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α 2–6-Neosialidase: A sialyltransferase mutant as a sialyl linkage-specific sialidase

John B. McArthur¹, Hai Yu¹, Nova Tasnima^{1,&}, Christie M. Lee¹, Andrew J. Fisher^{1,2}, and Xi Chen^{1,*}

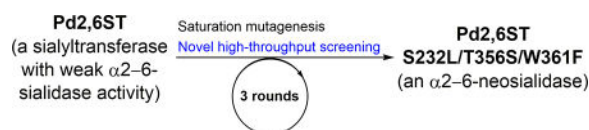
¹Department of Chemistry, University of California, Davis, CA 95616, USA

²Department of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA

Abstract

The lack of α 2–6-linkage specific sialidases limits the structural and functional studies of sialic acid-containing molecules. *Photobacterium damsela* α 2–6-sialyltransferase (Pd2,6ST) was shown previously to have α 2–6-specific, but weak, sialidase activity. Here we develop a high-throughput blue-white colony screening method to identify Pd2,6ST mutants with improved α 2–6-sialidase activity from mutant libraries generated by sequential saturation mutagenesis. A triple mutant (Pd2,6ST S232L/T356S/W361F) has been identified with 100-fold improved activity, high α 2–6-sialyl linkage selectivity, and ability in cleaving two common sialic acid forms *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). It is a valuable tool for sialoglycan structural analysis and functional characterization. The sequential saturation mutagenesis and screening strategy developed here can be explored to evolve other linkage-specific neoglycosidases from the corresponding glycosyltransferases.

Graphical abstract



Keywords

carbohydrate; enzyme engineering; sialic acid; sialidase; sialyltransferase

*Corresponding Author. xiichen@ucdavis.edu.

&Current Address: Department of Public Health, Drinking Water and Radiation Laboratory Branch, Richmond, CA 94805, USA

ORCID

John B. McArthur: 0000-0002-5032-4412

Hai Yu: 0000-0002-4378-0532

Nova Tasnima: 0000-0002-0028-4790

Christie M. Lee: 0000-0002-0212-5486

Andrew J. Fisher: 0000-0003-3488-6594

Xi Chen: 0000-0002-3160-614X

ASSOCIATED CONTENT

Supporting Information. Supplemental table and figures, and NMR spectra for Neu5Ac α 2–6Gal β X (1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

Sialidases are crucial tools for the structural and functional characterization of sialic acid-containing carbohydrates and glycoconjugates, including those presented in cellular extracts and physiological fluids,¹ on cellular surfaces,^{2–4} and in tissues.^{5–8} Sialidase treatment provides a convenient method for determining the presence of sialic acids,⁹ and it is mild enough to be useful for the functional evaluation of sialic acids on sensitive biological samples.¹⁰ For example, glycoproteins treated with a sialidase were rapidly cleared to the liver upon intravenous injection in rabbits, leading to the discovery that terminal sialic acids are critically important to the serum half-life of circulating therapeutic glycoproteins.¹¹ Similarly, α 2–3-selective sialidase treatment of lymphoid organ samples eliminated binding of mouse lymphocytes to the peripheral lymph node high endothelial venules, providing the first evidence that the endogenous ligands of L-selectin contained terminal α 2–3-linked sialic acid.¹² Sialidase treatment has also been used to enhance the immunogenicity of conjugated vaccines prepared from group B *Streptococcus* type V capsular polysaccharide, producing robust protection against lethal challenge by live group B *Streptococcus* in neonatal mice.¹³

Although powerful and broadly useful for the study or modification of carbohydrates, known sialidases possess either specificity towards α 2–3-linked sialic acid or a broad promiscuity towards sialic acid with α 2–3-, α 2–6-, and α 2–8-linkages.¹⁴ For example, commercially available sialidases from *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Vibrio cholerae*, as well as recombinant human cytosolic sialidase hNEU2, *Streptococcus pneumoniae* SpNanA, and *Bifidobacterium infantis* sialidase BiNanH2 can catalyze the cleavage of α 2–3/6/8-linked sialic acid. While commercially available sialidases from *Salmonella typhimurium* and *Streptococcus pneumoniae* SpNanB, and the sialidase activity of multifunctional *Pasteurella multocida* α 2–3-sialyltransferase PmST1 are selectively towards α 2–3-linked sialic acid. All of these sialidases can cleave *N*-acetylneuraminic acid (Neu5Ac, the most common sialic acid form),¹⁵ *N*-glycolylneuraminic acid (Neu5Gc, a non-human sialic acid form),¹⁵ and some of their C-9, C-5, and C-7 derivatives.^{16–22} The lack of α 2–6-linkage specific sialidases in the toolbox limits the functional studies of sialic acid-containing biomolecules. We aim to obtain a highly active α 2–6-linkage-specific sialidase with promiscuity in cleaving various sialic acid forms.

Previously we have shown that several bacterial sialyltransferases including those in the Carbohydrate Active Enzyme (CAZy)²³ glycosyltransferase GT80^{24–26} and GT54²⁷ families display linkage-specific sialidase and donor hydrolysis activities, although such activities were much lower than their glycosyltransferase activities. Recently, Withers et al. showed that these types of sialidase activities require cytidine 5'-monophosphate (CMP) and suggested a two-step mechanism beginning with the cleavage of the sialosidic linkage in the presence of CMP by a reverse sialyltransferase reaction to form CMP-sialic acid, followed by a forward sialyltransferase reaction using water as the acceptor substrate to form CMP and sialic acid (donor hydrolysis²⁶).²⁸ Here, we use enzyme engineering to improve this “neosialidase” activity of *Photobacterium damsela* α 2–6-sialyltransferase (Pd2,6ST) to useful rates while retaining its sialyl-linkage specificity.

