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X-Neu5Ac: A Novel Substrate for Chromogenic Assay of Neuraminidase Activity in Bacterial Expression Systems

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Abstract—A chromogenic substrate **1**, 5-bromo-4-chloroindol-3-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic acid (X-Neu5Ac), has been synthesized to facilitate the screening of bacterial colonies or plaques for the detection of either natural or mutant neuraminidase activity. Substrate **1** was hydrolyzed by neuraminidase isolated from *Clostridium perfringens* to release a halogenated indol-3-ol **2** that undergoes rapid aerobic oxidation to form the dark blue pigment, 5,5'-dibromo-4,-4'-dichloroindigo **3**. Preliminary kinetic studies indicate that this compound is a good substrate (K_m 0.89 x 10^{-3} M) for neuraminidase and is quite stable under identical conditions in the absence of enzyme. These results suggest that X-Neu5Ac **1** can be useful to screen for bacterially-encoded enzyme production directly on agar plates.

In recent years, many studies have focused on the functional role of cell surface gangliosides, which are thought to facilitate cell–cell recognition, antigenicity, and cell growth regulation.[1] Recent studies suggest that *N*-acetyl neuraminic acid (Neu5Ac) within gangliosides serves a central function in these roles. To obtain deeper insight into the functional roles of membrane gangliosides, the development of novel neuraminidase-like catalysts, specific for the gangliosides of interest, is desirable. One approach for obtaining such catalysts is to screen naturally occurring neuraminidases for the substrate specificity using microbial hosts. Recently neuraminidases have been cloned from several microorganisms, including *Clostridium sor dellii* G12,[2] *Clostridium perfringens* A99,[3] and *Bacteroides fragilis* TAL2480,[4] and hence site-directed mutagenesis should allow alteration of the natural specificity of these enzymes to generate suitable mutants for this purpose.[5] Herein we report a chromogenic substrate **1** that facilitates the screening of bacterial colonies or plaques for detection of either natural or mutant neuraminidase activity.

Chromogenic substrates are routinely used for histologic, cytologic, and spectroscopic analyses of enzyme activity. Among the commonly used chromophores, the 5-bromo-4-chloroindol-3-yl group is particularly useful for identifying enzyme activity.[6] Indigo is insoluble in aqueous solution and accumulates at the site of the reactions, thereby affording high detection sensitivity.[7] A chromogenic substrate **1**, 5-bromo-4-chloroindol-3-yl-*N*-acetyl-neuraminic acid (X-Neu5Ac), was designed, which consists of 5-bromo-4-chloro-3-hydroxyindole **2** linked to Neu5Ac via α glycosidic bond at C(2). Thus, substrate **1** would be hydrolyzed by either natural neuraminidase or mutant enzymes to release a halogenated indol-3-ol **2** that undergoes rapid aerobic oxidation to the dark blue pigment, 5,5'-dibromo-4,-4'-dichloroindigo **3** as shown in Figure 1.[8]

This paper describes the synthesis of X-Neu5Ac **1** and its use as a chromogenic substrate for neuraminidase.

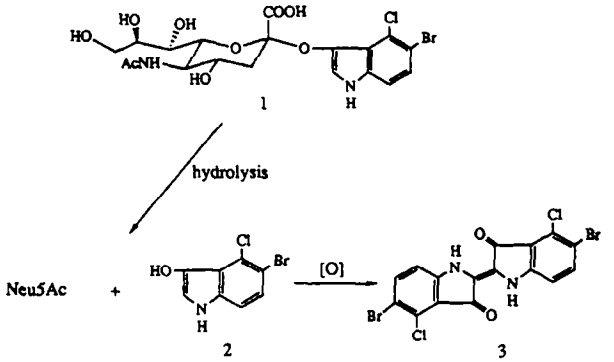

Figure 1. Simplified model for hydrolysis of chromogenic substrate **1**. The hydrolysis product **2** undergoes rapid aerobic oxidation to form indigo blue pigment **3**

Results and Discussion

Synthesis of X-Neu5Ac **1** was achieved from *N*-acetyl-neuraminic acid **4** via six steps using a method similar to that reported by Eschenfelder *et al.*,[9] as outlined in Figure 2. 5-Bromo-4-chloroindol-3-yl-1,3-diacetate was selectively deacetylated at the C(3)-position by treatment with 80% sulfuric acid.[10] The hydroxy groups of the *N*-acetylneuraminic acid were protected as acetates by treatment with acetic anhydride in pyridine, and then the carboxyl group was converted to the methyl ester with diazomethane in 93% yield. The completely protected neuraminic acid was treated with sat. hydrogen chloride in acetyl chloride to give the chloride **5**, which was then reacted with 1-acetyl-5-bromo-4-chloro-3-hydroxyindole in the presence of NaH in dry DMF to afford the corresponding glycoside **6** as the sole product in 41.3% yield. Deacetylation of the glycoside **6** with sodium methoxide in MeOH, followed by saponification with 1.0 M NaOH, gave the title compound **1** in 55% yield, which was purified by high-performance liquid chromatography (HPLC) on an RP-18 column. The α-configuration of the

