Supporting Information

Metabolic Labeling and Imaging of Native *Saccharomyces Cerevisiae* Glycan by Using *N*-Acetylglucosamine-6-Phosphate Analogs

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Supporting Figures and Tables

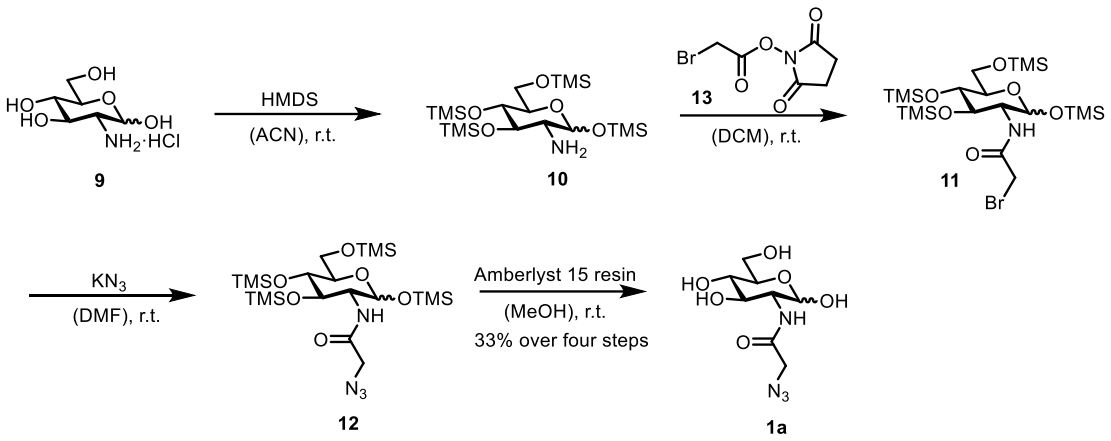


Figure S1. General strategies for compound **1a** synthesis.

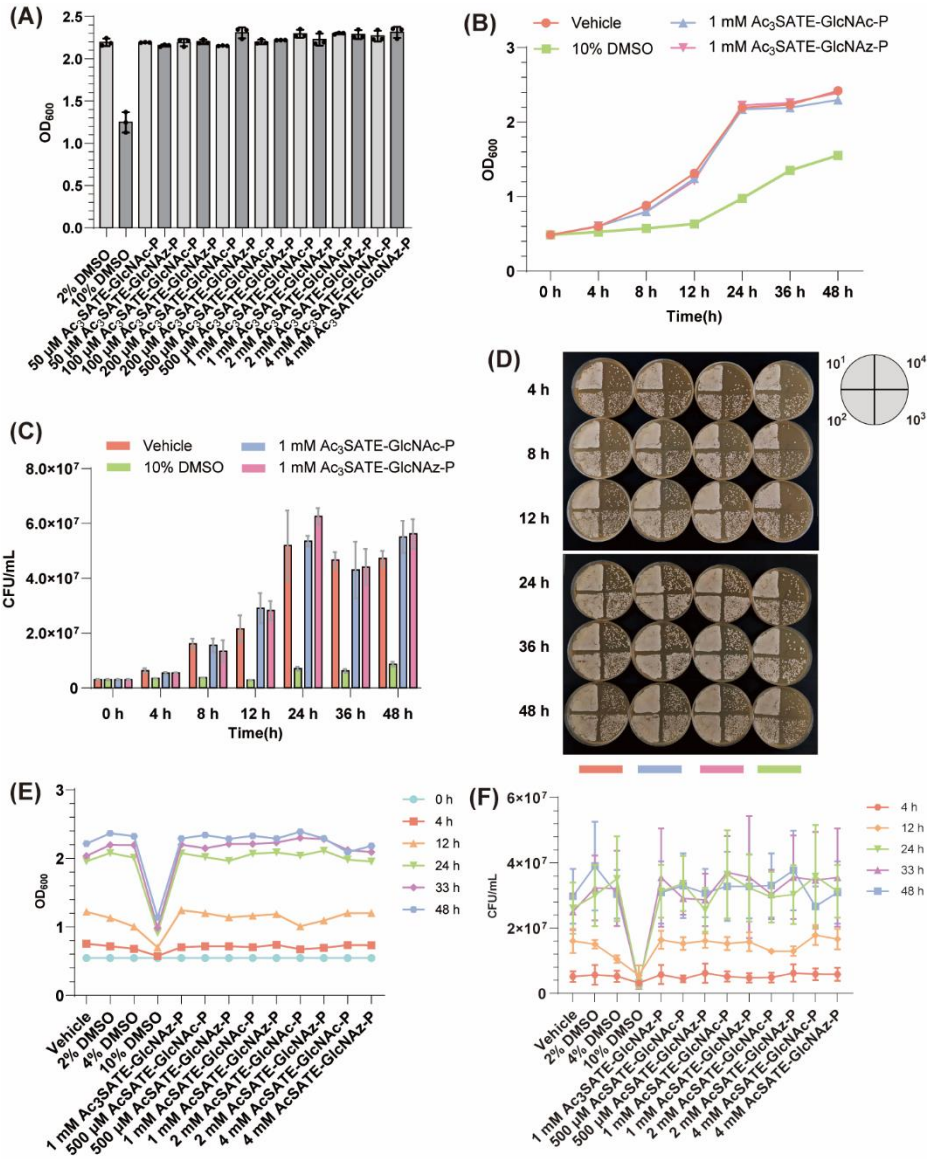


Figure S2. The sensitivity of *Saccharomyces cerevisiae* to the probes. Yeast suspensions were incubated with either the probes or DMSO in 2 mL of YPG liquid medium at 30℃ with shaking at 220 rpm. (A) Cell densities (OD_{600}) were measured after incubating the yeast suspensions with Ac_3SATE-GlcNAz-P (50 μM-4 mM), Ac_3SATE-GlcNAc-P (50 μM-4 mM), 2% DMSO, or 10% DMSO for 48 hours. (B) Growth curves were monitored by incubating the yeast suspensions with 1 mM Ac_3SATE-GlcNAz-P, 1 mM Ac_3SATE-GlcNAc-P, or 10% DMSO over a period of 0-48 hours. (C) Statistical analysis of the average colony-forming unit (CFU/mL) counts from (B) was conducted for the incubation period of 0-48 hours. (D) Photographs were taken with a camera following the colony-forming unit counting assay as described in Section 2.5. (E) Cell densities (OD_{600}) were measured after incubating the yeast suspensions with 1 mM Ac_3SATE-GlcNAz-P, AcSATE-GlcNAz-P (500 μM-4 mM), AcSATE-GlcNAc-P (500 μM-4 mM), or 2%–10% DMSO over a time course of 0-48 hours. (F) Statistical analysis of the average CFU/mL counts from (E) was performed for the incubation period of 0-48 hours. Error bars indicate standard errors calculated from at least three independent replicates.

