Supplementary Information for:

Human neuraminidase isoenzymes show variable activities for 9-*O*-acetyl-sialoside substrates

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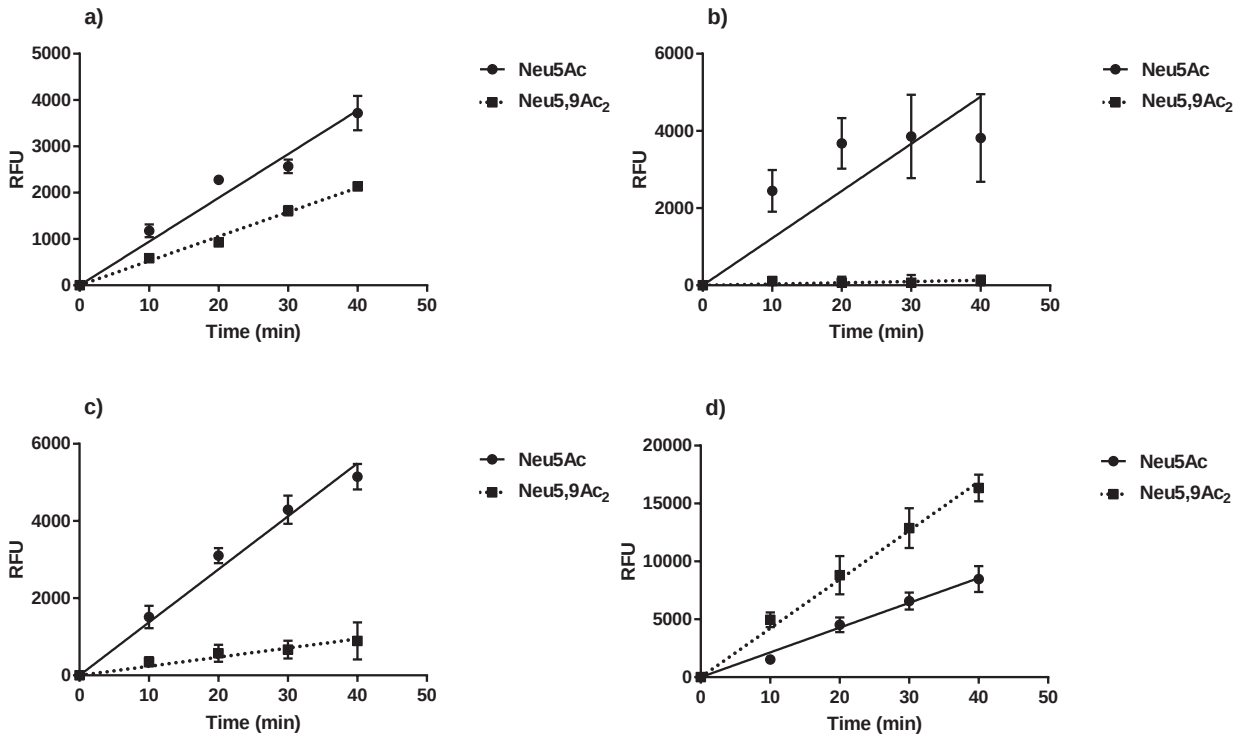


Figure S1. Kinetics of hNEU cleaving 4MU-Neu5Ac (solid line) and 4MU-Neu5,9Ac2 (dashed line) for a) NEU1, b) NEU2, c) NEU3, d) NEU4. Slopes were fit by linear regression forced through zero with triplicate experiments. Error bars denote standard error.

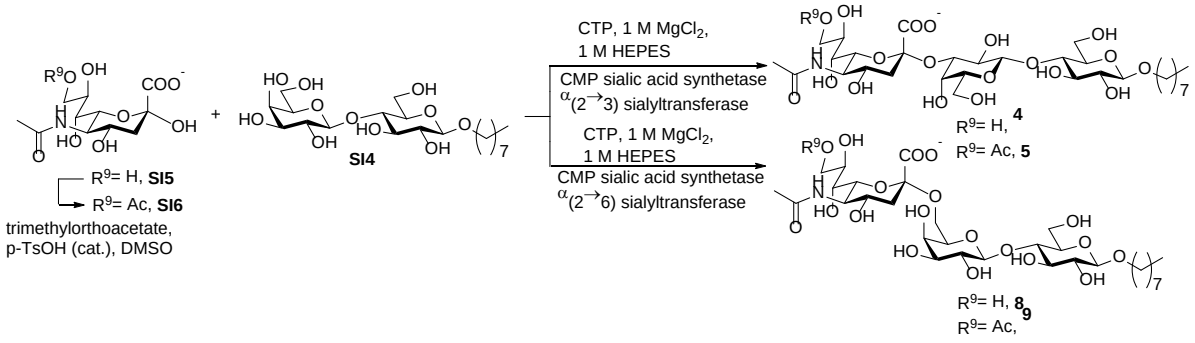


Figure S2. One-pot-two-enzyme synthesis of Neu5Ac (4,5) and Neu5,9Ac2 (8,9) octyl GM3 derivatives.

