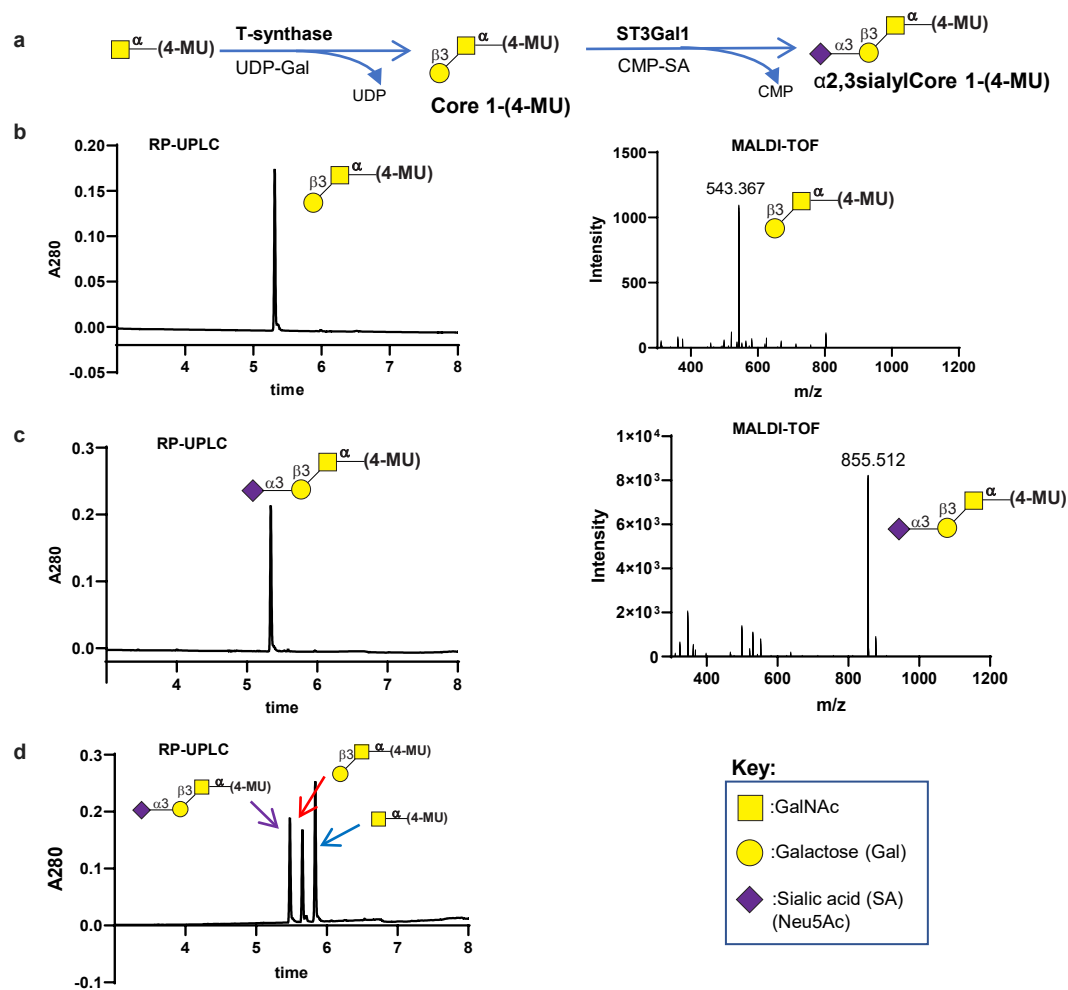




Supplementary Figure 1. Synthesis of Core 1- and α 2,3sialylCore 1-(4-MU). **a.** Schematic strategy of synthesis of fluorogenic substrates. **b.** RP-UPLC and MALDI-TOF-MS analysis of Core 1-(4-MU) (MW: 541.5 Da calculated, 543.367 Da observed). **c.** RP-UPLC and MALDI-TOF-MS analysis of α 2,3SialylCore 1-(4-MU) (MW: 832.8 Da calculated, 855.512 Da ($M+H+Na$)⁺ observed). **d.** Further RP-UPLC analysis of the mixture of synthesized α 2,3sialylCore 1-(4-MU), Core 1-(4-MU), and acceptor GalNAc- α -(4-MU).

10 20 30 40 50 60 70 80 90

POGase_TB/1-1839 -- MRSSQRSA...ALTAGAALAIIGGITAPAFGVITYTSPITGMSVAYADVETTEGAGNPAKLVLDDGNTDTYWHWKWNAKDLPHKLVLVDLGSEV
POGase_TP/1-1758 MNMRVSRRLGA...VLVTGAALALSGVAAPAYSVITYTSPLPQAEITAVYTDVEETEGEGDNGPLDRVLDDGKTEYWHWKWQGTQDPLPHQFVIDLGSSV
POGase_AS/1-1508MGA...SVAVGVLAMMGAPA...IAAKGYEYTAIPQDMETAYVSVVEETEGEGANGPIEKILDDGRNTYWHWKWNAKDLPHSFVVLGSEV
POGase_KS/1-1354
POGase_BN/1-1556 -- MRFKAAGVLAAGVVLVGAAGLPASP...LAPATAEFPVYTPIAQGMSVNVASVEKQALFNGAPELVLDGNKDTYWHWKWAGGVLDPLPHLTVRLGDPE

100 110 120 130 140 150 160 170 180 190

POGase_TB/1-1839 SVEGLGRIVLTPROSSNGSGRFGNFNVSVSTAEACANFEAYASKDSVDFESVADESIPAAPDAAGKLAFLVNVDFETAAARCAVNTINSTWGGNDSAEQVAA
POGase_TP/1-1758 SVKDLGRITLTPROSSNGSGRFDFTVSVSNDGSCAKAETAYADLST...PFTFVKEASIPVAPPEAGTLASVDVDFNPTARCVKVDIKSTWGGNGMPEVSA
POGase_AS/1-1508 VEGELGRIDLLAROSSNSGRVNEYQVWAVDTETDETAFA.....SAEPVATGNVP...ADSFKTEPLEITFDRLVANCVKVQYDSSWGGNNAPAEQVGS
POGase_KS/1-1354
POGase_BN/1-1556 EPVEIGRIALTTPROSSNGSGRVAEYSVQTSTAEACQADF.....TEVAKGSVA...ATVNPKEDEVVDFEAVSARCVKVVYNASWGGNNTEKVAI

200 210 220 230 240 250 260 270 280 290

POGase_TB/1-1839 ALSEFAAFTAAEN.....EP...GTEEPAAFLFVVIPEGAPEITGQGLTVHTRAFPDVVYDLGKQMPKGFQDALTSVNI DGKATPVVVGDSVSAAD
POGase_TP/1-1758 SLAEFQAFVTKASGEESGEEED...QEETTPSEPLEISIEGAVEISDGLATVTRHPYFPQVVDYRLGKQLAGKFGDALKEIQ DGKSVSNVVGKRVVGGED
POGase_AS/1-1508 SLAEFNAYTAVAEEDPDEPGDG.....PVVPIENAVGITDGLTAVLHPDFPDVVGYVLNDKEIAGKFGDALDSIAINGEARPVTVGELQVAA
POGase_KS/1-1354 ALLISTMATGAAALPRAVPGGGAQAASVDPLAVTPDGPARTISDGLTVTRTHPDPFQVVDYRLGKQLAGRYGGALTSITVGGVAPVTVGTPTTSA
POGase_BN/1-1556 TLAEFNAYTVTDEEP...GEPG.....TGPELVVPGDAIELTSDGLSVLRHKKFPQVVDYRVGDAQLAGKYGDALTQVITDEKPYDVTGVDVTASGS

300 310 320 330 340 350 360 370 380 390

POGase_TB/1-1839 DKTAVTYPVSLP...SVS...GSFDDVVISVKDQVLDYRITNITDPEKNINRIQIPGLDLVSLDSVADATAMVGGARINTNRANPDSFLTVA...STADTTARV
POGase_TP/1-1758 DKSSVTYPVSPIS...SIKGGSFNVVL SVKEQVLDYRITNIEDPDAQINRIHIPNLDLVSLNAAKDAKALLGGAHIDTNRNRSGDMWFVDA...KSFDVHRTA
POGase_AS/1-1508 D...QVITYPIITGDALEGVSNVVSVADSLTYKITDIRDPNGLVNIIEIPNLDLVSVTDAQ...PGASVYGARVSVDRNVSGDNHVLVSGANIGNTPSVNSA
POGase_KS/1-1354 D...SVSPVITFD...QLPGASLTAVISVKDGAMTYALRKITDQRRLLNRJAIPDLDLVSVSATD...PKSQIMATTLVSDRGKPGDKLITVA...GAEEQAKD
POGase_BN/1-1556 S...EATYPLSLA...ALGVDLSVKVSLADGVLTYSLPEVKDPGSKVHRIVIPNLDLVSVTGAAA...SITVSTLGVDRTKAADTTLQV...KAKEGTATG

