Enzymatic synthesis of colorimetric substrates to determine α-2,3- and α-2,6-specific neuraminidase activity.

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1. Experimental procedure

1.1 Reagents

5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) was purchased from Carbosynth, Compton, Berkshire, UK.; *S. tiphymorium* neuraminidase, *A. ureafaciens* neuraminidase, CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), fetuin and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich; *C. perfigens* neuraminidase was purchased from New England Biolabs. Inc. Buffer reagents were obtained from Thermo Fisher Scientific, lactose from Acros Organics, LB medium from Foremedium UK.

2. Expression and purification of TcTS and ST6Pd0160

The expression and purification of TcTS was made according to the methodology reported by Ariokaa S, et al.¹ The purified enzyme was concentrated and stored at -80°C until further use.

The expression and purification process for ST6Pd0160 enzyme was carried out as published by Sun et al.² The enzyme was re-suspended in Tris-HCl buffer (20 mM, pH 7.5) containing 10% glycerol, and stored at 4 °C.

2. Sialic acid linkage determination.

The method reported by Alley William R. et al.³ was modified as follows: 40 μ l of 0.5 M DMT-MM in ammonium chloride solution (0.5 M, pH 6.5) were added to a dried sample and the mixture was incubated at 65 °C for 2.5 h. Powdered NaOH was suspended in acetonitrile and washed thoroughly with dimethyl sulfoxide (DMSO) prior further use. The sample was freeze dried, solubilized in 100 μ l of DMSO/MeI/water 70:25:5 and added to NaOH. The mixture was allowed to react for 15 min. The liquid was collected, treated with additional 25 μ l of MeI and reapplied to the NaOH reactor for additional 15 min. The sample was collected in a clean 2 ml tube and the NaOH washed with 2 x 150 μ l acetonitrile. The combined organic solution was washed with 1 ml of 0.5 M NaCl and the product extracted with 400 μ l of chloroform. The chloroform solution was dried under nitrogen flow and the product solubilised in 20 μ l of methanol for MALDI-MS analysis. The sample was mixed with DHB (20 mg/ml, 1:6) spotted onto a MALDI target and let to dry at room temperature. Mass spectra were recorded on a Bruker Ultraflex II MALDI-ToF/ToF mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument was operated with an accelerating voltage of 25 kV in positive reflector mode and mass range 300-1100 Da (Figure S2).

Synthesis and characterization of 5-bromo-4-chloro-3-indolyl N-acetyl-α-neuraminyl-(2→3)-β-Dgalactopyranoside (4)

50 ml of X-Gal (1 mM) in phosphate buffer (50 mM, pH 7) containing fetuin (1.5 mM) were treated with 20 mg of purified TcTS and the mixture was incubated for 4 h at 25 °C. To eliminate unreacted X-Gal, 2.0 ml of *Kluyveromyces lactis* galactosidase (Sigma) were added and the solution was incubated for 15 min at 37 °C. Enzymes and fetuin were removed by ultrafiltration (10 kDa), the filtrate was freeze dried and the product purified by HPLC using Luna C18(2), 100 Å, 250 x 10 mm column with UV detection at 245 nm (Eluents: A – 50 mM ammonium formate, B – acetonitrile, flow rate 4 ml/min, 0-10 min isocratic 10 % B, 10-30 min linear gradient 10 to 30 % B). The final product **4** was characterised by analytical HPLC using Luna C18(2), 100 Å, 250 x 2 mm column with UV detection at 245 nm (Eluents: A – 50 mM ammonium formate pH 4.5, B – acetonitrile, flow rate 0.4 ml/min, 0-10 min isocratic 10 % B, 10-30 min linear gradient 10 to 30 % B). Low-resolution as well as high-resolution mass spectra (HRMS) were obtained with Waters Micromass LCT TOF mass spectrometer (Figure S5-A). HRMS (ESI+); m/z calc for C₂₅H₃₂BrClN₂O₁₄ [M+Na]⁺ 721.0618, found 721.0591.

Synthesis and characterization of 5-bromo-4-chloro-3-indolyl N-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranoside (5)

To a solution of X-Gal (1 mM) and CMP-Neu5Ac (10 mM) in 50 ml Tris/HCl buffer (50 mM, pH 7.5) was added purified ST6Pd0160 (15 mg) and the mixture was incubated for 2 h at 37 °C. To eliminate unreacted X-Gal 2.0 ml of *Kluyveromyces lactis* galactosidase (Sigma) were added and the solution was incubated for additional 15 min at 37 °C. The enzymes were removed by ultrafiltration (10 kDa), the filtrate was freeze dried and the product purified and characterised by HPLC using the same conditions as for **4** (Figure S4-C). Low-resolution as well as high-resolution mass spectra (HRMS) were obtained with Waters Micromass LCT TOF mass spectrometer (Figure S5-B): HRMS (ESI+); m/z calc for $C_{25}H_{32}BrClN_2O_{14}$ [M+Na]⁺ 721.0618, found 721.0641.

5. Neuraminidase activity determination

All reactions were performed in a total volume of 20 μ l. Compound **4** was used as substrate at final concentration of 50 nM, **5** was used at 25 nM. Commercial neuraminidases were used as follow: 1.5 units of *Artrobacter ureafaciens* neuraminidase, 75 U from *Salmonella typhimurium* neuraminidase, 150 U of *Clostridium perfringens* neuraminidase. All samples were incubated according to the manufacturer instructions. The reactions were stopped by heating (100 °C) for different times. 1 mU of *Kluyveromyces lactis* β -galactosidase was added to each reaction and the samples were incubated for 15 min. Absorbance was measured at 655 nm on an infinite M200 microplate reader (TECAN).

Bacillus pumilus and *Arthrobacter aurescens* were grown overnight in 5 ml of LB media at 25 °C. Then the cells were harvested by centrifugation and lysed using Bugbuster lysis solution (Novagen). 10 μ l of soluble extract were used to perform each reaction at 20 μ l final volume in phosphate buffer (50 mM, pH 7.0). β -

galactosidase from of *Kluyveromyces lactis* (1mU) were added to each reaction, the samples were incubated for 15 min at 37 C and absorbance was measured at 655 nm.



Figure S1 MALDI–ToF MS spectra of **3** ($[M+H]^+$ 431.6 m/z) and enzymatically sialylated products **4** and **5** ([M+Na]+ 744.8 m/z).



Figure S2 Linkage determination in 3´SiaLac (Neu5Ac- α 2,3Gal- β 1,4Glc 1) and 6´SiaLac (Neu5Ac- α 2,6Gal- β 1,4Glc 2) after amidation/lactonization followed by permethylation.



Figure S3 Absorbance spectrum of 5,5'-dibromo-4,4'-dichloro-indigo (8).



Figure S4 HPLC trace analysis of X-Gal (A) and sialylated X-Gal 4 (B) and 5 (C).



Figure S5 Mass spectra of sialylated products 4 and 5.

References

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