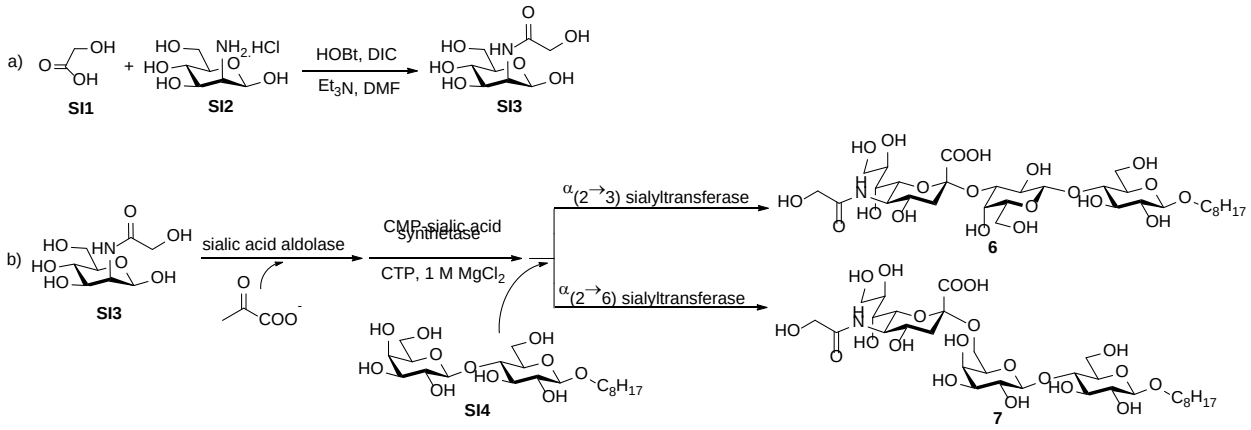
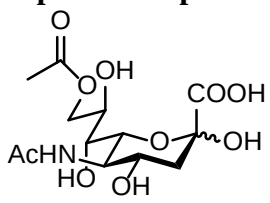


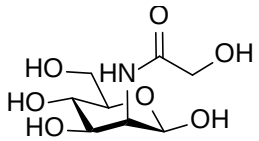
Figure S3. Synthetic route to Neu5Gc octyl GM3 derivatives (6,7). a) Generation of N-glycolyl-d-mannosamine. b) One-pot-three-enzyme synthesis of Neu5Gc octyl GM3 derivatives.

Experimental procedures for the synthesis of SI6, SI13, and SI14.



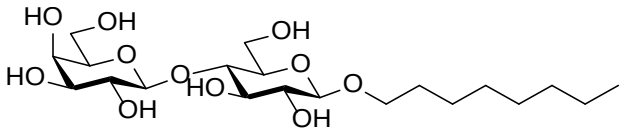
5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (SI6):

Starting from *N*-acetylneuraminic acid, **(SI6)** was prepared in one step, as previously reported.[1] ^{1}H and ^{13}C NMR data were consistent with previous reports.[1, 2]



***N*-glycolyl-D-mannosamine (SI3):**

N-glycolyl-D-mannosamine was prepared as previously reported, with minor modifications.[3] Briefly, glycolic acid (100 mg, 1.31 mmol) was added to a mixture of mannosamine hydrochloride (**SI2**) (338.5 mg, 1.57 mmol) and triethylamine (0.22 mL, 1.57 mmol) in anhydrous DMF. The reaction was cooled to 0 °C and DIC (0.24 mL, 1.57 mmol) and HOBt (194.7 mg, 1.44 mmol) were added sequentially. After stirring overnight the reaction mixture was filtered and concentrated under reduced pressure. Crude product was purified with iatrobeads ($CHCl_3$:MeOH, 2:1) to yield 238 mg (77 %) of (**SI3**) as an off-white solid. ^{1}H and ^{13}C NMR data were consistent with previous reports.[3]



Octyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (SI4):

Starting from β-lactose, octyl β-D-galactopyranosyl-(1→4)-β-D-glycopyranoside was prepared as previously reported.[4] ^{1}H and ^{13}C NMR data were consistent with previous reports.[4]

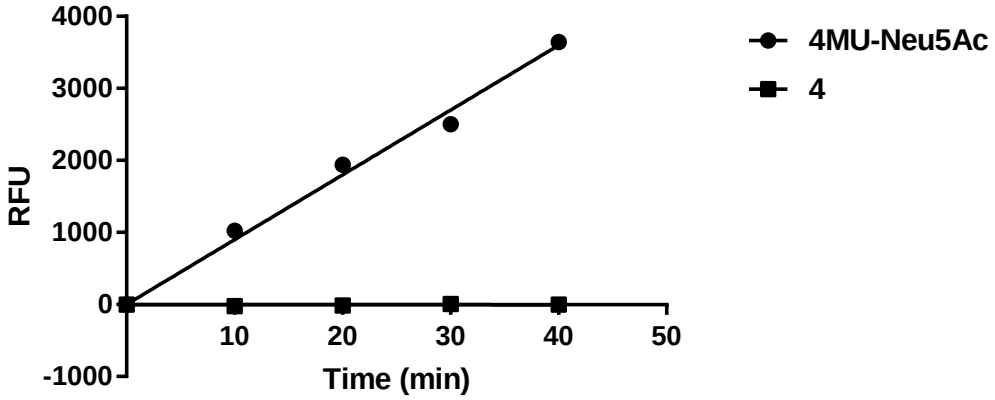


Figure S4. Control experiment showing that NEU1 does not cleave octyl sialyllactoside **4**. 4MU-Neu5Ac is a positive control. The result is consistent with previous reports that NEU1 does not cleave glycolipid substrates,[5] and is consistent with the majority of the neuraminidase activity in the crude HEK293E cell lysate being NEU1.