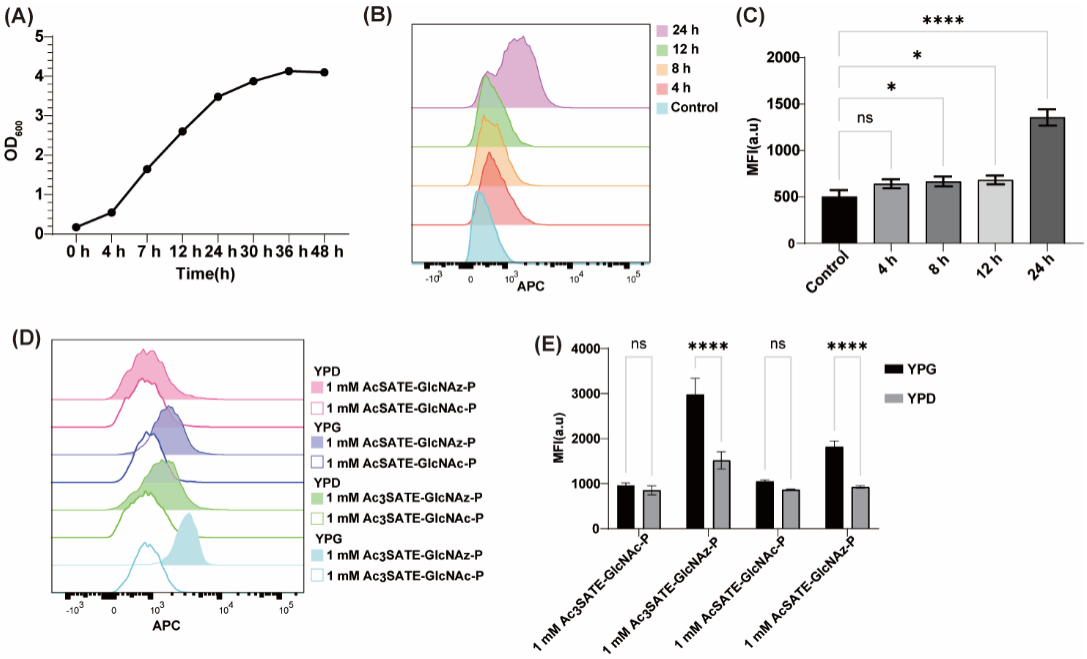


Figure S3. Comparison of the metabolic labeling effects of Ac_3SATE-GlcNAz-P and AcSATE-GlcNAz-P on yeast in YPD and YPG liquid media. (A) Yeast growth curves in 2 mL of YPD liquid medium were monitored over a 48-hour period. (B) Flow cytometry analysis of yeast cells incubated with 1 mM Ac_3SATE-GlcNAz-P or 2% DMSO (control) in 2 mL of YPD liquid medium for 4-24 hours, followed by CuAAC reaction with alkyne-Cy5. (C) Statistical analysis of the median fluorescence intensity from (B). (D) Flow cytometry analysis of yeast cells incubated with 1 mM Ac_3SATE-GlcNAz-P, 1 mM AcSATE-GlcNAz-P, 1 mM Ac_3SATE-GlcNAc-P (negative control), or 1 mM AcSATE-GlcNAc-P (negative control) in 2 mL of YPD or YPG liquid media for 48 hours, followed by CuAAC reaction with alkyne-Cy5. (E) Statistical analysis of the median fluorescence intensity from (D). Statistical analyses were performed using one-way ANOVA for (C) and two-way ANOVA for (E), with at least three replicates. Significance levels were defined as follows: ns (not significant), *P < 0.05, ****P < 0.0001.

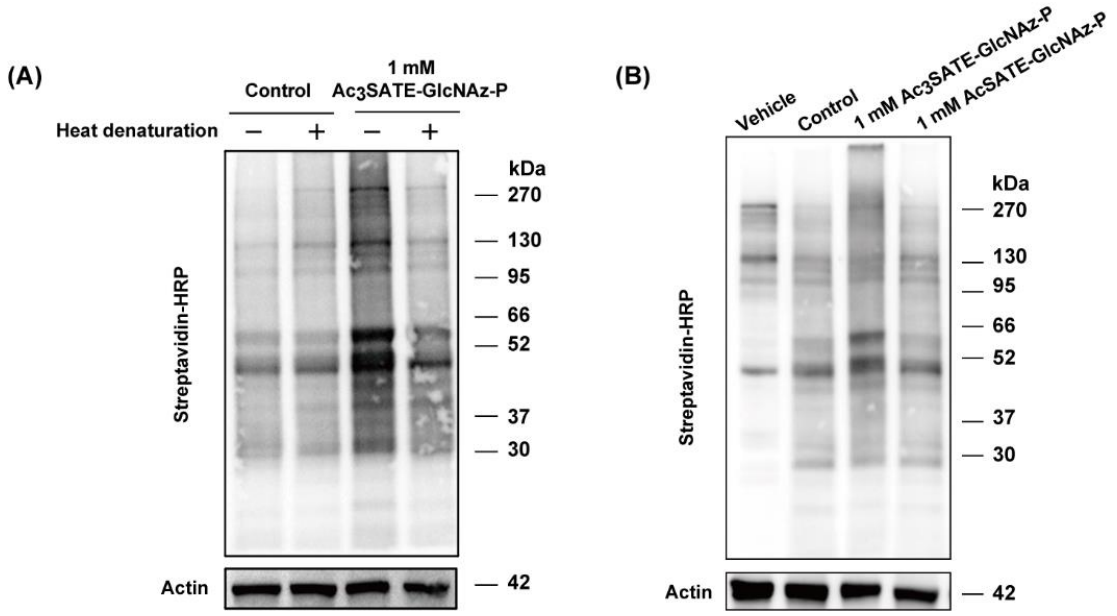


Figure S4. Non-specific *S*-glyco-modification induced by Ac_3SATE-GlcNAz-P and AcSATE-GlcNAz-P. (A) Yeast lysates, before and after heat denaturation, were incubated with an equal volume of DMSO (Control) or 1 mM Ac_3SATE-GlcNAz-P for 2 hours at 37℃, followed by a CuAAC reaction with alkyne-PEG_4-biotin. The biotinylated glycoproteins were analyzed using SDS-PAGE and detected through blotting with streptavidin-HRP. (B) Yeast lysates were heat-denatured and then incubated with 1 mM Ac_3SATE-GlcNAz-P, 1 mM AcSATE-GlcNAz-P, or an equal volume of DMSO for 2 hours at 37℃, followed by either a CuAAC reaction with alkyne-PEG_4-biotin (Control, 1 mM Ac_3SATE-GlcNAz-P, 1 mM AcSATE-GlcNAz-P) or no click reaction (Vehicle). The biotinylated glycoproteins were subjected to SDS-PAGE and detected by blotting with streptavidin-HRP.

Table S1. Proteins identified with high confidence by mass spectrometry from Ac_3SATE-GlcNAz-P-treated *S. cerevisiae* were analyzed. The experiments were conducted in triplicate, and the union of the three datasets was considered the total dataset. A protein was classified as high confidence if the total number of assigned spectra was at least fivefold higher in Ac_3SATE-GlcNAz-P samples compared to DMSO blank samples and non-specific labeling control samples. Additionally, only proteins that are localized to the membrane system, bud neck, vacuole, or those classified as secreted proteins were included, as these locations have been previously verified to be suitable for the presence of N-glycoproteins based on glycosite identification.[1] All identified proteins contained the consensus sequence for N-linked glycosylation, N-!P-[S/T] (where !P represents any residue except proline). The resulting data were subsequently compared to the dataset from Breidenbach et al.[1-2], Parzych et al.[3], Wild et al.[4], Mima et al.[5], Kumar et al.[6], Endrizzi et al.[7], Poljak et al.[8], Kung et al.[9].

Accession	Description	Location	N-!P-[S/T]	Citation
Q12100	Probable serine/threonine-protein kinase RTK1	Bud neck	Yes	
P53217	Uncharacterized membrane protein YGR026W	Cell membrane	Yes	
P53739	Flippase kinase 1 FPK1	Cell membrane	Yes	
P38146	GTP-binding protein YPT10	Cell membrane	Yes	
Q00246	GTP-binding protein RHO4	Cell membrane	Yes	
Q12675	Phospholipid-transporting ATPase DNF2	Cell membrane	Yes	
P33754	Translocation protein SEC66	Endoplasmic reticulum membrane	Yes	[1,8]
P17967	Protein disulfide-isomerase PDI1	Endoplasmic reticulum membrane	Yes	[1,8]
P25574	ER membrane protein complex subunit EMC1	Endoplasmic reticulum membrane	Yes	[1]
P25371	Probable ATP-dependent permease ADP1	Endoplasmic reticulum membrane	Yes	[1]
P33775	Dolichyl-phosphate-mannose--protein mannosyltransferase PMT1	Endoplasmic reticulum membrane	Yes	[1]
Q04080	GPI transamidase component GPI17	Endoplasmic reticulum membrane	Yes	[1,9]
P33767	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit WBP1	Endoplasmic reticulum membrane	Yes	[1,4,8]