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indole moiety in **1** was determined by the fact that **1** was cleavable by the neuraminidase as shown later. Furthermore, in the NMR spectrum, it was supported by the chemical shift (δ, 2.82) for the signal of H-3e, that is consistent with the value (δ, 2.8) of α-nitrophenyl glycoside of *N*-acetylneuraminic acid.[9]

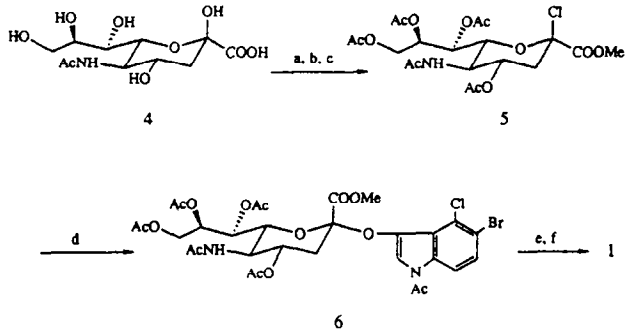

Figure 2. Reagents and conditions: (a) Ac_2O/pyridine; (b) CH_2N_2/ Et_2O; (c) HCl/AcCl; (d) 1-acetyl-5-bromo-4-chloro-3-hydroxyindole/-NaH/DMF; (e) cat. NaOMe/MeOH; (f) 1.0 M aq. NaOH

The hydrolysis of the chromogenic substrate **1** with neuraminidase isolated from *Clostridium perfringens* was examined. Incubation of **1** with the enzyme gave *N*-acetylneuraminic acid along with the blue color pigment of 5,5'-dibromo-4,4'-dichloroindigo, which is shown in a composite, time-dependent, UV-visible absorption spectrum in Figure 3. The reaction rate was assayed by monitoring indigo formation by visible absorption at 620 nm. The reaction consisted of 40 mU neuraminidase and 0.25–4.0 mM substrate in 100 mM potassium acetate, pH 5.5, including 0.03 % bovine serum albumin, and was incubated at 20 °C.[12] The hydrolysis demonstrated saturation kinetics,[13] and a Lineweaver–Burke analysis of the initial rates afforded a K_m value of 0.89 x 10^{-3} M (Figure 4). In the absence of neuraminidase, control experiments showed 0.4 % and 0.08 % spontaneous degradation of **1** at pH 5.5 and 7.0, respectively, after 20 h

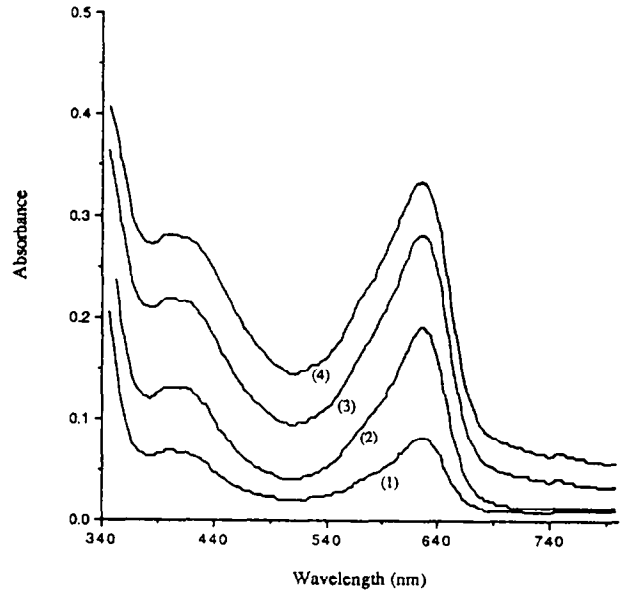

Figure 3. Composite, time-dependent, UV-visible absorption spectra obtained during the hydrolysis of X-Neu5Ac (4 mM) by neuraminidase (40 mU) in 100 mM potassium acetate, pH 5.5, including 0.03 % BSA: reaction time, line (1) 25 min, (2); 60 min, (3); 100 min, (4); 120 min

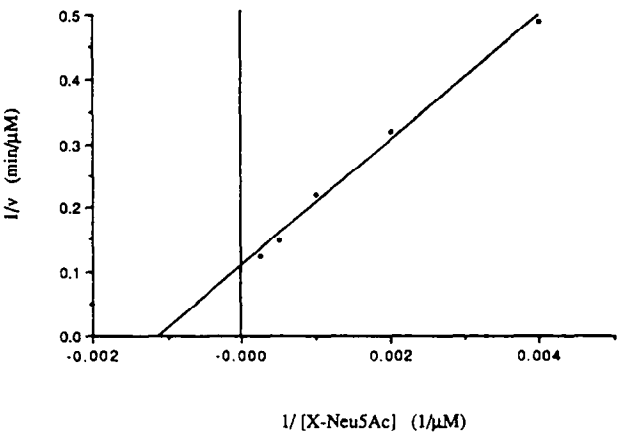

Figure 4. Lineweaver–Burke plot of the hydrolysis of X-Neu5Ac by neuraminidase in 100 mM potassium acetate, pH 5.5, including 0.03% BSA; [X-Neu5Ac] = 0.25, 0.5, 1.0, 2.0, 4.0 mM

at 20 °C. Furthermore, Neu5Ac **1** was resistant to the action of β-galactosidase from *E. coli* when incubated with 3.0 U of the enzyme in 100 mM phosphate, pH 7.0. These results suggest that X-Neu5Ac **1** can be used to screen for bacterially-encoded enzyme production directly on agar plates.