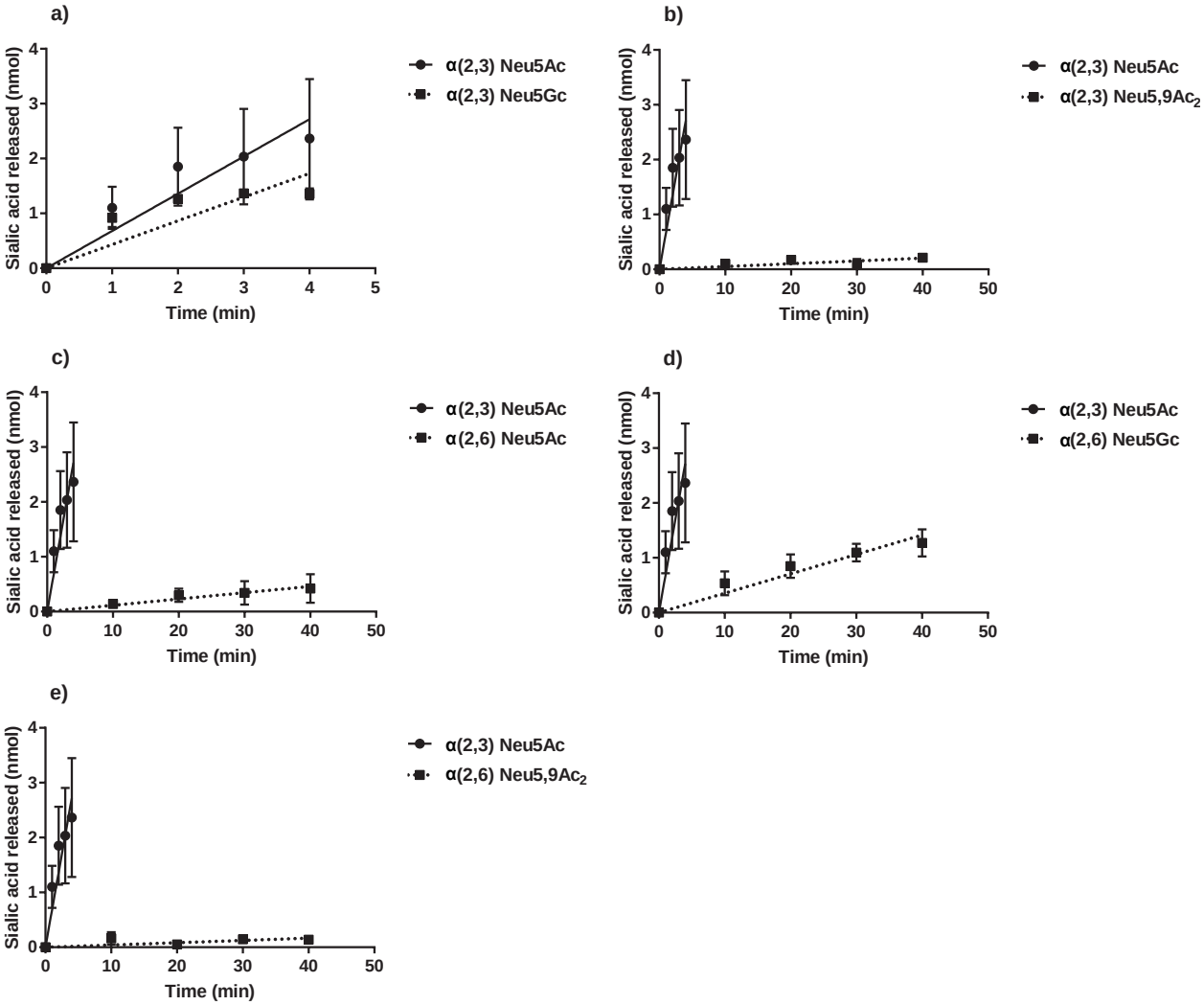


Figure S5. Kinetics of NEU2 cleaving octyl sialyllactosides (5-9 – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (4 – solid line, matched control replotted on every panel). Slopes were fit by linear regression forced through zero of triplicate experiments, error bars denote standard error.