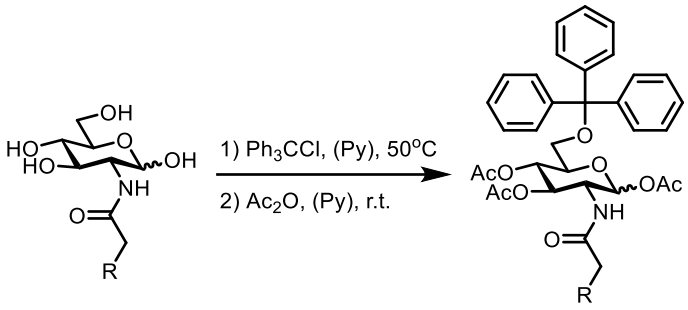
P39007	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3	Endoplasmic reticulum membrane	Yes	[1,4,8]
P41543	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit OST1	Endoplasmic reticulum membrane	Yes	[1,4,8]
P12684	3-hydroxy-3-methylglutaryl-coenzyme A reductase HMG2	Endoplasmic reticulum membrane	Yes	[1,8]
P23642	Mannan polymerase I complex VAN1	Endoplasmic reticulum membrane	Yes	UniProt
P36057	Signal recognition particle receptor subunit beta SRP102	Endoplasmic reticulum membrane	Yes	
P40207	Ergosterol biosynthesis protein ERG29	Endoplasmic reticulum membrane	Yes	
P53045	C-4 methylsterol oxidase ERG25	Endoplasmic reticulum membrane	Yes	
P51533	ATP-dependent permease PDR10	Membrane	Yes	UniProt
P38616	Protein YGP1	Secreted	Yes	[1,2]
Q06490	Thiamine biosynthesis protein THI22	Secreted	Yes	
P38109	Vacuolar serine-type carboxypeptidase ATG42	Vacuole	Yes	[1,3]
P37302	Aminopeptidase Y APE3	Vacuole	Yes	[1,8]
P09232	Cerevisin PRB1	Vacuole	Yes	[1]
P27614	Carboxypeptidase S CPS1	Vacuole	Yes	[1]
P00729	Carboxypeptidase Y PRC1	Vacuole	Yes	[1,5,6,7,8]
P07267	Saccharopepsin PEP4	Vacuole	Yes	[1]
P48016	Periplasmic acid trehalase ATH1	Vacuole	Yes	UniProt
P20107	Vacuolar zinc transporter ZRC1	Vacuole	Yes	
P38247	Protein SLM4	Vacuole	Yes	
P39923	Nitrogen permease regulator NPR2	Vacuole	Yes	
Q99385	Vacuolar calcium ion transporter VCX1	Vacuole	Yes	

1. Materials and Methods for Compound Synthesis

1.1 General materials and methods

All the solvent and chemicals used for compound synthesis were of reagent grade or higher, commercially available and used without further purification unless otherwise noted. Reactions were monitored by using thin layer chromatography (TLC) on silica gel HSHF 254 pre-coated plates or AGF 254 neutral pre-coated plates. TLC plates were first visualized under shortwave UV light (λ = 254 nm) and then stained by dipping in *p*-anisaldehyde (PAA) solution, followed by heating. Reaction mixtures were purified by using column chromatography on 200-300 mesh silica gel. ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and ^{1}H-^{13}C HSQC spectra were recorded on a Bruker Avance Ⅲ 400 MHz or Bruker Avance NEO 600 MHz spectrometer in $CDCl_3$ or CD_3OD. NMR chemical shifts were referenced to the solvent residual peak for $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C) and CD_3OD (3.31 ppm for ^{1}H and 49.00 ppm for ^{13}C). HRMS data were recorded using a Waters MALDI SYNAPT mass spectrometer with electrospray ionization (ESI) as the ion source.

1.2 Synthesis of compound 2a/2b



1a: R=N_3
1b: R=H

2a: R=N_3
2b: R=H

Compound **1a** was prepared according to previously established procedures[10].

Synthesis of compound 2a：

Compound **1a** (2 g, 7.62 mmol, 1 eq) was dissolved in anhydrous pyridine (Py, 40 mL) and trichloromethyl benzene (Ph_3CCl, 5.32 g, 19.08 mmol, 2.5 eq) was added to the solution. The mixture was stirred slowly at 50℃ for 4 hours, with the reaction monitored by TLC (DCM/MeOH = 8:1). Upon completion, acetic anhydride (Ac_2O, 5.7 mL, 60.96 mmol, 8 eq) was added under ice bath conditions. The mixture was then stirred at room temperature overnight. Once TLC (PE/EA = 2:1) indicated the reaction was complete, the mixture was extracted using a citric acid aqueous solution and DCM. The DCM layer was collected, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by silica gel chromatography using PE/EA as the eluent, yielding compound **2a** (3.8 g, 80% yield) as a white viscous solid. Compound **2a** (α/β = 1:0.57), R_f = 0.45 (PE/EA = 2:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.35 (m, 9.42H), 7.31 – 7.15 (m, 14.13H), 6.50 – 6.38 (m, 1.57H), 6.29 (d, *J* = 3.6 Hz, 1H), 5.73 (d, *J* = 8.6 Hz, 0.57H), 5.34 (t, *J*

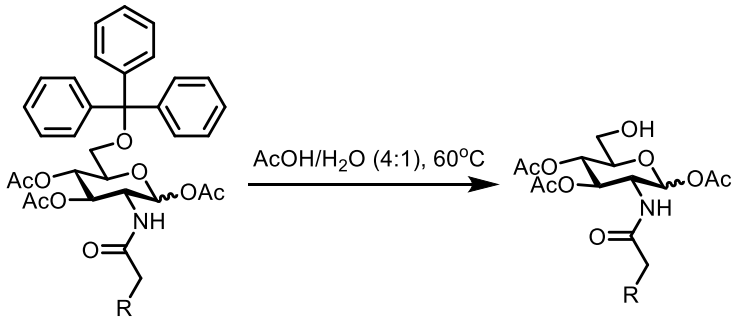
= 9.8 Hz, 1H), 5.28 – 5.16 (m, 1.57H), 5.15-5.06 (m, 0.57H), 4.48 (ddd, *J* = 3.6, 8.8, 10.7 Hz, 1H), 4.31 – 4.20 (m, 0.57H), 3.92 (s, 2H), 3.90 – 3.85 (m, 2.14H), 3.71 – 3.59 (m, 0.57H), 3.34 – 3.23 (m, 1.57H), 3.09 – 2.96 (m, 1.57H), 2.16 (s, 3H), 2.12 (s, 1.71H), 2.01 (s, 3H), 1.99 (s, 1.71H), 1.72 (s, 1.71H), 1.70 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 171.19, 169.51, 168.91, 168.89, 168.72, 167.14, 167.01, 143.63, 143.62, 128.87, 128.85, 127.95, 127.94, 127.18, 127.16, 92.70, 90.65, 86.85, 86.73, 74.48, 72.70, 71.43, 71.04, 68.32, 67.97, 62.03, 61.57, 53.45, 52.77, 52.62, 51.63, 21.07, 21.06, 20.88, 20.77, 20.59, 20.54. m/z (HRMS$^+$) $[M+NH_4]^+$ 648.2661 ($C_{33}H_{38}N_5O_9^+$ requires 648.2664).

***Synthesis of compound 2b*:**

Compound **1b** is commercially available.

