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Supporting Information

An α2,3-Sialyltransferase from *Photobacterium phosphoreum* with Broad Substrate Scope: Controlling Hydrolytic Activity by Directed Evolution

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Supporting Information

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Cloning, expression, and purification of 2,3SiaT$_{pph}$

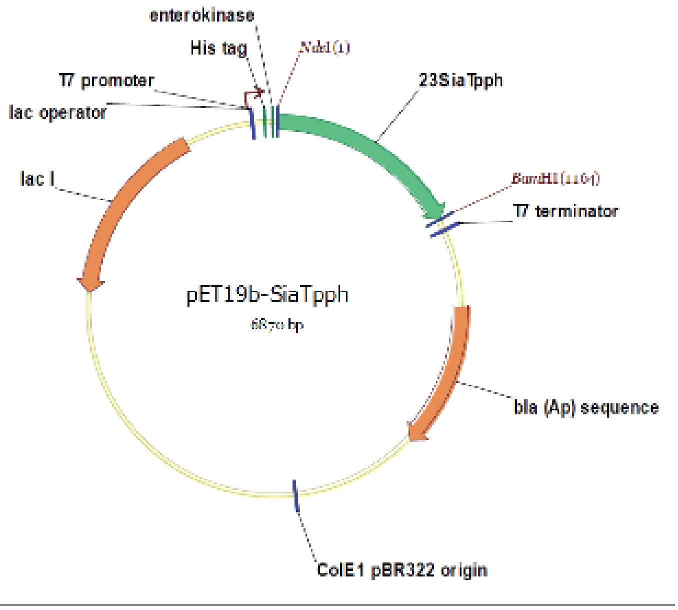


Figure S1. Plasmid construction for expression of 2,3SiaT$_{pph}$.

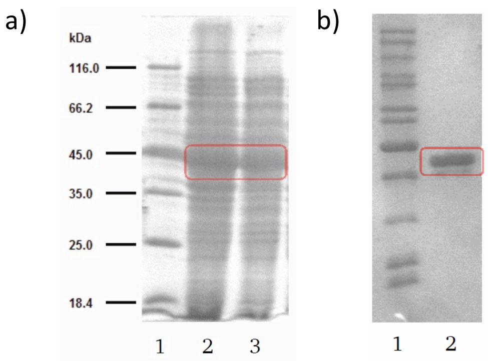


Figure S2. Cloning and expression of 2,3SiaT from *Photobacterium phosphoreum*. a) Expression level of the recombinant SiaT$_{pph}$ in BL21(DE3) analyzed by SDS-PAGE. Lanes 1: marker; 2: crude cell extract; 3: supernatant of crude cell extract. b) Purification of 2,3SiaT$_{pph}$ by Ni-affinity chromatography. Lanes 1: marker; 2: purified 2,3SiaT$_{pph}$.

Table S1. Primers for site directed mutagenesis library (Agilent method)

SiaT-L120NNK_F	AGTCATTATTAACGGCAATACGnnkTGGGCTGTCGACGTGGTAAAC
SiaT-L120NNK_R	GTTTACCACGTCGACAGCCCAmnnCGTATTGCCGTTAATAATGACT
SiaT-W121NNK_F	GTCATTATTAACGGCAATACGCTGnnkGCTGTCGACGTGGTAAACATTATC
SiaT-W121NNK_R	GATAATGTTTACCACGTCGACAGCmnnCAGCGTATTGCCGTTAATAATGAC
SiaT-D148NNK_F	AACCGAGATCGAACTGAACTTCTATGATnnkGGTAGCGCCGAGTAT
SiaT-D148NNK_R	ATACTCGGCGCTACCmnnATCATAGAAGTTCAGTTCGATCTCGGTT
SiaT-S150WVC_F	CGAACTGAACTTCTATGATGATGGTwvcGCCGAGTATGTTC
SiaT-S150WVC_R	GAACATACTCGGCGbwaCCATCATCATAGAAGTTCAGTTCG
SiaT-A151NNK_F	CTGAACTTCTATGATGATGGTAGCnnkGAGTATGTTCGCCTGTATGATTTC
SiaT-A151NNK_R	GAAATCATACAGGCGAACATACTCmnnGCTACCATCATCATAGAAGTTCAG
SiaT-R155NNK_F	GATGATGGTAGCGCCGAGTATGTTnnkCTGTATGATTTCTCTCGC
SiaT-R155NNK_R	GCGAGAGAAATCATACAGmnnAACATACTCGGCGCTACCATCATC
SiaT-N190NNK_F	CATCAACGGTACTCAGCCGTTCGATnnkAGCATTGAAAACATCTATGG
SiaT-N190NNK_R	CCATAGATGTTTTCAATGCTmnnATCGAACGGCTGAGTACCGTTGATG
SiaT-T278WVC_F, SiaT-N279WVC_F	CACGAAAACTTCATCTTCATTGGCwvcwvcTCTGGTACTGCCACTGCCGAGCAA
SiaT-T278WVC_R, SiaT-N279WVC_R	TTGCTCGGCAGTGGCAGTACCAGAGbwgbwgCCAATGAAGATGAAGTTTTCGTG
SiaT-H317NNK_F	TGGCACCAATTCTGGTACTGCnnkTGCCGAGCAACAAATCGATAT
SiaT-H317NNK_R	ATATCGATTTGTTGCTCGGCAmnnGCAGTACCAGAATTGGTGCCA
SiaT-S359 WVC, S360WVC_F	CAGATGCCGTGGGTGGTATGGGTwvcwvcCGTTTTCTTCTCACTGCCAAAGAC
SiaT-S359 WVC, S360WVC_R	GTGTTTGGCAGTGAGAAGAAAACGbwgbwgACCCATACCACCCACGGCATCTG
SiaT-L364NNK_F	ACCGACATTGAGAATAACGCCnnkATCCAGGTCATGATTGAGCTG
SiaT-L364NNK_R	CAGCTCAATCATGACCTGGATmnnGGCGTTATTCTCAATGTCGGT

Table S2. Primers for second generation combination variants (NEB method)

1) SiaT-150S/T, SiaT151L/M_F	ascmtgGAGTATGTTCGCCTGTATG
2) SiaT-D148S/L_R	acccraATCATAGAAGTTCAGTTCG
3) SiaT-S150S/T, A151_F	ascgccGAGTATGTTCGCCTGTATG
4) D148_R	accatcATCATAGAAGTTCAGTTCG

Primer mix: A = 1+2, B = 1+4, C = 2+3, D = 3+4

Table S3. Templates and primer mix combination for second generation combination library

Primer mix →	A	B	C	D	A	B	C	D
Position ↓	template: native gene				template: S359T/S360T			
D148	L/S	D	L/S	D	L/S	D	L/S	D
S150	S/T	S/T	S/T	S/T	S/T	S/T	S/T	S/T
A151	L/M	L/M	A	A	L/M	L/M	A	A

Table S4. Primers for preparation of specific individual combination variants (NEB method)

SiaT-A151D_F	TGATGGTAGCgatGAGTATGTTCG
SiaT-A151D_R	TCATAGAAGTTCAGTTCGATCTCGG
SiaT-E342A_F	AATCCCGTTCgcgGCCCTGATTATG
SiaT-E342A_R	TTGTTATAAATCTCGATCATGTTATG
SiaT-S359T_F	TGGTATGGGTaccAGCGTTTTCTTCTCAC
SiaT-S359T_R	CCCACGGCATCTGGCAGAG
SiaT-L387A_F	GAATAACGCCgcgATCCAGG
SiaT-L387A_R	TCAATGTCGGTGTCGCTTTTATAG

Table S5. Primer and template combination for specific individual combination variants

template	**primer**	**product**
w.t.	SiaT-E342A_F/R	E342A
A151D	SiaT-E342A_F/R	A151D/E342A
A151D	SiaT-L387A_F/R	A151D/L387A
S359T/S360T	SiaT-A151D_F/R	A151D/S359T/S360T
A151D/S359T/S360T	SiaT-L387A_F/R	A151D/S359T/S360T/L387A

Table S6. Analysis of distances between 13 selected amino acid residues and substrates or water molecules in the active site, and distances between water molecules and the centers of nucleophile attack during transfer or potential hydrolysis (sialoside C2 and phosphate, respectively).

AA position / Codon type	Contact distances [Å] [a]					
L120 / NNK	CD2,GalO2: 4.7	CD2, GalO3: 5.0	CD2, GalO4: 4.1			
W121 / NNK	NE1, GalO4: 3.4	NE1, GalO5: 3.7	NE1, GalO6: 3.6			
D148 / NNK	OD2, GalO3: 2.1	OD2, GalO4: 2.4	OD2, SiaO4: 3.4	OD2, wat4: 4.5		
	OD1, GalO3: 3.7	OD1, GalO4: 1.9	O, SiaO4: 4.0	O, wat4: 2.7		
S150 / WVC	OG, SiaO1: 4.8	OG, SiaO4: 3.5	OG, PO2: 4.2	OG, wat1: 2.8	OG, wat2: 4.6	
	N, SiaO1: 4.3	N, SiaO4: 2.1	OG, SiaN5: 1.7			
A151 / NNK	N, SiaO10: 3.9	N, SiaN5: 3.0	N, SiaN5: 3.0		CB, GalO3: 4.3	
R155 / NNK	NH2, GalO1: 6.5	NE, GalO2: 7.8	NE, SiaO10: 7.3	NH2, GluO1: 5.1		
N190 / NNK	ND2,GalO1: 7.2	ND2,GalO2: 9.1	ND2,GluO1: 6.4			
T278 / WVC	OG1, wat3: 2.8	OG1, wat2: 6.1	OG1, SiaO9: 4.7	OG1, SiaO8: 5.5		
N279 / WVC	N, SiaO9: 6.6	O, SiaO9: 7.0	ND2, SiaO9: 5.8	OD1, SiaO9: 5.5		
H317 / NNK	NE2, PO3: 4.1	NE2, PO4: 6.3	NE2, PA: 5.6	NE2, SiaO8: 4.3	NE2, SiaO9: 5.4	N, CMPN4: 4.7
S359 / WVC	OG, PO2: 3.5	OG, PO3: 3.7	OG, PO4: 3.7	OG, PO4: 3.9	OG, wat3: 4.0	
					N, wat3: 4.3	N, wat2: 4.8
S360 / WVC	N, PO2: 1.3	N, PO3: 3.7	N, PA: 2.7	N, PO4: 3.4	N, wat1: 3.6	N, wat2: 5.6
	OG, PO2: 1.8	OG, PO3: 3.4	OG, PA: 2.2	OG, PO4: 3.2	OG, wat1: 3.8	OG, wat4: 5.3
	OG, PO1: 1.8					
L387 / NNK	CD2, wat1: 4.7	CD2, wat2: 4.0		CD1, SiaC11: 4.1	CD2, SiaC11: 5.2	CD2, PA: 8.7

	Distances between water molecules and phosphate/SiaC2			
	PA, wat1: 4.4	PA, wat2: 6.4	PA, wat3: 5.7	PA, wat4: 6.2
	SiaC2A, wat1: 5.1	SiaC2A, wat2: 6.9	SiaC2A, wat3: 7.2	SiaC2A, wat4: 4.8

[a] Based on the 3D alignment model, as determined by PyMOL analysis. Selections contain: CD2 (contact in AA), GalO2 (contact to substrate and water); C: contact atom underlined, CD2: PyMOL atom descriptor. For clarity, PyMOL descriptors were amended by prefix Sia for Neu5Ac moiety, Gal for galactosyl unit and Glu for glucose unit in lactose.

Table S7. Thermodynamic stability of 2,3SiaT$_{pph}$ determined by nanoDSF.

enzyme variant	melting point T_m [°C]
parent	47.1
A151D	45.4
L387A	44.8
S359T/S360T	48.0

HPTLC screening

Reaction samples were analyzed by high performance thin layer chromatography (HPTLC; *CAMAG Automatic TLC Sampler 4*) on Merck silica gel plates 60 F_{254}. For each time point measurement two identical 10 μL-samples were sprayed and developed with different solvent mixtures to discriminate partially overlapping bands and quantify the amount of product (α2,3-SiaLac) and hydrolysis side product (Neu5Ac). The basic solvent mix consisted of *n*-propanol : H_2O : NH_3(20%) = 7 : 3 : 1, and acidic solvent mix of *n*-butanol : acetone : AcOH : H_2O = 35 : 35 : 7 : 23. After anisaldehyde staining a quantitative analysis was performed (*CAMAG TLC Scanner 4*) against standards for SiaLac and Neu5Ac as positive controls, resulting in absolute concentrations of the substrates and products.