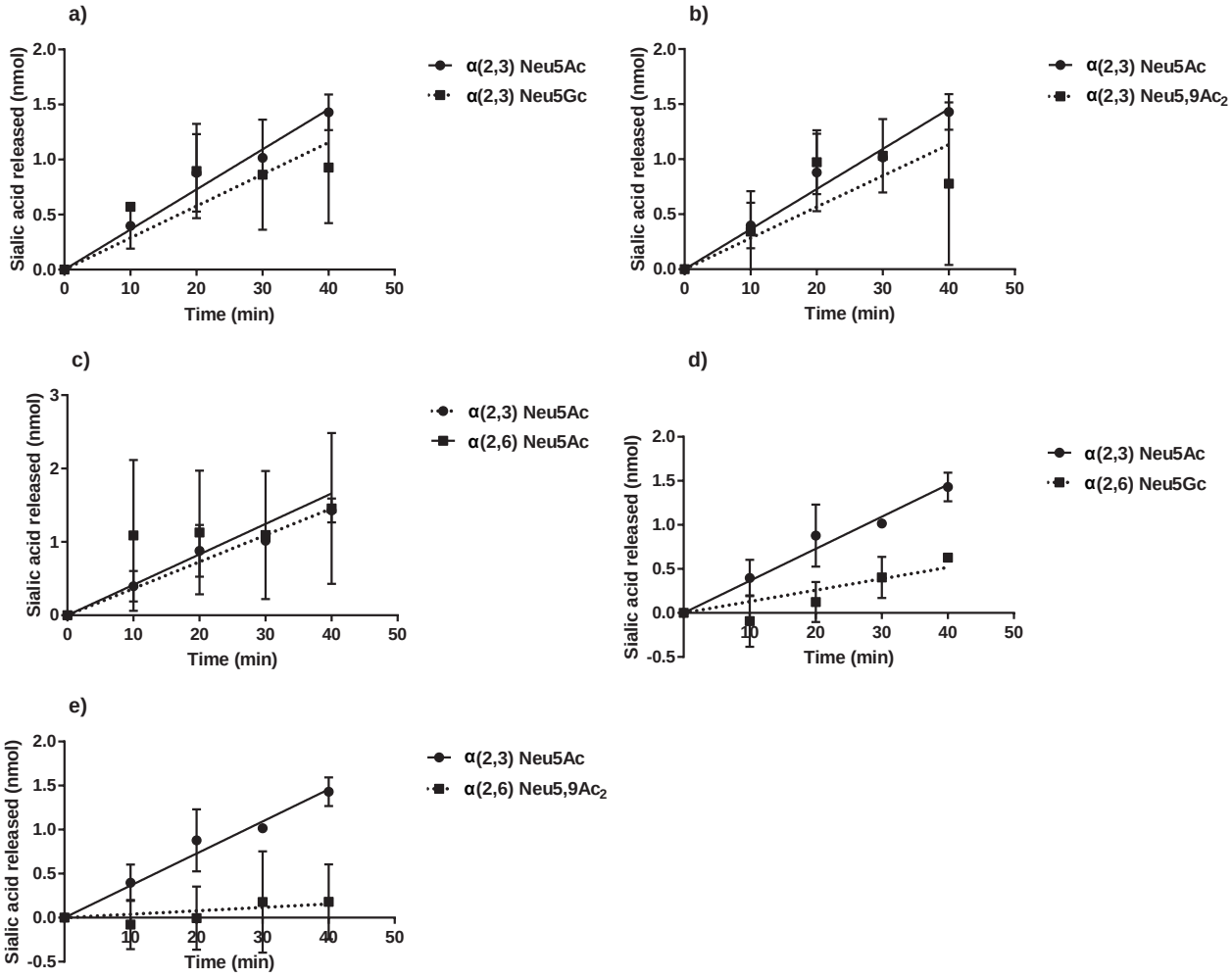


Figure S6. Kinetics of NEU3 cleaving octyl sialyllactosides (5-9 – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (4 – solid line, matched control replotted on every panel). Slopes were obtained by linear regression forced through zero of triplicate experiments, error bars denote standard error.