400 410 420 430 440 450 460 470 480 490

POGase_TB/1-1839 VWMVAAGNGLAAGFETNAIDDOAVSGT...SGNDRYTVQVREQNGNIGITISPYEWVHRGGAVVQYDDGSGIGEEPDPFIQVKITADANADGAVDWDA
POGase_TP/1-1758 AWVMTASGHGLAAGFETNAIDDOAKQNT...SGNDRYVLAVRDEBGT KVGSVSPYEWVYRGGA VKKYAEFGGIGTEPDPFIKVKITDDANDGVDVWDGSA
POGase_AS/1-1508 AWMIITANTDEFAGFDGNAVVDKTSKGG...NADTFKFKYRLERVDGKVATVSPGGEVYRAGAVRAYDDGTGIGDPNDPIYSVKFTEDANDQKVDWDGA
POGase_KS/1-1354 DWVMTANDSGLAAGFETNAIDGNTTSVG...ANANRFARHRTVDGVRVGSVAAAEYTRADAISTYDDGSGIGVDDPDPRIITVKITDDANDGVDVWDGA
POGase_BN/1-1556 GYMAAINDQALAAGLMSNAVSDNISAGPRHGSARWIANVTTIGDAPVGTISFGPVVHRGT...ADLGIGPEEAPYQVKIVADANKDGAVDWDA

500 510 520 530 540 550 560 570 580 590

POGase_TB/1-1839 AIAATRDVLRPFVGMNDTKNITVITRIPFNIVSQATHPFLRTLDDTKRISLATDNLGGQVLLKGYYQSEG...DIAAGDYGNMYNERAGGLADLKLVDAGKAWNA
POGase_TP/1-1758 AIAATRDVLRPFVGMNDTKNIRVITRIPFNIVSQATHPFLRTLDDTKRISLATDNLGGQVLLKGYYQSEG...DIAAGDYGNMYNERAGGLADLKLVDAGKAWNA
POGase_AS/1-1508 AIALRDIKPRANSAGDVENYVSRIPFNIVSQAAHPFLRTLDDTKRISLATDNLGGQVLLKGYYQSEG...DIAAGDYAGHYNERAGGLADLKLVDAGKAWNA
POGase_KS/1-1354 AIAATRDILTFIGQDDVKNYVIPRIPFNIVSQATHPFLRTLDDTKRISLATDNLGGQVLLKGYYQSEG...DIAAGDYGNMYNERAGGLADLKLVDAGKAWNA
POGase_BN/1-1556 AIAATRDILTFATNGODEVKNKVIIPRIPFNIVSQATHPFLRTLDDTKRVALEIDNLGGQVMLKGYYQSEG...DIAADPYAGHYNERAGGLADLKLVDAGKAWNA

600 610 620 630 640 650 660 670 680 690

POGase_TB/1-1839 TFGIHNATESYSEAKCFSDGVNYSVDD...AANGVAAPCELRMPFRLAWGWMNQAYKMNQKDLATGNVLRKRLADLRKDFKDSNMLNWLNYDYIYYESGWW
POGase_TP/1-1758 TFGIHNATESYSEAKCFSDGKNGFVDKDEHENGQAAPCELRMPFRLAWGWMNQAYKMNQKDLATGNVLRKRLADLRKDFKDSNMLNWLNYDYIYYKKGWW
POGase_AS/1-1508 TFGIHNATESYSEAYAFDD.....LLQMPFRLAWGWMNQAYKMNQKDLATGNVLRKRLADLRKDFKDSNMLNWLNYDYIYYPRGWE
POGase_KS/1-1354 TFGVHVNATESYSEANAFSD.....LLQMPFRLAWGWMNQAYKMNQKDLATGNVLRKRLADLRKDFKDSNMLNWLNYDYIYYPRGWE
POGase_BN/1-1556 NIGVHVNVATESYSEAHAFSD.....LLRMPFRLAWGWMNQAYKMNQKDLATGNVLRKRLADLRKDFKDSNMLNWLNYDYIYYPRGWE

700 710 720 730 740 750 760 770 780 790

POGase_TB/1-1839 AEAFAWRQQELGFRILA...EYAYSLPTLSTWSHWANDDEFGGTTNGKLSSTLIRFVENSYRDTFNPD...PMLGNTNRYREFEGWAGNVNNTFTIGGIWQKNLP
POGase_TP/1-1758 AEAFSRMQEEGWRILA...EYAYSLPTLSTWSHWANDDEFGGTTNGKLSSTLIRFVENSYRDTFNPD...PMLGNTNRYREFEGWAGNVNNTFTIGGIWQKNLP
POGase_AS/1-1508 ANRFSQEV...SDGWRILA...EYAYSLPTLSTWSHWANDDEFGGTTNGKLSSTLIRFVENSYRDTFNPD...PMLGNTNRYREFEGWAGNVNNTFTIGGIWQKNLP
POGase_KS/1-1354 GNRFAVE...LKGWRILA...EYAYSLPTLSTWSHWANDDEFGGTTNGKLSSTLIRFVENSYRDTFNPD...PMLGNTNRYREFEGWAGNVNNTFTIGGIWQKNLP
POGase_BN/1-1556 GRLGAEL...LKGWRILA...EYAYSLPTLSTWSHWANDDEFGGTTNGKLSSTLIRFVENSYRDTFNPD...PMLGNTNRYREFEGWAGNVNNTFTIGGIWQKNLP

800 810 820 830 840 850 860 870 880 890

POGase_TB/1-1839 AKFLQDSIMSWTKPQAGRVVFANGTEVTSAGTAVGGYDSDATDRVVTYDGTATVNG...ATYLLPWKDGGEEDRLYYNPNSEATWKLINAWASQSLKLF
POGase_TP/1-1758 AKFLQDSIMSWKDSGTEPGKITFKNGTEVTSALRKVSGFDVAPRVITYDGTATVNG...GDYLLPWKDGGEEDRLYYNPNSEATWKLINAWASQSLKLF
POGase_AS/1-1508 AKFLQDSIMSW...PGKITFKNGTEVTSALRKVSGFDVAPRVITYDGTATVNG...GDYLLPWKDGGEEDRLYYNPNSEATWKLINAWASQSLKLF
POGase_KS/1-1354 AKFLQDSIMRWE...DKRIAFENGTVTSALRKVSGFDVAPRVITYDGTATVNG...GRYLLPWKDGGEEDRLYYNPNSEATWKLINAWASQSLKLF
POGase_BN/1-1556 AKFLQDSIMTWT...DGKITFKNGTEVTSALRKVSGFDVAPRVITYDGTATVNG...GAYLLPWTDGA...KRLYHYNPKGGASTWELTEAWQGSLLTFL

900 910 920 930 940 950 960 970 980 990

POGase_TB/1-1839 FKLTATGAEVTDLPVSDGSVTIPATDGGATAYVLYPSSAVRAPATPNWGGEGTADPGFFSGTLDSDYVTDGATVATSERGNYDAFGEGAGSISQEI
POGase_TP/1-1758 YELTDGAEVATIPVNNGEVSLPVTK...PSTAYVLYPSSDLRKISTPNWGGEGTADPGFFSGTLDSDYVTDGATVATSERGNYDAFGEGAGSISQEI
POGase_AS/1-1508 YKLTDTGAEVSDIAVAGSVSLPATE...ADTAYVLYPSSAVRAPATPNWGGEGTADPGFFSGTLDSDYVTDGATVATSERGNYDAFGEGAGSISQEI
POGase_KS/1-1354 YKLTDTGAEVADVSGGRVNLPA...EATAYVLYPSSAVRAPATPNWGGEGTADPGFFSGTLDSDYVTDGATVATSERGNYDAFGEGAGSISQEI
POGase_BN/1-1556 FKLTDTGAEVGVDAVATGGKVTINAD...AGTAYVLYPSSAVRAPATPNWGGEGTADPGFFSGTLDSDYVTDGATVATSERGNYDAFGEGAGSISQEI