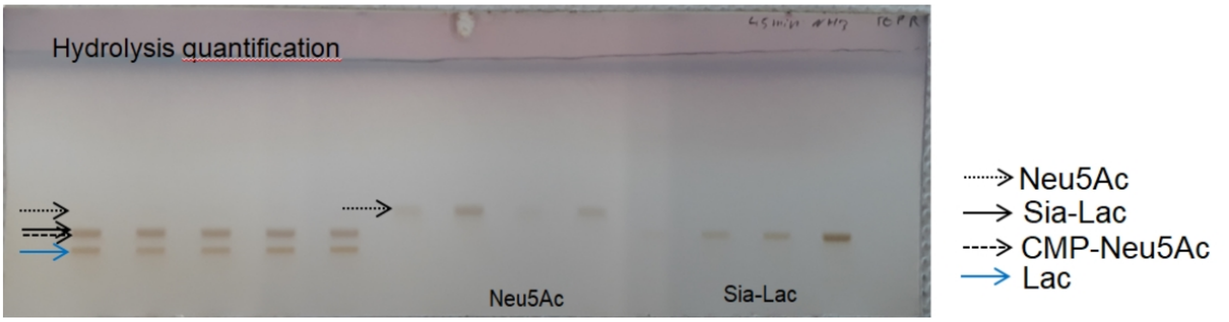


Figure S3. Elution by *n*-propanol:H_2O:NH_3 = 7:3:1 for unbiased integration of Neu5Ac.

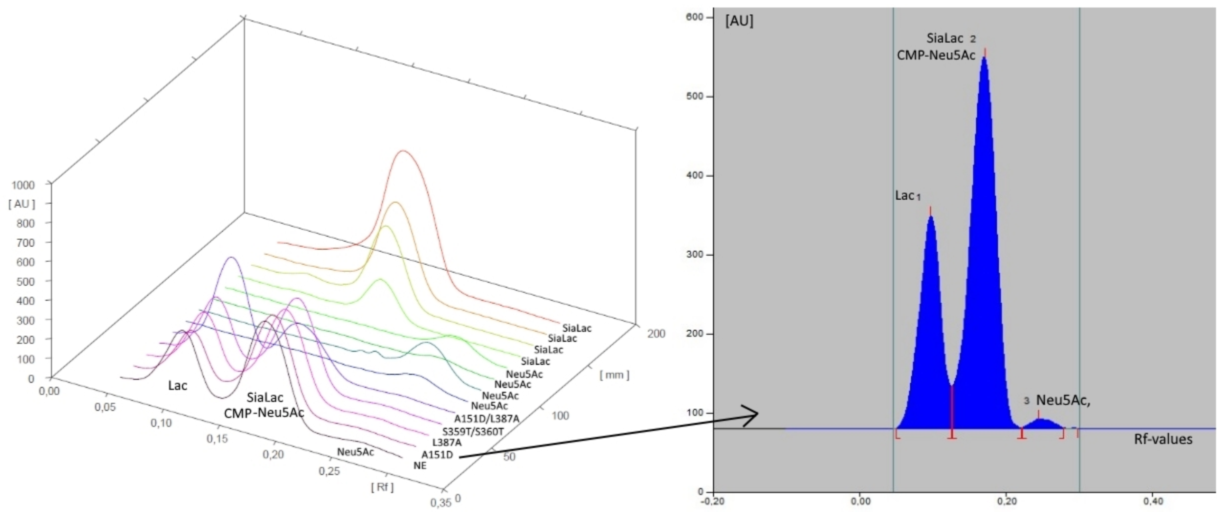


Figure S4. Quantitative analysis of sample from Figure S3; inset shows integration of lane 2.

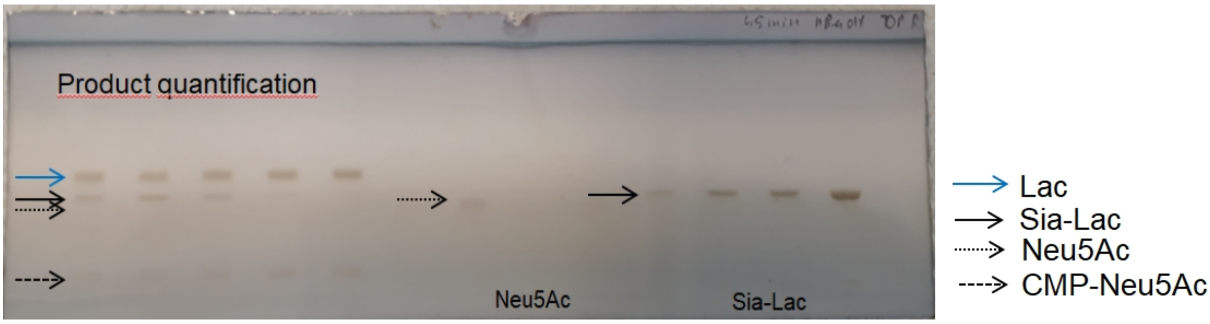


Figure S5. Elution by *n*-butanol:acetone:H_2O:AcOH = 35:35:23:7 for unbiased integration of SiaLac.

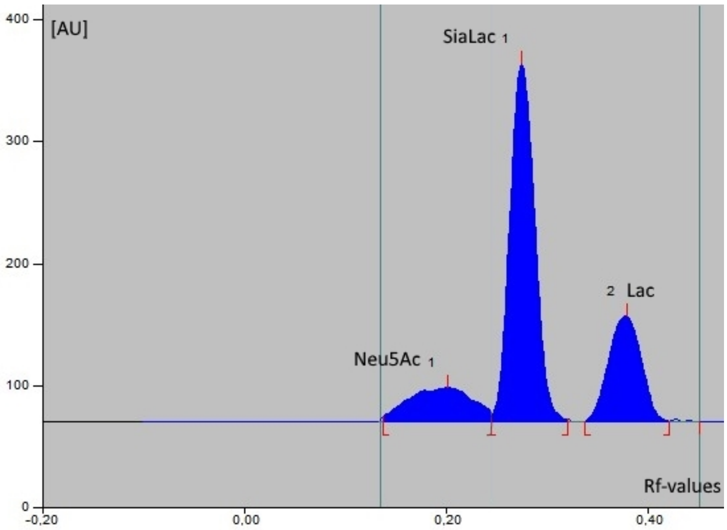


Figure S6. Quantitative analysis for lane 1.

Table S8. HPTLC analysis of sialyltransfer *vs.* hydrolysis using 2,3SiaT$_{pph}$ (NE) and variants (duplicate runs).

1.	NE		C8 (A151D)		C7 (L387A)		G7 (S359T/S360T)		2x (A151D/L387A)	
	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %
10,00	9,36	2,99	14,43	0,57	7,18	1,31	2,65	3,91	1,29	0,71
45,00	16,87	2,40	22,64	2,85	14,79	3,30	6,89	2,25	1,66	2,87
120,00	27,85	2,33	31,73	2,10	25,57	2,73	19,57	2,38	4,53	1,66
240,00	50,38	7,97	55,82	5,52	44,94	6,89	39,48	4,75	7,34	5,57
360,00	70,76	6,18	73,50	3,58	65,20	3,91	68,11	3,66	15,71	2,57
1320,00	89,29	10,71	93,03	6,97	88,11	8,56	91,41	8,59	29,68	14,57

2.	NE		C8 (A151D)		C7 (L387A)		G7 (S359T/S360T)		2x (A151D/L387A)	
	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %
10,00	6,96	1,73	8,39	0,60	5,57	1,22	2,90	1,81	1,92	2,16
45,00	19,46	1,85	22,39	1,02	16,67	1,33	12,48	1,67	5,39	1,60
120,00	30,62	2,01	34,26	1,09	33,57	1,94	30,65	3,47	9,87	1,29
240,00	46,10	4,55	47,12	2,14	45,90	2,10	47,47	2,25	16,32	2,19
360,00	51,86	5,30	55,78	2,05	57,83	1,70	65,08	3,06	22,27	4,59
1320,00	89,08	10,92	93,95	6,05	91,27	6,49	93,71	9,76	39,73	11,26

Average	NE		C8 (A151D)		C7 (L387A)		G7 (S359T/S360T)		2x (A151D/L387A)	
	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %
10,00	8,16	2,36	11,41	0,58	6,38	1,27	2,77	2,86	1,60	1,44
45,00	18,17	2,13	22,51	1,94	15,73	2,32	9,68	1,96	3,53	2,24
120,00	29,24	2,17	33,00	1,60	29,57	2,34	25,11	2,92	7,20	1,48
240,00	48,24	6,26	51,47	3,83	45,42	4,49	43,48	3,50	11,83	3,88
360,00	61,31	5,74	64,64	2,82	61,51	2,80	66,59	3,36	18,99	3,58
1320,00	89,19	10,81	93,49	6,51	89,69	7,53	92,56	9,18	34,70	12,92

error	wt		C8 (A151D)		C7 (L387A)		G7 (S359T/S360T)		2x (A151D/L387A)	
	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %	product %	hydrolysis %
10	1,20	0,63	3,02	0,02	0,81	0,04	0,12	1,05	0,32	0,72
45	1,29	0,27	0,13	0,91	0,94	0,98	2,80	0,29	1,86	0,64
120	1,39	0,16	1,26	0,51	4,00	0,40	5,54	0,54	2,67	0,19
240	2,14	1,71	4,35	1,69	0,48	2,40	4,00	1,25	4,49	1,69
360	9,45	0,44	8,86	0,76	3,68	1,11	1,52	0,30	3,28	1,01
1320	0,11	0,11	0,46	0,46	1,58	1,04	1,15	0,58	5,02	1,65

NMR study of sialidase activity

Table S9. Data for Figure 4: Quantification of sialidase activity of 2,3SiaT$_{pph}$ and variants by ^{1}H NMR (500 MHz) *in situ* monitoring of Neu5Ac liberation from α2,3-SiaLac.[a]

	Native SiaT	A151D	L387A	S359T/S360T
18h	10.40	0.48	0.29	0.27
20h	11.72	0.50	0.32	0.38
22h	13.13	0.55	0.33	0.40
24h	13.23	0.60	0.34	0.46
42h	19.16	0.83	0.48	0.71
48h	20.45	0.92	0.65	0.66

[a] Conditions: 14.9 mM α2,3-SiaLac, 520 μL Tris-buffered (20 mM) D_2O, 10 μg enzyme, 30°C, 48h.

Control for CMP independent sialidase activity

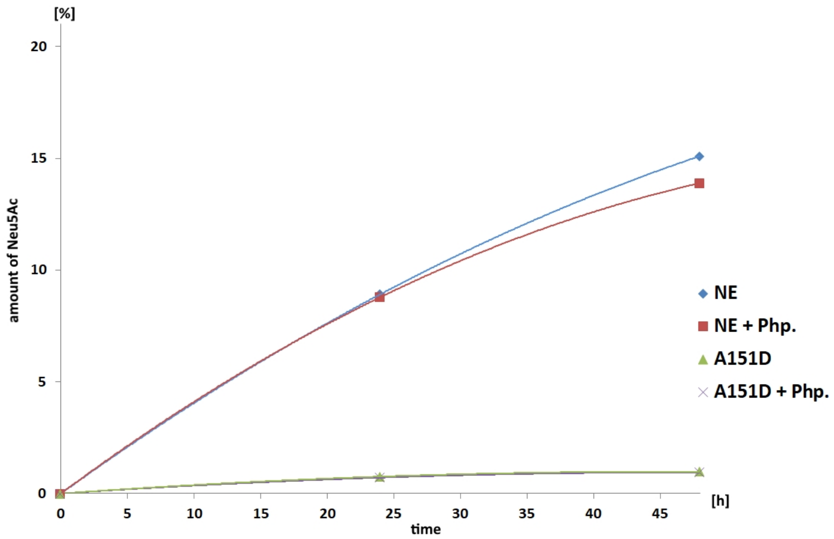
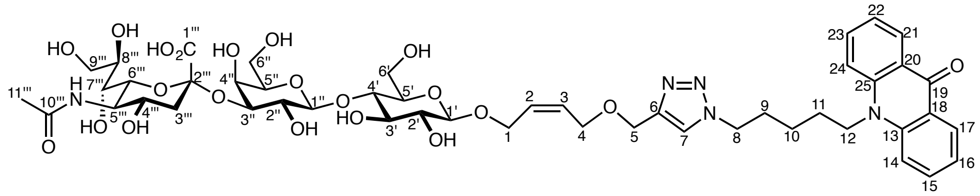


Figure S7. Control experiment for sialidase activity in the presence of alkaline phosphatase (Php) by *in situ*-NMR spectroscopy demonstrating hydrolytic Neu5Ac release from GM3 in the absence of CMP. Conditions: 14.9 mM α2,3-SiaLac, 520 μL Tris-buffered (20 mM) D_2O, 10 μg enzyme, 30°C, 48h. Control: + 20 U alkaline phosphatase. NE = native enzyme.