RESULTS AND DISCUSSION

Development of a Blue-white Membrane-blot High-throughput Screening Method

To allow easy identification of mutants with improved α 2–6-sialidase activity, a novel blue-white membrane-blot high-throughput screening method was developed. To do this, a 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal or Gal β X)-like α 2–6-sialoside probe Neu5Ac α 2–6Gal β X (**1**) (Scheme 1) was designed and synthesized. The screening works similarly to a plate-based high-throughput method for linkage-specific sialidase substrate specificity studies using *para*-nitrophenyl sialyl galactosides (Sia α 2–3/6/8Gal β pNP) as substrates.^{16, 20} Enzymatic cleavage of the α 2–6-linked sialic acid on the Neu5Ac α 2–6Gal β X probe by active Pd2,6ST mutants expressed in *E. coli* BL21(DE3) cells forms Gal β X (or X-Gal). The terminal galactose (Gal) residue is then rapidly hydrolyzed by endogenous β -galactosidase expressed in *E. coli* BL21(DE3) cells to yield the indole aglycone. This aglycone spontaneously dimerizes and forms a bright blue precipitate (Scheme 1). To avoid potential problems with membrane impermeability of the probe and minimize the amount of the probe used, colonies are not screened directly on agar plates but are instead lifted onto nitrocellulose filters, induced to express the mutant proteins, lysed over chloroform vapors, and screened by soaking the nitrocellulose filter in the Neu5Ac α 2–6Gal β X solution. The ease and high throughput of this assay allow mutant libraries to be screened as quickly as they can be generated. Therefore, each round of mutagenesis ends upon identification of an improved variant, and further mutagenesis is performed on the improved variant to provide libraries for the next round of saturation mutagenesis.

Selection of Mutation Sites Based on Crystal Structures of Sialyltransferases

Considerable structural information is available for GT80 sialyltransferases, including the binary complex structure (PDB ID: 4R84) of 15Pd2,6ST(N) with CMP-3F(*a*)Neu5Ac,²⁹ the ternary complex structure (PDB ID: 2Z4T) of *Photobacterium* sp. JT-ISH-224 α 2–6-sialyltransferase (or 16Psp2,6ST) with CMP and acceptor lactose,³⁰ and the ternary complex structure (PDB ID: 2IHZ) of *Pasteurella multocida* sialyltransferase 1 (24PmST1) with donor analog CMP-3F(*a*)Neu5Ac and lactose.³¹ Analysis of these structures identified four (Asp229, Ser232, Trp361, and Ala403 in the substrate binding site) of the six residues ultimately chosen for mutagenesis (Figure 1). Thr356 and Ile425 were also chosen based on previously described mutants of PmST1 and Pd2,6ST, respectively, with increased sialyltransferase activity.³²

The first two residues targeted for mutagenesis were Asp229, the catalytic aspartate, and Trp361, a tryptophan sitting underneath the lactose and hydrogen bonded to the 7-OH of CMP-3F(*a*)Neu5Ac in PmST1 (PDB ID: 2IHZ). Mutating Asp229 was a test of the proposed mechanism, as any detectable sialidase activity from mutants at this position would be evidence that the proposed catalytic function of Asp229 was incorrect. No improved variants were found from this library. In comparison, several colonies from the W361X library became noticeably blue after approximately 2 hours (Figure S1). All of these colonies were found to have the same W361F mutation.

From the W361F mutant, libraries S232X and A403X were generated. Mutations of Ser232 and the homologous residue in related enzymes have been shown to affect a wide variety of properties including donor hydrolysis and sialidase activities, donor specificity, and acceptor specificity.^{26, 33} Ala403 aligns with PmST1 residue Arg313, which has been found to affect sialidase activity.³⁴ From the A403X library, the colonies that turned blue first were those retaining Ala403. However, in the S232X library, several colonies turned noticeably blue after only 20 minutes (Figure S1). These colonies were sequenced and all were found to have the S232L mutation.

From the S232L/W361F mutant, the next library screened was T356X. Mutations at this site were previously found to improve the sialyltransferase activity of PmST1.³² Interestingly, this site is positioned near the nucleotide binding region of the active site and does not interact with any part of the sialoside. This library was screened at pH 7.0 and with no supplemented CMP in order to slow the reaction down and improve visual detection of the fastest color development. Two colonies turned light blue with overnight incubation and were found to encode the T356S mutation (Figure S1). From the S232L/T356S/W361F mutant, the I425X library was generated. This site was found to also improve sialyltransferase activity of Pd2,6ST in the same work that identified the importance of Thr356.³² However, no improved α 2–6-neosialidase variants were found from this library.

The Effect of CMP and Observation of CMP-Neu5Ac formation

The effect of CMP concentration on Pd2,6ST S232L/T356S/W361F triple mutant α 2–6-neosialidase activity (Figure S2) indicated that the presence of 0.5 mM CMP was close to optimum. At this CMP concentration, the formation of CMP-Neu5Ac as an intermediate during the cleavage of Neu5Ac α 2–6Lac β MU was detected by high resolution mass spectrometry (Figure S3). This provided additional evidence for the two-step, reverse sialylation followed by CMP-sialic acid hydrolysis process proposed for the sialidase activity of GT80 family multifunctional sialyltransferases.²⁸

The pH Profile of Pd2,6ST S232L/T356S/W361F Neosialidase

The pH profile study of the neosialidase activity of the Pd2,6ST S232L/T356S/W361F mutant was carried out using Neu5Ac α 2–6Lac β MU as the substrate (Figure 2). The optimal pH was found to be between 5.2 and 6.0. This agreed with previous pH profiles of sialyltransferase-catalyzed sialidase activity,²⁴ suggesting that the engineering process did not significantly alter the optimal pH.

Kinetics Studies

Three Pd2,6ST mutants including W361F, S232L/W361F, and S232L/T356S/W361F were kinetically characterized for neosialidase activity using Neu5Ac α 2–6Lac β MU as the substrate (Table 1). The use of this probe with a different aglycon was a precaution to avoid mistaking improved recognition of the indole in **1** for improved neosialidase activity.³⁶ Gratifyingly, the Pd2,6ST S232L/T356S/W361F triple mutant displayed 100-fold improved α 2–6-sialidase activity compared to the wild-type enzyme. The high activity of the Pd2,6ST triple mutant was due to almost entirely an increase in k_{cat} . However, the kinetic constants for the intermediate mutants show that each mutation had a greatly different effect on k_{cat}

and K_M . The W361F mutation resulted in a 2.35-fold increase in sialidase activity via a decrease in k_{cat} but a larger decrease in K_M . Addition of the S232L mutation had little effect on K_M but greatly enhanced k_{cat} and provided the largest single-round gain in activity. The additional T356S mutation provided another large gain for k_{cat} but also increased the K_M nearly to that of the wild-type enzyme. Relative to the activity of human NEU2 (hNEU2),^{17, 20} an α 2–3/6/8-sialidase, the Pd2,6ST S232L/T356S/W361F neosialidase displayed nearly 22-fold higher activity on a similar Neu5Aca2–6-containing probe. The donor hydrolysis activity of the triple mutant was found to have increased 341-fold from the wild-type (Table 2).