1000 1010 1020 1030 1040 1050 1060 1070 1080 1090

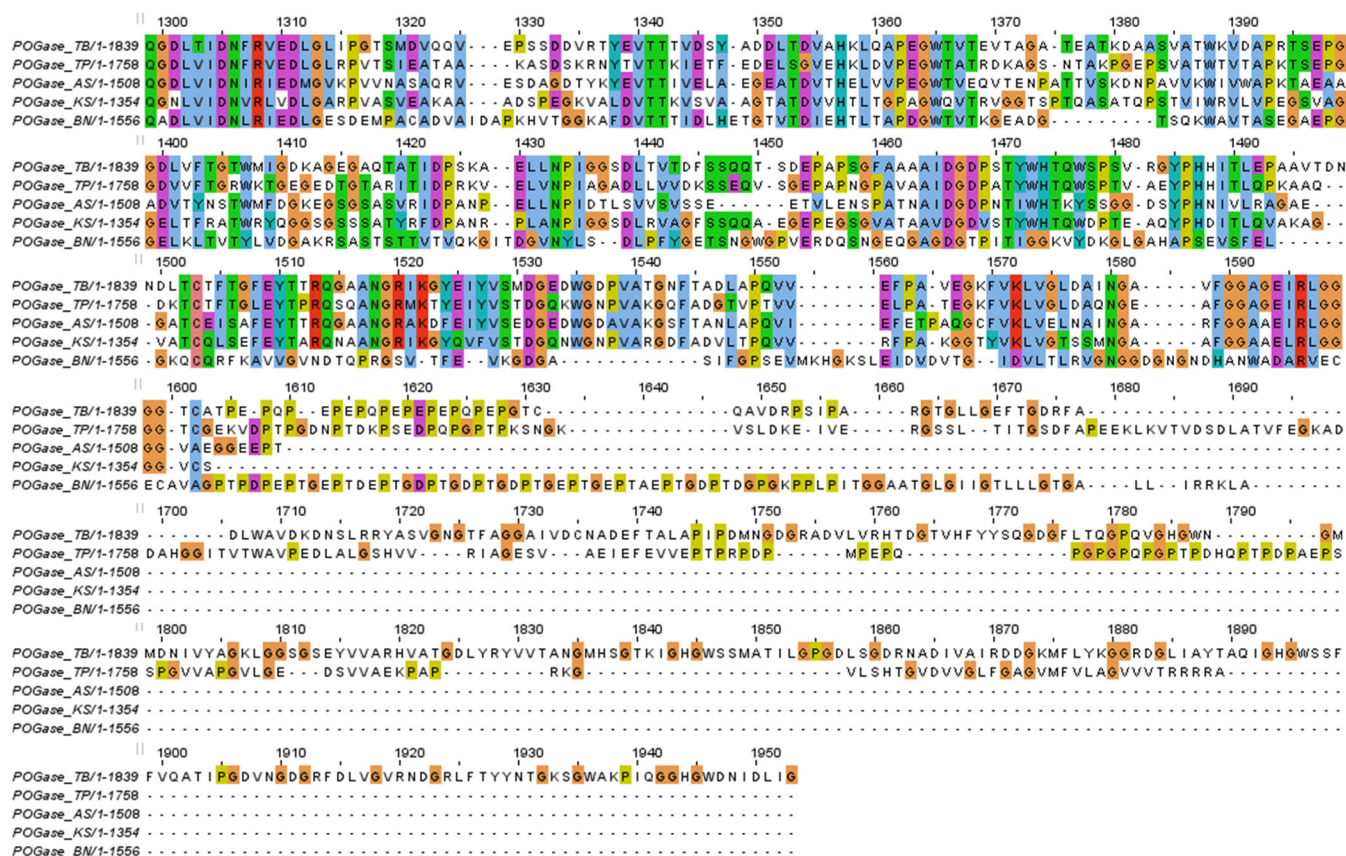
POGase_TB/1-1839 ITLPEGDYSAWAWVEIEPGKTRFVSVSVDGKDVVTPGDYKDVVDGVAITITITSGAINATASDEKFKTRFQRPVRFHITDGKAMTFAITVG...EGDATVAVD
POGase_TP/1-1758 IKLPAAGDYSAWAWVEIEPGKTRFVSVSVDGKDVVTPGDYKDVVDGVAITITITSGAINATASDEKFKTRFQRPVRFHITDGKAMTFAITVG...EGDATVAVD
POGase_AS/1-1508 ITLPEGDYSAWAWVEIEPGKTRFVSVSVDGKDVVTPGDYKDVVDGVAITITITSGAINATASDEKFKTRFQRPVRFHITDGKAMTFAITVG...EGDATVAVD
POGase_KS/1-1354 LRLPAAGDYSAWAWVEIEPGKTRFVSVSVDGKDVVTPGDYKDVVDGVAITITITSGAINATASDEKFKTRFQRPVRFHITDGKAMTFAITVG...EGDATVAVD
POGase_BN/1-1556 LRLPAAGDYSAWAWVEIEPGKTRFVSVSVDGKDVVTPGDYKDVVDGVAITITITSGAINATASDEKFKTRFQRPVRFHITDGKAMTFAITVG...EGDATVAVD

1100 1110 1120 1130 1140 1150 1160 1170 1180 1190

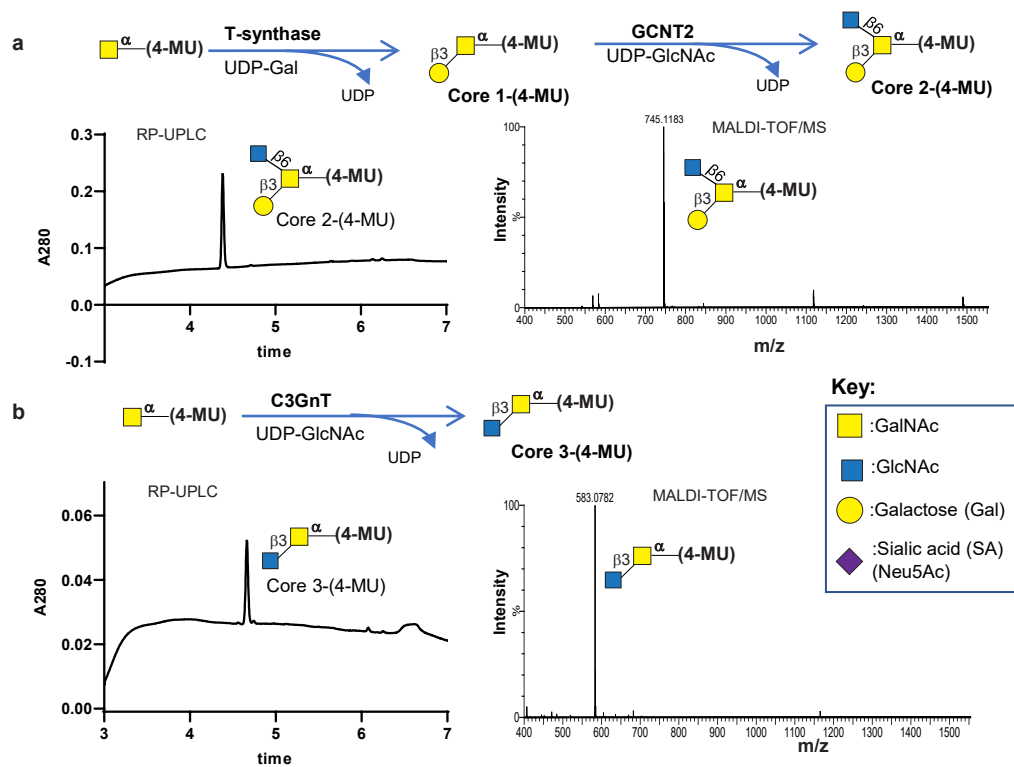
POGase_TB/1-1839 VDDLRLMVAFKET...DKAPITDAITAFNTFEDTGYWPFVTSQT...GDARTQLALRNEPYSQSGWVGIRNGQN...TEAGDKLIDNVLDGWSLLAHQENGGI
POGase_TP/1-1758 VDDLRLVREKEI...DKNPTSETIVFNTEFDITGYWPFVTSQANN...GDARTQLALRNEPYSQSGWVGIRNGQN...AEAKKKYLDNVLDGWSLLAHQENGGI
POGase_AS/1-1508 VDDLRLVPOARADKNA...AETIYFNTEFDITGYWPFVTSQANN...GDARTQLALRNEPYSQSGWVGIRNGQN...AEAKKKYLDNVLDGWSLLAHQENGGI
POGase_KS/1-1354 VDDLRLVPOWQVPAKTGA...VTFEDFEDVDTGYWPFVTSQANN...GDARTQLALRNEPYSQSGWVGIRNGQN...AEAKKKYLDNVLDGWSLLAHQENGGI
POGase_BN/1-1556 VDDLRLVVEFTPGKDPKTEGVTFEDFEDVDTGYWPFVTSQANN...GDARTQLALRNEPYSQSGWVGIRNGQN...AEAKKKYLDNVLDGWSLLAHQENGGI