An efficient approach to generating mutant enzymes with altered substrate specificity involves augmenting the rational design of mutagenesis based on protein engineering, X-ray structural analyses or molecular modelings, with direct screens for activity. The development of chromogenic substrates, such as the substrate described here, allows for a simple activity assay of large numbers of mutants at the stage of bacterial expression.

Experimental Section

5-Bromo-4-chloroindol-3-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic acid **1**

To a solution of 1-acetyl-5-bromo-4-chloro-3-hydroxyindole (298 mg, 1.0 mmol)[10] in DMF (2.5 mL) was added sodium hydride (40 mg, 60 % in oil, 1.0 mmol) at 0 °C. Methyl 5-acetoamide-4,7,8,9-tetra-*O*-acetyl-2-chloro-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulo-pyranosoate **5** (533 mg, 1.0 mmol) was added at 0 °C and stirred for 20 h at room temperature. The reaction mixture was concentrated *in vacuo*, and the residue was taken up into AcOEt and succesively washed with 10 % citric acid aqueous solution and water. The organic layer was dried over $MgSO_4$, and was concentrated. The residue was purified by silica gel column chromatography (column: 20 x 450 mm, eluted with toluene–ethyl acetate (1:3)) to give methyl 2-α-(1-acetyl-5-bromo-4-chloroindol-3-yl)-5-acetoamide-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-D-2-nonulopyranosoate **6** (315 mg, 41.3 %). A mixture of **6** (260 mg, 341 μmol) and 28 % sodium methoxide (45 μL) in methanol (15 ml) was stirred for 2 h at room temperature and was neutralized with Amberlyst-15. The mixture was filtered, concentrated and the residue was

dissolved in water (3 mL), after which 1.0 M sodium hydroxide (600 µL) was added. The solution was stirred for 30 min at room temperature and was neutralized with Amberlyst-15. The mixture was filtered, concentrated, and purified by HPLC (Cosmosil 5C18, 10 x 250 mm, acetonitrile/50 mM ammonium acetate buffer, gradient 10–40 % acetonitrile (15 min), flow rate: 3 mL/min, retention time: 15 min). After lyophilization, the remaining ammonium acetate was removed by HPLC (Cosmosil 5C18, 10 x 250 mm, acetonitrile–water, gradient 10–80 % acetonitrile (10 min), flow rate: 3 mL/min). Yield: 100 mg (54.5 %). ^{1}H-NMR (δ, ppm): 1.81 (t, J = 11.9 Hz, 1H), 1.93 (s, 3H), 2.86 (dd, J = 12.3, 4.5 Hz, 1H), 3.45–3.52 (m, 2H), 3.57–3.80 (m, 5H), 7.15 (s, 1H), 7.16 (d, J = 8.8 Hz, 1H), 7.30 (d, J = 8.8 Hz, 1H), HRMS (FAB) calcd for $C_{19}H_{23}O_9Na^{35}Cl^{79}Br$ 537.0276, found 537.0267 $[M+H]^+$

Enzyme Assay

Neuraminidase from *Clostridium perfringens* (1.1 units / mg solid, unit definition was determined with *N*-acetyl neuramin-lactose as substrate) and β-galactosidase from *Escherichia coli* (500 units / mg protein, unit definition was determined with *o*-nitrophenyl-β-D-galactopyranoside as substrate) were obtained from SIGMA, U.S.A. Incubations were conducted at 20 °C in a total volume of 1.0 mL of 100 mM potassium acetate, pH 5.5, containing 0.03 % bovine serum albumin. The reactions were initiated by adding 100 µL of various concentrations of a stock solution of substrate, and were stopped by placing the reaction tubes in a boiling water bath for 1.5 min. After the reaction mixtures were allowed to undergo aerobic oxidation, the formation of indigo blue pigment was measured at 620 nm (ε = 29,700) with visible detection, using a HITACHI 150-20 spectrophotometer. The concentration of 5-bromo-4-chloro-3-hydroxyindole **2** liberated as a result of the enzymatic hydrolysis was estimated by measuring the concentration of indigo formed after complete aerobic oxidation. Initial rates were determined from the first 5–10 % of the reaction for a given range of substrate concentration. The kinetic constants were obtained from the Lineweaver–Burke plots.

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