The Substrate Specificity of Pd2,6ST S232L/T356S/W361F Neosialidase

The sialidase substrate specificity of the Pd2,6ST S232L/T356S/W361F triple mutant was investigated by high-performance liquid chromatography (HPLC) analysis using probes containing varied sialyl linkages (α 2–3/6/8), different sialic acid forms including Neu5Ac, Neu5Gc, and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn), and various internal glycans (Gal β pNP and GalNAc β pNP). The Pd2,6ST S232L/T356S/W361F triple mutant was selective towards α 2–6-linked sialic acid while retaining some promiscuity to the sialic acid form and internal glycan (Figure 3). For example, among Neu5Aca2–3/6Gal β pNP (compounds **2** and **3**) and Neu5Aca2–8Neu5Aca2–3Gal β pNP (compound **4**) tested, only Neu5Aca2–6Gal β pNP (**2**) was a suitable sialidase substrate for the Pd2,6ST S232L/T356S/W361F. Activities towards Neu5Aca2–3Gal β pNP (**3**) and Neu5Aca2–8Neu5Aca2–3Gal β pNP (**4**) were 400-fold and 303-fold, respectively, lower than Neu5Aca2–6Gal β pNP (**2**) (Table S1), suggesting the high selectivity of the Pd2,6ST-derived neosialidase towards α 2–6-sialyl linkage. The triple mutant was able to cleave α 2–6-linked Neu5Gc in Neu5Gca2–6Gal β pNP (**6**) at 12% of the activity of Neu5Aca2–6Gal β pNP (**2**), although its sialidase activity towards Kdn α 2–6Gal β pNP (**5**) containing an α 2–6-linked Kdn was 1,020-fold lower than **2**. Quite interestingly, the neosialidase activity of the Pd2,6ST S232L/T356S/W361F triple mutant was 1.9-fold higher towards Neu5Aca2–6GalNAc β pNP (**7**) than for Neu5Aca2–6Gal β pNP (**2**).

Recognition of Egg Yolk Sialoglycopeptide

To demonstrate the utility of Pd2,6ST S232L/T356S/W361F towards more complex glycoconjugates, the neosialidase was tested against egg yolk sialoglycopeptide, a hexapeptide with a biantennary complex-type N-linked glycan containing α 2–6-linked sialic acid on each antenna. Detection of the desialylated glycopeptide by HPLC (Figure S4) and high resolution mass spectrometry (Figure S5) confirmed that the engineered neosialidase can recognize and cleave α 2–6-linked sialic acid from complex sialylated glycoconjugates.

Concluding Remarks

The reprogramming of natural enzymes for non-natural functions is an important area of interest for enzyme engineering.³⁷ By exploiting the reversibility of glycosyltransferase activity and the evolvability of glycosyltransferase substrate promiscuity, we have demonstrated that glycosyltransferases can be conveniently engineered into efficient neoglycosidases with specificities not known to exist in nature. This strategy will likely

provide a valuable source of new enzymes to supplement known exoglycosidases, particularly for the selective cleavage of sugar residues from natural product glycosides or complex carbohydrates.

The Pd2,6ST-derived neosialidase developed here catalyzes the removal of sialic acid with high selectivity towards α 2–6-linkages and promiscuity towards both Neu5Ac and Neu5Gc via a mechanism different from all known sialidase mechanisms. The engineered mutant will be a valuable addition to glycobiology, assisting in the elucidation of sialoglycan structure and function.

We are pleasantly surprised to discover three beneficial mutations across just six investigated residues within the active site of Pd2,6ST. This implies that the sialyltransferase activity of the enzyme is quite robust towards active site mutations and that the discrimination of nucleophilic water is quite sensitive to mutations. However, the Pd2,6ST triple mutant did not display the expected α 2–6-sialidase activity towards Kdn even though Pd2,6ST was efficient in synthesizing Kdn α 2–6-containing sialosides in high yield.³⁸ This data suggests that the mutations that improve neosialidase towards Neu5Ac-containing probe Neu5Ac α 2–6Gal β X (1) may have also altered the substrate specificity towards Kdn-containing compound Kdn α 2–6Gal β pNP (5).

The blue-white screening method used for the neosialidase engineering can be easily modified for the engineering of other neoglycosidases using suitable X-based probes. The throughput and simplicity of this method make the engineering of neoglycosidases practical for non-specialists in the absence of expensive equipment such as automated liquid handling systems and microplate reader spectrophotometers. It is particularly convenient that the disaccharide-X probes can be synthesized from commercially available monosaccharide-X building blocks using wild-type glycosyltransferase activity.

In conclusion, Pd2,6ST S232L/T356S/W361F neosialidase has been generated by sequential saturation mutagenesis and screening using a high-throughput blue-white colony assay. This triple mutant displays over 100-fold improved sialidase catalytic efficiency relative to the wild-type enzyme while retaining linkage selectivity of the wild-type sialyltransferase activity. The mutant can catalyze the cleave of α 2–6-linked sialic acid in egg yolk sialoglycopeptide efficiently. This enzyme is a useful new tool for studying the structure and function of sialoglycans, and the engineering strategy may be proven useful to researchers interested in obtaining enzymes with glycosidase specificities not already known to exist in nature.

METHODS

Materials

Chemicals were purchased and used as received. NMR spectra were recorded in the NMR facility of the University of California, Davis, on a Bruker Avance-800 NMR spectrometer (800 MHz for ^1H , 200 MHz for ^{13}C). Chemical shifts are reported in parts per million (ppm) on the δ scale. High resolution (HR) electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry

Facility in the University of California, Davis. *N*-Acetylneuraminic acid (Neu5Ac) was from Inalco (Italy). Cytosine 5'-triphosphate (CTP) was purchased from Hangzhou Meiya Pharmaceutical Co. Ltd. X-Gal was purchased from Sigma. Egg yolk sialoglycopeptide was purchased from TCI America. Neu5Ac α 2-6Gal β pNP (2), Neu5Ac α 2-3Gal β pNP (3),¹⁶ Kdn α 2-6Gal β pNP (5),¹⁶ Neu5Gc α 2-6Gal β pNP (6),¹⁶ and Neu5Ac α 2-6GalNAc β pNP (7),¹⁶ Neu5Ac α 2-8Neu5Ac α 2-3Gal β pNP (4),²⁰ and Neu5Ac α 2-6Lac β MU²⁴ were synthesized as described previously. *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)³⁹ and *Photobacterium species* α 2-6-sialyltransferase (Psp2,6ST)⁴⁰ were expressed and purified as reported previously.

