Published in final edited form as:

Carbohydr Res. 2012 October 1; 359: 92–96. doi:10.1016/j.carres.2012.06.009.

Synthesis of novel *N*-acetylneuraminic acid derivatives as substrates for rapid detection of influenza virus neuraminidase

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Abstract

Two novel *N*-acetylneuraminic acid derivatives, luciferyl *N*-acetylneuraminic acid (**1**) and luciferyl 4,7-di-O-methyl-*N*-acetylneuraminic acid (**2**), were designed and synthesized as substrates for the rapid detection of influenza virus neuraminidase. The sensitivity and specificity of the assays with compound **1** or **2** as the substrate for detection of neuraminidases from influenza virus (H1N1 and H5N1) and bacteria (*A. ureafaciens* and *C. perfringens*) were evaluated. Compound **1** was sensitive to neuraminidases from both influenza virus and bacteria. Biochemiluminescent assays with this compound with H1N1 and H5N1 neuraminidases were approximately 20- and 16-fold more sensitive, respectively, than the fluorescent method with the commercial substrate 4-MUNANA. In contrast, compound **2** was only sensitive to the neuraminidases from influenza virus, showing approximately 10- and 8-fold greater sensitivity than 4-MUNANA for the detection of H1N1 and H5N1 neuraminidases, respectively. The data showed that compound **2** could be used in assays that detect of influenza viral neuraminidase.

Keywords

Rapid detection; Influenza virus neuraminidase; *N*-acetylneuraminic acid derivatives; Bioluminescence

Influenza poses serious threats to public health. Each year, influenza epidemic leads to great number of hospitalization and deaths throughout the world.¹ The H1N1 influenza pandemic of 2009 has added weight to concerns regarding the influenza.² As medical sciences advance, several promising drugs are now available for the prophylaxis or treatment of influenza.^{3–5} Because anti-influenza medications are most effective when taken by the patients only within 48 h of the onset of signs and symptoms,⁶ early detection of the influenza virus is important for the treatment or prevention control. Traditionally, the detection of influenza virus is performed with three methods,^{7–12} including virus isolation in cell culture, various types of immunoassays, and molecular assays such as polymerase chain reaction (PCR) assays. Among these three methods, virus isolation in cell culture is the

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‘gold standard’ for influenza virus detection.⁸ However, the results can only be obtained after 2 to 14 days, which is too late for effective medical intervention. Although faster when compared to the cell culture method, immuno- and molecular assays have a number of drawbacks, which make these assays less than an ideal influenza diagnostic tool. As an immunoassay detects one or more influenza antigens, it is generally less sensitive, particularly for a new variant with mutated antigenic epitopes. A molecular assay, on the other hand, involves the use of complicated techniques, expensive equipments and well-trained technicians, preventing these assays from being widely adopted for use in point-of-care settings, particular in the developing countries.^{9–12}

In recent years, a new class of influenza diagnostics was demonstrated. Rather than detecting an antigen or nucleic acid marker, these diagnostics, represented by the ZstatFlu and ZstatFlu-II tests, detected the neuraminidase activity of influenza A or B virus.^{13–15} ZstatFlu and ZstatFlu-II tests exploit small molecular derivatives of *N*-acetylneuraminic acid coupling with a chromogenic or luminescent substance to directly detect influenza viral neuraminidase activity, exhibiting excellent specificity towards influenza virus A and B.^{16–18} Bioluminescence-based assays have been used in many applications because of their comparatively high sensitivity. Among the biochemiluminescent substrates, luciferin in the firefly luciferin-luciferase system is best known and well characterized.^{19,20} Therefore, it is expected that firefly luciferin group was introduced into *N*-acetylneuraminic acid derivatives to enhance the sensitivity of rapid test for influenza viral neuraminidase, hence influenza. Here, we report the synthesis of *N*-acetylneuraminic acid derivatives, including luciferyl *N*-acetylneuraminic acid (**1**) and luciferyl 4,7-di-*O*-methyl-*N*-acetylneuraminic acid (**2**). The sensitivity and specificity of **1** and **2** for detection of neuraminidases from influenza virus (H1N1 and H5N1) and bacteria (*A. ureafaciens* and *C. perfringens*) were also evaluated.

