Supporting Information

Table of Contents

Table S1. Relative sialidase activities of Pd2,6ST S232L/T356S/W361F towards different sialosides.

Compound number	Compound abbreviation	Relative sialidase activity
2	Neu5Acα2–6Gal*β*pNP	1.0
3	Neu5Acα2–3Gal*β*pNP	2.5×10^{-3}
4	Neu5Acα2–8Neu5Acα2–3Gal*β*pNP	3.3×10^{-3}
5	Kdnα2–6Gal*β*pNP	9.8×10^{-4}
6	Neu5Gcα2–6Gal*β*pNP	1.2×10^{-1}
7	Neu5Acα2–6GalNAc*β*pNP	1.9

Figure S1. Sequential saturation mutagenesis and blue-white colony screening of Pd2,6ST mutants for enhanced α2–6-sialidase activity.

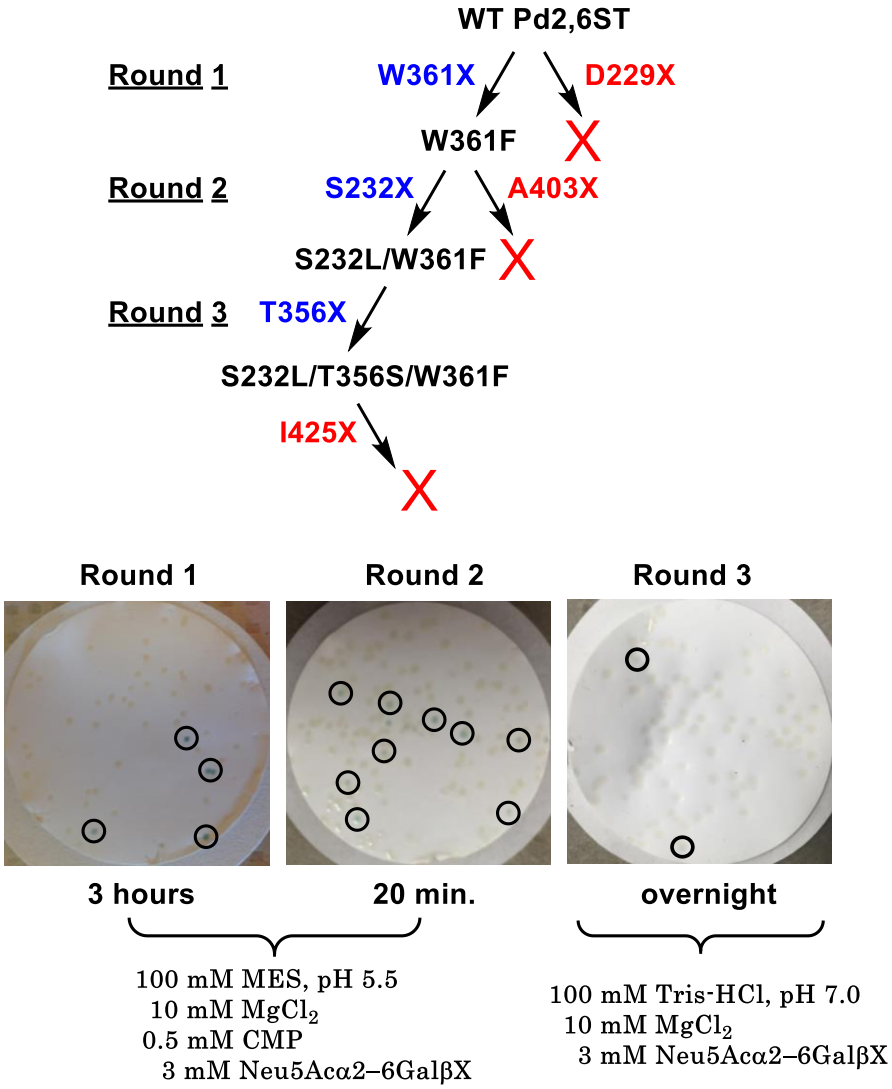


Figure S2. Effect of CMP concentration (0.1, 0.2, 0.5, 1, 2, 5, 10, and 25 mM) on Pd2,6ST S232L/T356S/W361F neosialidase reaction rate.