Mutagenesis

Pd2,6ST libraries were constructed using either the Q5 Mutagenesis Kit (D229X and W361X) or the QuikChange II Site Directed Mutagenesis kit using the following primers: D229X_f: NNKGGTTCTTCTGAATATGTAAGTTTATATCAATGG, D229X_r: ATCATACAACTAATATGAGAAATTTTCACCTTCTCG, S232X_f: 5' AATTTCTCATATTAGTTTGTATGATGATGGTTCTNNKGAATATGTAAGTTTATATCAA TGGAAAGATACAC 3', S232X_r: 5' GTGTATCTTTCCATTGATATAAACTTACATATTCMNNAGAACCATCATCATACAAAC TAATATGAGAAATT 3', T356X_f: 5' ACAATATCACAATCCCCACTACCAAACCTTTATTTTTNNKGGCACAACAACCTTTG CTG 3', T356X_r: 5' CAGCAAAAGTTGTTGTGCCMNNAAAATAAAGTTTGGTAGTGGGGATTGTGAATA TTGT 3', W361X_f: 5' NNKGTGGGGGGGAAACG 3', W361X_r: 5' AGTTGTTGTGCCGTAAAAATAAAGTTTGG 3', A403X_f: 5' GACTACGATCTATTTTTCAAGGGGCATCCTNNKGGTGGCGTTATTAACG 3', A403X_r: 5' CGTTAATAACGCCACCMNNAGGATGCCCCCTTGAAAAATAGATCGTAGTC 3', I425X_f: 5' TGATATGATCAATATCCAGCCAAGNNKTCATTTGAGGTCTTGATGATGACGG 3', and I425X_r: 5' CCGTCATCATCAAGACCTCAAATGAMNNCTTGGCTGGAATATTGATCATATCA 3'. The assembled DNA was transformed into *E. coli* 10 G electrocompetent cells (Lucigen). Ten percent of the transformed cells were plated on LB agar plates supplemented with ampicillin in order to determine the number of total transformants. The remaining transformed cells were diluted into fresh LB media (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ NaCl) supplemented with ampicillin, grown overnight at 37 °C 250 rpm, and the plasmid DNA was isolated. This DNA was transformed into homemade chemically competent *E. coli* BL21(DE3) cells.

One-pot Two-enzyme synthesis of Neu5Ac α 2-6Gal β X (1)

A reaction mixture in a total volume of 20 mL containing Tris-HCl buffer (100 mM, pH 8.5), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranosides (X-Gal, 50 mg, 0.122 mmol), Neu5Ac (57 mg, 0.184 mmol), CTP (97 mg, 0.184 mmol), DMF (7%), MgCl₂ (20 mM), NmCSS³⁹ (2.5 mg), and Psp2,6ST⁴⁰ (4.0 mg) were incubated in a shaker at 30 °C for 18 h. The reaction was stopped by adding 20 mL of 95% ethanol followed by incubation at 4 °C

for 30 minutes. After centrifugation, the supernatant was concentrated and purified using a C18 column on a CombiFlash Rf 200i system eluted with a gradient of 0–100% acetonitrile in water for 20 minutes and a 30 mL min⁻¹ flow rate. The fractions containing the desired product were collected and dried to give Neu5Aca2–6GalβX as a white powder (81 mg, 92%). ¹H NMR (800 MHz, MeOD) δ 7.18 (d, *J* = 8.8 Hz, 1 H), 7.14 (bs, 1 H), 7.04 (d, *J* = 8.8 Hz, 1 H), 4.58 (d, *J* = 8.0 Hz, 1 H), 3.93–3.40 (m, 11 H), 2.77 (d, *J* = 12.8 and 4.8 Hz, 1 H), 1.92 (s, 3 H), 1.53 (t, *J* = 12.0 Hz, 1 H); ¹³C NMR (200 MHz, MeOD) δ 173.96, 173.13, 136.65, 133.36, 125.39, 123.78, 117.98, 113.36, 111.81, 111.11, 104.08, 100.40, 73.96, 73.37, 72.93, 71.52, 71.17, 68.84, 68.49, 68.08, 62.98, 62.42, 52.66, 41.18, 21.14. HRMS (ESI) *m/z* calcd for C₂₅H₃₁BrClN₂O₁₄ [M–H]⁻ 697.0653, found 697.0609.

Library Screening

Mutant libraries were transformed to BL21(DE3) chemically competent cells and plated on LB-agar plates supplemented with ampicillin. Following overnight incubation at 37 °C, colonies were lifted onto 0.45 μm 47 mm Mixed Cellulose Esters Surfactant-Free Membrane Filters (Millipore). These nitrocellulose filters were carefully placed colony-side up on LB-agar plates supplemented with ampicillin and 0.1 mM IPTG, and these plates were incubated for 3 hours at 37 °C. Meanwhile, the original LB-agar plates were incubated for 3–5 hours at 37 °C until the colonies regrew and then stored at 4 °C as master plates. The filters were then suspended over chloroform vapors for 10 minutes, briefly air dried, and were placed colony-side up on 55 mm Whatman filter paper soaked with 0.5 mL of the assay solution. For the first two rounds, the assay solution contained 3 mM Neu5Aca2–6GalβX, 0.5 mM CMP, 100 mM MES pH 5.5, and MgCl₂ (10 mM). For the third and fourth rounds, the assay solution contained Neu5Aca2–6GalβX (3 mM), Tris-HCl (pH 7.0, 100 mM), and MgCl₂ (10 mM). Reactions were conducted at 37 °C with regular examination of the filters for the development of blue color.