1200 1210 1220 1230 1240 1250 1260 1270 1280 1290

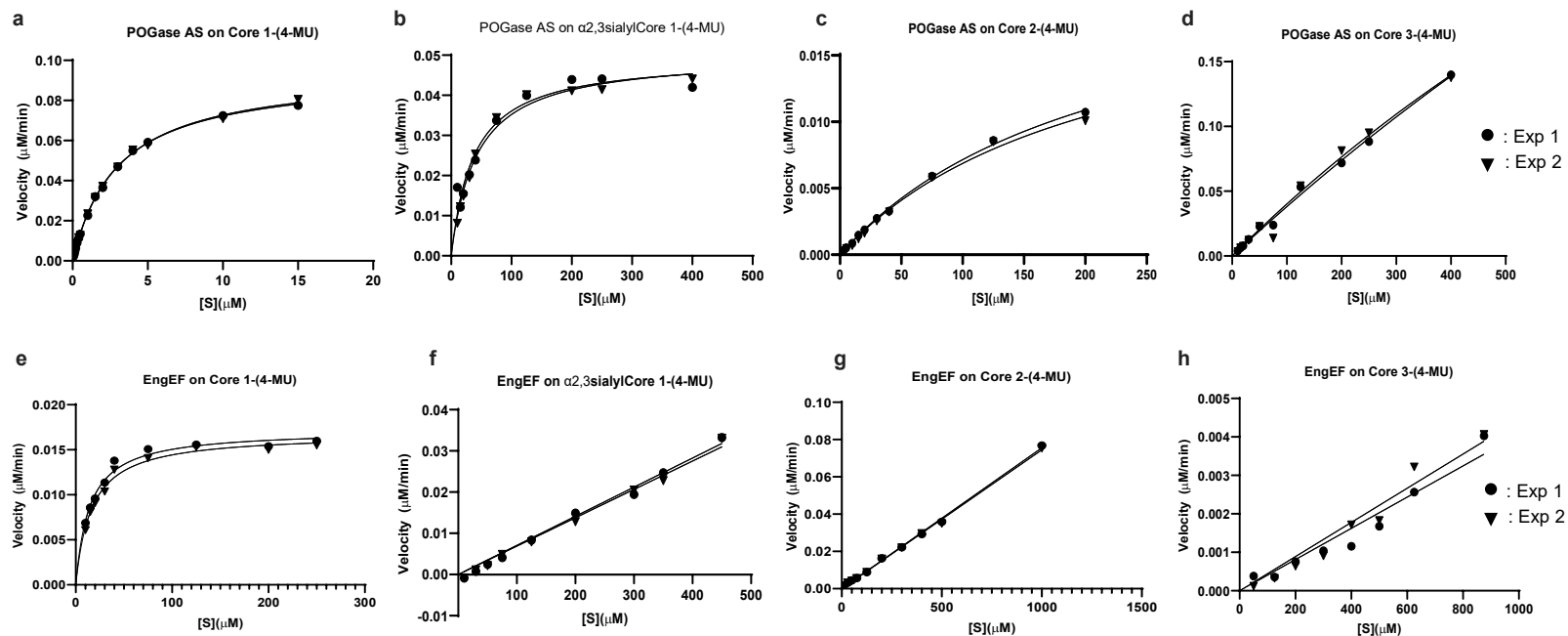
POGase_TB/1-1839 ILRTTASVPLEANRTYKVSFDYQAGYDDGYQLVIGHDESTGDSWKEVIDTRNPIDSRGTGWQDADGNAGKGTSTFEMQFRA...SSQPTFIGIVKAGNHYD
POGase_TP/1-1758 ILRTTASVPMKTHHTYRTVTFDYQAGYDDGYQIVAGHDEATDAAWTVIDIKRWELKSGARGKGTWDAQGRRGSGTQRFTEIVA...GDHPSFQVVAADSDAVSGLR
POGase_AS/1-1508 ILRTANGSFPMMLKGYRTVTFDYQAGYDDGYFVVGVDPTANGKKEVQNTAAMARGESWEG...TGTEVTFKDIJAVTDNPTFFGIIKKLGGRRV
POGase_KS/1-1354 ILRTTAAALPQVGHSTYRTVTFDYQAGYDDGYSVVLGKDTKNDAAWKEIERTVPLPDARGKWRLLD...QVSGTKQFSLFSLDTATPFGVITKSGNIG
POGase_BN/1-1556 ILRTIPSIDFKPGHKYRISMDHOSGAGKYQOMVLAIRVANSEAASTIVTKKPIGEV...EIAVLVSHFVAGCGQYWFGEKIIAGGS



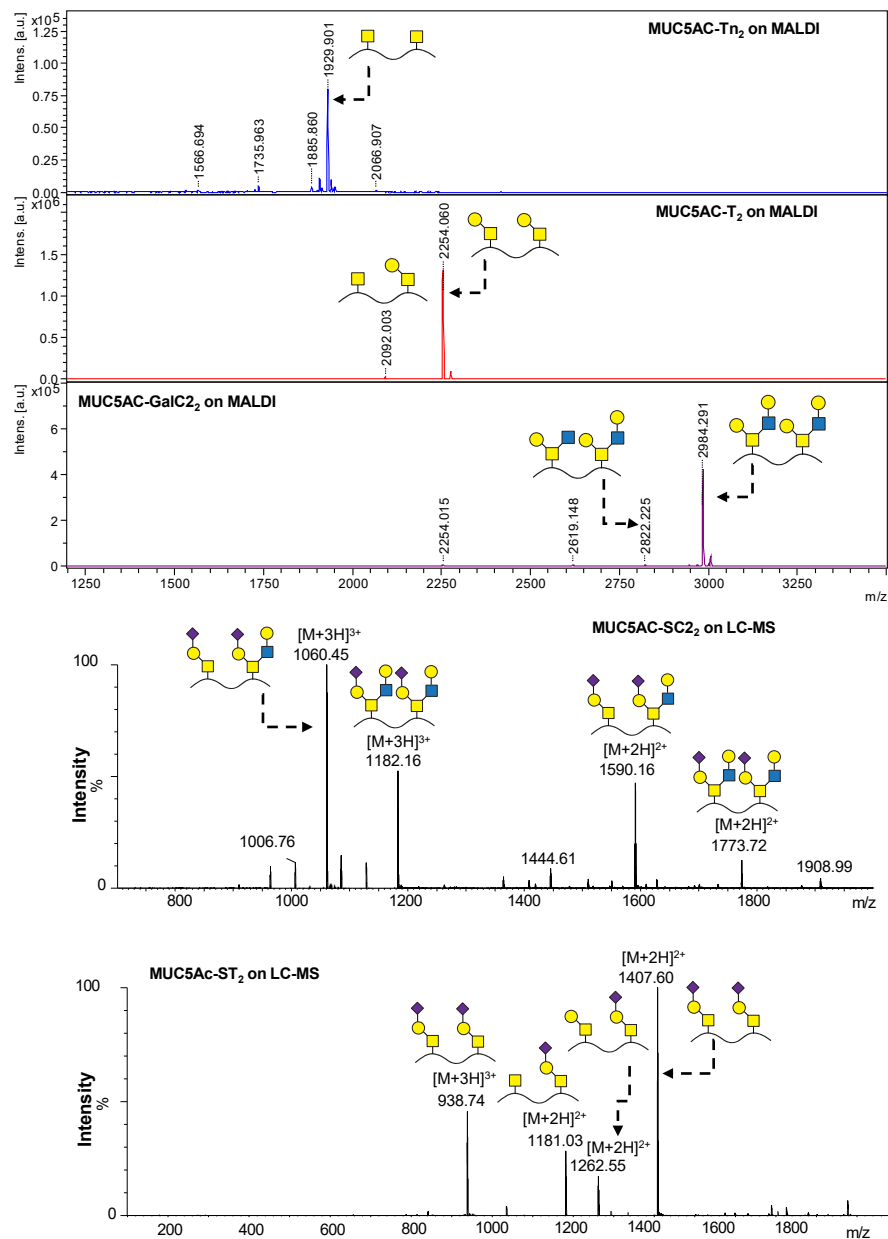
Supplementary Figure 2. Multiple sequence alignment of the 5 POGases identified in this study. Sequences were aligned with Clustal Omega and alignment was viewed using Jalview. For ease of analysis, residues were annotated and colored according to their clustal grouping. Motif 1 (red), motif 2 (blue), and motif 3 (black) along with the DDE catalytic triad residues (yellow boxed) are all indicated.