General protocol for synthesis of fluorescence-labeled *neo*-sialoconjugates using native 2,3SiaT$_{pph}$

CTP (25.2 mg; 2 eq.) and the corresponding sialic acid (Neu5Ac **1** or analogs **2**-**6;** 1.5 eq.) were dissolved in 5 mL Tris-HCl buffer (50 mM, pH 8.6) containing 0.5 M NaCl, 0.1% Triton×100 and 20 mM $MgCl_2$. Reaction was started by addition of 5 mg CSS from *N. meningitis* and inorganic pyrophosphatase (2 U). When CMP activation was completed, lactoside **7** (20 mg; 1 eq.), 0.5 mg recombinant 2,3SiaT$_{pph}$ and alkaline phosphatase (160 U) were added and the pH was readjusted. When the reaction was finished, 30 mL cold methanol (–20 °C) was added to stop the reaction. Protein precipitate was removed by filtration. The filtrate was passed over a plug of reverse-phase C_{18}-silica and the product eluted with 20-50% gradient of aqueous methanol. The solution was concentrated to 10 mL and separated by Biogel P-2 (Bio-Rad, Germany) column chromatography (3 × 100 cm) using 5 mM Tris buffer (pH 8.5). The product fractions were collected and lyophilized for characterization.

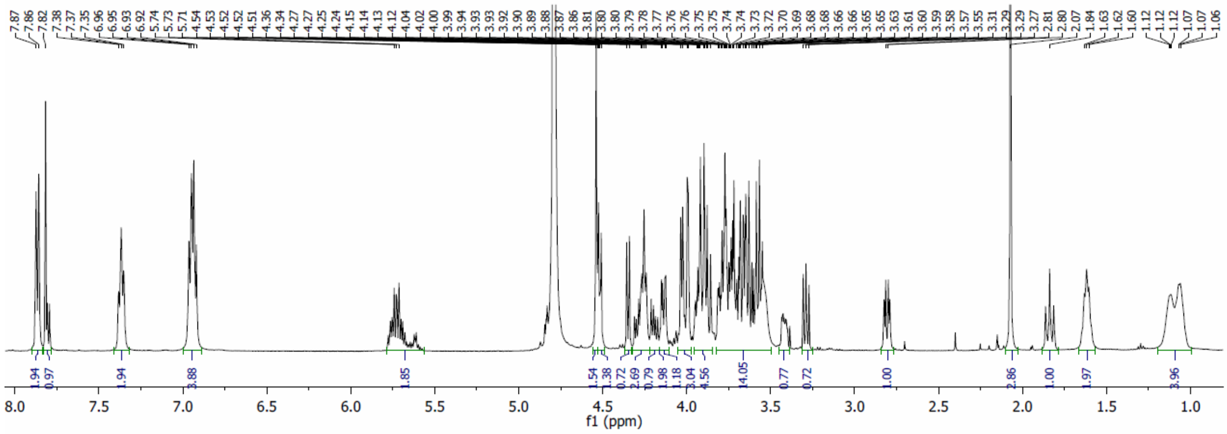


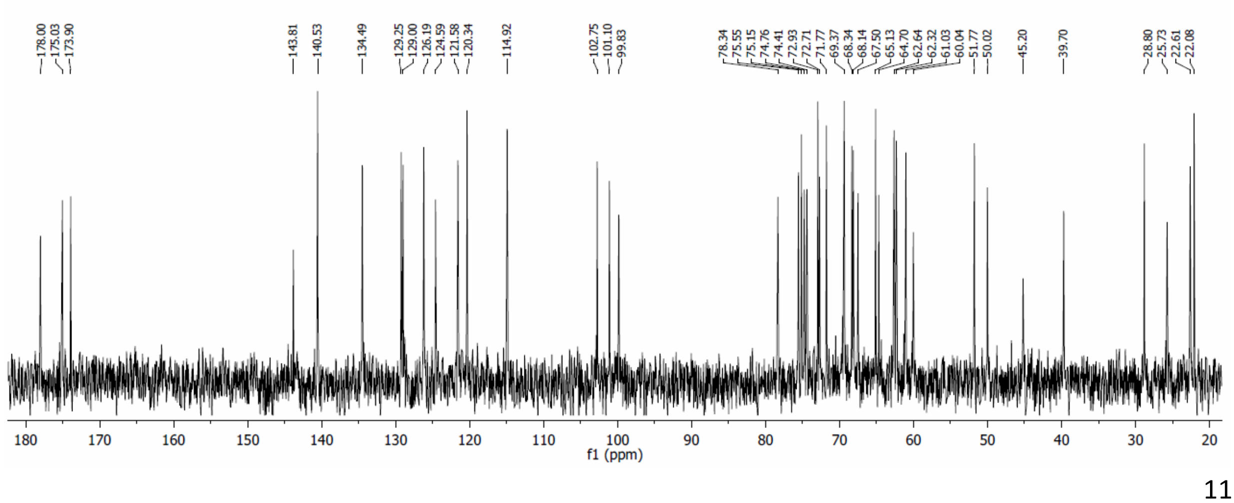
α2,3-Neu5Ac-Lac-Acr (**8**) from 13.1 mg Neu5Ac; yield 23.7 mg pale yellow solid (84%).

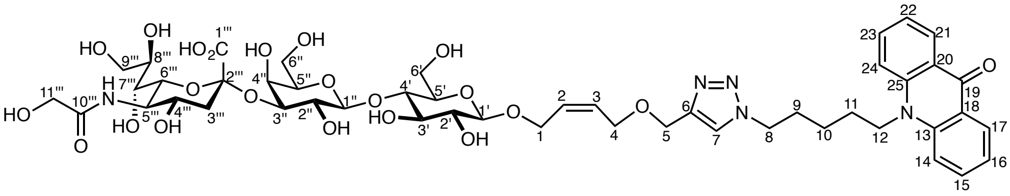
^{1}H NMR (500 MHz, D_2O) δ = 7.87 (d, J = 8.1 Hz, 2H, 17-, 21-H), 7.82 (s, 1H, 7-H), 7.37 (t, J = 7.1 Hz, 2H, 15-, 23-H), 6.98 – 6.90 (m, 4H, 24-, 14-, 16-, 22-H), 5.79 – 5.56 (m, 2H, 2-, 3-H), 4.54 (s, 2H, 5-H), 4.52 (d, J = 8.1 Hz, 1H, 1''-H), 4.35 (d, J = 8.0 Hz, 1H, 1'-H), 4.32 – 4.22 (m, 3H, 1a-, 8-H), 4.22 – 4.16 (m, 1H, 1b-H), 4.16 – 4.11 (m, 1H), 4.04 – 3.98 (m, 3H, 3''-, 2-, 4-H), 3.96 – 3.85 (m, 5H, 4''-, 6'''-, 9'''a-, 6'a-, 5'''-H), 3.82 – 3.50 (m, 13H, 6'b-, 6''-, 8'''-, 7'''-, 9'''b-, 3'-, 4'''-, 4'-, 2''-, 8-, 5''-H), 3.42 (ddd, J = 9.6, 4.7, 2.5 Hz, 1H, 5'-H), 3.29 (dd, J = 9.1, 8.1 Hz, 1H, 2'-H), 2.81 (dd, J = 12.4, 4.6 Hz, 1H, 3'''a-H), 2.07 (s, 3H, 11'''-H), 1.84 (t, J = 12.1 Hz, 1H, 3'''b-H), 1.67 – 1.57 (m, 2H, 9-H), 1.19 – 0.99 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D_2O) δ = 178.00 (C-19), 175.03 (C-10'''), 173.90 (C-1'''), 143.81 (C-6), 140.53 (C-13, -25), 134.49 (C-15, -23), 129.25 (C-2), 129.00 (C-3), 126.19 (C-17, -21), 124.59 (C-7), 121.58 (C-16, -22), 120.34 (C-18, -20), 114.92 (C-14, -24), 102.75 (C-1''), 101.10 (C-1'), 99.83 (C-2'''), 78.34 (C-4'), 75.55 (C-3''), 75.15 (C-5''), 74.76 (C-5'), 74.41 (C-3'), 72.93 (C-2'), 72.71 (C-8'''), 71.77 (C-6'''), 69.37 (C-2''), 68.34 (C-7'''), 68.14 (C-4'''), 67.50 (C-4''), 65.13 (C-4), 64.70 (C-1), 62.64 (C-9'''), 62.32 (C-5), 61.03 (C-6''), 60.04 (C-6'), 51.77 (C-5'''), 50.02 (C-8), 45.20 (C-12), 39.70 (C-3'''), 28.80 (C-9), 25.73 (C-11), 22.61 (C-10), 22.08 (C-11''').

HRMS ESI$^+$: m/z calcd for $[C_{48}H_{66}N_5O_{21}]^+$ = 1048.4245; found 1048.4255.





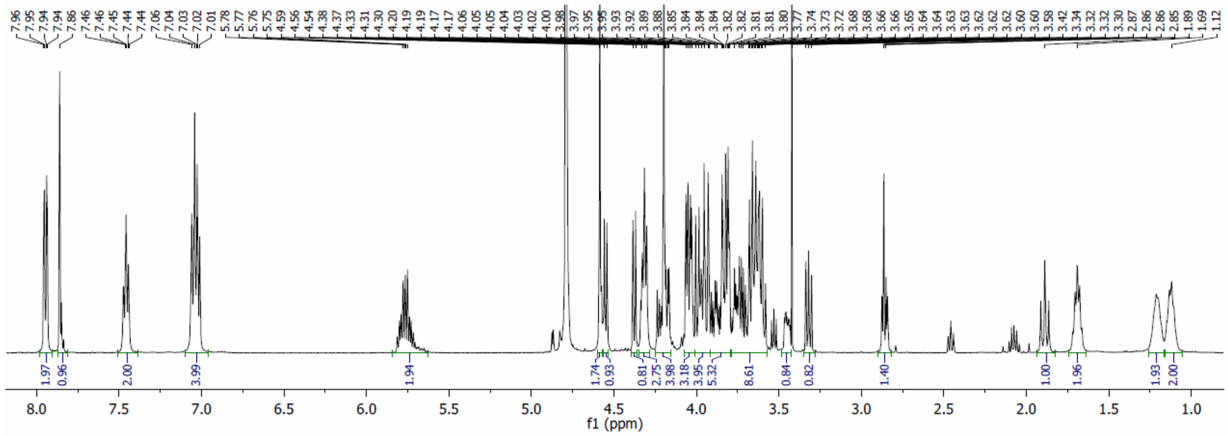


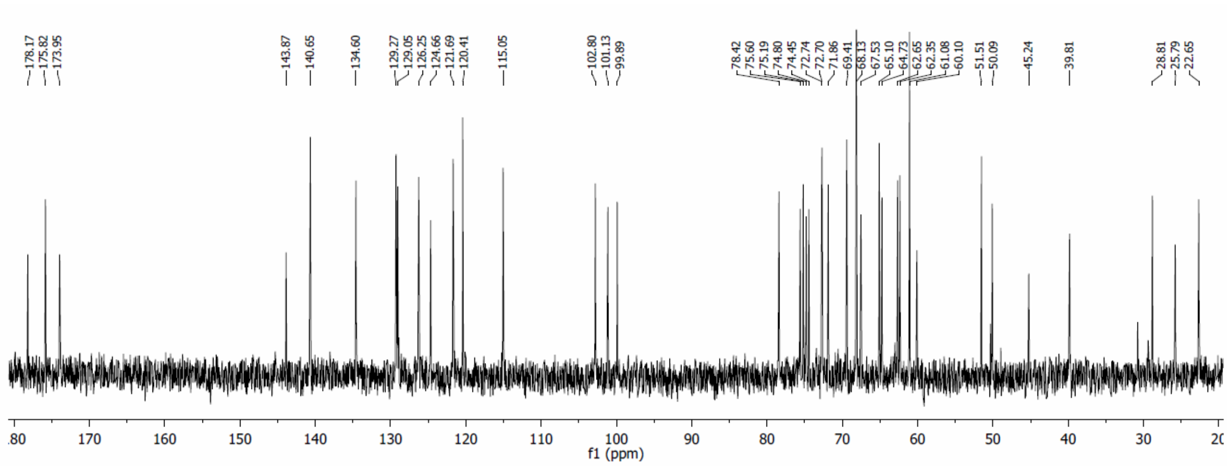
α**2,3-Neu5Gc-Lac-Acr** (**9**) from 13.7 mg Neu5Gc; yield 22.1 mg pale yellow solid (77%).