Overexpression and Purification

Flasks containing 1 L of autoclaved LB media supplemented with ampicillin (100 μg mL⁻¹) were inoculated with 1 mL of overnight cultured *E. coli* BL21(DE3) cells harboring the mutant plasmids. The 1 L cultures were grown at 37 °C until OD_{600 nm} reached 0.6 to 1.0, then expression was induced with isopropyl β-D-1-thiogalactoside (IPTG) to a final concentration of 0.1 mM and the cells shaken at 20 °C overnight. Cells were harvested in a Sorvall Legend RT centrifuge at 4000 rpm for 30 minutes, resuspended in 20 mL of Tris-HCl (pH 7.5, 100 mM) and lysed by sonication with the following method: amplitude at 65%, 10 s pulse on and 20 s pulse off for 18 cycles. The lysate was collected after centrifugation at 8000 rpm for 30 minutes and then loaded onto a Ni²⁺-NTA affinity column at 4 °C that was pre-equilibrated with 6 column volumes of binding buffer (50 mM Tris-HCl buffer, pH 7.5, 10 mM imidazole, 0.5 M NaCl). The column was washed with 10 column volumes of binding buffer and 10 column volumes of washing buffer (50 mM of Tris-HCl buffer, pH 7.5, 50 mM of imidazole, 0.5 M of NaCl) sequentially to wash away the nonspecific binding protein. The target protein was eluted using Tris-HCl buffer (50 mM, pH 7.5) containing 200 mM of imidazole and 0.5 M NaCl. Fractions containing the purified protein were combined and dialyzed against Tris-HCl buffer (20 mM, pH 7.5) supplemented

with 10% glycerol. The enzyme solutions were aliquoted, flash frozen in liquid N₂, and stored at -20 °C.

Neosialidase Kinetics

Reactions were performed in duplicate at 37 °C for 10 to 30 minutes with Tris-HCl (100 mM, pH 6.0), MgCl₂ (10 mM), CMP (0.5 mM), enzyme (7.0 μM Pd2,6ST W361F, 0.32 μM Pd2,6ST S232L/W361F, 0.070 μM Pd2,6ST S232L/T356S/W361F), and varying concentrations (0.5, 1.0, 2.0, and 5.0 mM) of Neu5Acα2-6LacβMU. Reactions were stopped by adding an equal volume of pre-chilled methanol. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed with a P/ACE™ MDQ capillary electrophoresis (CE) system equipped with a UV-Vis detector (Beckman Coulter, Fullerton, CA). The CE procedure utilized a 75 μm i.d. capillary, 25 KV/80 μA, 5 s vacuum injections, was monitored at 315 nm, and used sodium tetraborate (25 mM, pH 9.4) buffer as the running buffer. The apparent kinetic parameters were obtained by fitting the experimental data from duplicate assays into the Michaelis-Menten equation using Grafit 5.0.

Donor Hydrolysis Kinetics

Reactions were performed in duplicate at 37 °C for 10 to 30 minutes with Tris-HCl (100 mM, pH 8.5), MgCl₂ (10 mM), enzyme (0.030 μM Pd2,6ST S232L/T356S/W361F), and varying concentrations (2.0, 5.0, 10.0, and 20.0 mM) of CMP-Neu5Ac. Reactions were stopped by adding an equal volume of pre-chilled methanol. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed with a P/ACE MDQ capillary electrophoresis (CE) system equipped with a UV-Vis detector (Beckman Coulter, Fullerton, CA). The CE procedure utilized a 75 μm i.d. capillary, 25 KV/80 μA, 5 s vacuum injections, was monitored at 254 nm, and used sodium tetraborate (25 mM, pH 9.4) buffer as the running buffer. The apparent kinetic parameters were obtained by fitting the experimental data from duplicate assays into the Michaelis-Menten equation using Grafit 5.0.

pH Profile

Reactions were performed in duplicate at 37 °C for 30 minutes with a suitable buffer (100 mM MES from pH 4 to 6 or 100 mM Tris-HCl from pH 6.5 to 8.5), MgCl₂ (10 mM), Neu5Acα2-6LacβMU (1 mM), and CMP (0.5 mM). Reactions were stopped by adding an equal volume of pre-chilled methanol. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed with an Infinity 1290-II HPLC equipped with a UV-Vis detector (Agilent Technologies, CA). The HPLC procedure utilized a ZORBAX Eclipse Plus C18 Rapid Resolution HD 1.8 μm particle 2.1 × 50 mm column (Agilent Technologies, CA), an isocratic flow of 1 mL min⁻¹ for a 9% acetonitrile and 91% aqueous solution containing 0.1% TFA, and an injection volume of 2 μL. The 4-methylumbelliferone absorbance signal was monitored at 315 nm.

CMP Concentration Effect Assays

Reactions were performed in duplicate at 37 °C for 30 minutes in MES buffer (100 mM, pH 6.0) containing MgCl₂ (10 mM), Neu5Acα2-6LacβMU (1 mM), CMP with a concentration varying from 0.1 mM to 25.0 mM (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 25.0 mM), and Pd2,6ST S232L/T356S/W361F (0.130 μM). Reactions were stopped by adding an equal volume of pre-chilled methanol. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed using an Infinity 1290-II HPLC equipped with a UV-Vis detector (Agilent Technologies, CA). The HPLC procedure utilized a ZORBAX Eclipse Plus C18 Rapid Resolution HD 1.8 μm particle 2.1 × 50 mm column (Agilent Technologies, CA), an isocratic flow of 1 mL min⁻¹ for a 9% acetonitrile and 91% aqueous solution containing 0.1% TFA, and an injection volume of 2 μL. The 4-methylumbelliferone absorbance signal was monitored at 315 nm.

Desialylation of Egg Yolk Sialoglycopeptide

Reactions were performed at 37 °C for 60 minutes with MES buffer (100 mM, pH 6.0), MgCl₂ (10 mM), CMP (0.5 mM), Pd2,6ST S232L/T356S/W361F (0.0 or 13.0 μM), and egg yolk sialoglycopeptide (1 mM). Reactions were stopped by thermal denaturation of the enzyme at 60 °C for 10 minutes. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Chromatographic separation and detection were achieved with an Infinity 1290-II HPLC equipped with a UV-Vis detector (Agilent Technologies, CA). The HPLC procedure utilized a ZORBAX Bonus-RP Rapid Resolution HD 1.8 μm particle 2.1 × 100 mm column (Agilent Technologies, CA), a gradient flow of 0.7 mL min⁻¹ of 0.3 to 8% acetonitrile over 6 minutes in aqueous solution containing 0.1% TFA, and an injection volume of 1 μL. The peptide bond absorbance signal was monitored at 214 nm. High resolution (HR) electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis.