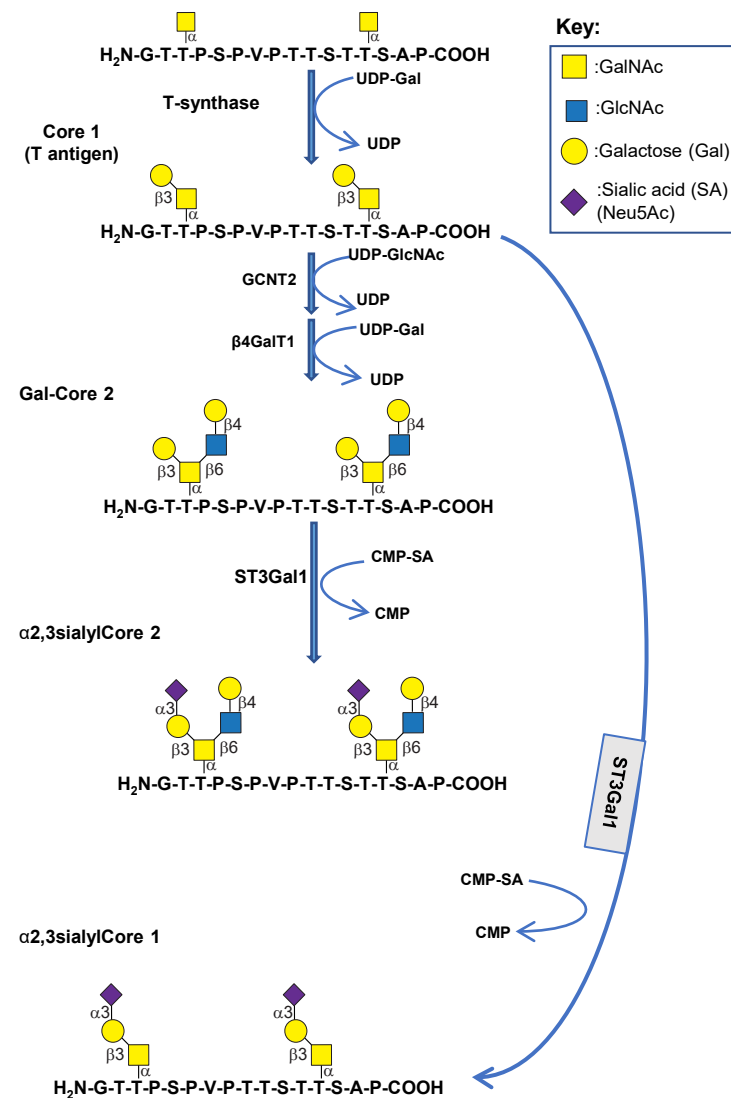
Supplementary Figure 3. Synthesis of Core 2- and Core 3-(4-MU). **a.** RP-UPLC and MALDI-TOF-MS analysis of Core 2-(4-MU) (MW: 744.7 Da calculated, 745.1 Da observed). **b.** RP-UPLC and MALDI-TOF-MS analysis of Core 3-(4-MU) (MW: 582.6 Da calculated, 583.1 Da observed).



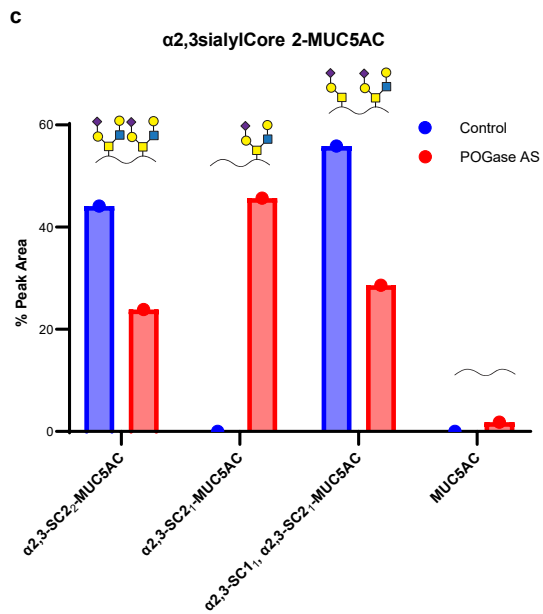
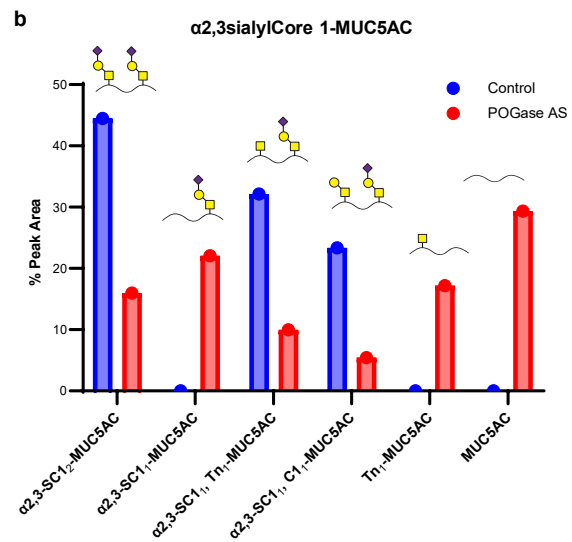
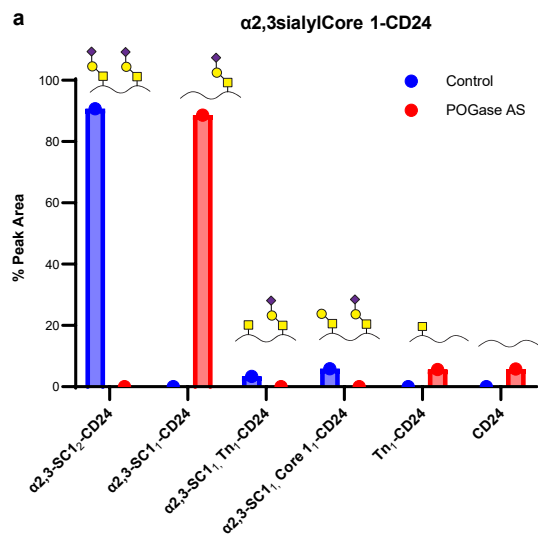
Supplementary Figure 4. Kinetic characterization of POGase AS. **a.** $[Et] = 61 \text{ pM}$. **b.** $[Et] = 610 \text{ pM}$. **c.** $[Et] = 61 \text{ pM}$. **d.** $[Et] = 610 \text{ pM}$. **e.** $[Et] = 688 \text{ pM}$. **f.** $[Et] = 6.88 \text{ nM}$. **g.** $[Et] = 3.44 \text{ nM}$. **h.** $[Et] = 3.44 \text{ nM}$. $[Et]$: The total concentration of enzyme, POGase AS in different kinetic studies. Source data are provided as a Source Data file.