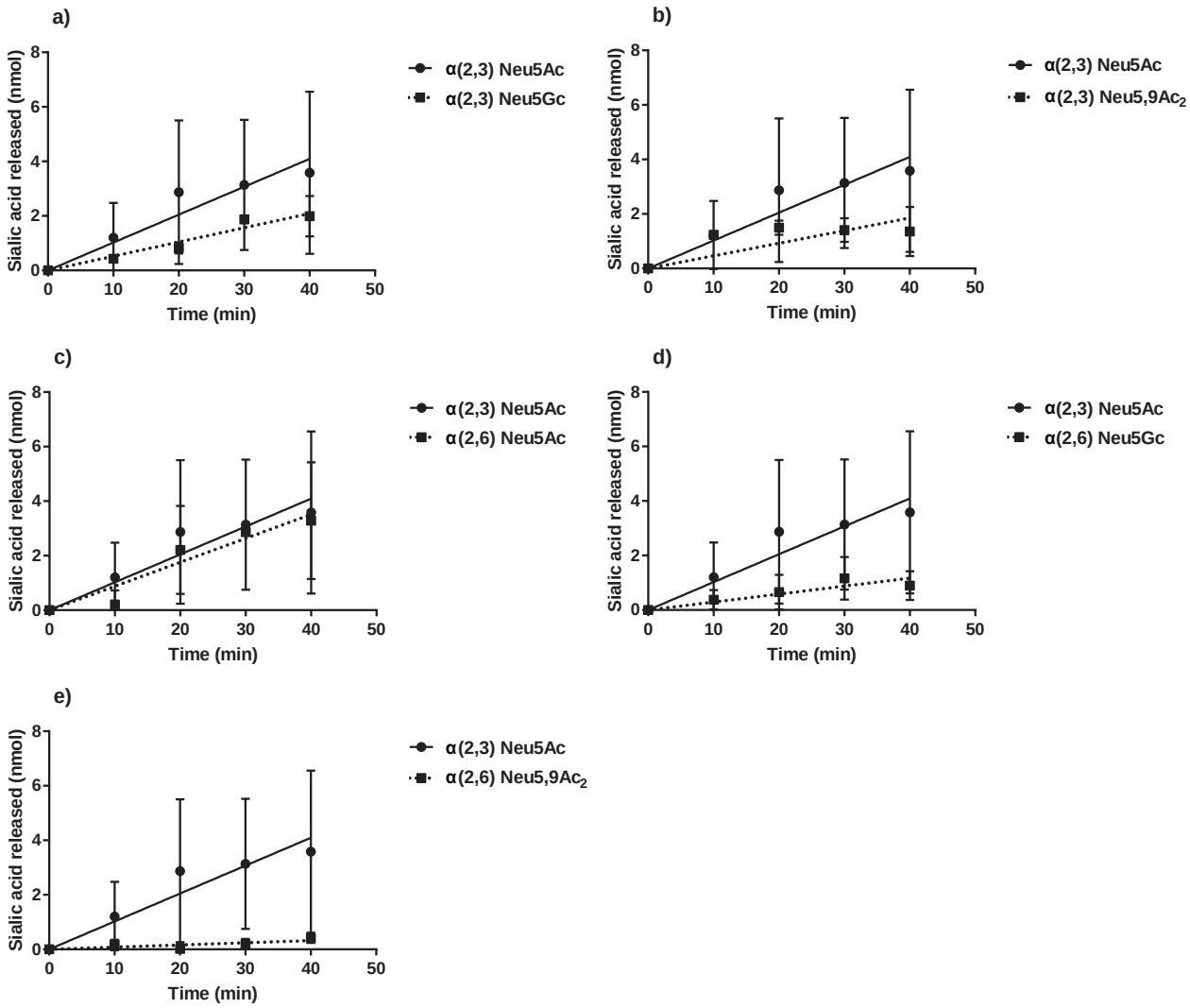


Figure S7. Kinetics of NEU4 cleaving octyl sialyllactosides (5-9 – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (4 – solid line, matched control replotted on every panel). Slopes were obtained by linear regression forced through zero of triplicate experiments, error bars denote standard error.

Table S1. Summary of kinetics assays showing the effect of sialic acid modification on hydrolysis with hNEU. Rates were obtained by taking points every 10 min for 40 min (or every 1 min for 4 min compounds 4 and 6 only); followed by a linear regression of triplicate experiments. Data is in the form of relative rates, ± standard error, normalized to α(2→3) Neu5Ac.

Assay	*4MU*		*Octyl sialyllactosides*					
Compound	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**
NEU1	1.00 ± 0.05	0.56 ± 0.01						
NEU2	1.0 ± 0.2	0.026 ± 0.006	1.0 ± 0.1	0.011 ± 0.001	0.6 ± 0.1	0.052 ± 0.004	0.008 ± 0.001	0.006 ± 0.003
NEU3	1.00 ± 0.04	0.17 ± 0.01	1.00 ± 0.04	1.1 ± 0.2	0.8 ± 0.1	0.35 ± 0.07	0.8 ± 0.1	0.11 ± 0.04
NEU4	1.00 ± 0.03	1.97 ± 0.04	1.00 ± 0.09	0.85 ± 0.08	0.51 ± 0.04	0.28 ± 0.04	0.5 ± 0.1	0.08 ± 0.02

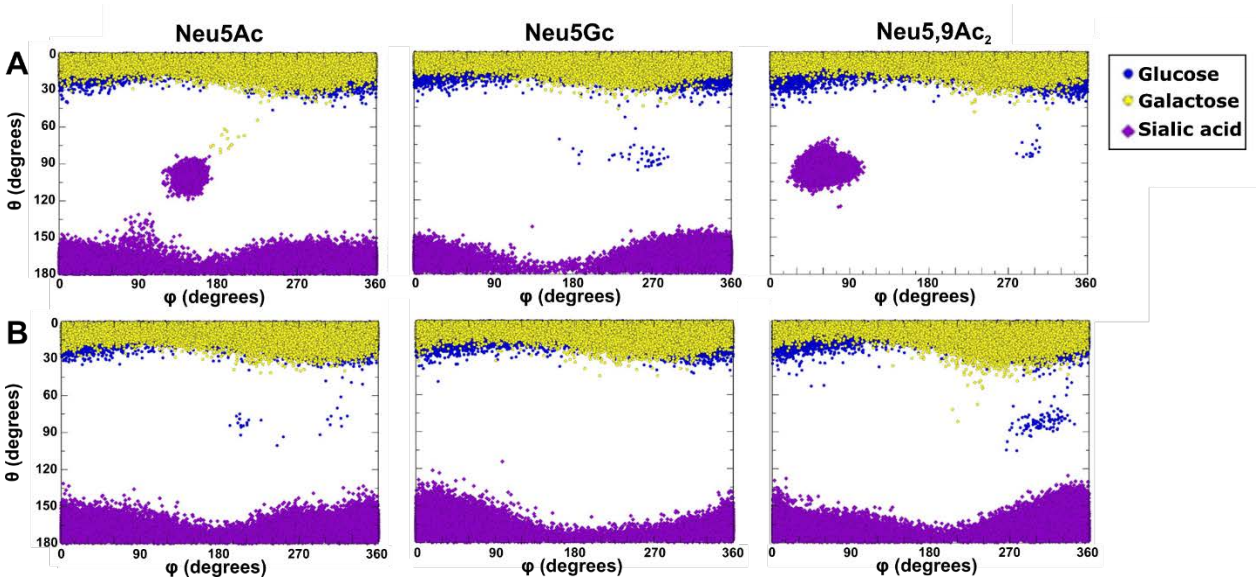


Figure S8. Ring conformations from 25-ns MD simulations for GM3 trisaccharides of 4, 6, and 8 bound to a) NEU2 and b) NEU3. See also Table S2 and Table S3.