Linkage Specificity Assays for the Pd2,6ST Mutants

Reactions were performed in duplicate at 37 °C for 30 minutes in MES buffer (100 mM, pH 6.0), MgCl₂ (10 mM), CMP (0.5 mM), and 1 mM substrate. Enzyme concentrations were 0.030 μM for **2**, 0.30 μM for **7**, 3.0 μM **6**, and 30.0 μM for **3–5**. These conditions provided testing at initial rates (1.2–24% yield) for each substrate. Reactions were stopped by adding an equal volume of pre-chilled methanol. The mixtures were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed with an Infinity 1290-II HPLC equipped with a UV-Vis detector (Agilent Technologies, CA). The HPLC procedure utilized a ZORBAX Eclipse Plus C18 Rapid Resolution HD 1.8 μm particle 2.1 × 50 mm column (Agilent Technologies, CA), an isocratic flow of 1 mL min⁻¹ for a 9% acetonitrile and 91% aqueous solution containing 0.1% TFA, and an injection volume of 2 μL. The *para*-nitrophenyl absorbance signal was monitored at 315 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CE	capillary electrophoresis
CMP	cytidine 5'-monophosphate
CTP	cytosine 5'-triphosphate
ESI	electrospray ionization
Kdn	2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
IPTG	isopropyl β -D-1-thiogalactoside
Neu5Ac	<i>N</i> -acetylneuraminic acid
Neu5Gc	<i>N</i> -glycolylneuraminic acid
NmCSS	<i>Neisseria meningitidis</i> CMP-sialic acid synthetase
Pd2,6ST	<i>Photobacterium damsela</i> α 2-6-sialyltransferase
PmST1	<i>Pasteurella multocida</i> sialyltransferase 1 (24PmST1)
pNP	<i>para</i> -nitrophenyl
NMR	nuclear magnetic resonance
ppm	parts per million
Psp2,6ST	<i>Photobacterium species</i> α 2-6-sialyltransferase
Sia	sialic acid
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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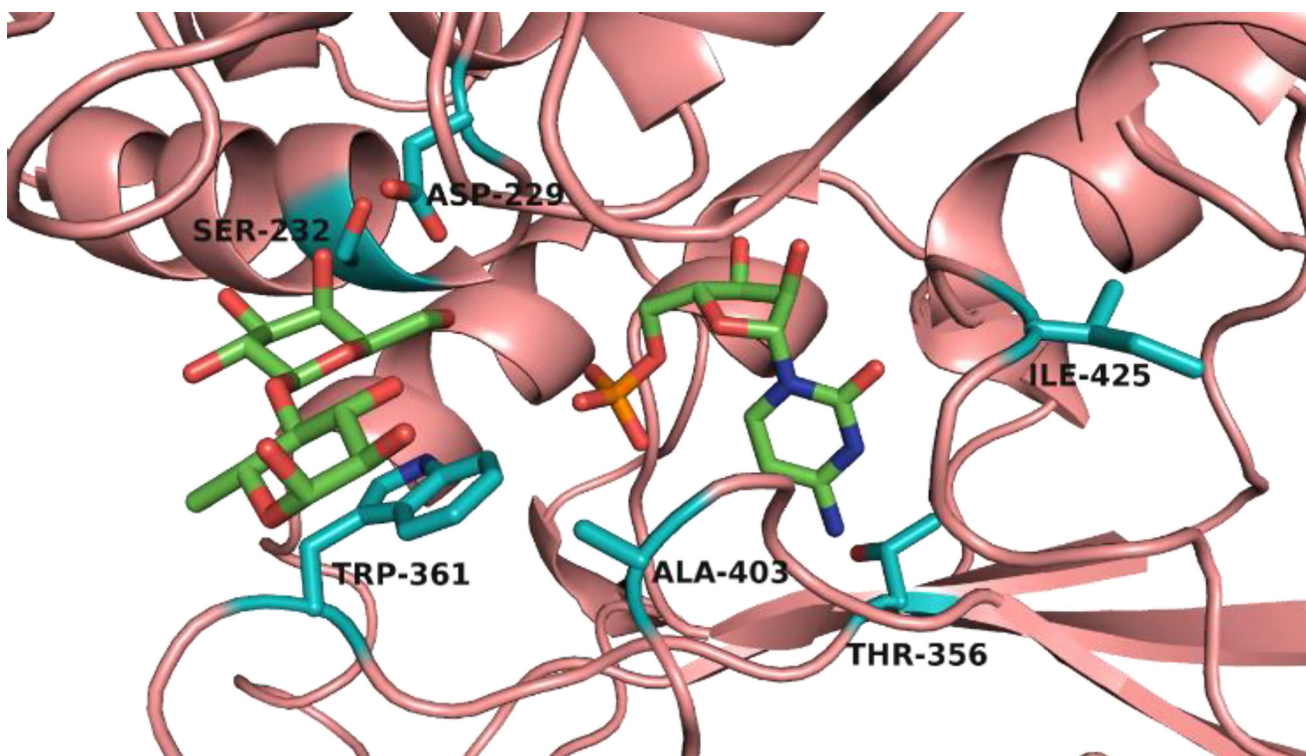


Figure 1. The substrate binding site of 15Pd2,6ST(N) structure modelled based on the co-crystal structure of 16Psp2,6ST (PDB ID: 2Z4T) with CMP and lactose (represented with green-colored carbons). Structural modelling was performed with SWISS-MODEL. The six sites chosen for mutagenesis are represented with teal-colored carbons.