Compound **2b** was synthesized using the procedure employed for compound **2a**, yielding 74% as a white solid. Compound **2b** (α/β = 1:0.53), R_f = 0.4 (PE/EA = 1:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.36 (m, 9.18H), 7.30 – 7.17 (m, 13.77H), 6.27 (d, *J* = 3.6 Hz, 1H), 5.65 (d, *J* = 8.6 Hz, 0.53H), 5.62 – 5.55 (m 1.53H), 5.33 (t, *J* = 9.9 Hz, 1H), 5.22 (t, *J* = 9.6 Hz, 0.53H), 5.16 (dd, *J* = 9.5, 11.0 Hz, 1H), 5.02 (dd, *J* = 9.4, 10.7 Hz, 0.53H), 4.52 (ddd, *J* = 3.6, 9.1, 11.0 Hz, 1H), 4.37 – 4.27 (m, 0.53H), 3.86 (ddd, *J* = 2.3, 4.2, 10.3 Hz, 1H), 3.63 (ddd, *J* = 2.5, 4.4, 10.0 Hz, 0.53H), 3.34 – 3.21 (m, 1.53H), 3.06 (dd, *J* = 4.4, 10.6 Hz, 0.53H), 3.03 (dd, *J* = 4.2, 10.7 Hz, 1H), 2.15 (s, 3H), 2.14 (s, 1.59H), 2.02 (s, 3H), 2.00 (s, 1.59H), 1.93 (s, 3H), 1.92(s, 1.59H), 1.72 (s, 1.59H), 1.71 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.10, 171.52, 170.25, 170.12, 169.79, 168.88, 168.82, 168.69, 147.02, 143.65, 128.87, 128.86, 128.06, 127.94, 127.17, 127.14, 93.12, 91.08, 86.84, 86.71, 74.52, 73.16, 71.41, 71.34, 68.37, 68.07, 62.10, 61.67, 53.36, 51.47, 23.36, 23.22, 21.11, 21.09, 20.92, 20.82, 20.58, 20.55. m/z (HRMS$^+$) $[M+Na]^+$ 612.2238 ($C_{33}H_{35}NO_9Na^+$ requires 612.2204).

1.3 Synthesis of compound 3a/3b



2a: R=N_3
2b: R=H

3a: R=N_3
3b: R=H

Synthesis of compound 3a:

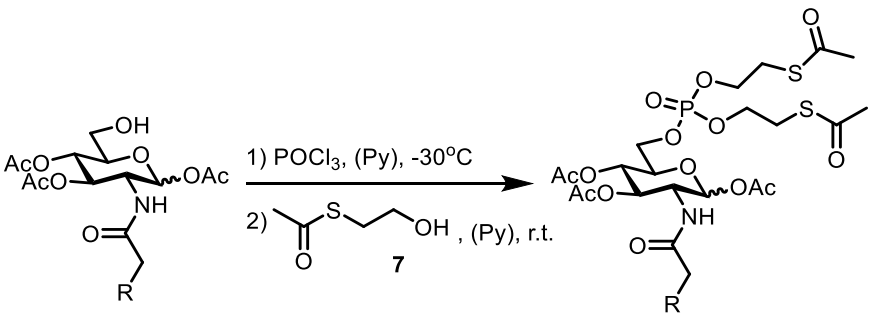
Compound **2a** (3.33 g, 5.28 mmol, 1 eq) was suspended in a mixture of acetic acid and water (32 mL:8 mL), heated to 60°C, and stirred slowly for 2 hours, with the reaction monitored by TLC (PE/EA = 1:4).

Upon completion, EA and an aqueous sodium chloride solution were added to the mixture. The EA layer was collected, and solid $NaHCO_3$ was added to the aqueous layer to adjust the pH to near neutral. The aqueous layer was then extracted multiple times with EA until TLC indicated the product was nearly absent. The EA layers were combined, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by silica gel chromatography with PE/EA as the eluent, yielding compound **3a** (1.78 g, 87% yield) as a white viscous solid. Compound **3a** (α/β = 1:0.46), R_f = 0.4 (PE/EA = 1:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.64 (d, *J* = 9.4 Hz, 0.46H), 6.49 (d, *J* = 8.8 Hz, 1H), 6.20 (d, *J* = 3.6 Hz, 1H), 5.80 (d, *J* = 8.8 Hz, 0.46H), 5.37 – 5.29 (m, 1.46H), 5.17 (t, *J* = 9.8 Hz, 1H), 5.07 (t, *J* = 9.6 Hz, 0.46H), 4.42 (ddd, *J* = 3.6, 8.8, 10.9 Hz, 1H), 4.28 – 4.16 (m, 0.46H), 3.93 (s, 2H), 3.89 (s, 0.92H), 3.86 – 3.80 (m, 1H), 3.78 – 3.73 (m, 0.46H), 3.72 – 3.64 (m, 1.46H), 3.62 – 3.55 (m, 1.46H), 2.19 (s, 3H), 2.10 (s, 1.38H), 2.08 (s, 3H), 2.07 (s, 1.38H), 2.06 (s, 3H), 2.04 (s, 1.38H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.77, 171.10, 170.39, 170.17, 169.67, 169.08, 167.32, 167.11, 92.31, 90.40, 75.17, 72.21, 72.20, 70.24, 68.32, 67.84, 61.10, 60.90, 53.33, 52.65, 52.49, 51.42, 21.06, 21.01, 20.85, 20.79, 20.77, 20.76. m/z (HRMS$^+$) $[M+NH_4]^+$ 406.1554 ($C_{14}H_{24}N_5O_9^+$ requires 406.1568).

Synthesis of compound 3b：

Compound **3b** was synthesized using the procedure employed for compound **3a**, yielding 80% as a white viscous solid. Compound **3b** (α isomer), R_f = 0.2 (PE/EA = 1:4). The NMR data was consistent with the literature[11]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.17 (d, *J* = 3.6 Hz, 1H), 5.67 (d, *J* = 9.1 Hz, 1H), 5.27 (t, *J* = 9.8 Hz, 1H), 5.15 (t, *J* = 9.8 Hz, 1H), 4.44 (ddd, *J* = 3.6, 9.1, 10.7 Hz, 1H), δ 3.80 (ddd, *J* = 2.3, 4.4, 10.1 Hz, 1H), 3.68 (dd, *J* = 2.3, 12.8 Hz, 1H), 3.57 (dd, *J* = 4.4, 12.8 Hz, 1H), 2.18 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.93 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.95, 170.15, 170.03, 168.96, 90.86, 72.20, 70.69, 68.06, 61.10, 51.31, 23.15, 21.06, 20.86, 20.74. m/z (HRMS$^+$) $[M+NH_4]^+$ 365.1545 ($C_{14}H_{25}N_2O_9^+$ requires 365.1554).

1.4 Synthesis of Ac_3SATE-GlcNAz-P (4a)/Ac_3SATE-GlcNAc-P (4b)



3a: R=N_3
3b: R=H

Ac_3SATE-GlcNAz-P 4a: R=N_3
Ac_3SATE-GlcNAc-P 4b: R=H

Compound **7** was prepared according to previously established procedures [12]. Potassium thioacetate (48 g, 420.3 mmol, 1.2 eq) was dissolved in acetone (300 mL) under ice bath. Subsequently, 2-bromoethanol

(25 mL, 350.2 mmol, 1eq) was added slowly, resulting in the formation of a white precipitate. The reaction mixture was stirred for 24 hours at room temperature. The product can be detected using TLC and stained with potassium permanganate as a staining agent (PE/EA = 1:1, R_f = 0.5). The mixture was filtered, and the filtrate was evaporated under appropriate conditions to remove acetone without losing the product. The resulting yellow crude liquid was extracted eight times with DCM and the saturated NaCl solution. The combined DCM layers were concentrated to afford a yellow liquid product **7** (30 g), which was used directly in the next step.