The synthetic routes for the preparation of novel *N*-acetylneuraminic acid derivatives **1** and **2** were outlined in Scheme 1. The intermediates of **1** and **2** were synthesized following a procedure according to the previous methods, Scheme S1 and S2 (Supporting Information). All corresponding spectroscopic data of the intermediates are consistent with those of reported compounds.^{21–30} The α configuration of **1** and **2** were ascertained by the chemical shift (δ 3.05 and 3.07) in the ¹H NMR spectrum for the signal of H-3eq which was diagnostic for the α configuration.^{16, 31} It was also supported by the fact that **1** and **2** were cleavable by the neuraminidases from influenza virus as shown later.^{32–33}

In the present assay, neuraminidase and luciferase reactions were performed successively. This two-step assay was performed as below: the neuraminidase reaction was conducted in the presence of neuraminidase and substrate in a buffer with an optimal pH, followed by the luciferase reaction, which was carried out in a detection mixture containing luciferase, ATP, and MgSO₄·7H₂O in Tris-Acetate buffer pH = 8.0, the optimal pH for luciferase reaction.^{24, 34} The second detection mixture was added to the first step reaction mixture to initiate the bioluminescence reaction, and the luminescence emission was immediately measured using a luminometer. In the first reaction, conjugate **1** or **2** was cleaved by neuraminidase to liberate luciferin, which was the substrate of luciferase in the second reaction mixture. Luciferin was subsequently oxidized by luciferase to give detectable luminescence signal. In this bioluminescence detection assay, these two conjugates themselves were not luciferase substrates as they lack 6'-OH group of luciferin for activity,^{35, 36} and the luciferase-catalyzed bioluminescence was dependent on neuraminidase activity, therefore substrates **1** and **2** enable the detection of the neuraminidase in the presence of luciferase.

The optimal pH for substrates **1** and **2** with neuraminidases from different sources were studied by varying the pH of neuraminidase reaction solution from 4.5 to 8.0. As shown in

Fig 1, the optimal pH was 6.5 for the reaction of neuraminidases from H1N1 and H5N1, and 5.0 for that of neuraminidases from *A. ureafaciens* and *C. perfringens*. The subsequent experiments were carried out at pH = 6.5 for influenza viral neuraminidase reaction and at pH = 5.0 for bacterial neuraminidase reactions. Additional experiments were performed to determine the optimal incubation time for reactions involving influenza viral neuraminidases. As shown in Fig. 2, the signal intensity increased rapidly in the first 15 minute, followed by a period of plateau lasting up to 40 minute. The reaction reached the maximum peak at about 15 minute, after which the light signal intensity remained fairly constant. Thus, fifteen minute was chosen to be the optimal incubation time.

Kinetic studies were performed with neuraminidases from H1N1 and H5N1. K_m and V_{max} values for **1** and **2** at pH 6.5 were estimated using a Lineweaver-Burk plot. The data was summarized in Table 1. Substrate **1** had a lower K_m value ($98.2 \pm 5.8 \mu\text{M}$) and a higher V_{max} value ($10.1 \pm 0.5 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) for H1N1 neuraminidase in comparison with K_m ($225.9 \pm 11.5 \mu\text{M}$) and V_{max} ($7.1 \pm 0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) of **2**. These results indicated that substrate **1** had a higher affinity for the tested neuraminidases than substrate **2**. This was also true for the substrates when tested with H5N1 neuraminidase. These findings are consistent with the fact that the chemical structure of substrate **1** more closely resembles that of a natural neuraminidase substrate as compared to substrate **2**, which is methylated on the neuraminic acid moiety.

In order to minimize the background signal, 0.3–0.4 K_m concentration of substrates was chosen. Under such condition, the background light emission was not sufficiently intense to produce false-positive results.⁷ Accordingly, the concentrations of 40 μM for substrate **1** and 80 μM for **2** were chosen for following experiments.