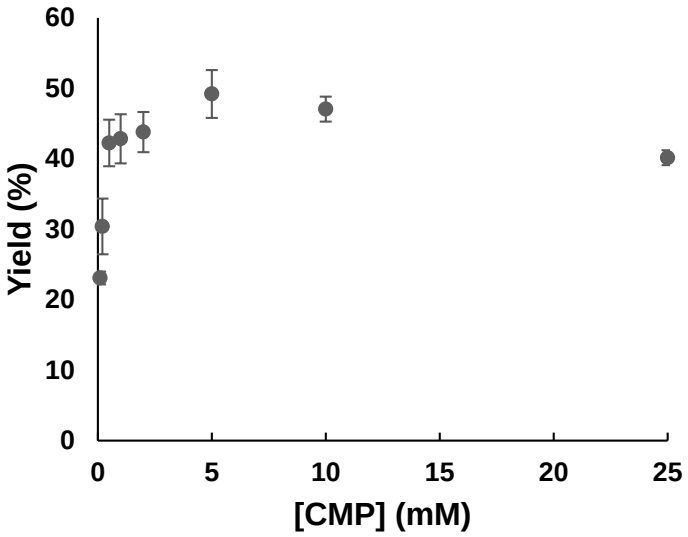


Figure S3. A) HRMS detection of intermediate CMP-Neu5Ac formation by the neosialidase activity of Pd2,6ST S232L/T356S/W361F. **B)** and **C)** are enlarged m/z regions in **A)**. Signals for M-1 molecular ions of Neu5Ac (Calcd. 308.0987, found 308.0976), CMP (Calcd. 322.0440, found 322.0435), CMP-Neu5Ac (Calcd. 613.1400, found 613.1356), and Neu5Acα2–6LacβMU (Calcd. 790.2411, found 790.2372) were observed.