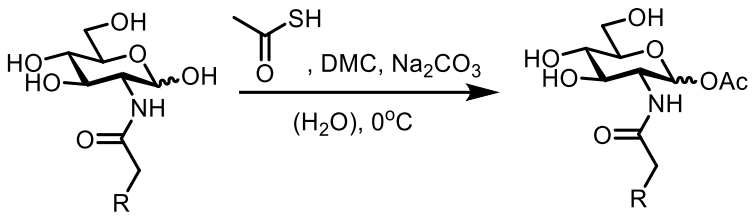
***Synthesis of Ac_3SATE-GlcNAz-P (4a)*:**

A 50 mL flask was charged with a moderate-activated 4 Å molecular sieve and anhydrous pyridine (7.5 mL). Phosphorus oxychloride ($POCl_3$, 0.4 mL, 7.74 mmol, 6 eq) was added under strictly anhydrous and anaerobic conditions and cooled to -30°C. Compound **3a** (0.5 g, 1.29 mmol, 1eq) was dissolved in anhydrous pyridine (2 mL) and added dropwise very slowly (about 2 hours). The reaction was then stirred at -30°C for an additional 0.5 hours. After TLC confirmed the reaction was complete, compound **7** (3 mL, 28.8 mmol, 20 eq) was added, and the mixture was stirred at room temperature overnight. TLC indicated the reaction had finished. The mixture was then filtered, followed by extraction with a citric acid aqueous solution and DCM. The aqueous layer was extracted twice with DCM. The DCM layers were combined, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by silica gel chromatography with PE/EA elution, yielding compound **4a** (0.24 g, 28% yield) as a yellow syrup. Compound **4a** (α isomer), R_f = 0.4 (PE/EA = 1:4). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.46 (d, *J* = 8.9 Hz, 1H), 6.20 (d, *J* = 3.6 Hz, 1H), 5.32-5.25 (m, 1H), 5.19 (t, *J* = 9.8 Hz, 1H), 4.46 (ddd, *J* = 3.6, 8.8, 10.8 Hz, 1H), 4.16 – 4.07 (m, 6H), 4.06 (m, 1H), 3.94 (s, 2H), 3.21-3.12 (m, 4H), 2.35 (s, 6H), 2.22 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 194.95, 194.93, 171.67, 169.27, 168.79, 166.99, 90.19, 70.42, 70.37, 67.43, 66.37, 65.47, 52.50, 51.26, 30.68, 29.22, 21.05, 20.82, 20.75; ^{31}P NMR (243 MHz, $CDCl_3$) δ -2.15; m/z (HRMS$^+$) $[M+NH_4]^+$ 690.1575 ($C_{22}H_{37}N_5O_{14}PS_2^+$ requires 690.1510).

***Synthesis of compound Ac_3SATE-GlcNAc-P (4b)*:**

Ac_3SATE-GlcNAc-P was synthesized following the procedure used for compound **4a**, yielding 30% as a yellow syrup. Compound **4b** (α isomer), R_f = 0.2 (PE/EA = 1:4). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.16 (d, *J* = 3.6 Hz, 1H), 5.60 (d, *J* = 9.1 Hz, 1H), 5.23 (t, *J* = 9.8 Hz, 1H), 5.16 (t, *J* = 9.8 Hz, 1H), 4.47 (ddd, *J* = 3.6, 9.1, 10.7 Hz, 1H), 4.15 – 4.08 (m, 6H), 4.02 (m, 1H), 3.20 – 3.14 (m, 4H), 2.36 (s, 6H), 2.19 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.93 (s, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 194.96, 194.94, 171.90, 170.09, 169.24, 168.74, 90.58, 70.76, 70.31, 67.52, 66.37, 65.54, 51.09, 30.69, 29.23, 23.19, 21.10, 20.87, 20.76; ^{31}P NMR (243 MHz, $CDCl_3$) δ -2.18; m/z (HRMS$^+$) $[M+NH_4]^+$ 649.1462 ($C_{22}H_{38}N_2O_{14}PS_2^+$ requires 649.1496).

1.5 Synthesis of compound 5a/5b



1a: R=N_3
1b: R=H

5a: R=N_3
5b: R=H

Compound **1a** was prepared according to previously established procedures[10].

Synthesis of Compound 5a：

The compound was synthesized following previously established procedures [13] , with slight modifications. Compound **1a** (1 g, 3.80 mmol, 1 eq) and Na_2CO_3 (604.1 mg, 5.70 mmol, 1.5 eq) were dissolved in 200 mL of water and placed in an ice bath for 10 minutes. Thioacetic acid (0.4 mL, 5.70 mmol, 1.5 eq) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 642.4 mg, 3.80 mmol, 1.0 eq, dissolved in 1.5 mL H_2O) were added sequentially to the reaction mixture at 0℃, with stirring for 15 minutes. Subsequently, Na_2CO_3 (604.1 mg, 5.70 mmol, 1.5eq, dissolved in 1.5 mL of H_2O) and thiourea (0.4 mL, 5.70 mmol, 1.5 eq) were added, followed by slow addition of DMC (642.4 mg, 3.80 mmol, 1.0 eq, dissolved in 1.5 mL of H_2O). This stepwise addition was repeated three more times, for a total of five times. The reaction mixture was washed three times with 200 mL of DCM each time. An appropriate amount of Amberlyst 15 ion-exchange resin was added to the aqueous layer, the pH was adjusted to 5-6, and rotary evaporation under reduced pressure was carried out at 34°C. The pH was continuously monitored, and additional resin was added as needed to maintain the pH between 5-6, until the solution was completely evaporated. After drying, MeOH was added, and the precipitated Na_2CO_3 was filtered off. The filtrate was concentrated, and the crude product was purified by silica gel column chromatography using DCM/MeOH elution, yielding 0.6 g (52% yield) of compound **5a** as a white solid.

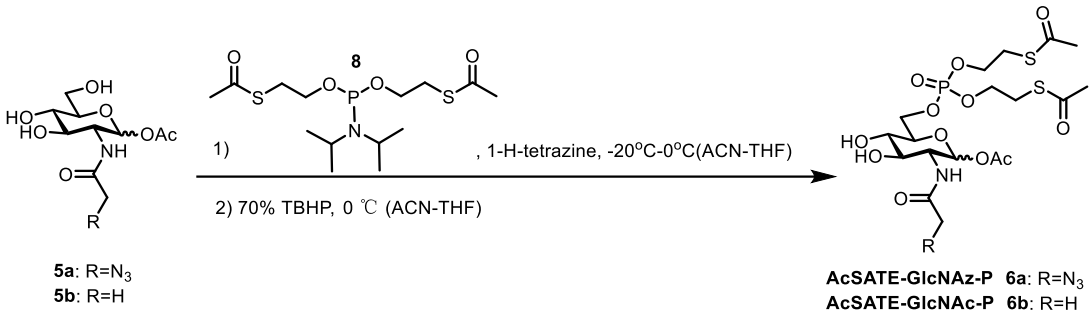
Compound **5a** (α/β = 1:0.54), R_f = 0.58 (DCM/MeOH = 4:1). ^{1}H NMR (600 MHz, CD_3OD) δ 6.14 (d, *J* = 3.6 Hz, 1H), 5.65 (d, *J* = 8.8 Hz, 0.54H), 4.04 (dd, *J* = 3.6, 10.7 Hz, 1H), 3.93 – 3.82 (m, 4.16H), 3.80-3.78 (m, 1H), 3.75-3.68 (m, 2.54H), 3.67 – 3.64 (m, 1H), 3.60 – 3.55 (m, 0.54H), 3.46 (t, *J* = 9.4 Hz, 1H), 3.41 – 3.38 (m, 1.08H), 2.13 (s, 3H), 2.03 (s, 1.62H); ^{13}C NMR (151 MHz, CD_3OD) δ 171.30, 171.09, 170.82, 170.69, 94.02, 92.04, 78.85, 76.19, 75.38, 72.11, 71.57, 71.50, 62.32, 62.25, 56.24, 54.45, 53.08, 52.56, 20.81, 20.75. m/z (HRMS$^+$) [M+Na] $^+$ 327.0952 ($C_{10}H_{16}N_4O_7Na^+$ requires 327.0911).