Luciferin exists in two chiral forms. It was known that only D-luciferin contributes to bioluminescence in a luciferase reaction. Thus, racemisation experiments were conducted to determine the extent of L-luciferin in the substrates. The racemisation experiments were performed by determining the liberated luciferin using fluorescence assay and coupled-bioluminescence assay with luciferase. As shown in Table 2, the initial rate of neuraminidase hydrolysis of **1** and **2** by bioluminescence assay was observed only 84% and 63% of those values observed under the same conditions of hydrolysis in a fluorescence assay. The difference between the fluorescence and bioluminescence assays indicated that 84% of the luciferin released for **1** and 63% of luciferin released for **2** were active in the light-emitting reaction, suggesting that a portion of the luciferin moiety in both substrates was partly racemised into L-form during synthesis.²³

Limit of detection studies were performed to compare the sensitivity of assays formulated with **1** and **2** for influenza viral neuraminidases with that of a fluorescent assay using substrate (4-MUNANA) under identical condition. The signal-to-noise ratio (S/N) is often used to indicate the sensitivity of an assay. Based on previously reported literatures,^{18, 34} the S/N ratio of 2 was chosen as the cut-off value for both bioluminescent and fluorescent assays. A sample with an S/N ratio equal to or greater than 2 (≥ 2) was considered positive. As shown in Table 3, for detection of H1N1 neuraminidase, the limits of detection for **1** and **2** were approximately 20- and 10-fold, respectively, more sensitive than that of an assay based on the 4-MUNANA. For detection of H5N1 neuraminidase, it was found that substrates **1** and **2** afforded approximately 16- and 8-fold, respectively, increases in sensitivity when compared to fluorescent assay. In conclusion, the bioluminescence method using substrate **1** or **2** was substantially more sensitive than the fluorescent assay using the 4-MUNANA for the detection of neuraminidase.

The specificities of **1** and **2** towards bacterial neuraminidases were also evaluated in present work. When the bacterial neuraminidase from *C. perfringens* or *A. ureafaciens* was used, even at a very high concentration (10^{-2} U/mL), substrate **2** produced less than 12% luminescence signal intensity of **1**. At a lower bacterial neuraminidase level (10^{-4} U/mL), substrate **1** still produce strong luminescence signal, indicating that substrate **1** can be cleaved efficiently by bacterial neuraminidase. In contrast, the signal intensity produced by substrate **2** was close to background. These results revealed that the substrate **2** was only sensitive to the neuraminidases from influenza virus (Fig. 3). Therefore, the substrate **2** is suitable for influenza virus detection.

In summary, two new *N*-acetylneuraminic acid derivatives **1** and **2** were synthesized as the substrates for the rapid detection of influenza viral neuraminidase. The K_m and V_{max} for substrates **1** and **2** of two influenza viral neuraminidases were determined. Furthermore, the evaluation of the sensitivity and specificity of substrates **1** and **2** for detection of the neuraminidases from influenza virus (H1N1 and H5N1) and bacteria (*A. ureafaciens* and *C. perfringens*) were performed. The results revealed that **1** was sensitive to both influenza viral and bacterial neuraminidases whereas **2** was only sensitive to influenza viral neuraminidases. Substrates **1** or **2** enabled assays that were substantially more sensitive for detection of influenza viral neuraminidases than a commercial fluorescent substrate 4-MUNANA. Because of enhanced sensitivity and specificity for detection of influenza viral neuraminidase, it appears that substrate **2** is suitable for use in influenza diagnosis.