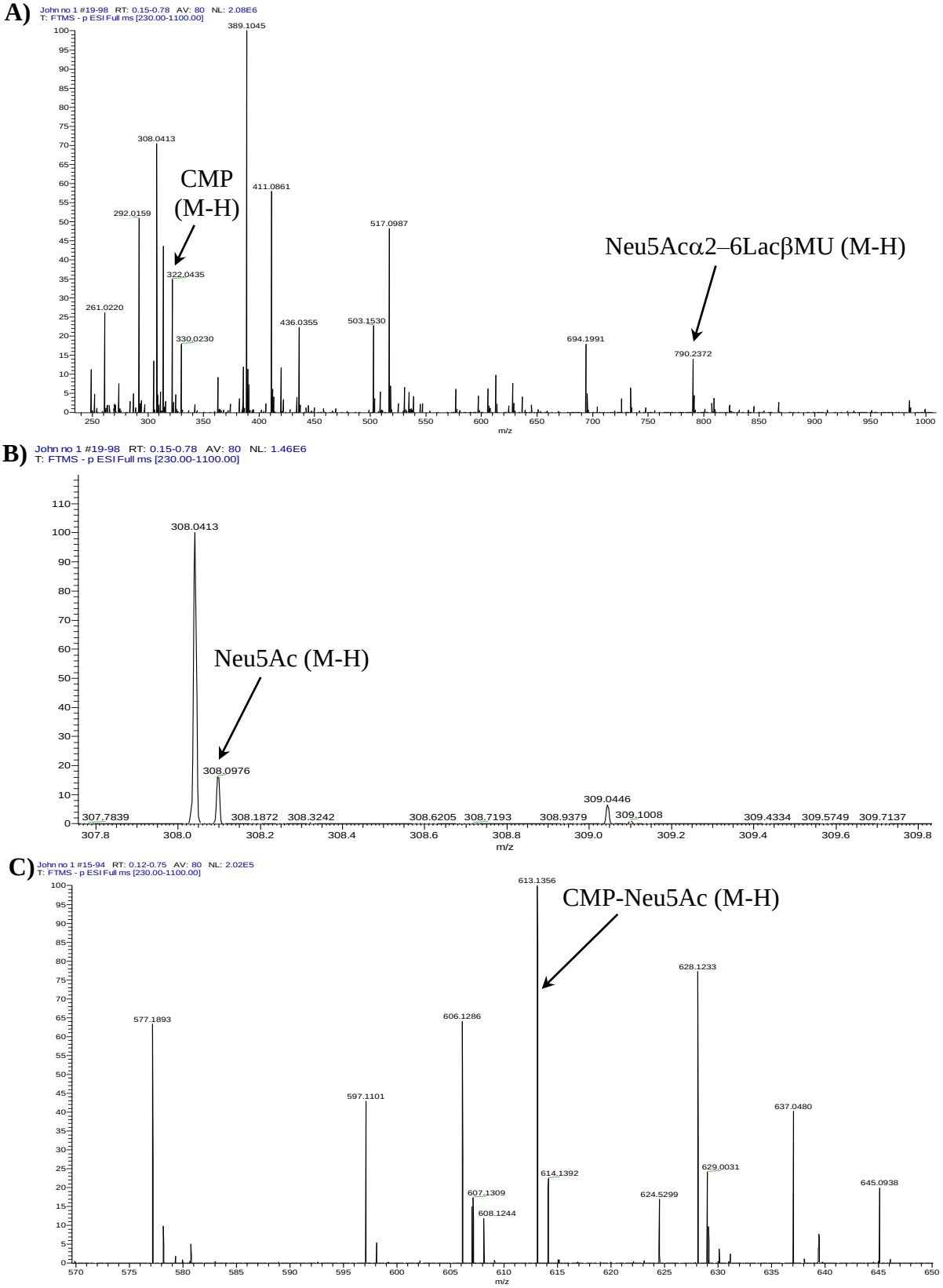


Figure S4. HPLC detection of Pd2,6ST S232L/T356S/W361F neosialidase activity toward egg yolk sialoglycopeptide. **A)** no-enzyme control; **B)** neosialidase enzymatic reaction; **C)** enzymatic reaction mixed with egg yolk sialoglycopeptide substrate standard.