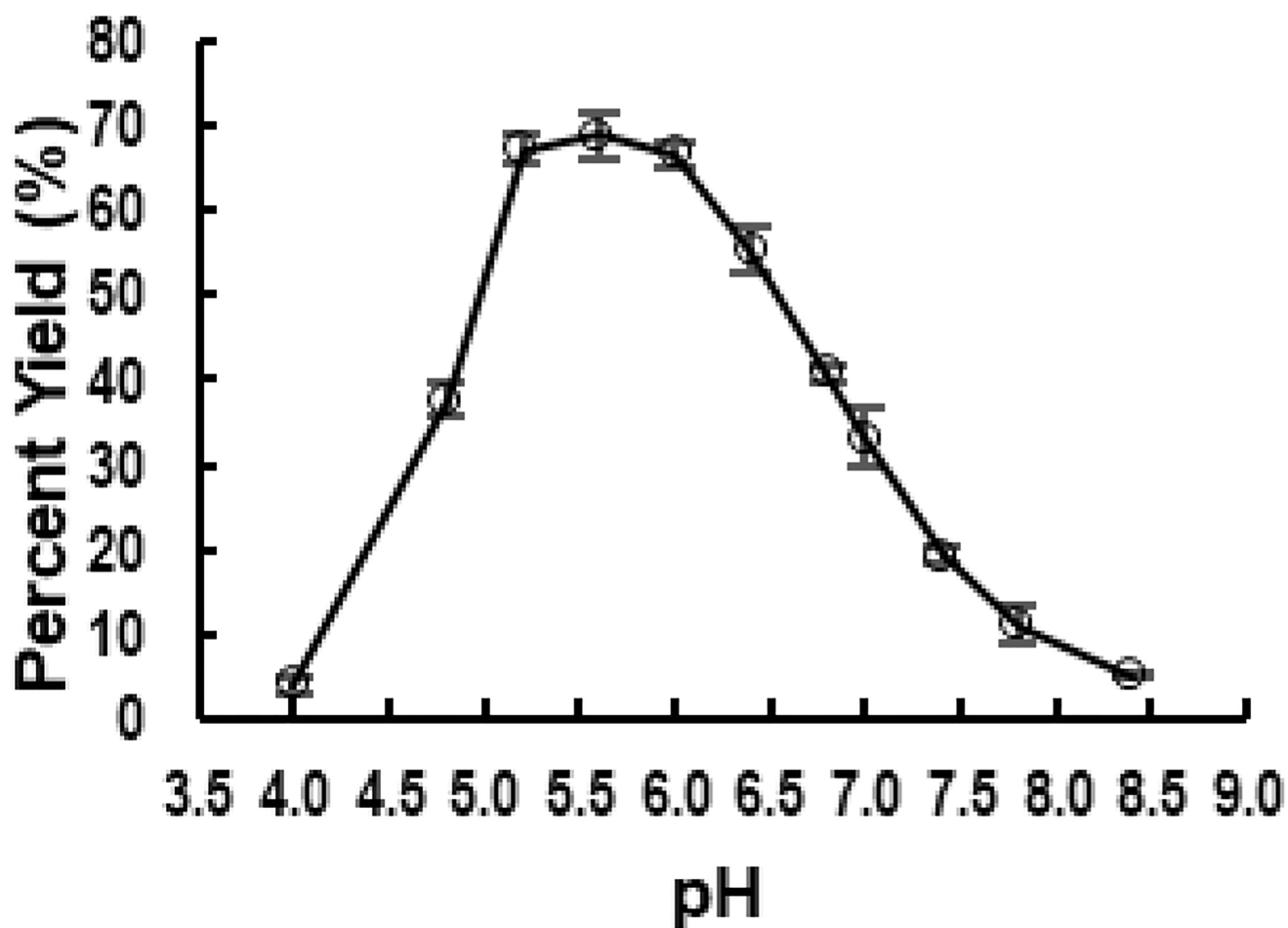


Figure 2.
The pH-profile of Pd2,6ST S232L/T356S/W361F neosialidase.