Experimental

1.1. General methods

The ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 600 spectrometer. Chemical shifts were given in ppm downfield to the signal of internal TMS. Mass spectra were obtained by a QTRAP-2000 LC/MS/MS System mass spectrometer. The elemental analysis was done on a Vario ELIII analyzer. Optical rotation was obtained with Autopol^R IV Automatic Polarimeter. Melting points were taken on a micro melting point XT4A Beijing Keyi electrooptic apparatus and were not corrected. Relative light unit (RLU) was measured on FLUOstar OPTIMA luminometer from BMG LABTECH. Fluorescence measurements were obtained in Gemini EM Fluorescence Microplate Reader. HPLC analysis was carried out on a Shimadzu SCL-10A HPLC system equipped with LC-6AD Pump, SPD-10A UV-VIS Detector, and it was performed on a Microsorb 60-8 C18 column (21.4×250 mm, $8 \mu\text{m}$) at 75 min elution time using a gradient of 5–20% acetonitrile in 1% ammonium acetate at 15 mL/min flow rate with detection wavelength set at 330 nm. Luciferase was purchased from Promega Corporation with specific activity 2.8×10^{-10} . D-Luciferin was purchased from Gold Biotechnology. 4-Methylumbelliferyl-*N*-acetylneuraminic acid (4-MUNANA) was obtained from Sigma Chemical Company. The bacterial neuraminidase from *Arthrobacter ureafaciens* (*A. ureafaciens*) was purchased from Roche Diagnostics Corporation. The bacterial neuraminidase from *Clostridium perfringens* (*C. perfringens*) was obtained from Sigma Chemical Company. One Unit (U) of bacterial neuraminidase was defined as the amount of enzyme that hydrolyzes 1 μmol *N*-acetylneuraminosyl-lactose in 1 min at pH 5.0 at 37°C. The influenza A virus H1N1 (A/California/04/2009) and H5N1 (A/Anhui/1/2005) neuraminidase were purchased from Sino Biological Inc. (China). One Unit (U) of influenza viral neuraminidases was defined as the amount of enzyme that cleave 1 nmol 2'-(4-Methylumbelliferyl)- α -D-*N*-acetylneuraminic acid per min at pH 7.5 at 37°C.

1.2. Synthesis of luciferyl *N*-acetylneuraminic acid (1)

A solution of luciferin (365 mg, 1.3 mmol) in 1 M NaOH (1.2 mL) in 100 mL four-necked flask was stirred for 10 min at 0 °C under N₂ in the dark. Then, the ice bath was removed and the reaction solution was allowed to warm up to the room temperature. Meanwhile a solution of the crude product **iii** and NaI·2H₂O (242 mg, 1.3 mmol) in acetone (8 mL) was stirred at room temperature for 15 min, then, the mixture of **iii** and another 1 M NaOH (2.5 mL) were simultaneously added dropwise to the above solution of luciferin within 15 min to ensure pH = 9–10. The mixture was saturated with nitrogen continuously and stirred for another 2 h in the dark, and evaporated to dryness under diminished pressure. The residue was dissolved in methanol (8 mL), and an addition of NaOH (2 mL) was added to the above mixture solution, which was kept stirring for 1 h. The reaction mixture was neutralized with 3 M HCl to pH = 4.5–5.0 and then extracted with CH₂Cl₂ (15 mL × 3). The recovered water layer was purified by reverse phase HPLC (*R*_t = 13.6 min) and lyophilized to afford white powder **1** (491 mg, 43%). Mp 199–200 °C. [α]_D²⁵ +160.7 (c 1.0, CH₃OH). ¹H NMR (600 MHz, CD₃OD) δ 1.87–1.94 (m, 1H, H-3ax), 2.04 (s, 3H, NAc), 3.05 (d, 1H, *J* = 10.7 Hz, H-3eq), 3.57–3.59 (m, 1H, H-thiazolyl SCH₂), 3.67–3.68 (m, 1H, H-thiazolyl SCH₂), 3.77–3.92 (m, 7H, H-4, 5, 6, 7, 8, 9, 9'), 5.27–5.30 (m, 1H, H-thiazolyl NCH), 7.47 (d, 1H, *J* = 7.9 Hz, H-phenyl 5'), 7.91–7.96 (m, 2H, H-phenyl 4', 7'). ¹³C NMR (151 MHz, CD₃OD) δ 23.18 (Me-NAc), 37.30 (thiazolyl SCH₂), 43.38 (C-3), 54.64 (C-5), 65.07 (C-9), 69.91 (C-7), 70.85 (C-6), 73.77 (C-4), 75.84 (C-8), 82.39 (thiazolyl NCH), 104.90 (C-2), 115.73, 124.22, 125.46, 138.23, 151.20, 156.39, 162.01, 166.76 (phenyl and thiazolyl), 173.66 (CO-NAc), 176.09 (COOH). MS (*m/z*): 570.2 [M-H][−]. Anal. Calcd for C₂₂H₂₅N₃O₁₁S₂: C, 46.23; H, 4.41; N, 7.35; S, 11.22. Found: C, 46.36; H, 4.45; N, 7.31; S, 11.12.