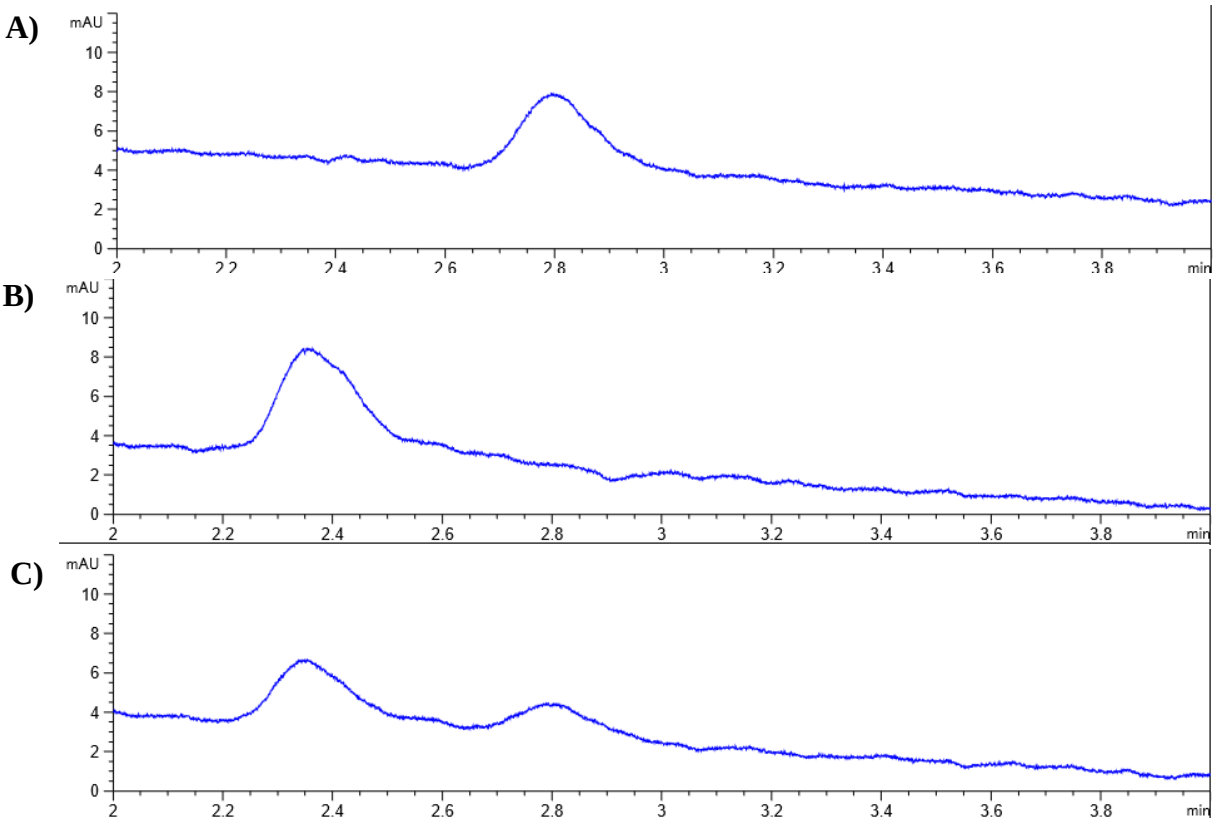
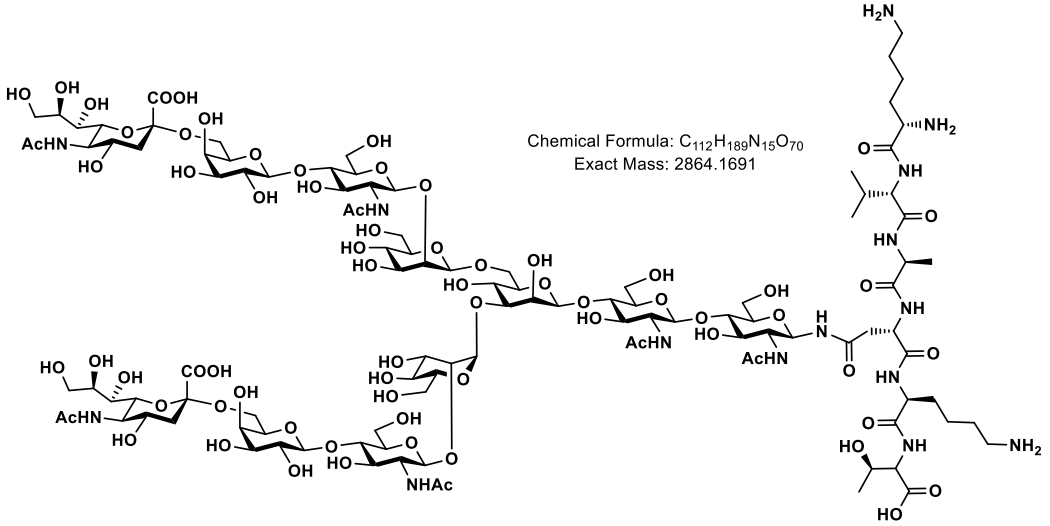
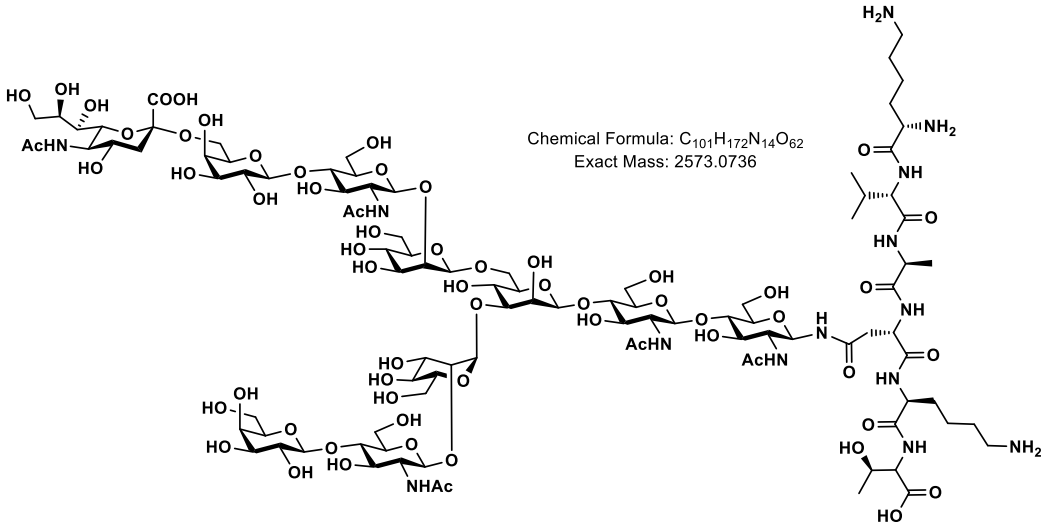