Synthesis of Compound 5b：

The synthesis of compound **5b** is similar to compound **5a**, yielding of 53% as a white solid. Compound **5b** (α isomer), R_f = 0.4 (DCM/MeOH = 4:1). The NMR data was consistent with the literature[14]. ^{1}H NMR (600 MHz, CD_3OD) δ 6.12 (d, *J* = 3.6 Hz, 1H), 4.00 (dd, *J* = 3.6, 10.7 Hz, 1H), 3.79 (dd, *J* = 2.3, 12.0 Hz, 1H), 3.72 – 3.67 (m, 2H), 3.65 (ddd, *J* = 2.3, 5.2, 10.0 Hz, 1H), 3.44 (t, *J* = 10.0 Hz, 1H), 2.13 (s, 3H), 1.96 (s,

3H); ^{13}C NMR (151 MHz, CD_3OD) δ 173.93, 171.28, 92.13, 76.16, 72.21, 71.66, 62.30, 54.37, 22.39, 20.81. m/z (HRMS$^+$) [M+Na]$^+$ 286.0912 ($C_{10}H_{17}NO_7Na^+$ requires 286.0897).

1.6 Synthesis of AcSATE-GlcNAz-P (6a)/AcSATE-GlcNAc-P (6b)



Compound **8** was prepared according to previously established procedures [13], with slight modifications. A 100 mL flask was charged with a moderate-activated 4 Å molecular sieve and anhydrous THF (60 mL). Compound **7** (4 g, 33.3 mmol, 1 eq) and triethylamine (TEA, 18.5 mL, 133.2 mmol, 4 eq) were added, thoroughly mixed, and cooled to 0°C in an ice bath for 10 minutes. *N*-dichlorophosphanyl-*N*-propan-2-ylpropan-2-amine (3.0 mL, 16.6 mmol, 0.5 eq) was added under strictly anhydrous and anaerobic conditions, resulting in the formation of a white precipitate. The reaction mixture was stirred at room temperature overnight. The product can be detected using AGF 254 neutral pre-coated plates and stained with ninhydrin staining solution (PE/EA/TEA = 20:1:1, R_f = 0.78). Subsequently, the mixture was filtered, and the filtrate was concentrated. The oily crude product was purified using silica gel column chromatography with a PE solvent containing 0.1% triethylamine, where the silica gel was pre-soaked in the same solvent. A high-purity yellow oily product **8** was obtained, which was suitable for direct use in the next reaction.

Synthesis of AcSATE-GlcNAz-P (6a)：

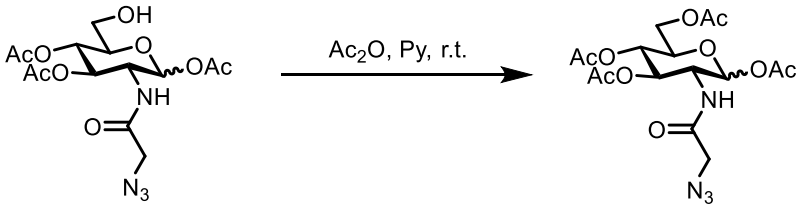
A 50 mL flask was charged with a moderate-activated 4 Å molecular sieve and compound **5a** (280 mg, 0.92 mmol, 1 eq) was dissolved in anhydrous THF (14 mL). Compound **8** (611 mg, 1.65 mmol, 0.3 M in anhydrous THF, 1.8 eq) was added, followed by the slow addition via syringe of 1-H-tetrazine (322 mg, 4.6 mmol, 0.4 M in anhydrous ACN, 5 eq) at -20°C. The mixture was then allowed to warm naturally to 0°C and reacted at 0°C overnight. TLC indicated the reaction was complete. Subsequently, 70% *tert*-Butyl hydroperoxide (TBHP, 0.62 mL, 4.6 mmol, 5 eq) was added to the mixture, and the reaction was stirred for an additional 2 hours at 0°C. The solvent was removed, and the crude product was purified by silica gel chromatography using DCM/MeOH as the eluent, affording compound **6a** (136 mg, 26% yield) as a colorless syrup. Compound **6a** (α/β = 1:0.4), R_f = 0.64 (EA/MeOH = 4:1). ^{1}H NMR (600 MHz, CD_3OD) δ 6.12 (d, *J* = 3.6 Hz, 1H), δ 5.66 (d, *J* = 8.8 Hz, 0.4H). 4.37 (ddd, *J* = 2.0, 6.4, 11.3 Hz, 0.4H).4.31 (ddd, *J* = 2.0, 6.4, 11.3 Hz, 1H), 4.28 – 4.21 (m, 1.4 H), 4.17 – 4.12 (m, 5.6H), 4.06 (dd, *J* = 3.6, 10.8 Hz, 1H), 3.94 – 3.81 (m, 4.2H), 3.74 (dd, *J* = 8.8, 10.8 Hz, 1H), 3.62 – 3.57 (m, 0.8H), 3.46 (dd, *J* = 8.8, 10.1 Hz,

1H), 3.40 (dd, *J* = 8.8, 9.9 Hz, 0.4H). 3.22 – 3.18 (m, 5.6H), 2.36 (m, 8.4H), 2.14 (s, 3H), 2.07 (s, 1.2H); ^{13}C NMR (151 MHz, CD_3OD) δ 196.44-196.39 (4C), 171.04, 170.95, 170.79, 170.67, 93.84, 91.85, 76.70, 75.16, 74.20, 72.06, 71.17, 71.04, 68.24, 68.17, 67.65, 56.10, 54.28, 53.08, 52.55, 30.48, 30.01, 20.80; ^{31}P NMR (243 MHz, CD_3OD) δ -2.19 (α), -2.25 (β); m/z (HRMS$^+$) $[M+NH_4]^+$ 606.1263 ($C_{18}H_{33}N_5O_{12}PS_2^+$ requires 606.1299).

Synthesis of AcSATE-GlcNAc-P (6b)：

The synthesis of compound **6b** is similar to compound **6a**, yielding of 23% as a colorless syrup. Compound **6b** (α isomer), R_f = 0.48 (EA/MeOH = 4:1). ^{1}H NMR (600 MHz, CD_3OD) δ 6.09 (d, *J* = 3.6 Hz, 1H), 4.31 (ddd, *J* = 2.0, 6.4, 11.3 Hz, 1H), 4.25 (ddd, *J* = 5.1, 7.6, 11.2 Hz, 1H), 4.14 (dt, *J* = 6.4, 7.7 Hz, 4H), 4.03 (dd, *J* = 3.6, 10.7 Hz, 1H), 3.83 (ddd, *J* = 2.0, 5.1, 10.1 Hz, 1H), 3.70 (dd, *J* = 8.8, 10.7 Hz, 1H), 3.45 (dd, *J* = 8.8, 10.1 Hz, 1H), 3.20 (t, *J* = 6.4 Hz, 4H), 2.36 (s, 6H), 2.14 (s, 3H), 1.96 (s, 3H); ^{13}C NMR (151 MHz, CD_3OD) δ 196.39, 196.36, 173.88, 171.02, 91.91, 74.15, 72.14, 71.22, 68.26, 67.63, 54.18, 30.48, 30.00, 22.39, 20.80; ^{31}P NMR (243 MHz, CD_3OD) δ -2.19; m/z (HRMS$^+$) $[M+NH_4]^+$ 565.1255 ($C_{18}H_{34}N_2O_{12}PS_2^+$ requires 565.1285).