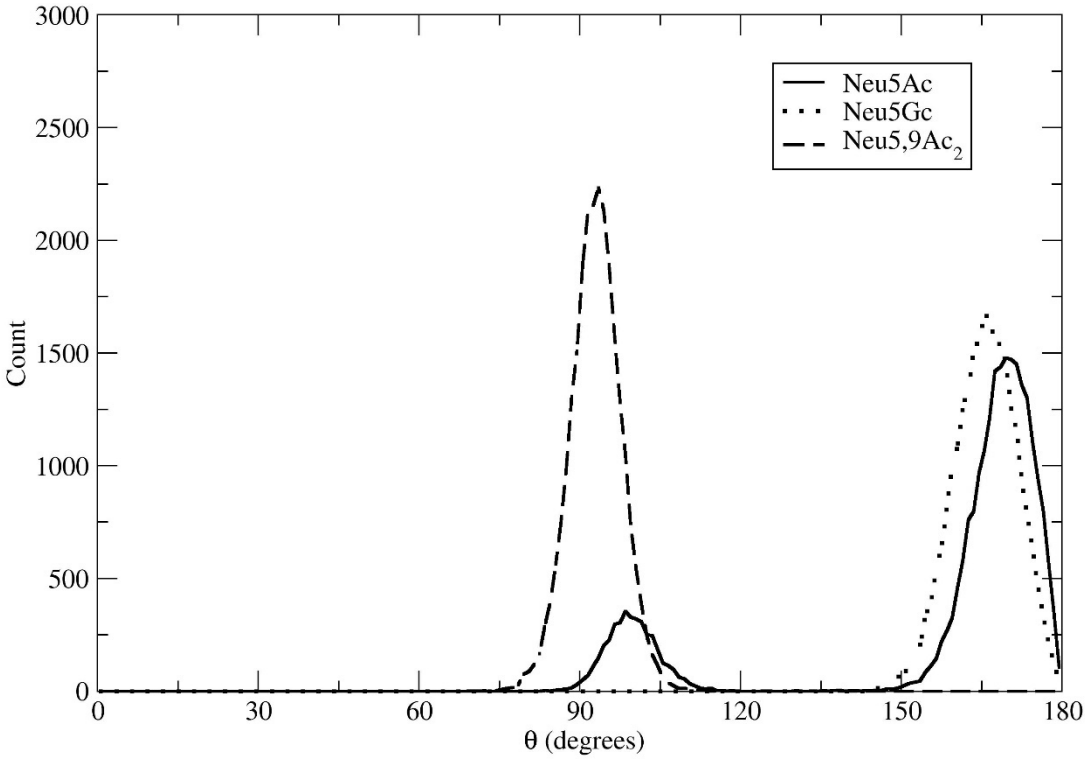


Figure S9. Histogram of ring conformations (along θ) from a 25-ns MD simulation for the GM3-analogs 4, 6, and 8 bound to NEU2. Neu5Ac in 4 is shown as a solid line and has two populations, one at θ = 99° (OS3, 18%) and a second at θ = 170° (2C5, 82%). Neu5Gc of 6 is shown as a dotted line and has one population at θ = 166° (2C5). Neu5,9Ac2 in 8 is shown as a line with long dashes and has one population at θ = 93° (B2,5). See also Figure S8.

Table S2. Pyranose ring conformations[6] and Cremer-Pople coordinates[7] from MD simulations of three GM3-analogs in the NEU2 binding site. See also Figure S8, Figure S9, and Figure S10.

GM3 analog	Pyranose ring	Ring conformation	ϕ (degrees)[a]	θ (degrees)[a]
	Glc	$^4C_1$	217 ± 117	11 ± 6
with Neu5Ac	Gal	$^4C_1$	208 ± 100	10 ± 6
	Neu	$^2C_5$	191 ± 119	158 ± 26
	Glc	$^4C_1$	204 ± 120	11 ± 7
with Neu5Gc	Gal	$^4C_1$	208 ± 90	9 ± 5
	Neu	$^2C_5$	247 ± 124	166 ± 6
	Glc	$^4C_1$	189 ± 122	11 ± 6
with Neu5,9Ac$_2$	Gal	$^4C_1$	215 ± 93	9 ± 5
	Neu	$B_{2,5}$	556 ± 13	93 ± 5

[a] The average value ± the standard deviation over the 25-ns MD trajectory are shown.

Table S3. Pyranose ring conformations[6] and Cremer-Pople coordinates[7] from MD simulations of three GM3 analogs in the NEU3 binding site. See also Figure S8 and Figure S11

GM3 analog	Pyranose ring	Ring conformation	ϕ (degrees)[a]	θ (degrees)[a]
	Glc	$^4C_1$	223.55 ± 101.25	10.91 ± 5.89
with Neu5Ac	Gal	$^4C_1$	223.88 ± 112.5	11.09 ± 6.32
	Neu	$^2C_5$	200.04 ± 128.42	168.09 ± 6.16
	Glc	$^4C_1$	228.12 ± 93.71	10.43 ± 5.59
with Neu5Gc	Gal	$^4C_1$	215.59 ± 119.55	11.09 ± 5.88
	Neu	$^2C_5$	111.68 ± 125.29	166.13 ± 7.26
	Glc	$^4C_1$	240.92 ± 78.38	11.58 ± 6.44
with $Neu5,9Ac_2$	Gal	$^4C_1$	212.76 ± 121.08	11.25 ± 7.3
	Neu	$^2C_5$	206.28 ± 130.93	166.02 ± 7.76

[a] The average value ± the standard deviation over the 25-ns MD trajectory are shown.