1.3. Synthesis of luciferyl 4,7-di-O-methyl-*N*-acetylneuraminic acid (2)

To a solution of **viii** in acetone (15 mL) was added NaI·2H₂O (483 mg, 2.6 mmol), and the mixture was stirred at room temperature for 15 min. Meanwhile, luciferin (730 mg, 2.6 mmol) was dissolved in 1 M NaOH (2.4 mL) in 100 mL four-necked flask at 0 °C under N₂ in the dark. After 10 min, the ice bath was removed and the reaction solution was allowed to warm up to the room temperature. Then the above mixture of **viii** and 5 mL of 1 M NaOH were simultaneously added dropwise to the solution of luciferin within 25 min to make pH = 9–10. The mixture was saturated continuously with nitrogen and stirred at room temperature for 2 h in the dark, then evaporated to dryness under diminished pressure. The addition of 1 M NaOH solution (4 mL) and methanol (15 mL) were added to the residue, which was kept stirring for another 1 h, and then neutralized with 3 M HCl to pH = 4.5–5.0. The mixture was extracted with CH₂Cl₂ (30 mL × 3) to eliminate impurities. The product was purified by reverse phase HPLC (*R*_t = 22.4 min) and lyophilized to afford white powder **2** (436 mg, 28%). Mp 204–205 °C. [α]_D²⁵ +167.7 (c 1.0, CH₃OH). ¹H NMR (600 MHz, CD₃OD) δ 1.78 (t, 1H, *J* = 11.8 Hz, H-3ax), 1.99 (s, 3H, NAc), 3.07 (dd, 1H, *J* = 12.2, 4.3 Hz, H-3eq), 3.30–3.32 (m, 3H, H-4, thiazolyl SCH₂), 3.39–3.42 (m, 4H, OMe-7, H-7), 3.44 (s, 3H, OMe-4), 3.64 (dd, 1H, *J* = 11.4, 4.8 Hz, H-9'), 3.72 (d, 1H, *J* = 9.3 Hz, H-9), 3.84 (dd, 1H, *J* = 10.4, 1.0 Hz, H-8), 3.90 (s, 1H, H-6), 4.06–4.12 (m, 1H, H-5), 5.19 (t, 1H, *J* = 9.1 Hz, H-thiazolyl NCH), 7.41 (d, 1H, *J* = 8.9 Hz, H-phenyl 5'), 7.85 (s, 1H, H-phenyl 7'), 7.91 (d, 1H, *J* = 8.9 Hz, H-phenyl 4'). ¹³C NMR (151 MHz, CD₃OD) α 23.63 (Me-NAc), 37.80 (thiazolyl SCH₂), 39.51 (C-3), 51.88 (C-5), 57.80 (OMe-4), 61.61 (OMe-7), 64.43 (C-9), 73.63 (C-7), 75.17 (C-6), 80.45 (C-8), 80.90 (C-4), 83.67 (thiazolyl NCH), 104.95 (C-2), 115.39, 123.93, 125.39, 138.18, 151.07, 156.35, 162.20, 165.95 (phenyl and thiazolyl), 173.97 (CO-NAc), 178.01 (COOH). MS (*m/z*): 598.2 [M-H][−]. Anal. Calcd for C₂₄H₂₉N₃O₁₁S₂: C, 48.07; H, 4.87; N, 7.01; S, 10.69. Found: C, 48.10; H, 4.89; N, 7.04; S, 10.64.