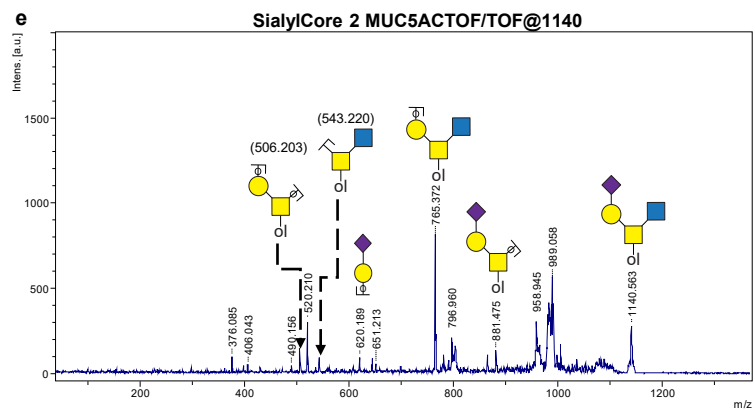
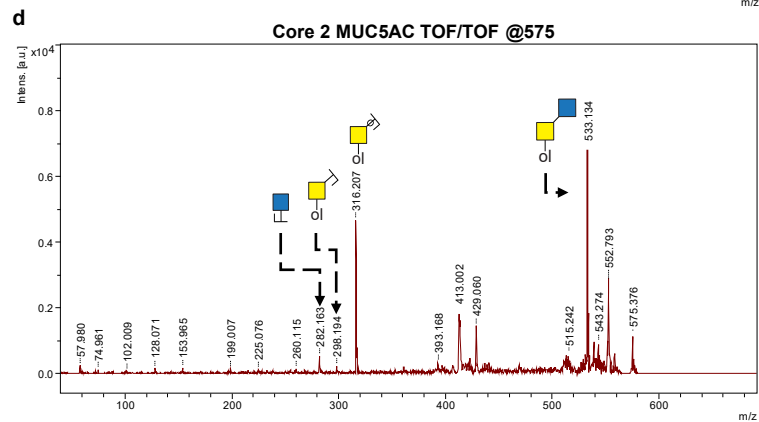
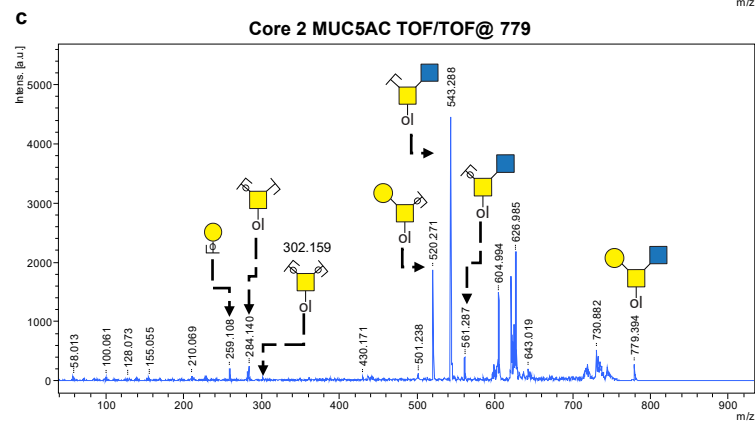
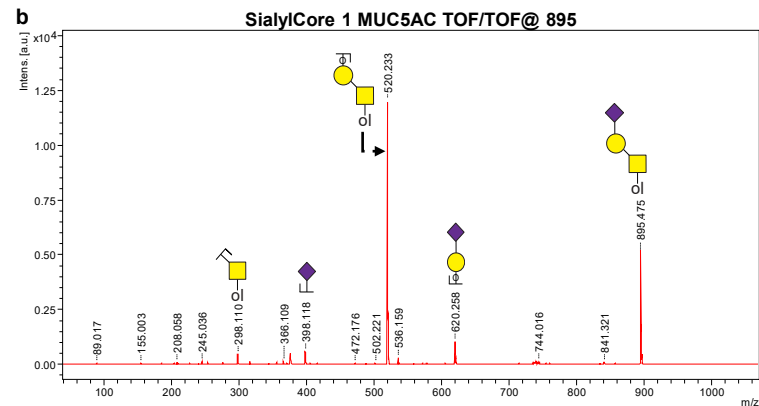
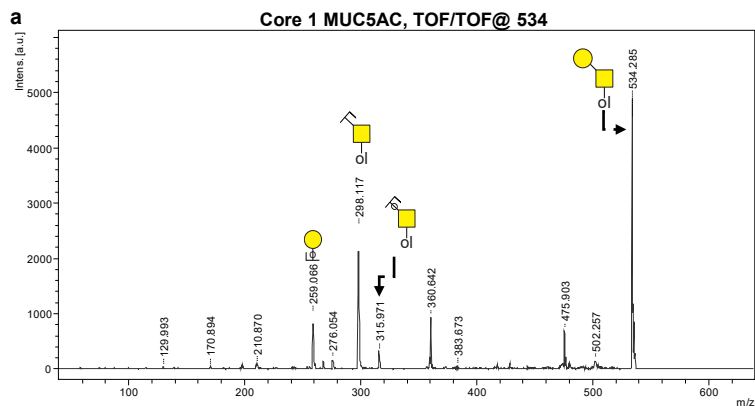
MUC5AC-Tn2



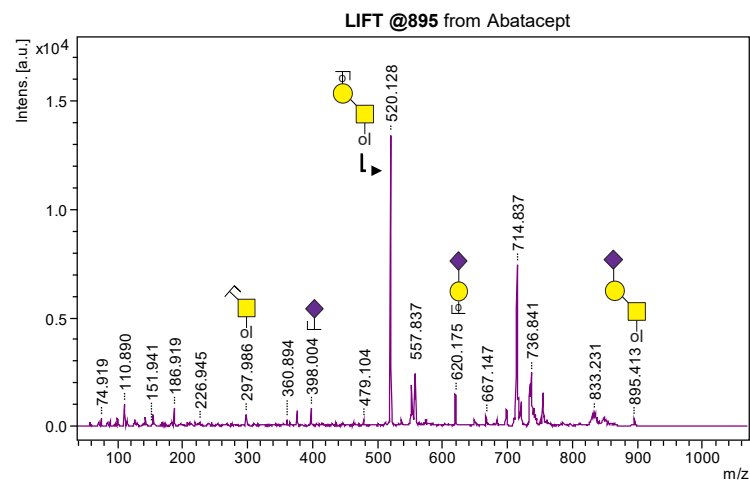
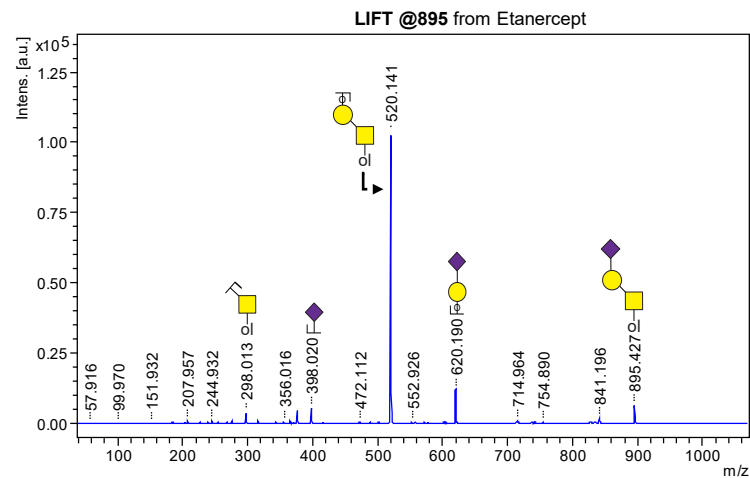
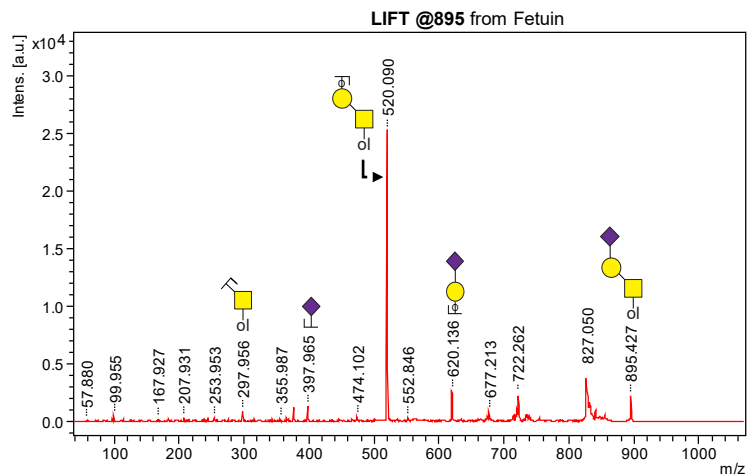
Supplementary Figure 5. Synthesis of α2,3sialylCore 1-MUC5AC, sialylCore 2-MUC5AC. α2,3sialylCore 1-MUC5AC, sialylCore 2-MUC5AC were synthesized with serial reactions by corresponding recombinant glycosyltransferases. The reactions were monitored and confirmed by either MALDI-TOF for neutral glycopeptides, or LC-MS for the sialylated glycopeptides.