1.7 Synthesis of Ac_4GlcNAz



3a **Ac_4-GlcNAz**

Compound **3a** (200 mg, 0.52 mmol, 1 eq) was dissolved in anhydrous pyridine (Py, 2 mL), and acetic anhydride (Ac_2O, 144 μL, 1.56 mmol, 3 eq) was added under ice bath conditions. The mixture was then stirred at room temperature overnight. Once TLC (PE/EA = 1:4) indicated the reaction was complete, pyridine was removed under reduced pressure. The resulting crude product was purified by silica gel chromatography using PE/EA as the eluent, yielding Ac_4GlcNAz (199 mg, 90% yield) as a white solid. Ac_4GlcNAz (α/β = 1:0.26), R_f = 0.8 (PE/EA = 1:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.45-6.41 (m, 1.26H), 6.20 (d, J = 3.7 Hz, 1H), 5.78 (d, J = 8.7 Hz, 0.26H), 5.32 – 5.26 (m, 1.26H), 5.21 (t, J = 9.8 Hz, 1H), 5.14 (t, J = 9.6 Hz, 0.26H), 4.45 (ddd, J = 3.7, 8.9, 10.8 Hz, 1H), 4.30-4.20 (m, 1.52H), 4.12 (dd, J = 2.3, 12.5 Hz, 0.26H), 4.07 (dd, J = 2.3, 12.4 Hz, 1H), 4.02 (ddd, J = 2.3, 4.1, 10.0 Hz, 1H), 4.31 – 4.20 (m, 2H), 4.07 (dd, J = 2.3, 12.4 Hz, 1H), 4.02 (ddd, J = 2.3, 4.1, 10.0 Hz, 1H), 3.83 (ddd, J = 2.2, 4.5, 9.8 Hz, 0.26H), 3.94 (s, 2H), 3.92 (s, 0.52H), 2.21 (s, 3H), 2.11 (s, 0.78H), 2.10 – 2.07 (2×s, 3.78 H), 2.07 – 2.02 (4×s, 7.56H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.70, 171.03, 170.85, 170.82, 169.47, 169.43, 169.29, 168.86, 167.16, 166.97, 92.29, 90.35, 72.99, 72.21, 70.39, 69.88, 67.64, 67.38, 61.63, 61.52, 53.31, 52.65, 52.50, 51.29, 21.08, 21.04, 20.89, 20.86, 20.75, 20.73. The NMR data was consistent with the literature[15]

2. Materials and Methods for Biochemical Assays

2.1 Strains, media and metabolic glycan labeling

Strains and media

Saccharomyces cerevisiae BY4742 was used in this study. The yeasts were grown in YPD media (1% yeast extract, 2% peptone, 2% glucose) or YPG media (1% yeast extract, 2% peptone, 2% glycerin). Solid media contained 2% agar.

Metabolic glycan labeling

A single colony was added to 10 mL of YPD liquid media and incubated at 30°C, 220 rpm overnight. The following day, 1 mL of the yeast cell culture was harvested and washed three times with sterile phosphate-buffered saline (1×PBS). The cells were then resuspended in fresh YPG liquid media (or YPD liquid media to compare the effects of different media on glycan metabolic labeling) with an initial OD_{600} of 0.15-0.2. **For the dose- and time-dependence experiments**, 2 mL of the cell suspension in a glass cell culture tube was treated with *N*-acetyl glucosaime-6-phosphate analogs (500 mM and 50 mM probe stock solutions, dissolved in DMSO) at the indicated concentrations for 48 hours, or with 1 mM probe for varying durations of time, followed by CuAAC click reaction. **For the inhibitory experiments**, 2 mL of cell suspension (or 1 mL for Nikkomycin Z inhibitory experiments) in a glass cell culture tube was treated with 1 mM Ac_3SATE-GlcNAc-P or 1 mM Ac_3SATE-GlcNAz-P for 12 hours. Tunicamycin (SC0393, Beyotime Biotechnology) or Nikkomycin Z (N463180, Aladdin) was then added to achieve the final indicated concentrations, and the cultures were incubated for an additional 36 hours, followed by CuAAC click reaction. **For the proteomic analysis by LC-MS**, 10 mL of cell suspension in a glass cell culture conical flask was treated with or without 1 mM Ac_3SATE-GlcNAz-P for 48 hours, followed by extraction of yeast protein.

2.2 Click reaction for *Saccharomyces cerevisiae* cells

Yeast cells (100 µL) treated with or without probes were harvested and washed once with YPG media, sterile water, and 1× PBS. Yeast cells were then incubated with 100 µL 1× PBS (pH = 7.4) containing 1 µM alkyne-Cy5 (HY-D0820, MedChemExpress), 2 mM $CuSO_4$, and 2.5 mM sodium L-ascorbate at 26 ℃ in the dark for 1 hour. Following incubation, yeast cells were centrifuged at 6000 g for 1 minute, washed with 1 mL 5 mM EDTA (dissolved in 1× PBS, pH = 6-7) for 15 minutes in the dark, centrifuged again, and washed once with 500 µL sterile water. Yeast cells were then prepared for imaging or flow cytometry.

2.3 Fluorescence imaging

The click-labeled yeasts were resuspended in 10 µL 1× PBS. Yeast samples (3 µL) were mounted on microscope slides, covered with a coverslip, and visualized using a Leica TCS SP8 confocal microscope

equipped with an HC PL APO CS 40×/0.85 CS objective. The Cy5 signal was excited using a 638 nm laser line (40%-50% laser excitation). The corresponding fluorescence emission was collected within a PMT detection window of 647-659 nm.

2.4 Flow cytometry

The click-labeled yeasts were resuspended in 500 μL 1×PBS. The samples were analyzed using a BD FACS Aria Ⅲ flow cytometer equipped with a 633 nm red laser for excitation. The Cy5 fluorescence signal was collected through APC channel (660/20 nm bandpass filter). A total of 10000-20000 viable cell events per sample were recorded. The flow cytometry data were processed with Flow Jo software (version 10.8.1). Data analysis was performed using GraphPad Prism (version 10.4.1).

2.5 Growth assay and colony-forming units counting assay

For growth assay, after diluting the overnight seed culture to 0.15-0.2 OD_{600} in YPG or YPD liquid media, 2 mL of cell suspension in a glass cell culture tube was added with or without the inclusion of probes or DMSO. The yeasts were then grown at 30℃, 220 rpm and the OD_{600} were measured using UV-visible spectrophotometer or microplate reader to assess probes inhibition. For the colony-forming units counting assay, yeast cells were collected at different incubation time points, and 30 μL 10-fold serial dilutions (10^1, 10^2, 10^3, and 10^4) of yeast cells were applied to distinct regions of YPD solid media in a 10 cm Petri dish. YPD plates were then incubated at 30 ℃ for 48 h before counting. CFU/mL = (C/V) × D, C is the average number of colonies counted on all replicate plates at a countable dilution, V is the plating volume, D is the dilution factor. Data analysis was performed using GraphPad Prism (version 10.4.1).

2.6 Extraction of *Saccharomyces cerevisiae* protein

After yeasts were treated with probes or DMSO in 2 mL YPG media for 48 hours. They were collected by centrifugation, washed once time with YPG media, sterile water and 1× PBS, and then frozen at -70°C. For protein labeling in yeast lysates, frozen yeast cells were resuspended in 100 μL of ice-cold RIPA lysis buffer (P0013K, Beyotime Biotechnology) containing 1×EDTA-free protease inhibitor cocktail (P1020, Beyotime Biotechnology) and 1 mM PMSF, with an equal amount of acid-washed glass beads (425–600 μm, G121929, Aladdin) added[16]. The yeasts in the 1.5 mL tube were then vortexed for 40 seconds at 3600 rpm using a BenchMate VM-D vortex mixer (Oxford Lab Products), followed by cooling for 5 minutes. This cycle was repeated 6 times. Then, 40 μL of RIPA lysis buffer was added, and the suspension was finally centrifuged at 10000 g for 20 minutes at 4℃. The supernatant was collected, and protein concentration was determined using the BCA protein assay kit (P0010S, Beyotime Biotechnology).