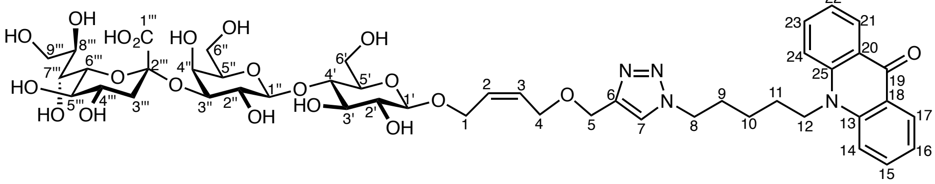
^{1}H NMR (500 MHz, D_2O) δ = 7.95 (dd, J = 8.1, 1.6 Hz, 2H, 17-, 21-H), 7.86 (s, 1H, 7-H), 7.46 (ddd, J = 8.7, 6.9, 1.6 Hz, 2H, 15-, 23-H), 7.10 – 6.96 (m, 4H, 24-, 14-, 16-, 22-H), 5.84 – 5.63 (m, 2H, 2-, 3-H), 4.59 (s, 2H, 5-H), 4.55 (d, J = 7.8 Hz, 1H, 1''-H), 4.38 (d, J = 8.0 Hz, 1H, 1'-H), 4.34 – 4.29 (m, 3H, 1a-, 8-H), 4.25 – 4.15 (m, 4H, 11'''-, 1b-, 3''-H), 4.07 – 3.79 (m, 12H, 6'a-, 5'''-, 6'b-, 6''-, 8'''-, 6'''-, 9'''b-, 3'-, 4'''-, 4''-, 2''-H), 3.79 – 3.57 (m, 9H, 6''b-, 3''-, 9'''b-, 12-, 7'''-, 3'-, 4'-, 2''-H), 3.45 (ddd, J = 9.7, 4.9, 2.5 Hz, 1H, 5'-H), 3.32 (dd, J = 9.2, 8.0 Hz, 1H, 2'-H), 2.90 – 2.82 (m, 1H, 3'''a-H), 1.88 (td, J = 12.1, 3.5 Hz, 1H, 3'''b-H), 1.69 (p, J = 7.0 Hz, 2H, 9-H), 1.20 – 1.06 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D_2O) δ = 178.17 (C-19), 175.82 (C-10'''), 173.95 (C-1'''), 143.87 (C-6), 140.65 (C-13, -25), 134.60 (C-15, -23), 129.27 (C-2), 129.05 (C-3), 126.25 (C-17, -21), 124.66 (C-7), 121.68 (C-16, -22), 120.41 (C-18, -20), 115.05 (C-14, -24), 102.80 (C-1''), 101.13 (C-1'), 99.89 (C-2'''), 78.42 (C-4'), 75.60 (C-3''), 75.19 (C-8'''), 74.80 (C-5'), 74.45 (C-5''), 72.74 (C-3'), 72.70 (C-2'), 71.86 (C-6'''), 69.41 (C-2''), 68.13 (C-7'''), 67.53 (C-4'''), 65.10 (C-4), 64.73 (C-1), 62.65 (C-9'''), 62.35 (C-5), 61.08 (C-6'', -11'''), 60.10 (C-6'), 51.51 (C-5'''), 50.09 (C-8), 45.24 (C-12), 39.81 (C-3'''), 28.81 (C-9), 25.79 (C-11), 22.66 (C-10).

HRMS ESI$^+$: m/z calcd for $[C_{48}H_{66}N_5O_{22}]^+$ = 1064.4194; found 1064.4206.





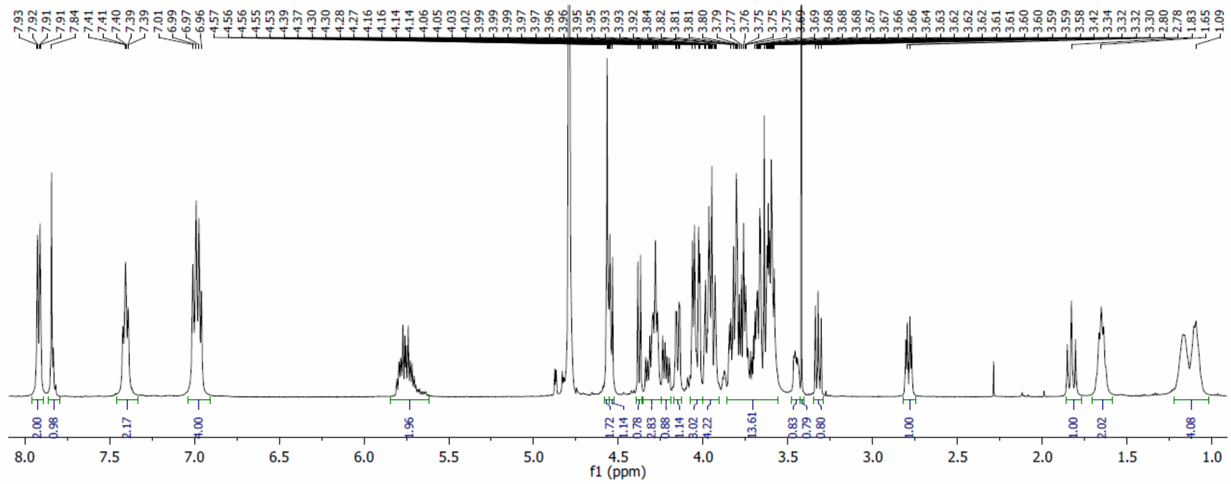


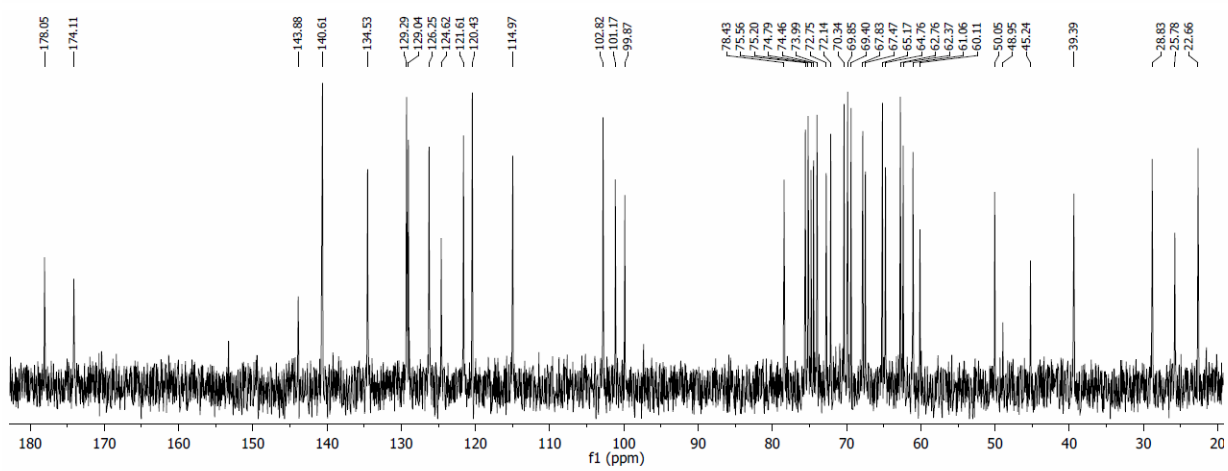
α2,3-KDN-Lac-Acr (**10**) from 11.5 mg KDN; yield 23.4 mg pale yellow solid (86%).

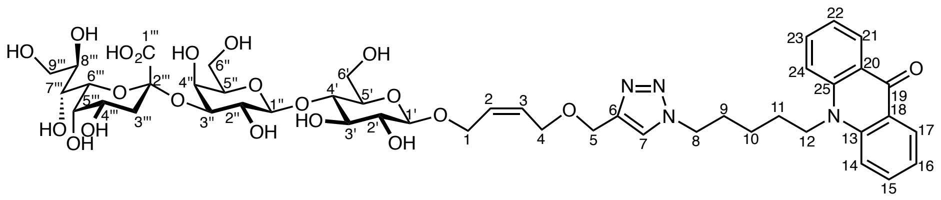
^{1}H NMR (500 MHz, D_2O) δ = 7.92 (dd, J = 8.0, 1.7 Hz, 2H, 17-, 21H), 7.84 (s, 1H, 7-H), 7.45 – 7.35 (m, 2H, 15-, 23-H), 7.05 – 6.92 (m, 4H, 14-, 24-, 16-, 22-H), 5.81 – 5.62 (m, 2H, 2-, 3-H), 4.56 (s, 2H, 5-H), 4.55 – 4.52 (m, 1H, 1''-H), 4.38 (d, J = 8.0 Hz, 1H, 1'-H), 4.35 – 4.25 (m, 3H, 1a-, 8-H), 4.22 (dd, J = 12.6, 6.8 Hz, 1H, 1b-H), 4.17 – 4.13 (m, 1H, H3''), 4.07 – 4.01 (m, 3H), 4.00 – 3.90 (m, 4H), 3.85 – 3.73 (m, 5H), 3.72 – 3.54 (m, 8H, 12-, 9'''b-, 4'''-, 8'''-, 3'-, 2''-, 4'-H), 3.45 (ddd, J = 9.7, 4.8, 2.5 Hz, 1H, 5'-H), 3.42 (s, 1H), 3.32 (dd, J = 9.2, 8.0 Hz, 1H, 2'-H), 2.79 (dd, J = 12.5, 4.6 Hz, 1H, 3'''a-H), 1.83 (td, J = 12.0, 2.8 Hz, 1H, 3'''b-H), 1.69 – 1.60 (m, 2H, 9-H), 1.24 – 1.02 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D_2O) δ = 178.05 (C-19), 174.11 (C-1'''), 143.88 (C-6), 140.61 (C-13, -25), 134.53 (C-15, -23), 129.29 (C-2), 129.04 (C-3), 126.25 (C-17, -21), 124.62 (C-7), 121.61 (C-16, -22), 120.43 (C-18, -20), 114.97 (C-14, -24), 102.82 (C-1''), 101.17 (C-1'), 99.87 (C-2'''), 78.43 (C-4'), 75.56 (C-3''), 75.20 (C-8'''), 74.79 (C-5'), 74.46 (C-5''), 73.99 (C-3'), 72.75 (C-2'), 72.14 (C-6'''), 70.34 (C-2''), 69.85 (C-7'''), 69.40 (C-4'''), 67.83 (C-5'''), 65.17 (C-4), 64.76 (C-1), 62.76 (C-9'''), 62.37 (C-5), 61.06 (C-6''), 60.11 (C-6'), 50.05 (C-8), 45.24 (C-12), 39.39 (C-3'''), 28.83 (C-9), 25.78 (C-11), 22.66 (C-10).

HRMS ESI^{+}: m/z calcd for $[C_{46}H_{63}N_4O_{21}]^{+}$ = 1007.3979; found 1007.3989.





