), 5.77 (d, 1H, J=8 Hz, anomeric H), 6.67—7.50 (m, 3H, aromatic H), 7.17 (br s, 1H), 7.88 (d, 1H, J=9 Hz, NHAc). IR ν_{max} cm^{-1}: 3350 (NH_2), 1745 (C=O), 1680 (CONH), 1620, 1535.

This coupled product (253 mg, 0.50 mmol) was then deacetylated with



luminol LUM-NAG

reagents: i, NaH, DMF; ii, 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine; iii, $NaOCH_3$, CH_3OH

Chart 1. Synthetic Route of LUM-NAG

1 M sodium methoxide (0.2 ml) in methanol (20 ml) at room temperature overnight. After being treated with Dowex 50W (H^+-form), the solvent was removed *in vacuo* and the residue was chromatographed on silica gel to afford 89 mg (47%) of LUM-NAG as a slightly yellow-colored crystalline solid. mp 178—180 °C (dec.). ^{1}H-NMR (δ, ppm): 1.70 (s, 3H, NHAc), 3.00—5.95 (m, 7H), 3.27 (br s, 5H, OH, NH_2), 6.60—8.40 (m, 4H, aromatic H). IR ν_{max} cm^{-1}: 3320 (OH, NH_2), 1660 (CONH), 1600, 1555. *Anal.* Calcd for $C_{16}H_{20}N_4O_7$: C, 50.53; H, 5.30; N, 14.73. Found: C, 50.48; H, 5.51; N, 14.60.

Photographic Detection of NAGase Chemiluminescent reactions were carried out on a food-wrapping film (polyvinylidene chloride) fixed on a Polaroid film holder and the resulting luminescence was imaged on Polaroid type 612 film.[7] A 5-μl aliquot of 0.1 M phosphate buffer (pH 6.3) containing LUM-NAG and NAGase was placed on the film, and to this spot was added 5 μl of Fe(III)–TCPP solution or POD. The chemiluminescence reaction was initiated by adding 5 μl of 0.1% H_2O_2. The reaction started immediately after mixing. The shutter of the film holder was opened in the dark and the film was exposed for 10 min.

Results and Discussion

Modification of Luminol The addition of equivalent mole of sodium hydride to luminol gave rise to the formation of the monoanion through the deprotonation in the hydrazide moiety, and this anion reacted smoothly with 1-chloro-1-deoxy-2,3,4,6-tetraacetyl-α-D-glucosamine[6] to give a nonluminescent product. After the removal of acetyl groups in the sugar moiety, LUM-NAG was obtained in a good yield. Although the exact position of the amino group could not be determined, it is more likely to be that closer to the sugar portion due to the stabilization of the monoanion of luminol through the hydrogen bond with its 3-amino group.

The release of luminol was observed by diluting 30 μl of LUM-NAG with 1 M hydrochloric acid to a volume of 0.5 ml (LUM-NAG concentration, 2.4 mM). The reversed-phase high-performance liquid chromatography (HPLC) of this solution showed that the acid labile glycosidic bond in LUM-NAG (retention time, 1.97 min) was cleaved completely into luminol (retention time, 3.07 min) and *N*-acetyl-D-glucosamine within 3 h at room temperature. The release was also observed by HPLC when 20 μl of LUM-NAG was incubated with 0.01 I.U. of NAGase in 0.2 ml of 0.1 M phosphate buffer (LUM-NAG concentration, 4 mM) at pH 6.3 and room temperature for 1 h.

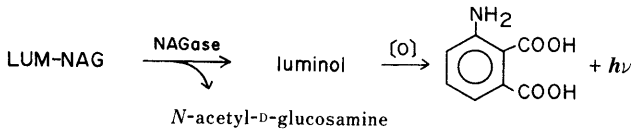


Chart 2. Principle of NAGase Assay by LUM-NAG

Chemiluminescence Detection of Enzyme The principle of the chemiluminescence detection for NAGase using LUM-NAG is illustrated in Chart 2, wherein the enzyme assay is based on the detection of the chemiluminescence of luminol which is released from the enzymatic hydrolysis of the substrate.