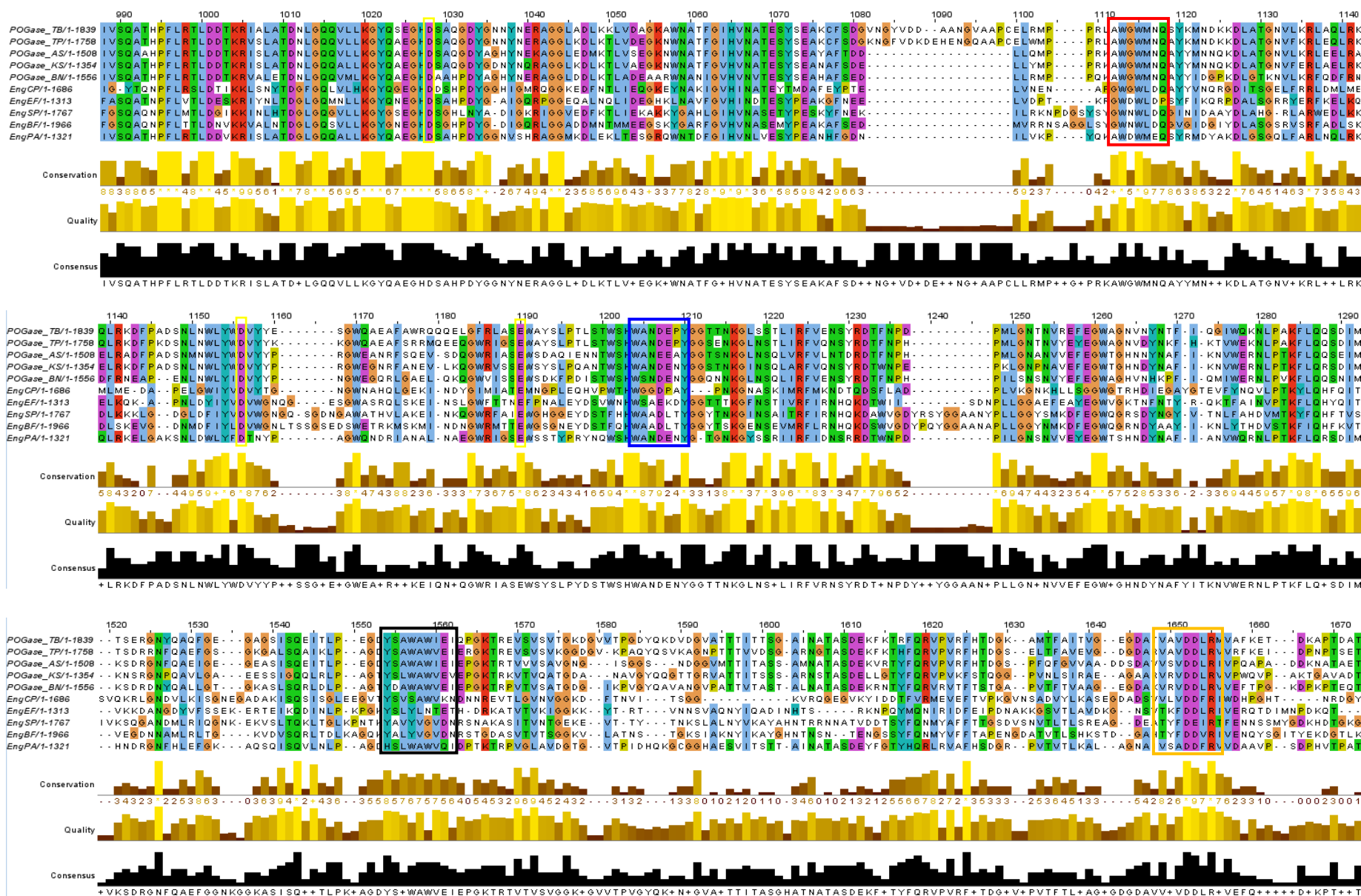
Supplementary Figure 6. Peak area from extracted ion chromatograms (XIC) of glycopeptide products after POGase AS treatment (n=1). a. $\alpha 2,3$ sialylCore 1-CD24, b. $\alpha 2,3$ sialylCore 1-MUC5AC, c. $\alpha 2,3$ sialylCore 2-MUC5AC. Source data are provided as a Source Data file.



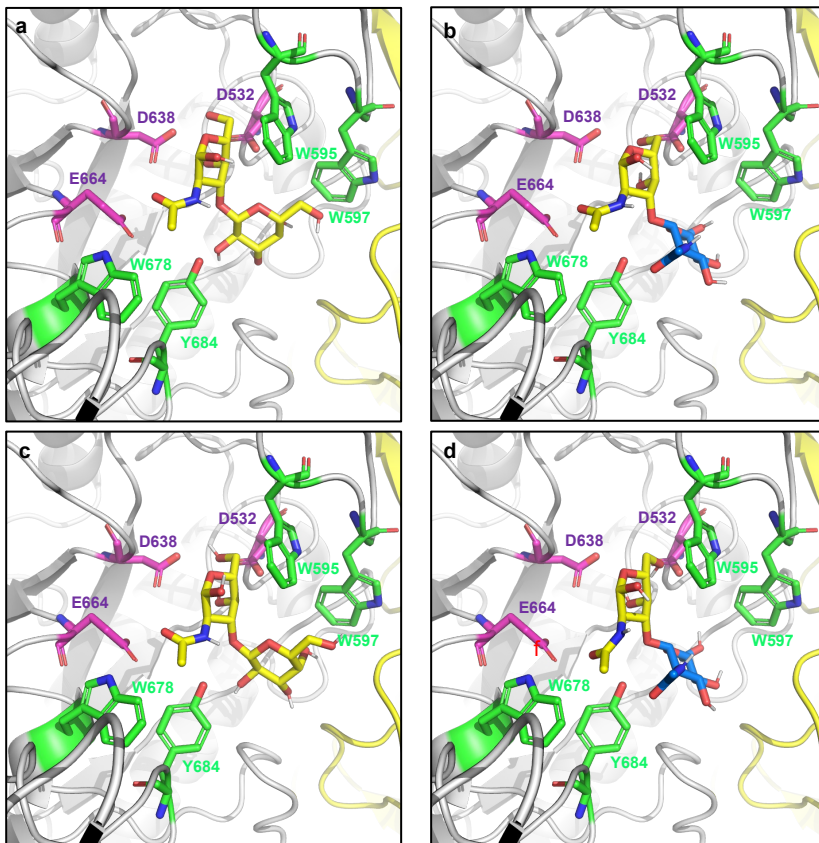
Supplementary Figure 7. LIFT-TOF/TOF-MS analysis of permethylated and reduced O-glycan species released by POGase from synthetic glycopeptide substrates. a. Core 1 O-glycan @ m/z 534 identified in Muc5AC, **b.** α 2,3-sialylCore 1 @ m/z 895, **c.** Core 2 O-glycan @ m/z 779, **d.** Core 6 O-glycan @ m/z 575, and **e.** α 2,3sialylCore 2 O-glycan @ m/z 1140 in MUC5AC. Source data are provided as a Source Data file.



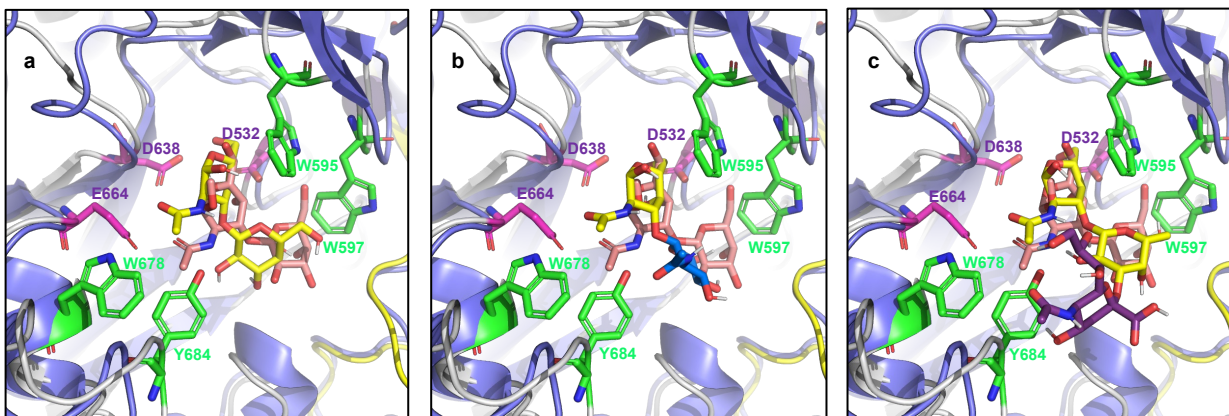
Supplementary Figure 8. LIFT-MS analysis of $\alpha 2,3$ sialylCore 1 O-glycans released from Fetuin, Etanercept and Abatacept by POGase AS. 895 m/z was selected for LIFT-MS/MS analysis to confirm the identity of the mono sialylCore 1 O-glycan peak by unique fragments at the reducing end. Mono substitution of the GalNAc core reveals the identity of $\alpha 2,3$ sialylCore 1 O-glycan. Source data are provided as a Source Data file.