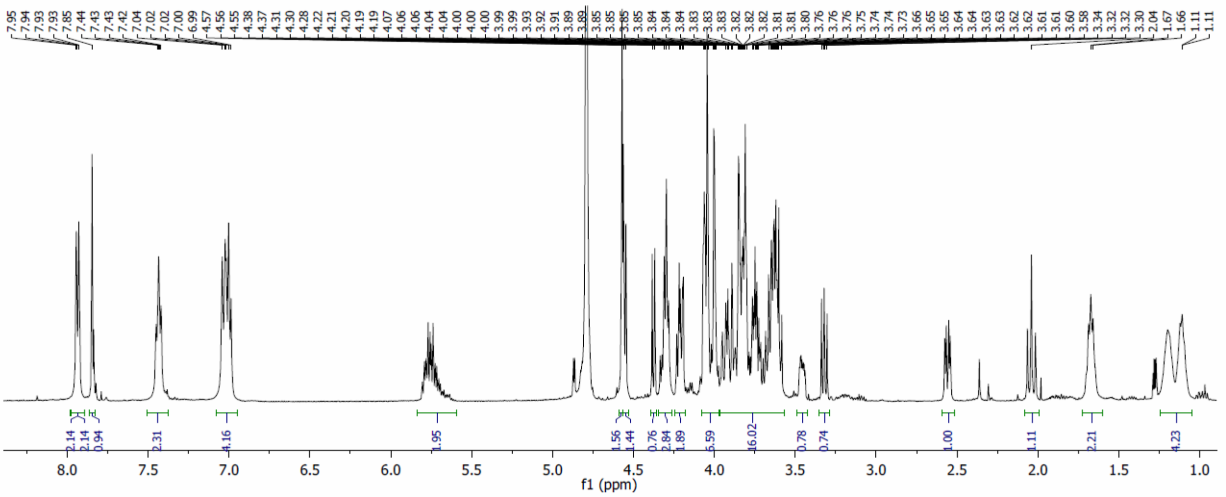


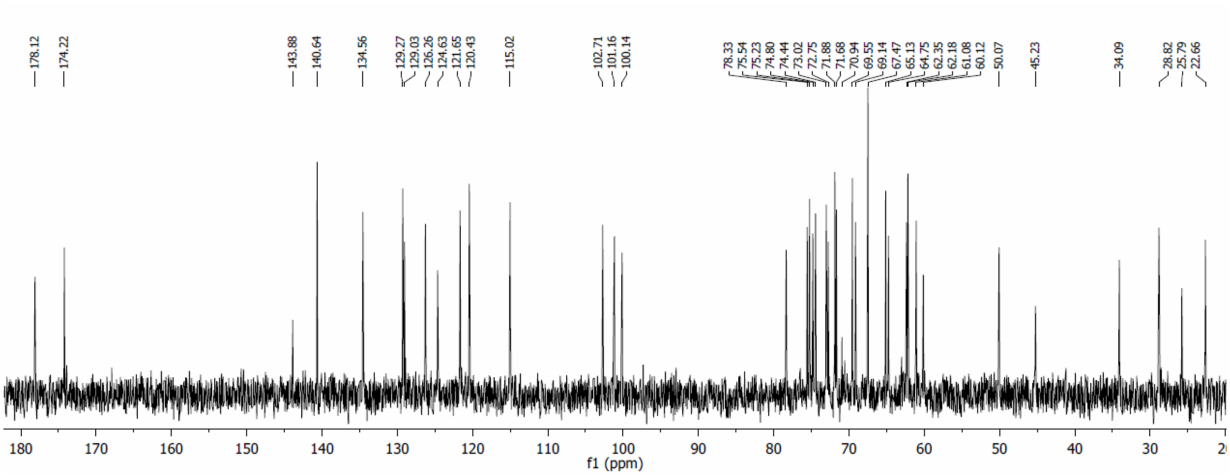
α**2,3-*epi*-KDN-Lac-Acr** (**11**) from 11.5 mg *epi*-KDN; yield 15.8 mg pale yellow solid (58%).

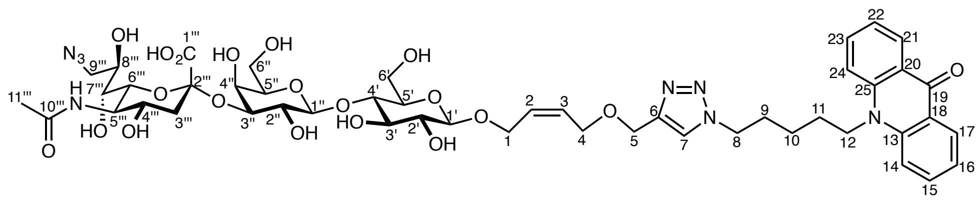
^{1}H NMR (500 MHz, D_2O) δ = 7.94 (dd, *J* = 8.0, 1.7 Hz, 2H, 17-, 21-H), 7.85 (s, 1H, 7-H), 7.51 – 7.38 (m, 2H, 15-, 23-H), 7.08 – 6.95 (m, 4H, 24-, 14-, 16-, 22-H), 5.80 – 5.62 (m, 2H, 2-, 3-H), 4.57 (s, 2H, 5-H), 4.55 (d, *J* = 7.7 Hz, 1H, 1''-H), 4.38 (d, *J* = 8.0 Hz, 1H, 1'-H), 4.32 – 4.28 (m, 3H, 1a-, 8-H), 4.25 – 3.97 (m, 9H, H-3''), 3.96 – 3.56 (m, 16H), 3.46 (ddd, *J* = 9.7, 4.9, 2.5 Hz, 1H, 5'-H), 3.32 (dd, *J* = 9.3, 8.0 Hz, 1H, 2'-H), 2.56 (dd, *J* = 12.2, 4.6 Hz, 1H, 3'''a-H), 2.04 (t, *J* = 12.4 Hz, 1H, 3'''b-H), 1.67 (p, *J* = 7.1 Hz, 2H, 9-H), 1.24 – 1.05 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D_2O) δ = 178.12 (C-19), 174.22 (C-1'''), 143.88 (C-6), 140.64 (C-13, -25), 134.56 (C.15, -23), 129.27 (C-2), 129.03 (C-3), 126.26 (C-17, -21), 124.63 (C-7), 121.65 (C-16, -22), 120.42 (C-18, -20), 115.02 (C-14, -24), 102.71 (C-1''), 101.16 (C-1'), 100.13 (C-2'''), 78.33 (C-4'), 75.54 (C-3''), 75.22 (C-8'''), 74.80 (C-5'), 74.44 (C-5''), 73.02 (C-3'), 72.75 (C-2'), 71.88 (C-6'''), 71.68 (C-2''), 69.55 (C-7'''), 69.14 (C-4'''), 67.47 (C-5'''), 65.13 (C-4), 64.75 (C-1), 62.35 (C-9'''), 62.18 (C-5), 61.08 (C-6''), 60.12 (C-6'), 50.07 (C-8), 45.23 (C-12), 34.09 (C-3'''), 28.82 (C-9), 25.79 (C-11), 22.66 (C-10).

HRMS ESI^{+}: *m/z* calcd for $[C_{46}H_{63}N_4O_{21}]^+$ = 1007.3979; found 1007.3983.





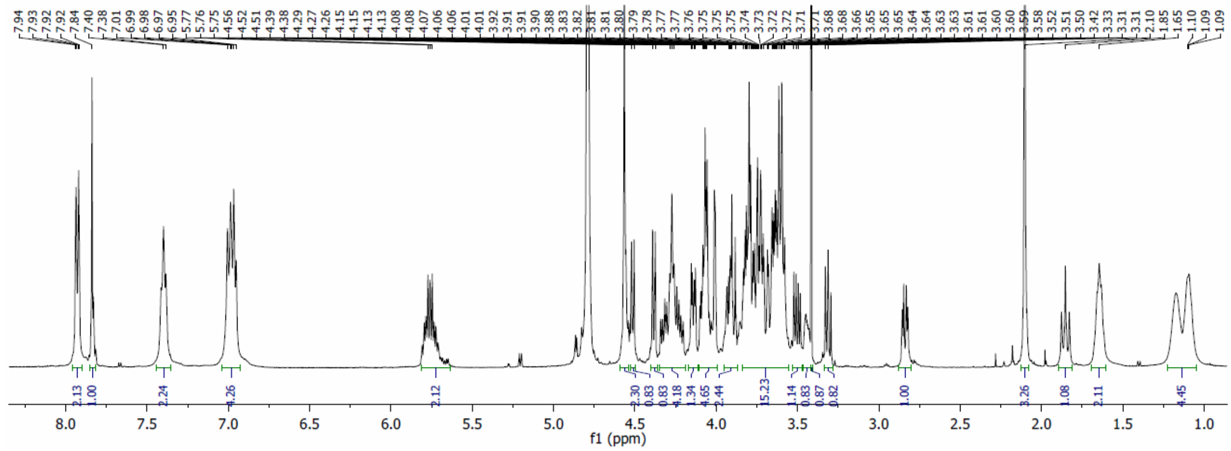


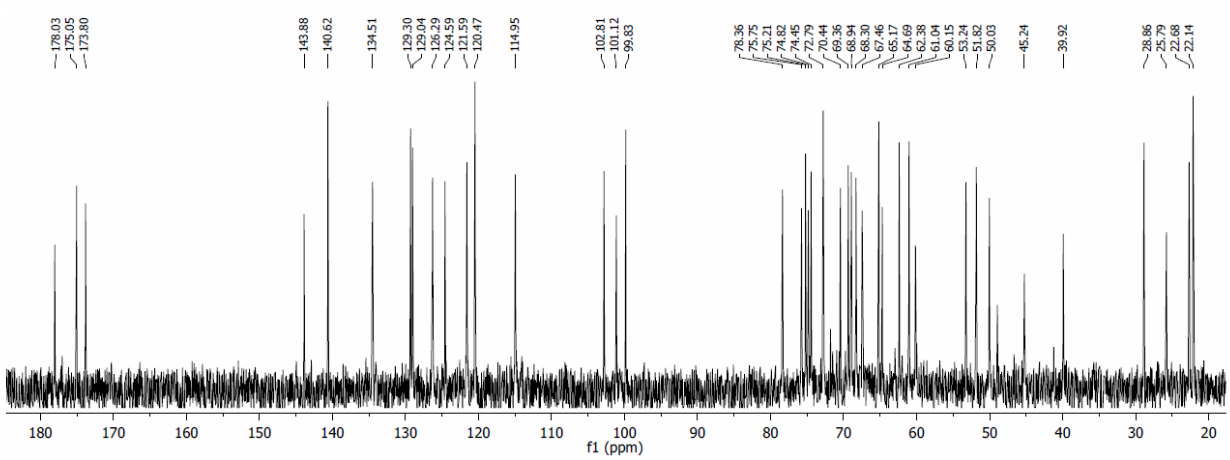
α2,3-Neu5Ac9N$_3$-Lac-Acr (**12**) from 14.1 mg Neu5Ac9N$_3$; yield 23.7 mg pale yellow solid (82%).

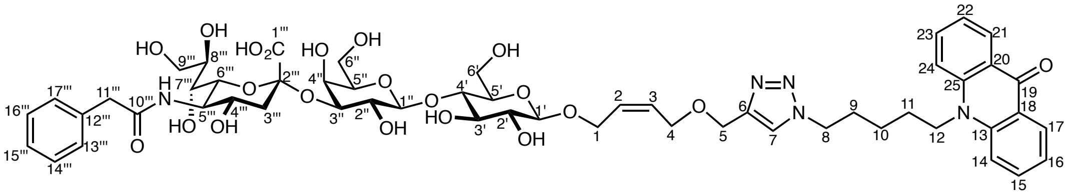
^{1}H NMR (500 MHz, D$_2$O) δ = 7.96 – 7.90 (m, 2H, 17-, 21-H), 7.84 (s, 1H, 7-H), 7.44 – 7.36 (m, 2H, 15-, 23-H), 7.04 – 6.93 (m, 4H, 24-, 14-, 16-, 22-H), 5.81 – 5.63 (m, 2H, 2-, 3-H), 4.56 (s, 2H, 5-H), 4.51 (d, J = 7.9 Hz, 1H, 1''-H), 4.38 (d, J = 7.9 Hz, 1H, 1'-H), 4.35 – 4.19 (m, 4H, 1'-, 1a-, 8-H), 4.17 – 4.11 (m, 1H, H-3''), 4.10 – 3.99 (m, 5H, 6'''-, 4-, 6''a-, 8'''-H), 3.95 – 3.87 (m, 2H, 5'''-, 6'a-H), 3.84 – 3.56 (m, 15H), 3.50 (dd, J = 13.2, 6.6 Hz, 1H, 9'''b-H), 3.46 – 3.42 (m, 1H, 5'-H), 3.31 (dd, J = 9.1, 8.0 Hz, 1H, 2'-H), 2.84 (dd, J = 12.4, 4.6 Hz, 1H, 3'''a-H), 2.10 (s, 3H, 11'''-H), 1.85 (t, J = 12.1 Hz, 1H, 3'''b-H), 1.65 (t, J = 7.4 Hz, 2H, 9-H), 1.23 – 1.05 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D$_2$O) δ = 178.03 (C-19), 175.05 (C-10'''), 173.80 (C-1'''), 143.88 (C-6), 140.62 (C-13, -25), 134.51 (C-15, -23), 129.30 (C-2), 129.04 (C-3), 126.29 (C-17, -21), 124.59 (C-7), 121.59 (C-16, -22), 120.47 (C-18, -20), 114.95 (C-14, -24), 102.80 (C-1''), 101.12 (C-1'), 99.83 (C-2'''), 78.36 (C-4'), 75.75 (C-3''), 75.21 (C-8'''), 74.82 (C-5'), 74.45 (C-5''), 72.78 (C-3'), 70.44 (C-2'), 69.36 (C-6'''), 68.94 (C-2''), 68.30 (C-7'''), 67.46 (C-4'''), 65.17 (C-4), 64.69 (C-1), 62.38 (C-5), 61.04 (C-6'''), 60.16 (C-6'), 53.24 (C-9'''), 51.82 (C-5'''), 50.03 (C-8), 45.25 (C-12), 39.92 (C-3'''), 28.86 (C-9), 25.79 (C-11), 22.68 (C-10), 22.14 (C-11''').