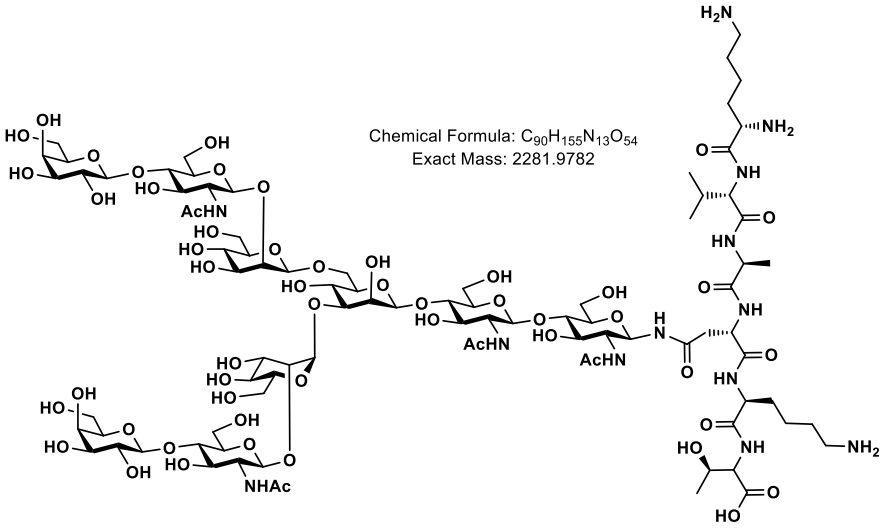
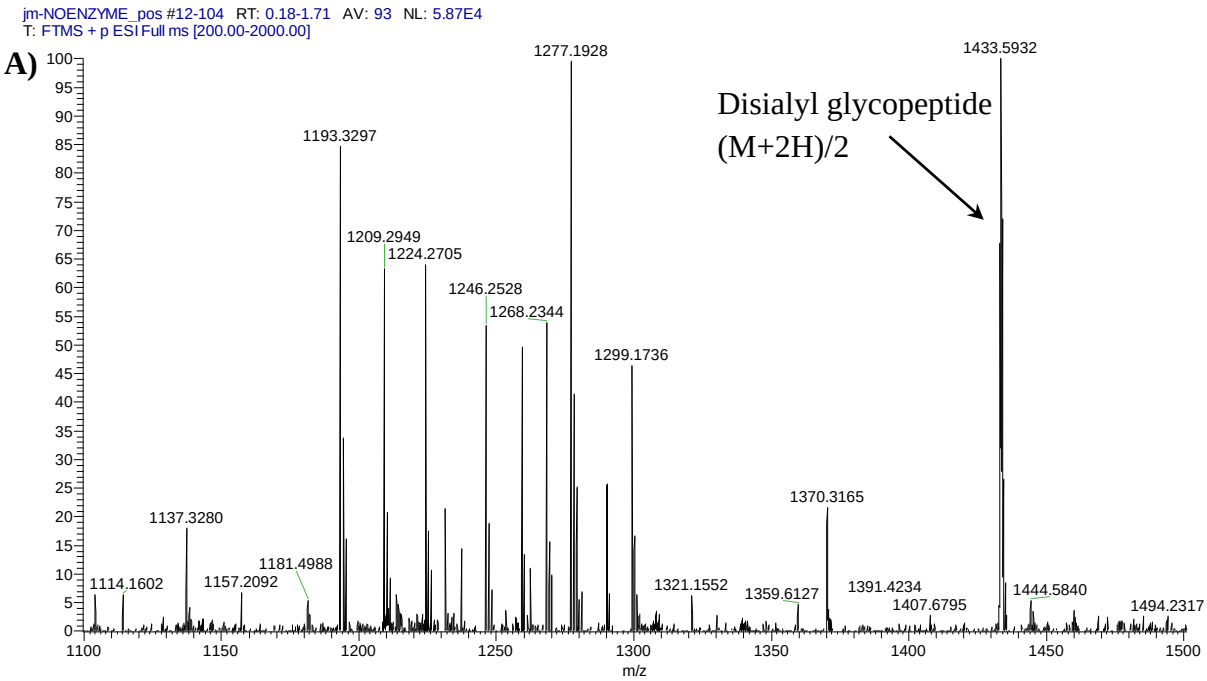