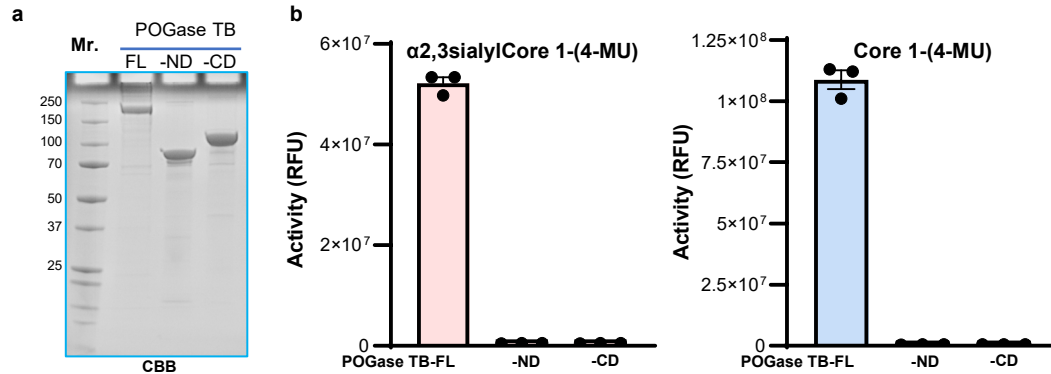
Supplementary Figure 9: Multiple sequence alignment of the GH101 Domain of 5 POGases and 5 known O-glycanases: Sequences were aligned with Clustal Omega and alignment was viewed using Jalview. For ease of analysis, residues were annotated and colored according to their clustal grouping. Motif-1 (red box), and Motif-3 (black box) along with the DDE catalytic triad residues (yellow boxes) are all indicated. In orange, the transition point between GH101 domain and other domains is indicated. The quality (how likely a residue is found at all in that position), consensus (Sequence representing most common residue in aligned position) and conservation (how likely residues with similar properties are found in that position) is shown to show conservation/divergence.



Supplementary Figure 10: Core 1 and Core 3 O-glycans docked to the active site of POGase AS—Comparing VinaCarb (a/b) and (c/d) AutoDock Results. **a.** Core 1 O-glycan (Yellow) docked between the WW/WY domains (Green) with the α GalNAc oriented toward the DDE catalytic triad (Magenta), docked with Vina Carb. **b.** Core 3 O-glycan (Yellow and Blue) docked between the WW/WY domains (Green) with the α GalNAc oriented toward the DDE catalytic triad (Magenta), docked with Vina Carb. **c.** Core 1 O-glycan (Yellow) docked between the WW/WY domains (Green) with the α GalNAc oriented near the DDE catalytic triad, docked with AutoDock Vina. **d.** Core 3 O-glycan (Yellow and Blue) docked between the WW/WY domains (Green) with the α GalNAc oriented near the DDE catalytic triad, docked with AutoDock Vina.



Supplementary Figure 11: Aligning Vina Carb Results to 5A56 reveals glycan docked poses aren't significantly altered from that of the experimentally derived structure with a T antigen (Core 1) agonist.



Supplementary Figure 12: Expression and enzymatic activity assays of POGase TB and its domains: **a.** Expression and purification of the full-length POGase TB (FL), its N-terminal domain (-ND), and C-terminal domain (-CD) were expressed in *E. coli* and purified. The purified proteins were run on SDS-PAGE, and gel was stained with Coomassie blue. **b.** Enzymatic activity Assay: the activity of recombinant POGase TB-FL and -ND, and -CD (500ng proteins) was assayed using 40 μ M of $\alpha 2,3\text{sialylCore 1-(4-MU)}$ substrate (left panel) and Core 1-(4-MU) (right panel) for 1 hour (n=3) at 30 μ L reaction mixtures, respectively. After adding 100 μ L 1M Glycine-NaOH (pH10) (stop solution) and mixing well, the RFU of free 4-MU was measured in the umbelliferone mode using the platereader. Data are presented as mean values $\pm$ SEM (n=3). Source data are provided as a Source Data file.

Supplementary Table 1. Putative endo- α -N-Acetylgalactosaminidase candidates screened in this study

<u>Candidate#</u>	<u>Organism</u>	<u>GenBank</u>	<u>Candidate#</u>	<u>Organism</u>	<u>GenBank</u>
01	Clostridiales bacterium CHKCI001	SCJ97620.1	24	Actinomyces bovis	SPT54038.1
02	Clostridiales bacterium CHKCI001	CUN20024.1	25	[Ruminococcus] torques	CUN81354.1
03	Streptococcus acidominimus	SNV33305.1	26	uncultured Ruminococcus sp.	SCH51847.1
04	Streptococcus suis	CYV18626.1	27	Streptococcus pneumoniae	COG72222.1
05	Hungatella hathewayi	CUP62238.1	28	Streptococcus pneumoniae	COM85265.1
06	Streptococcus suis	CYU53783.1	29	Clostridiales bacterium CHKCI001	CVI65756.1
07	Streptococcus suis	CYV10025.1	30	Clostridiales bacterium CHKCI001	CVI65639.1
08	Streptococcus suis	CYV24804.1	31	Trueperella pyogenes	AZR00042.1
09	Streptococcus suis	CYV25301.1	32	Neoeactinobaculum massiliense	WP_124039591.1
10	Streptococcus suis	CYV69930.1	33	Schaalia hyovaginalis	WP_154476515.1
11	Streptococcus suis	CYW34645.1	34	Actinomyces sp.	HHT41109.1
12	Streptococcus suis	CYV05123.1	35	Gleimonia hominis	PMC85471.1
13	Streptococcus suis	CYX81346.1	36	Cutibacterium avidum	RFT46484.1
14	Bifidobacterium reuteri DSM 23975	KFI88282.1	37	Knoellia sp. DB2414S	WP_171241682.1
15	Bifidobacterium longum subsp. longum JCM 1217	AAX44931.1	38	Bowdeniella nasicola	WP_073716010.1
16	Bifidobacterium longum subsp. longum CECT 7347	CCK34131.1	39	Knoellia remsis	WP_106296018.1
17	Bifidobacterium longum subsp. suis	KFI72916.1	40	Tessaracoccus massiliensis	WP_052459929.1
18	Streptococcus pneumoniae	CKE04844.1	41	bacterium 1xD8-27	NBJ77735.1
19	Bifidobacterium tsurumiense	KFJ08221.1	42	Tessaracoccus rhinocerotis	WP_143938588.1
20	[Ruminococcus] torques	CUO40056.1	43	Brachybacterium tyrofermentans	WP_193115799.1
21	uncultured Ruminococcus sp.	SCI35173.1	44	Corynebacterium xerosis	SLM98462.1
22	Trueperella bernardiae	KTF04411.1	45	Cutibacterium acnes KPA171202	AAT83312.1
23	Bifidobacterium bombi DSM 19703	KFF31755.1	46	Brachybacterium alimentarium	RCS90382.1

Supplementary Table 2. POGases with significant activity to cleave α 2,3sialylCore 1 O-glycans.

Name	Clone#	Organism	GeneBank ID
POGase TB	22	<i>Trueperella bernardiae</i>	KTF04411.1
POGase TP	31	<i>Trueperella pyogenes</i>	AZR00042.1
POGase AS	34	<i>Actinomyces</i> sp.	HHT41109.1
POGase KS	37	<i>Knoellia</i> sp. DB2414S	WP_171241682.1
POGase BN	38	<i>Bowdeniella nasicola</i>	WP_073716010.1

Supplementary Table 3: Glycopeptides Purchased from Vendors

Name	Sequences	O-glycans	Mass	Vendor
MUC5AC-3/13	GTT*PSPVPTTSTT*SAP-OH	GalNAcα- (Tn antigen)	1907.6	AnaSpec
CD24(41-51)-Tf(41, 51)	T*SNSGLAPNPT*-OH	Galβ1-3GalNAcα- (Core 1)	1788.77	Sussex Research
CD24(41-51)-sTf(41,51)	T*SNSGLAPNPT*-OH	Neu5Acα2-3Galβ1-3GalNAcα- (α2,3SialylCore 1)	2371.3	Sussex Research
MUC1(138-157)- Tn(144,150,151)	VTSA PDT*RPAPGS*T*APPAHG-OH	GalNAcα- (Tn antigen)	2496.59	Sussex Research

Supplementary Table 4. Comparisons of Motif-1, -2, and -3 sequences between POGases and known O-glycanases.

Name	Motif-1	Motif-2	Motif-3	Representative
POGases	AWGWMNQ	WANEAY	YSAWAWV/IEI	POGase AS
O-glycanases	GWD/NWLDN	WSAEKDY	YSLYLNTET	EngEF

Supplementary Table 5: Comparison of the two algorithms for docking

O-Glycans	AutoDock Vina	Vina-Carb	Chi Energy Applied	Anomeric C to DDE triad (Å)
Core 1	-5.7 ± 0.06 kcal/mol	-5.5 ± 0.15 kcal/mol	0.0	6.0, 5.7, 7.1 (ns)
Core 3	-5.5 ± 0.06 kcal/mol	-5.5 ± 0.1 kcal/mol	0.0	6.4, 6.1, 7.5 (ns)
α2,3SialyCore 1	-	-5.3 ± 0.06 kcal/mol	4.5 ± 0.0	6.5, 5.6, 6.9 (ns)
5a56	-	-	-	5, 4.2, 6.4

Supplementary Materials:

Movie_1: Predicted 3D Structure of POGase AS

Movie_2: Evolutionary Coupled Residues in/or Near Motif 1 (AWGWMNQ) within POGase AS