HRMS ESI$^+$: m/z calcd for $[C_{48}H_{65}N_8O_{20}]^+$ = 1073.4310; found 1073.4321.





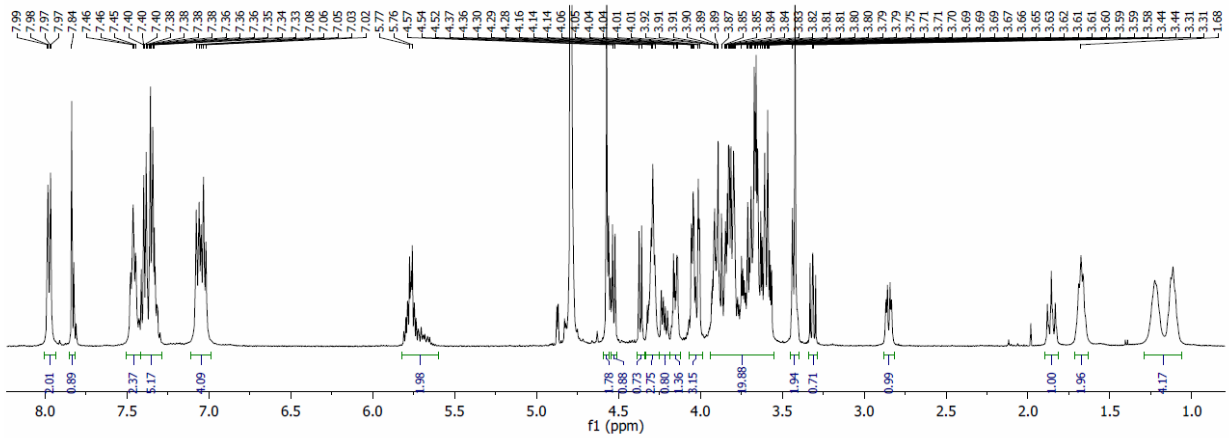


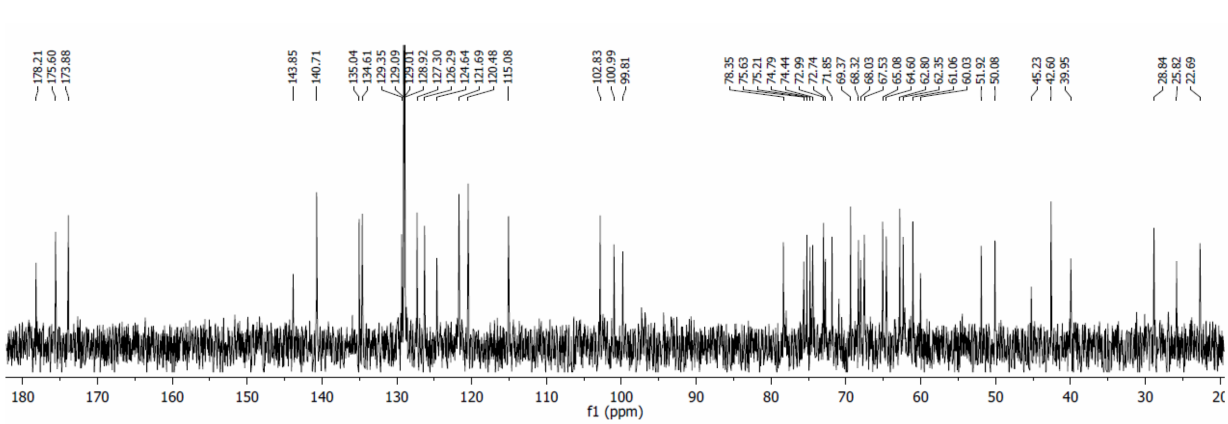
α2,3-Neu5NPhAc-Lac-Acr (**13**) from 16.1 mg Neu5NPhAc; yield 14.5 mg pale yellow solid (48%).

^{1}H NMR (500 MHz, D_2O) δ = 7.98 (dd, J = 7.9, 1.7 Hz, 2H, 17-, 21-H), 7.84 (s, 1H, 7-H), 7.45 (dt, J = 10.7, 8.3 Hz, 2H, 15-, 23-H), 7.42 – 7.28 (m, 5H, Har), 7.11 – 6.99 (m, 4H, 24-, 14-, 16-, 22-H), 5.82 – 5.60 (m, 2H, 2-, 3-H), 4.57 (s, 2H, 5-H), 4.53 (d, J = 7.8 Hz, 1H, 1''-H), 4.37 (d, J = 7.9 Hz, 1H, 1'-H), 4.33 – 4.25 (m, 3H, 1a-, 8-H), 4.22 (dd, J = 12.6, 6.5 Hz, 1H, 1b-H), 4.18 – 4.12 (m, 1H, 3''-H), 4.07 – 3.99 (m, 3H), 3.94 – 3.55 (m, 20H), 3.45 – 3.40 (m, 2H, 7'''-, 5'-H), 3.31 (dd, J = 9.2, 8.0 Hz, 1H, 2'-H), 2.88 – 2.81 (m, 1H, 3'''a-H), 1.85 (td, J = 11.8, 3.7 Hz, 1H, 3'''b-H), 1.68 (p, J = 6.3 Hz, 2H, 9-H), 1.29 – 1.06 (m, 4H, 11-, 10-H).

^{13}C NMR (126 MHz, D_2O) δ = 178.21 (C-19), 175.60 (C-10'''), 173.88 (C-1'''), 143.85 (C-6), 140.71 (C-13, -25), 135.04 (C-15, -23), 134.61 (C-12'''), 129.34 (C-14''''), 129.09 (C-2), 129.01 (C-3), 128.91 (C-13''', -15'''), 127.30 (C-12''', -16'''), 126.29 (C-17, -21), 124.64 (C-7), 121.69 (C-16, -22), 120.48 (C-18, -20), 115.08 (C-14, -24), 102.82 (C-1''), 100.99 (C-1'), 99.81 (C-2'''), 78.35 (C-4'), 75.62 (C-3''), 75.21 (C-8'''), 74.79 (C-5'), 74.44 (C-5''), 72.99 (C-3'), 72.74 (C-2'), 71.85 (C-6'''), 69.37 (C-2''), 68.32 (C-7'''), 67.53 (C-4'''), 65.08 (C-4), 64.60 (C-1), 62.80 (C-9'''), 62.35 (C-5), 61.05 (C-6''), 60.03 (C-6'), 59.84 (C-12), 51.92 (C-5'''), 50.08 (C-8), 42.60 (C-11'''), 39.95 (C-3'''), 28.84 (C-9), 25.82 (C-11), 22.69 (C-10).

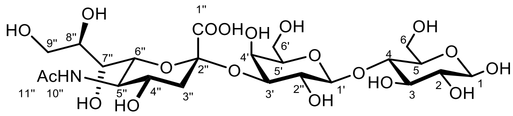
HRMS ESI$^+$: m/z calcd for $[C_{54}H_{70}N_5O_{21}]^+$ = 1124.4558; found 1124.4572.





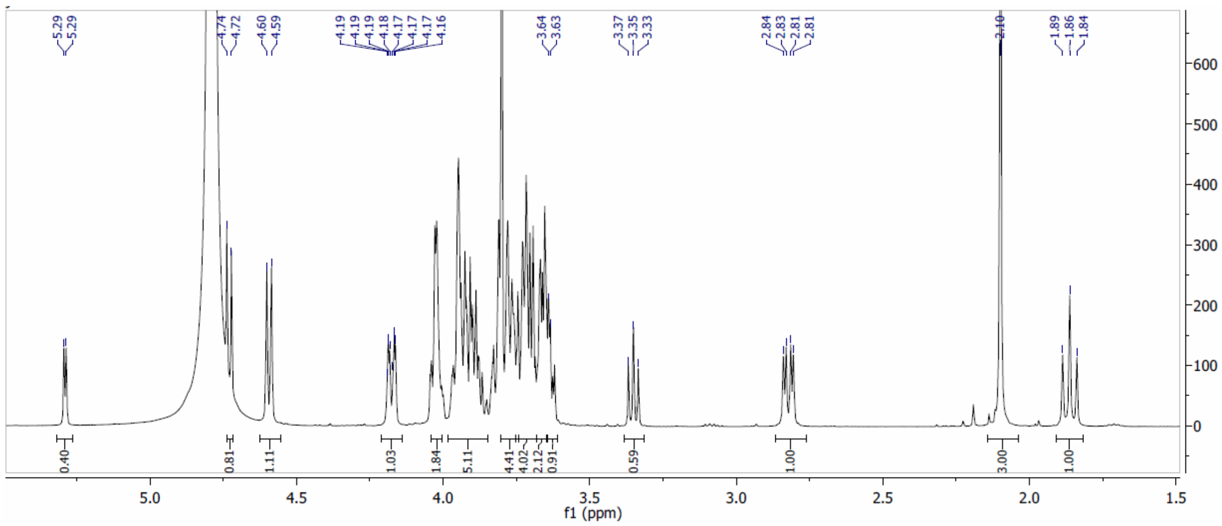
Synthesis of α2,3-SiaLac (GM3 trisaccharide, 14) by native 2,3SiaT$_{pph}$ and engineered 2,3SiaT$_{pph}$ (A151D)

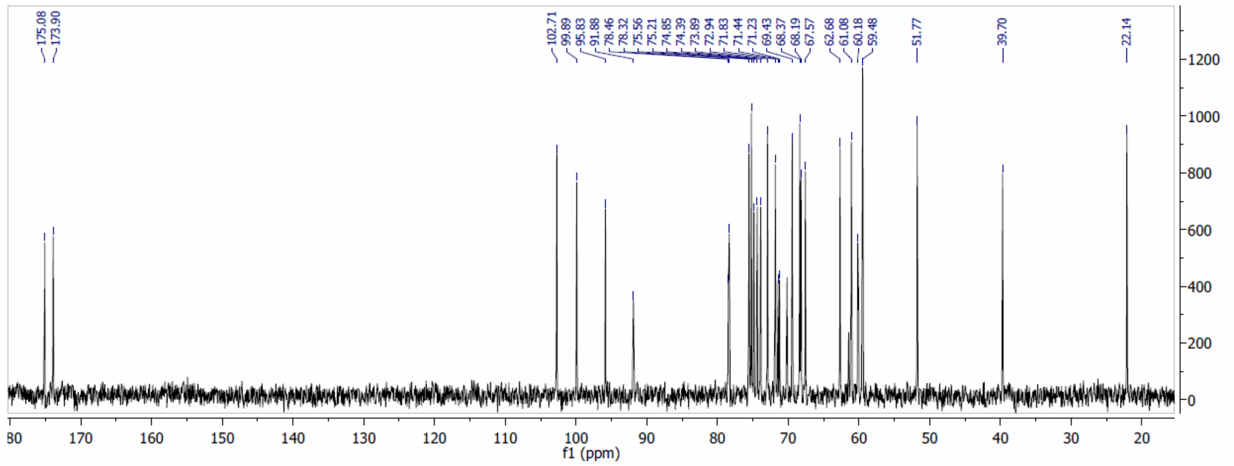
Neu5Ac (50 mg, 1 eq.) and CTP (117.2 mg, 1.5 eq.) were dissolved in 12 mL Tris-HCl buffer (50 mM, pH 8.5) containing 20 mM $MgCl_2$. After addition of inorganic pyrophosphatase (10 U) the reaction mixture was re-adjusted to pH 8.5 and started by adding 10 mg CSS, while keeping the pH constant by autotitration. After 2 h at 37 °C TLC analysis indicated the disappearance of Neu5Ac, then lactose (174.7 mg, 3 eq.), native 2,3SiaT$_{pph}$ or 2,3SiaT$_{pph}$ (A151D) (0.5 mg) and alkaline phosphatase (319 U) were added and incubation continued overnight. When conversion was completed, an equivalent volume of cold methanol (–20 °C) was added to stop the reaction. Protein precipitate was removed by centrifugation, methanol evaporated under reduced pressure, and the remaining solution directly purified over Biogel P-2 (Bio-Rad, Germany; 3 × 100 cm column). The product fractions were collected and lyophilized for NMR analysis. Product **14** was obtained as colorless solid (88.2 mg, 86% for native 2,3SiaT$_{pph}$ / 93.4 mg, 91% for 2,3SiaT$_{pph}$ (A151D)).