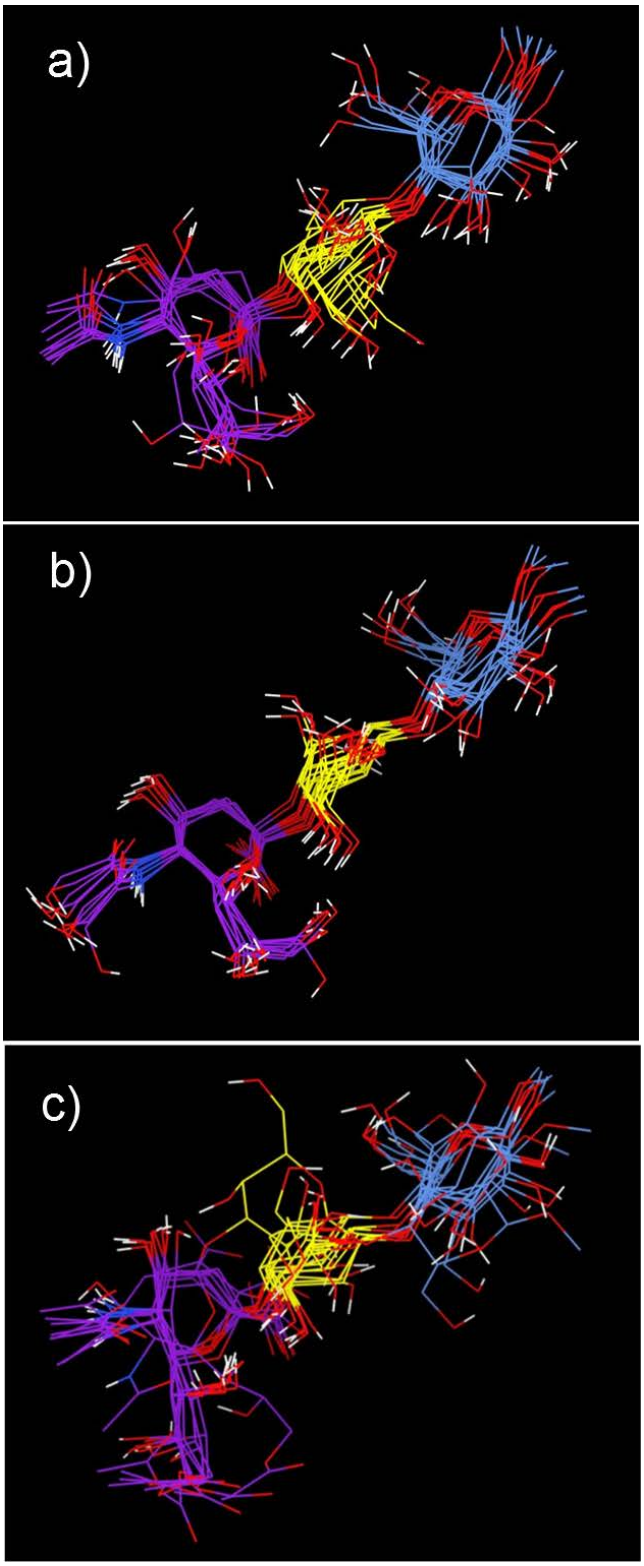


Figure S10. An overlay of 10 conformations of the trisaccharide of a) 4 b) 6 and c) 8 from the MD simulation in the NEU2 active site. The protein is omitted for clarity.