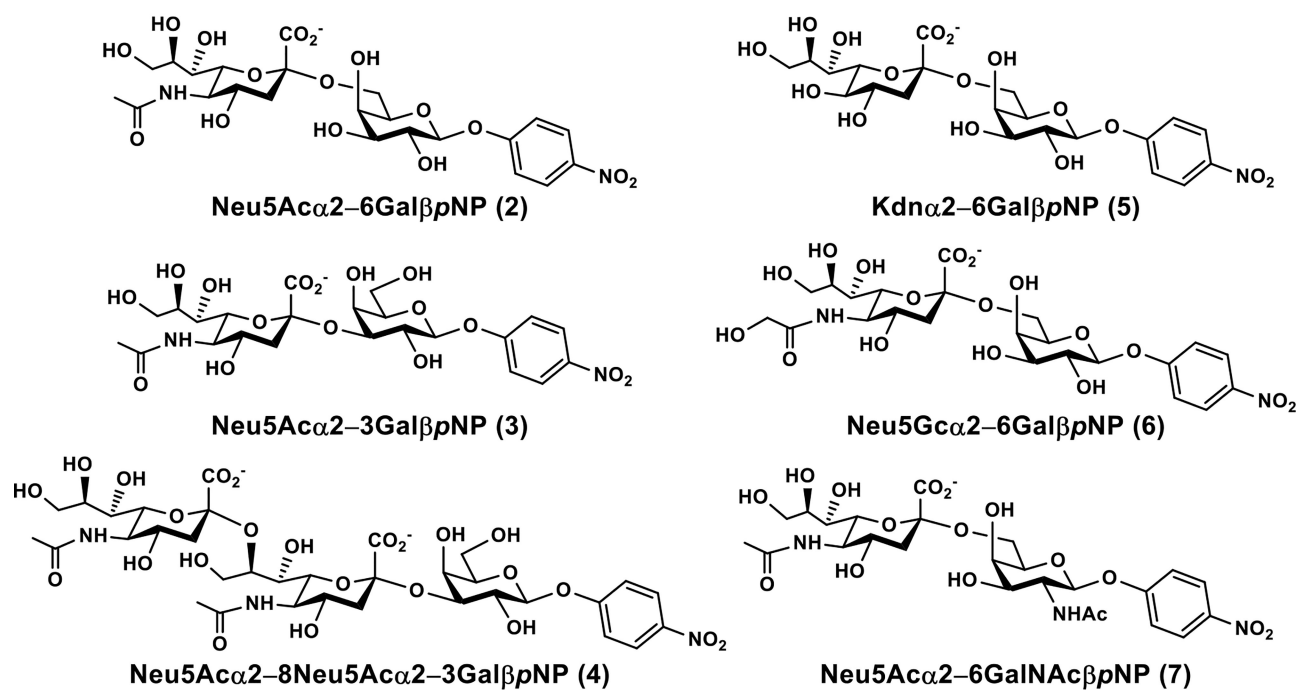
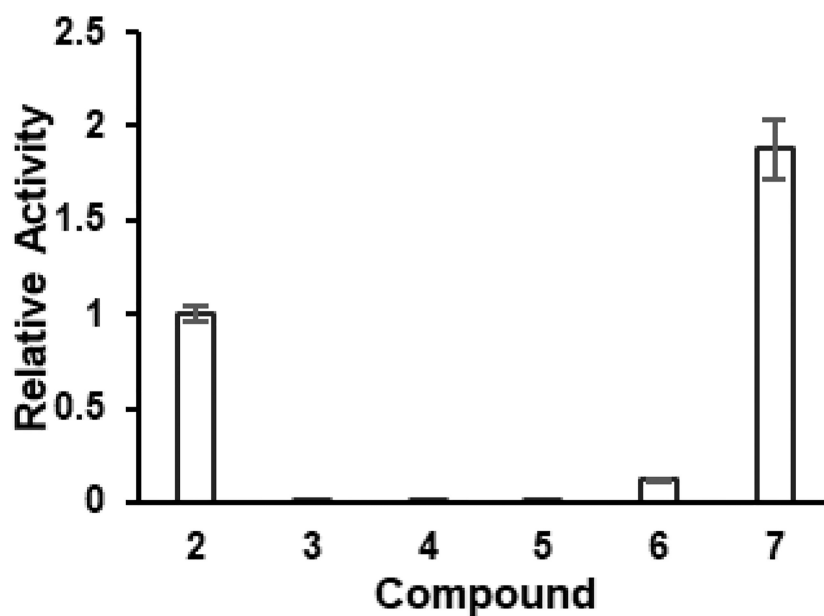
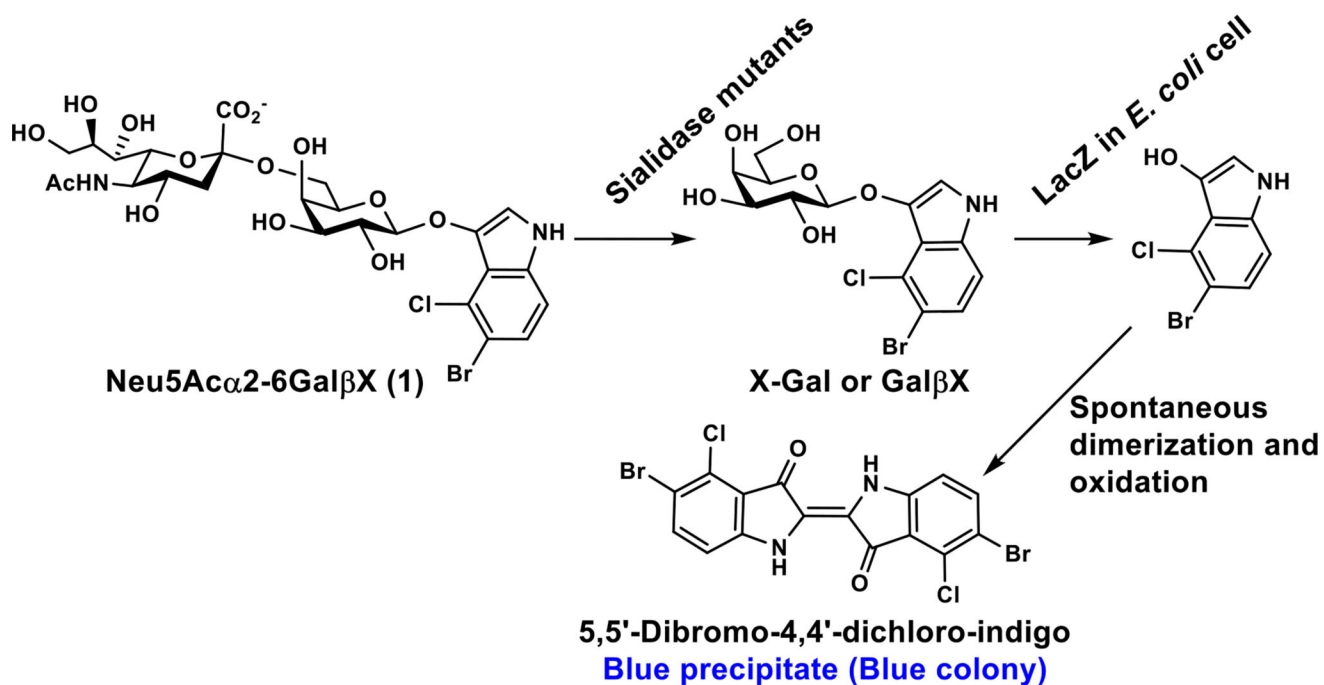


Figure 3.

Relative activities of Pd2,6ST S232L/T356S/W361F towards sialosides (2–7) with various sialic acid forms, internal glycans, and sialyl linkages. Error bars represent standard deviations from duplicated assay results.

**Scheme 1.**

High-throughput α 2-6-sialidase activity screening for LacZ-containing *E. coli* cells expressing sialidase mutants.

Table 1

Kinetic parameters for Pd2,6ST mutant neosialidase activity in the presence of 0.5 mM CMP.

Enzymes and mutants	$k_{cat}(\text{min}^{-1})$	$K_M(\text{mM})$	k_{cat}/K_M ($\text{min}^{-1}\text{mM}^{-1}$)
Pd2,6ST ^[a]	8.2 ± 0.3	7.6 ± 0.5	1.1
Pd2,6ST W361F	2.5 ± 0.2	1.0 ± 0.3	2.6
Pd2,6ST S232L/W361F	84 ± 4	1.1 ± 0.1	76
Pd2,6ST S232L/T356S/W361F	$(7.0 \pm 0.2) \times 10^2$	6.3 ± 0.2	1.1×10^2
hNEU2 ^[b]	10.8 ± 0.6	2.1 ± 0.2	5.1

^[a]Reported previously.²⁴^[b]Reported previously using Neu5Ac α 2-6Gal β pNP as the substrate.¹⁷

Table 2

Kinetic parameters for the CMP-Neu5Ac hydrolysis activities of Pd2,6ST and Pd2,6ST S232L/T356S/W361F neosialidase.

Enzymes and mutants	$k_{cat}(\text{min}^{-1})$	$K_M(\text{mM})$	k_{cat}/K_M ($\text{min}^{-1}\text{mM}^{-1}$)
Pd2,6ST ^[a]	$(4.0\pm 0.9)\times 10^2$	45±14	8.8
Pd2,6ST S232L/T356S/W361F	$(1.1\pm 0.01)\times 10^4$	3.7±0.1	3.0×10^3

^[a]Reported previously.³⁵