Figure 1 shows the photographic detection of NAGase by LUM-NAG using Fe(III)–TCPP complex as a catalyst. A 50-μl aliquot of LUM-NAG was incubated in 1 ml of 0.1 M phosphate buffer at pH 6.3 (LUM-NAG concentration, 2 mM) with 0—20 μl of NAGase (5.0 I.U./ml) at 25 °C for 30 min, and the enzymatic reaction was terminated by the addition of 2 ml of 0.1 M boric acid–0.2 M potassium hydroxide buffer (pH 12.6). A 5-μl aliquot of this solution was taken for the chemiluminescence detection with Fe(III)–TCPP complex and 0.1% hydrogen peroxide, which was done by photographic detection.

The chemiluminescence was observed only with the enzyme added, indicating that luminol was released from the substrate by enzymatic hydrolysis. The possibility that the chemiluminescence observed was due to the enhancement effect by the enzyme and not to the enzyme-catalyzed hydrolysis of the substrate was ruled out by the following experiment: The same experiment as above was undertaken except that the incubation at pH 6.3 was excluded. A 2-ml portion of 0.1 M boric acid–0.2 M potassium hydroxide buffer was added, without incubation, to 1 ml of 0.1 M phosphate buffer (pH 6.3) containing 50 μl of LUM-NAG (final concentration, 2 mM) and 20 μl of NAGase (5.0 I.U./ml); the 5-μl aliquot taken was then subjected to photographic detection with the Fe(III)–TCPP complex and 0.1% hydrogen peroxide in the same manner as above. Chemiluminescence was not detected under these conditions.

Since the enzyme was found to be detected chemilumines-

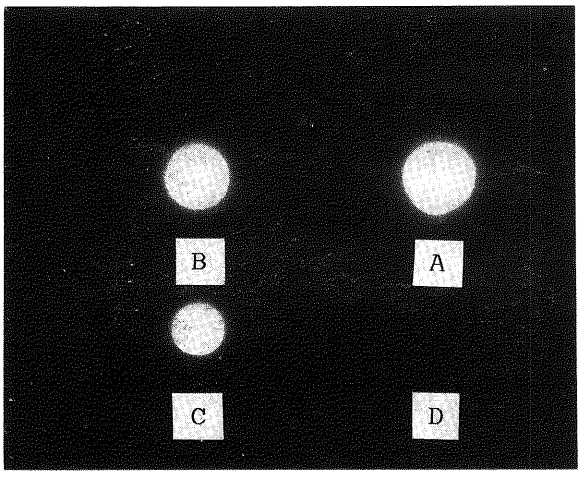


Fig. 1. Photographic Detection of NAGase by LUM-NAG Using Fe(III)–TCPP Complex as a Catalyst

Amount of NAGase added: A, 0.10 I.U.; B, 0.05 I.U.; C, 0.02 I.U.; D, blank.

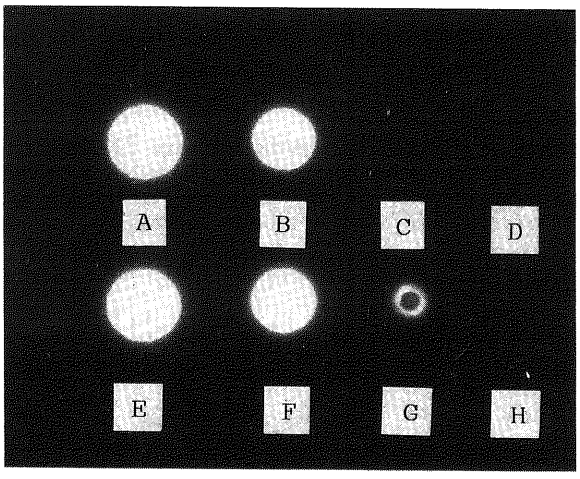


Fig. 2. Photographic Detection of NAGase by LUM-NAG Using POD as a Catalyst

Upper Spots (A—D) are with *p*-iodophenol added (0.1 mg). Amount of NAGase added: A and E, 50 mI.U.; B and F, 5 mI.U.; C and G, 0.5 mI.U.; D and H, blank.