1.4. Preparation of the detection mixture

The detection mixture was prepared as 100 μL aliquots containing 20 $\mu\text{g/mL}$ luciferase, 5 mM ATP, 0.25 mM BSA, 1 mM DTT, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM CaCl_2 , 220 mM mannitol, 30 mM sucrose, and 8 mM NaN_3 in 25 mM Tris-Acetate buffer, pH = 8.0. This pH value is close to the optimum for the luciferase reaction.^{24, 34}

1.5. Determination of kinetic constants

Kinetic constants of neuraminidases from H1N1 and H5N1 for substrates **1** and **2** were determined as follows. The luciferin concentration against the emission of light was firstly fixed as a calibration curve for the fluorescence measurements. The luciferin was dissolved in 50 μL of buffer at pH 6.5. Fluorescence was measured ($\lambda_{\text{ex}} = 355 \text{ nm}$ and $\lambda_{\text{em}} = 538 \text{ nm}$) with Gemini EM Fluorescence Microplate Reader after 100 μL of 0.5 M glycine-NaOH, pH = 10.3. Each measurement was carried out in triplicate.

Kinetic constants were determined using 10 – 1000 μM of substrates **1** or **2** in a total volume of 50 μL buffer at pH = 6.5 containing 0.25 mM BSA and 10^{-1} U/mL of influenza viral neuraminidase.^{18, 37} The reaction mixtures in 96-well plate were incubated at 25 $^\circ\text{C}$ for 5 min. Then 100 μL solution of 0.5 M glycine-NaOH (pH = 10.3) was added. Fluorescence readings were recorded ($\lambda_{\text{ex}} = 355 \text{ nm}$ and $\lambda_{\text{em}} = 538 \text{ nm}$) with Gemini EM Fluorescence Microplate Reader. Each measurement was performed in triplicate. The kinetic constants K_m and V_{max} were calculated from the Lineweaver-Burk plots.

1.6. Performance of racemisation experiments

Quantifying luminometrically active D-luciferin in **1** and **2** was carried out. 50 μL of luciferin solution or reaction mixture containing neuraminidase and substrate was applied to the 96-well plate. Then 100 μL of the detection mixture was added. The luminescence signal was immediately determined by a FLUOstar OPTIMA luminometer using solutions of D-luciferin serving as standards. Initial rates of neuraminidase hydrolysis of **1** and **2** was measured by bioluminescence assay.^{23, 24} Meanwhile, the initial rates of neuraminidase were estimated under the same conditions of hydrolysis in a fluorescence assay as described above for determination of kinetic constants conditions.

1.7. Determination of the optimal pH and incubation time

Determination of the optimal pH value was carried out using 10^{-1} U/mL of influenza viral neuraminidases or 10^{-2} U/mL of bacterial neuraminidases in a total volume of 50 μL containing 0.25 mM BSA, 40 μM of **1** or 80 μM of **2**. The pH value was varied from 4.5 to 8.0 with incubation of 15 min at 25 $^\circ\text{C}$. Subsequently, the detection mixture was added and the luminescence signal was immediately measured in a FLUOstar OPTIMA luminometer. The assay of the optimal incubation time was performed using 10^{-1} U/mL of influenza viral neuraminidases in a total volume of 50 μL (pH 6.5) which contained 0.25 mM BSA, 40 μM of **1** or 80 μM of **2** with incubation time varying from 5 to 40 min. The detection mixture was then added and the luminescence intensity was measured with a FLUOstar OPTIMA luminometer. Each measurement above was conducted in triplicate.