^{1}H NMR (500 MHz, D_2O) δ = 5.29 (d, J = 3.7 Hz, 0.4H, 1α-H), 4.73 (d, J = 8.1 Hz, 0.6H, 1β-H), 4.59 (d, J = 7.9 Hz, 1H, 1'β-H), 4.18 (ddd, J = 9.9, 1.9, 2.9 Hz, 1H, 3'-H), 4.06-3.99 (m, 1.8H, 4'-, 6α-H), 3.98-3.85 (m, 5H, 6"-, 5"-, 5-, 6'-H), 3.82-3.74 (m, 4H, 6β-, 5'-, 4-H) 3.74-3.68 (m, 4H, 4"-, 3-, 9"-H), 3.68-3.65 (m, 2H, 7"-, 8"-H), 3.64-3.61 (m, 1H, 2'-H), 3.35 (dd, J = 8.5 Hz, 1H, 2-H), 2.82 (dd, J = 12.4, 4.6 Hz, 1H, 3"-H),2.10 (s, 3H, 11"-H), 1.86 (dd, J = 12.4, 12.1 Hz, 1H, 3"-H).

^{13}C NMR (126 MHz, D_2O) δ = 175.08 (C-1''), 173.90 (C-10''), 102.71 (C-1'), 99.89 (C-2''), 95.83 (C-1β), 91.88 (C-1α), 78.46(C-4α), 78.32 (C-4β), 75.56 (C-3'), 75.21 (C-5'), 74.85(C-2α), 74.39 (C-2β), 73.89 (C-4''), 72.94 (C-3), 71.83 (C-6''), 71.44 (C-5α), 71.23 (C-5β), 69.43 (C-2'), 68.37(C-8''), 68.19 (C-7''), 67.57 (C-4'), 62.68 (C-9''), 61.08 (C-6'), 60.18 (C-6α), 59.48 (C-6β), 51.77(C-5''), 39.70 (C-3''), 22.14 (C-11'').



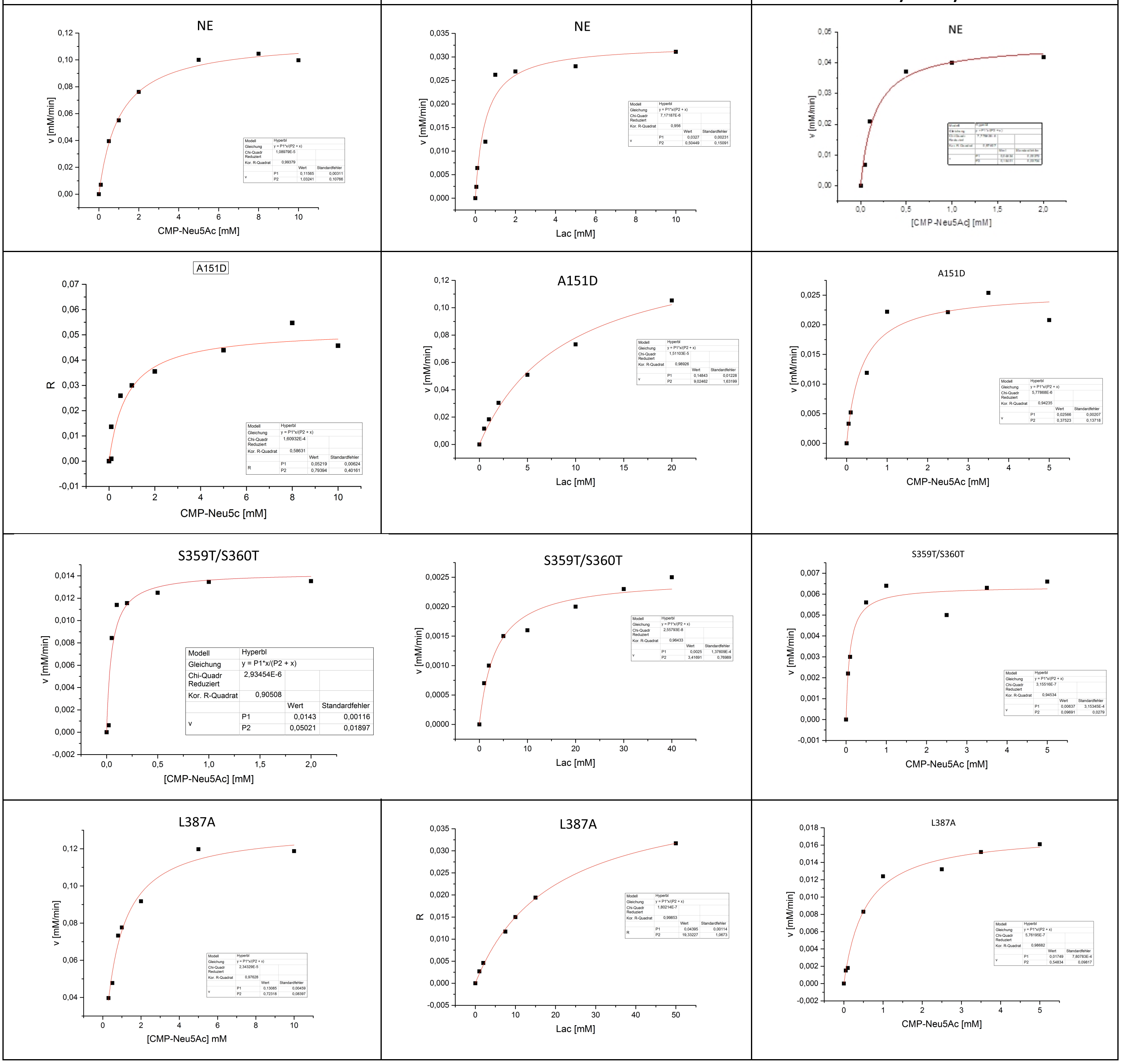


175.08
173.90
102.71
99.89
95.83
91.88
78.46
78.32
75.56
75.21
74.85
74.39
73.89
72.94
71.83
71.44
71.23
69.43
68.37
68.19
67.57
62.68
61.08
60.18
59.48
51.77
39.70
22.14
f1 (ppm)

Exemplary kinetic profiles for native enzyme (NE) and best variants

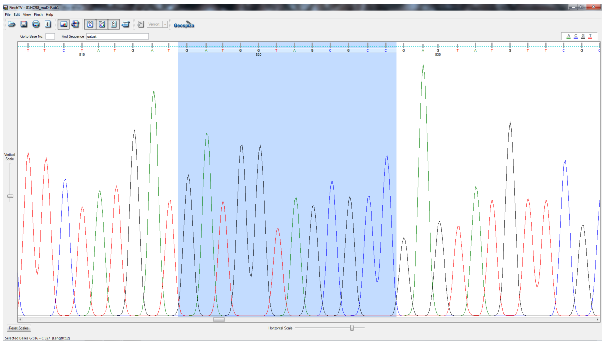
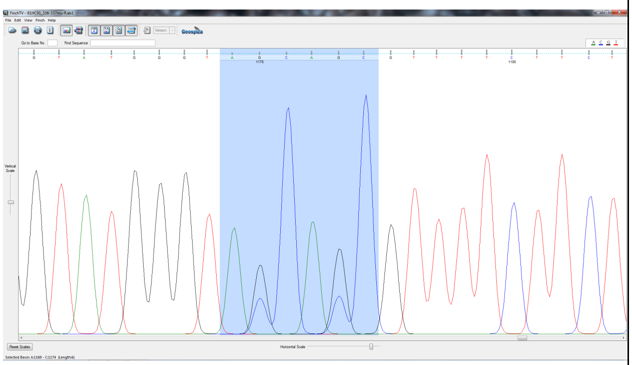
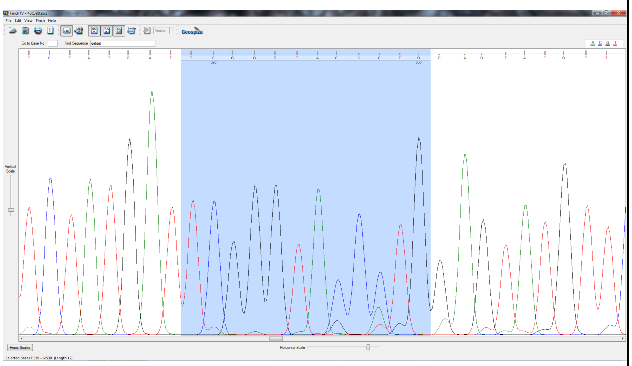
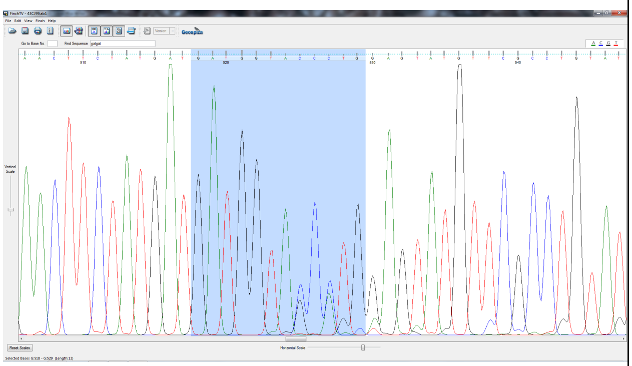
Enzymatic assays were performed as described. Experimental data were fitted to the Michaelis–Menten equation by nonlinear regression using Origin software (v9.1G, OriginLab) to derive apparent kinetic parameters.