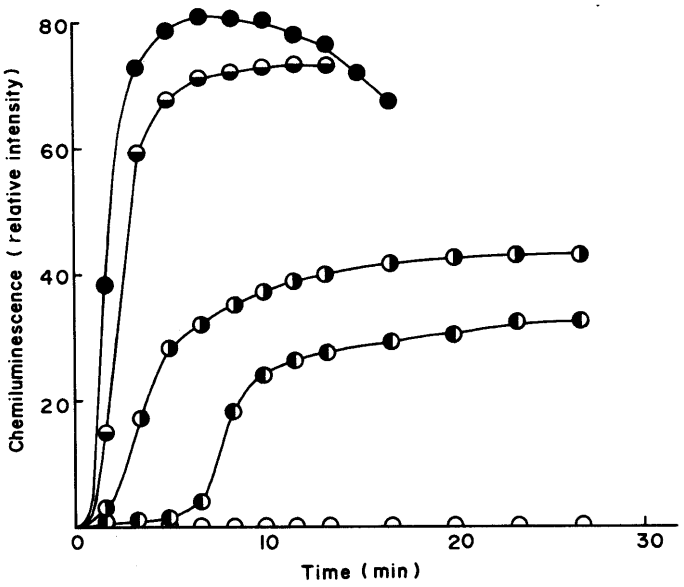


Fig. 3. Time Course of the Enzyme-Triggered Chemiluminescent Reaction at pH 6.3

Amount of NAGase added: (●) 100 mI.U.; (◒) 50 mI.U.; (◑) 10 mI.U.; (◐) 5 mI.U.; (○) blank.

cently using this substrate, we next attempted to carry out the chemiluminescent reaction at neutral pH where the enzymatic hydrolysis could occur simultaneously. POD was used instead of Fe(III)–TCPP complex. To each of 10-μl aliquots of 0.1 M phosphate buffer (pH 6.3) containing LUM-NAG (2 mM) and NAGase (0—50 mI.U.) were added 5 μl of POD (10 mI.U.) and 5 μl of 0.1% hydrogen peroxide to initiate the chemiluminescent reaction, which was imaged on instant film for 10 min. The results are shown in Fig. 2, in which the effect of the addition of *p*-iodophenol was also investigated. Although *p*-iodophenol is known to enhance the POD-catalyzed light emission,[8] the additions gave negative effects in these cases (upper row). The detection limit for NAGase under these conditions is 0.5 mI.U., which is more sensitive than that when Fe(III)–TCPP complex is used.

This POD-catalyzed enzyme triggering of chemiluminescence under the rate-assay conditions at neutral pH was then measured using a luminometer. Fifty microliters of each of POD (0.1 I.U.) and 0.1% hydrogen peroxide were added to 1 ml of 0.1 M phosphate buffer (pH 6.3) mixed with 10 μl of LUM-NAG (final concentration, 0.4 mM) and 0—100 μl of NAGase (1.0 I.U./ml) in a measuring cuvette. The measurement of the resulting chemiluminescence was started immediately after the mixing and was continued for more than 10 min at 23 °C. Figure 3 indicates the time courses of the reactions. The sigmoidal curves reflect the concentration change of luminol which is supplied from the enzymatic hydrolysis of the substrate and is consumed simultaneously through the chemiluminescent reaction yielding 3-aminophthalic acid.

It appears that the enzymatic hydrolysis is the rate-determining step and that the release of luminol in a certain amount triggers the chemiluminescent reaction. It is also noted that the light intensity gradually decreases after reaching the maximum intensity and that the time required to reach the maximum light intensity is proportional to the amount of enzyme added.

The enzyme-triggering of chemiluminescence using luminol could also be achieved by acylating the 3-amino group,[9] which greatly reduces the luminescence, but the background luminescence raises the lowest detection limit. Although the detection limit of NAGase by the present method is not very satisfactory, this unique approach has no background problems, and therefore offers some advantages over the conventional method by increasing the chemiluminescent quantum yield of the cyclic hydrazide; efforts are currently being made in this direction.

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