1.8. Evaluation of sensitivity of fluorescence substrate

The limit of detection for influenza viral neuraminidases was determined using 4-MUNANA as substrate. Neuraminidase activity was assayed with 10^{-9} - 10^{-2} U/mL of influenza viral neuraminidases, 0.25 mM BSA and 80 μM of 4-MUNANA in total volume of 50 μL (pH = 6.5). Incubations were conducted at 25 $^\circ\text{C}$ for 15 min.^{38–40} The reaction was terminated by the addition of 100 μL of 0.5 M glycine-NaOH (pH = 10.3). The fluorescence of released 4-methylumbelliferone was measured with a Gemini EM Fluorescence Microplate Reader

using excitation and emission wavelengths of 360 and 460 nm, respectively. Control experiments were performed in the absence of neuraminidases using the same conditions.

1.9. Evaluation of sensitivity of **1** and **2**

The sensitivity assay with **1** or **2** as the substrate was evaluated using 10^{-9} - 10^{-2} U/mL of influenza viral neuraminidases in a total volume of 50 μ L which contained 0.25 mM BSA, 40 μ M of **1** or 80 μ M of **2**. The neuraminidase reaction was conducted in 96-well plate at 25 °C for 15 min, followed by addition of 100 μ L of detection mixture to initiate the luciferase reaction. Luminescence emission was immediately measured by a FLUOstar OPTIMA luminometer. Control experiments without neuraminidase in the reaction were also carried out using the identical assay conditions.

1.10. Evaluation of specificity of **1** and **2**

The specificity assay with **1** and **2** as the substrates was performed using 10^{-4} - 10^{-2} U/mL of bacterial neuraminidases in a total volume of 50 μ L at pH = 5.0 in 96-well plate containing 0.25 mM BSA, 40 μ M of **1** or 80 μ M of **2**. After incubation at 25 °C for 15 min, 100 μ L of detection mixture was added, and the luminescence signal was immediately determined by a FLUOstar OPTIMA luminometer. Control experiments were conducted under identical conditions except for the absence of neuraminidases. Additional control experiments were performed in the presence of neuraminidase but no luciferase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Natural Science Foundation of Changchun City, China (No. 2009107), and NIH Grants (Nos. AI082728; AI077117).

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Figure 1.
The optimal pH curve of **1** and **2** for neuraminidases. The data was expressed as the mean.

Figure 2.
The optimal incubation time of 1 and 2 for neuraminidases. The data was expressed as the mean.

Figure 3.

The specificity detection of **1** and **2** towards bacterial neuraminidases. The assay was conducted as outlined under Experiment section. The mixture containing the detection mixture, **1** or **2** except for neuraminidase was used as control 1 and control 2, respectively. The control 3 containing neuraminidases, **1**, **2** and the detection mixture without luciferase showed no luminescence, therefore it was not seen in the figure.

Scheme 1.

The schematic synthesis of substrates **1** and **2**.

Table 1

Kinetic constants K_m and V_{max} for substrates of influenza viral neuraminidases.

Neuraminidase source	Substrate	K_m (μM)	V_{max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
H1N1	1	98.2 ± 5.8	10.1 ± 0.5
	2	225.9 ± 11.5	7.1 ± 0.1
H5N1	1	111.9 ± 8.4	8.9 ± 0.4
	2	251.4 ± 15.8	6.7 ± 0.2

^aThe constants are expressed as the mean $\pm$ SD.

Table 2Rates of neuraminidase hydrolysis of **1** and **2**.

Neuraminidase source	Substrate	Conc. (μM)	pH	Initial rate ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
H1N1	1	200	6.5	4.3 ± 0.31^a
				3.6 ± 0.12^b
	2	200	6.5	3.5 ± 0.09^a
				2.2 ± 0.05^b

^aFluorescence assay.^bBioluminescence assay.

Table 3

The limit of detection for influenza viral neuraminidases using different substrates

Neuraminidase source	Substrates		
	1	2	4-MUNANA
H1N1 (U/mL)	0.46×10^{-4}	0.93×10^{-4}	9.47×10^{-4}
H5N1 (U/mL)	0.65×10^{-4}	1.31×10^{-4}	10.42×10^{-4}