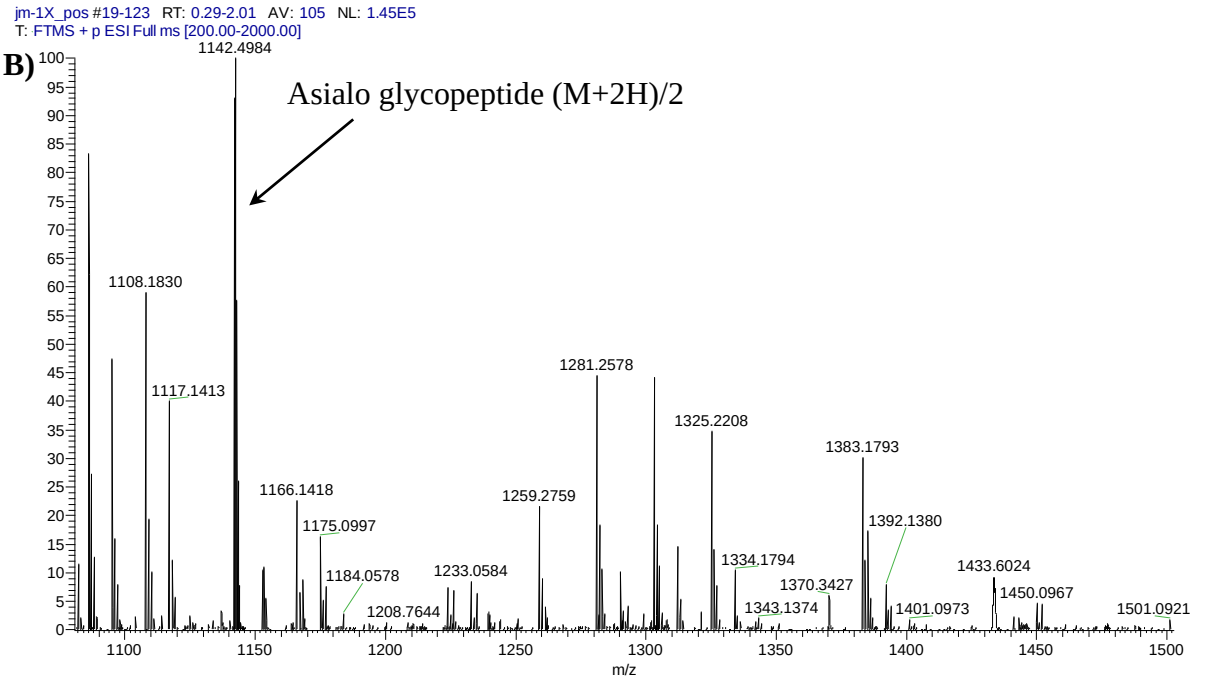
Figure S5. HRMS detection of Pd2,6ST S232L/T356S/W361F neosialidase activity towards egg yolk sialoglycopeptide. Signals for (M+3)/2 ions were observed for the disialyl (Calcd. 1433.0924, found 1433.0910) in the no-enzyme control (**A**) and asialo glycopeptides (Calcd. 1141.9969, found 1141.9967) in the neosialidase reaction (**B**). Monosialylated glycopeptide was not detected. Structures of the glycopeptides are shown.








jm-NOENZYME_pos #12-104 RT: 0.18-1.71 AV: 93 NL: 5.87E4
T: FTMS + p ESI Full ms [200.00-2000.00]
A)
Disialyl glycopeptide
(M+2H)/2
1277.1928
1433.5932
1193.3297
1209.2949
1224.2705
1246.2528
1268.2344
1299.1736
1370.3165
1137.3280
1181.4988
1114.1602
1157.2092
1321.1552
1359.6127
1391.4234
1407.6795
1444.5840
1494.2317
m/z


jm-1X_pos #19-123 RT: 0.29-2.01 AV: 105 NL: 1.45E5
T: FTMS + p ESI Full ms [200.00-2000.00]
B)
Asialo glycopeptide (M+2H)/2
1142.4984
1108.1830
1117.1413
1281.2578
1325.2208
1383.1793
1166.1418
1259.2759
1175.0997
1392.1380
1184.0578
1233.0584
1334.1794
1433.6024
1370.3427
1450.0967
1208.7644
1343.1374
1401.0973
1501.0921
m/z

^{1}H and ^{13}C NMR spectra of Neu5Acα2–6GalβX (**1**)