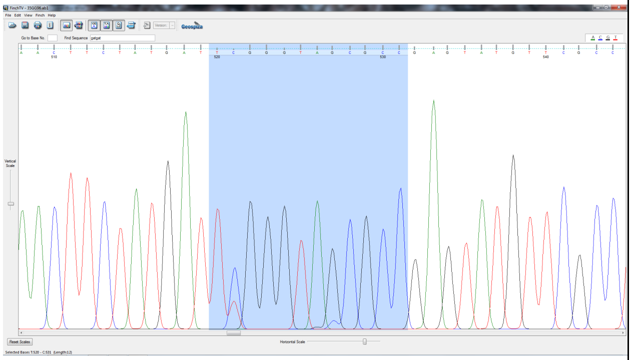
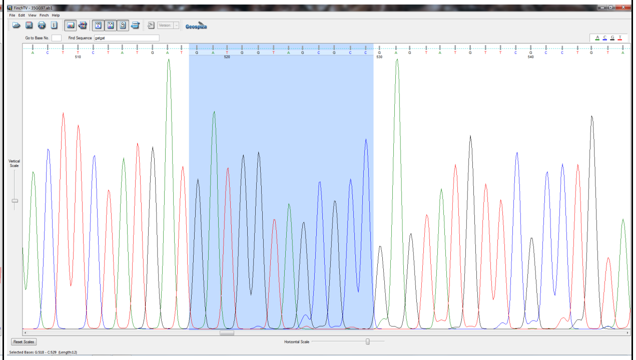
CMPNeu5Ac	Lactose	Hydrolysis



Quality control of second-generation combination library by sequencing

Because positions 148, 150 and 151 are too close to each other to assure sufficient homogenous primer binding, positions 148-151 were first deleted with adjacent primers. Into the resulting template the desired mutated sequence was introduced by using appropriate overhang primers. The PCR product resulting from the first rounds contained the newly introduced sequence, which was used as template for further amplification cycles. The resulting plasmid mixes were sequenced to assure successful mutagenesis. For ambiguous sequence data single clones were sequenced to assure a positive result. All combinations were found:

81HC98 w.t. Template	81HC91 359 Ser,Thr / 360 Ser,Thr 359/360 Template
	
wtA 43CJ98 148TCG/TTG (Ser/Leu), 150AGC/ACC (Ser/Thr), 151C(T)TG/ATG (Leu/Met)	wtB 43CJ99 148GAT (Asp), 150AGC/ACC (Ser/Thr), 151CTG/ATG (Leu/Met)
	

wtC 35GG96 148TCG/TTG (Ser/Leu), 150AGC/ACC (Ser/Thr), 151GCC (Ala) 	wtD 35GG97 148GAT (Asp), 150ACC/AGC (Ser/Thr), 151GCC (Ala) 

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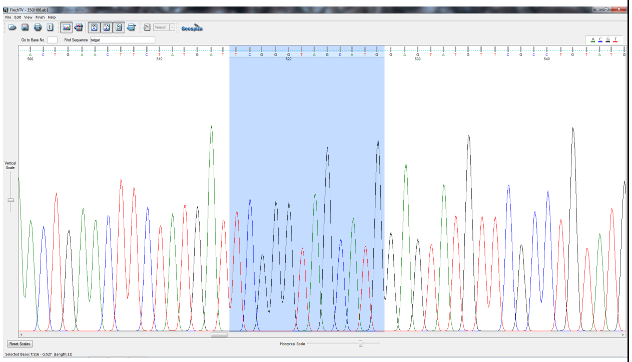
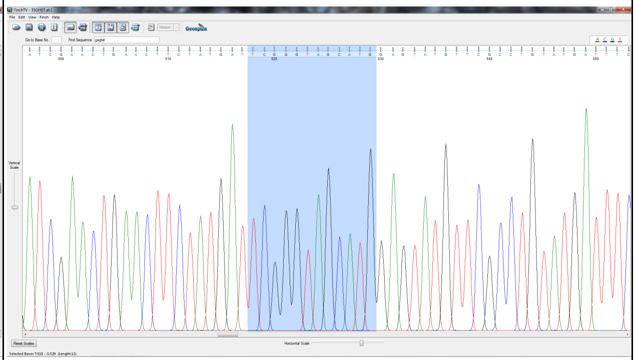
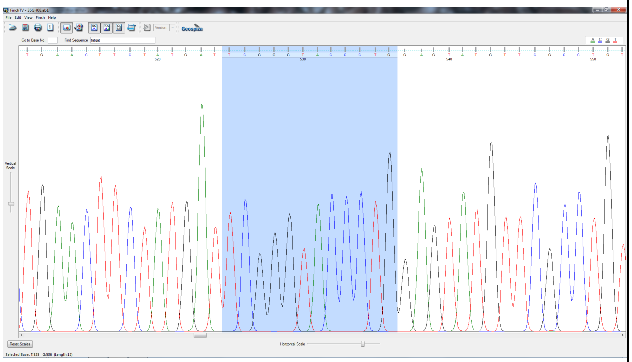
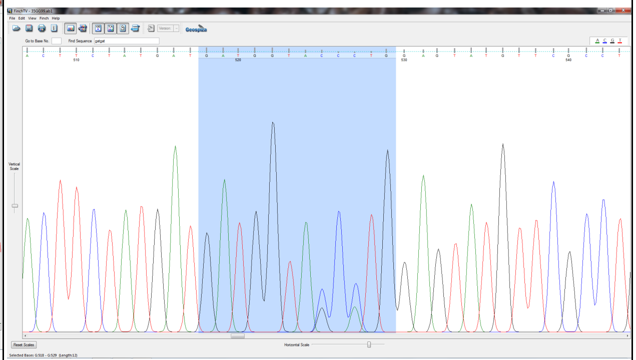
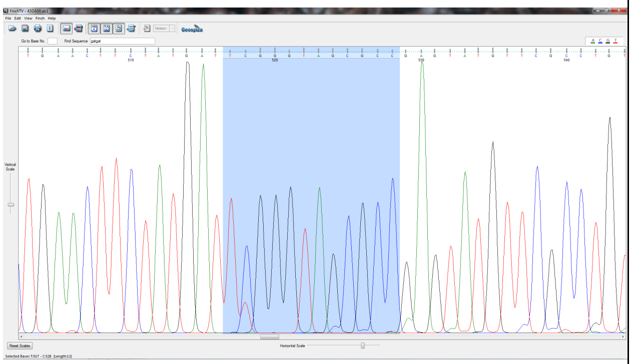
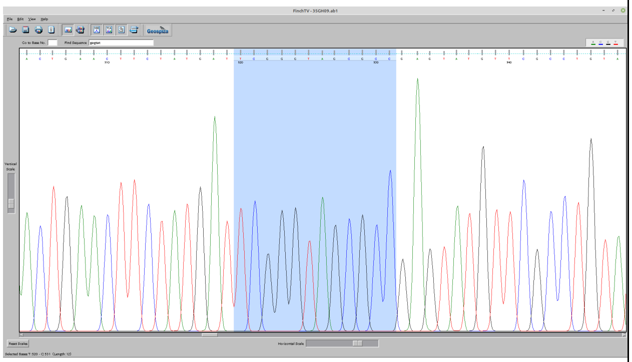
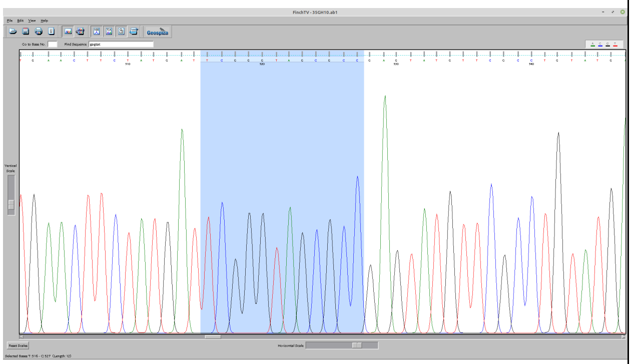
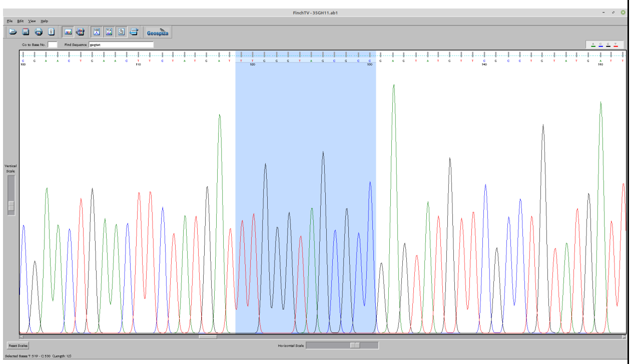
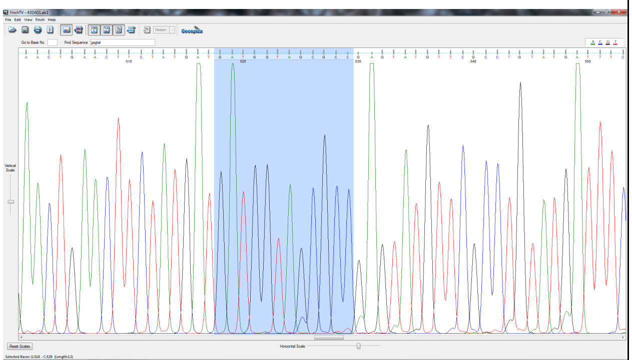
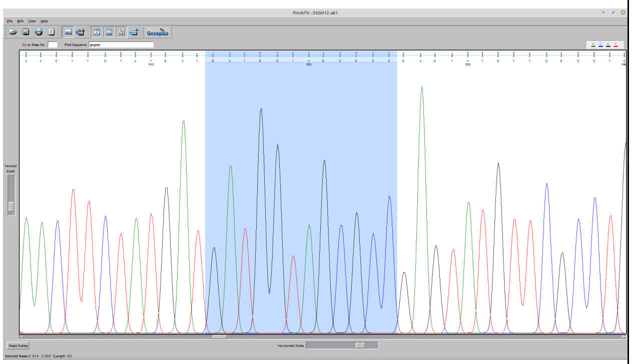
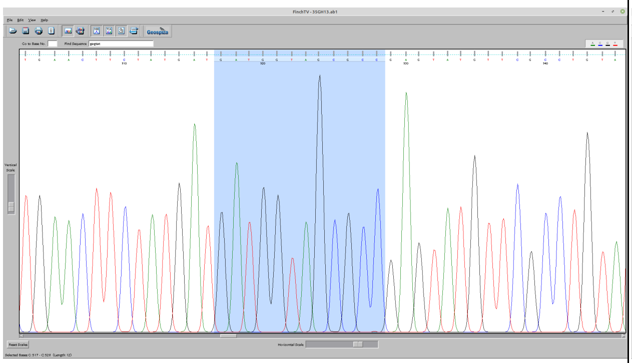
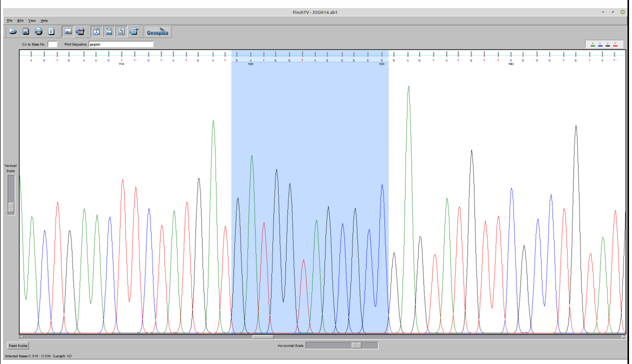
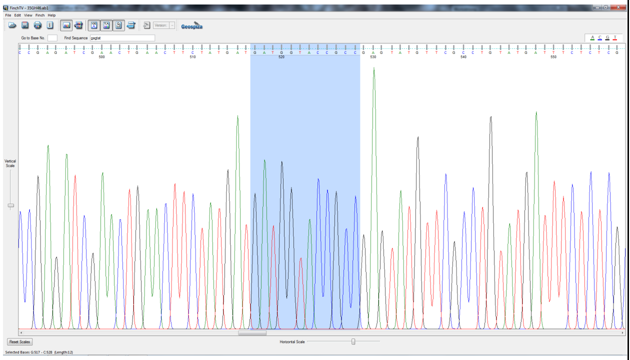
muA single clone 35GH06 148 TCG (Ser), 150 AGC (Ser), 151, ATG (Met) 	muA single clone 35GH07 148 TCG (Ser), 150 AGC (Ser), 151 ATG (Met) 
muA single clone 35GH08 148 TCG (Ser), 150 ACC (Thr), 151 CTG (Leu) 	muB 35GG99 148 GAT (Asp), 150 AGC/ACC (Ser/Thr), 151 CTG/ATG (Leu/Met) 

muC 43DA00 148 TCG/TTG (Ser/Leu), 150 AGC/ACC (Ser/Thr), 151 GCC (Ala) 	muC 35GH09 148Ser, 150Ser, 151Ala 
muC 35GH10 148 Ser, 150 Ser, 151 Ala 	muC 35GH11 148 Leu, 150 Ser, 151Ala 

muD 43DA01 148 GAT (Asp), 150 AGC/ACC (Ser/Thr), 151 GCC (Ala) 	muD single clone 35GH12 148 GAT (Asp), 150 ACC (Thr), 151 GCC (Ala) 

muD single clone 35GH13 148 GAT (Asp), 150 AGC (Ser), 151 GCC (Ala) 	muD single clone 35GH14 148 Asp, 150 Thr, 151 Ala 
35GH46 muD single clone 148 Asp, 150 Thr, 151 Ala 	muD single clone 35GH47 148 GAT (Asp), 150 AGC (Ser), 151 GCC (Ala)