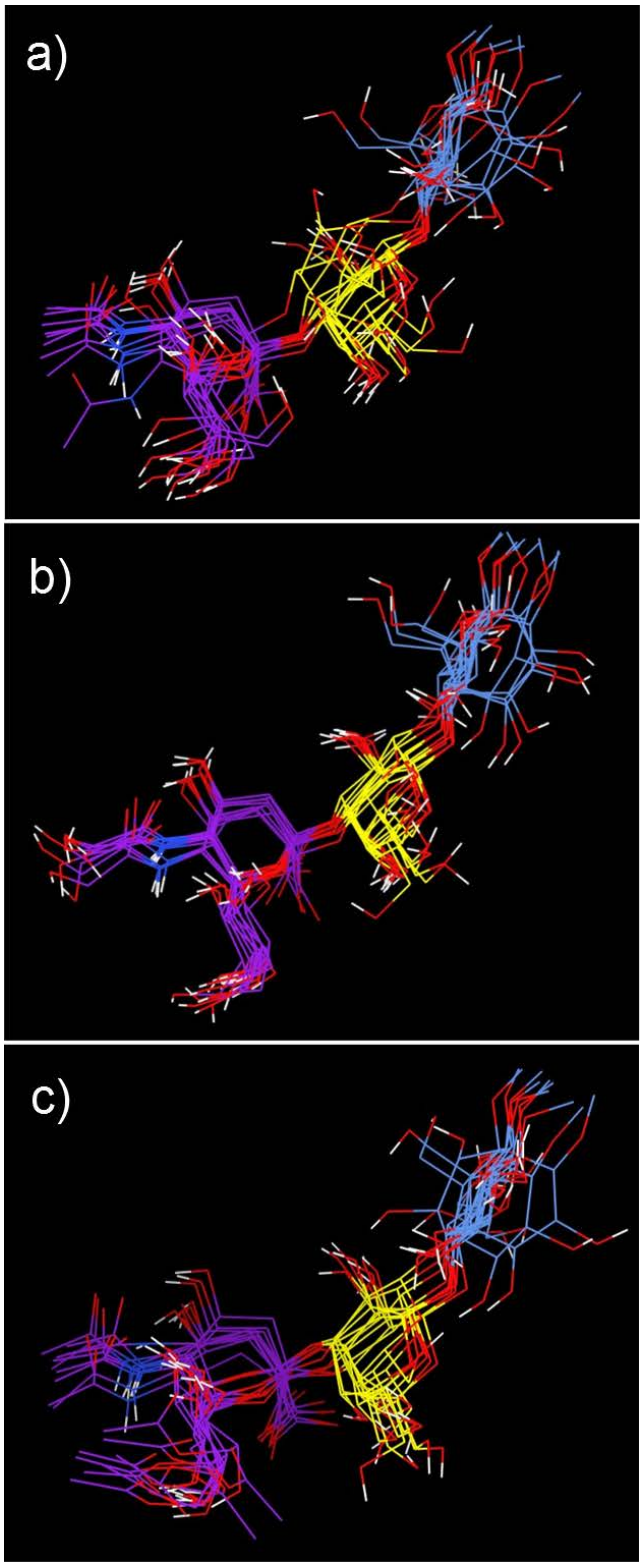


Figure S11. An overlay of 10 conformations of the trisaccharide of a) 4 b) 6 and c) 8 from the MD simulation in the NEU3 active site. The protein is omitted for clarity.

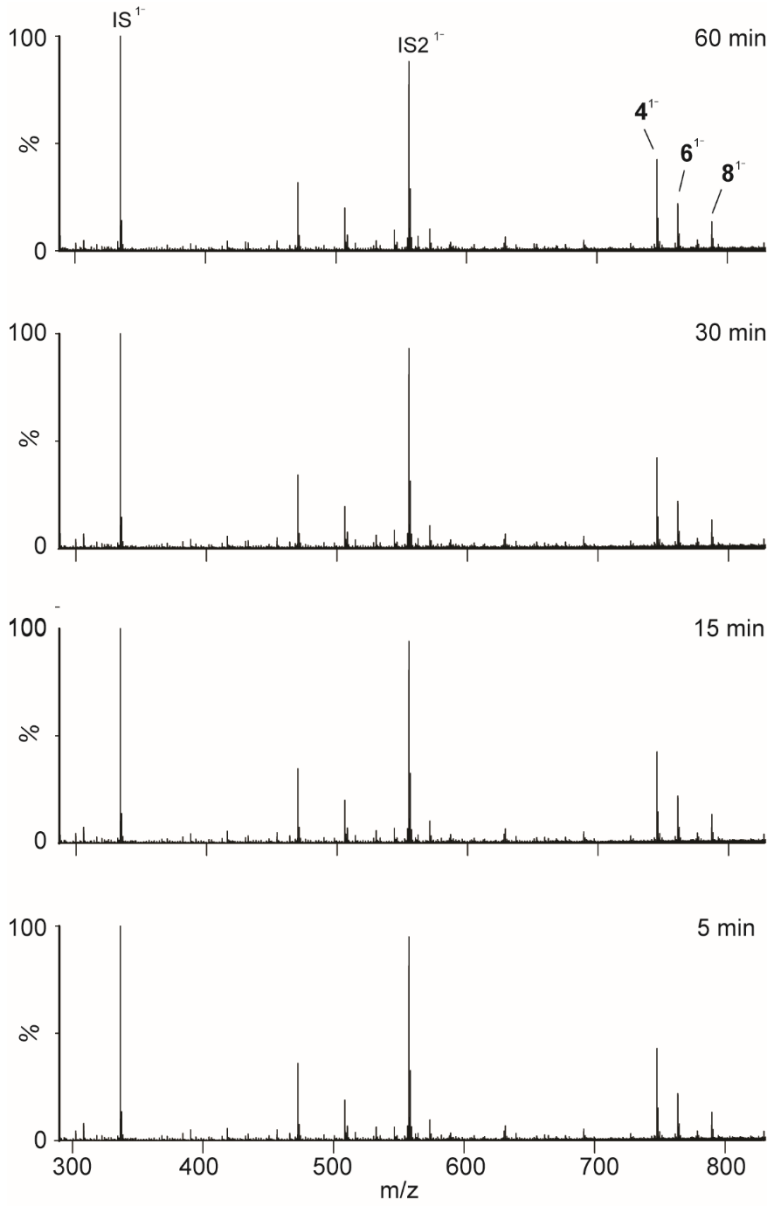


Figure S12. Negative control ESI-MS experiments done in the absence of enzyme. Data shows that sialic acid cleavage cannot be attributed to fragmentation of the substrate.

References:

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