2.7 Click reaction for glycoprotein

Protein lysates (prepared according to Section 2.6) were precipitated with methanol-chloroform-water (3:0.75:2) under ice bath conditions, followed by centrifugation at 12000 g for 5 minutes. The samples were washed once with 500 μL of cold methanol and then resuspended in PBS containing 0.4% SDS (w/v) to achieve a concentration of approximately 2 mg/mL. Subsequently, the protein lysates were incubated with click reaction buffer (final concentrations: 100 μM alkyne-PEG_4-biotin (3309612A, Adamas), 0.5 mM $CuSO_4$, 1 mM BTTAA (8011038F, Adamas) and 2.5 mM freshly prepared sodium L-ascorbate) for 2 hours at 26℃. After the reaction, eight volumes of cold methanol were added, and the proteins were precipitated overnight at -70℃. The reaction mixtures were then centrifuged at 10000 g for 10 minutes at 4 °C and then washed with cold methanol twice. The supernatant was discarded, and the protein samples were air-dried before dissolution in 40 μL of 1× PBS containing 0.4% SDS (w/v). This preparation was used for either western blot analysis or enrichment of glycoprotein

2.8 Western blotting analysis

Protein samples prepared via click reaction were mixed with loading buffer and boiled at 90°C for 10 minutes prior to SDS-PAGE on 10% Tris-glycine gels. Subsequently, proteins were transferred to a 0.45 μm Immobilon-P PVDF membrane (IPVH00010, Millipore). Blots were then blocked with 2.5% (w/v, g/mL) BSA in 0.1% Tween 20 in Tris-buffered saline (1×TBST) at 30°C for 1 hour. After blocking, the blots were washed three times in 1× TBST for 5 minutes each and then incubated overnight at 4°C with HRP-conjugated beta actin monoclonal antibody (1:6000 dilution in 1× TBST. HRP-60008, Proteintech) or streptavidin-HRP (1:6000 dilution in 1× TBST. A0305, Beyotime Biotechnology). The second day, the blots were washed again three times in 1× TBST for 5 minutes each and visualized using BeyoECL Plus (P0018, Beyotime Biotechnology) on a Tanon 5200 Multi system.

2.9 Enrichment of glycoprotein and PNGase F-deglycosylation

50 μL prewashed streptavidin agarose (P2159, Beyotime Biotechnology) were incubated with 500 μL 2.5% BSA in 1×TBST buffer for 3 hours at 30°C, washed three times with 1×PBS buffer, suspended in 1× PBS and then added to protein samples prepared via click reaction and dissolved in 0.4% SDS-PBS (approximately 2 mg/mL). The total volume was 120 μL. The system was incubated on a shaker at 4°C overnight. Subsequently, the streptavidin agarose beads were washed sequentially with 0.2% (w/v) SDS in 1×PBS, 8 M urea in 1×PBS, 2.5 M NaCl in 1×PBS, 500 mM ammonium bicarbonate (ABC), 250 mM ABC, and 50 mM ABC. After washing, 50 μL 1× reaction buffer (P2318, Beyotime Biotechnology) was added to the beads, and the suspension was divided into two equal-volume groups (each group with a volume of 40 μL). One group was supplemented with 4 μL PNGase F (P2318, Beyotime Biotechnology), while the other group was not. Both groups were incubated on a shaker at 37°C for 24 hours. The beads were then washed three times with 50 mM ABC and incubated with loading buffer at 90°C for 10 minutes. After cooling, the samples were centrifuged at 600 g for 5 minutes. The supernatant samples were subsequently subjected to Western blotting analysis using Streptavidin-HRP.

2.10 In vitro *S*-glyco-modification

For the group of heat denaturation, after removing cell debris, the supernatant of yeast lysate was incubated at 90°C in a metal bath for 15 minutes. The sample was then centrifuged at 10000 g for 10 minutes at 4℃, and the resulting protein precipitate was resuspended in 0.4% SDS in 1×PBS to achieve a concentration of approximately 2 mg/mL. The resuspended solution was subsequently incubated at 37°C for 2 hours in the presence of probes or an equal volume of DMSO. For the group of non-heat denaturation, the supernatant of yeast lysate was directly incubated at 37°C for 2 hours with probes or an equal volume of DMSO, without undergoing the heat treatment step. The protein samples were then precipitated with methanol-chloroform-water (3:0.75:2) under ice bath conditions. After centrifugation at 10000 g for 5 min, the protein precipitate was washed with cold methanol three times and resuspended with 0.4% SDS in 1×PBS. Click reaction and western blotting analysis were conducted according to the above steps.

2.11 Proteomic analysis by LC-MS

After the yeasts were treated with 1 mM Ac_3SATE-GlcNAz-P or DMSO in 10 mL YPG media (total 4 bottles) for 48 hours. They were collected by centrifugation, washed once time with YPG media, sterile water and 1× PBS, and then frozen at -70°C. For the labeling of glycan-labeled proteins, frozen cells were resuspended in 500 μL ice-cold RIPA lysis buffer containing 1×EDTA-free protease inhibitor cocktail and 1 mM PMSF, and an equal amount of acid-washed glass beads (425–600 μm) was added. The yeasts in the 1.5 mL tube were vortexed for 40 seconds at 3600 rpm using a BenchMate VM-D vortex mixer (Oxford Lab Products), followed by cooling for 5 minutes. This cycle was repeated 6 times. Then, 200 μL of RIPA lysis buffer was added, and the suspension was finally centrifuged at 10000 g for 30 mintures at 4℃. After removing the debris, the protein concentration was determined by the BCA protein assay kit (P0010S, Beyotime Biotechnology). For the blank group and the metabolic labeling experimental group, 500 μL protein lysates (2 mg/mL) were incubated with the click reaction buffer (100 μM alkyne-PEG_4-biotin, 0.5 mM $CuSO_4$, 1 mM BTTAA, and 2.5 mM freshly prepared sodium L- ascorbate; final concentration) for 2 hours at 26℃. The S-glyco-modification labeling group subjected to heat denaturation, and the click reaction was performed after incubation with 1 mM Ac_3SATE-GlcNAz-P at 37°C for 2 hours. Protein lysates were precipitated with methanol-chloroform-water (3:0.75:2) under ice bath conditions, then centrifuged at 10000 g for 5 minutes, washed three times with cold methanol. The protein was frozen at -70°C for mass spectrometry analysis.

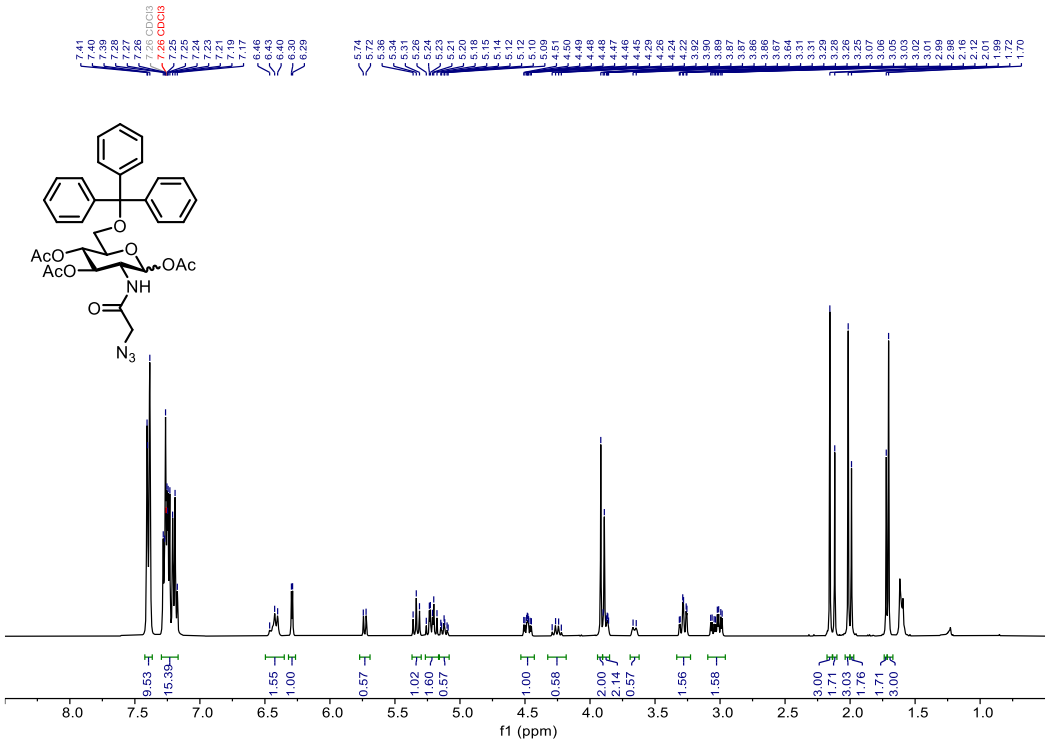
Protein samples were dissolved in PBS containing 2% SDS, quantified, and diluted tenfold to reduce the SDS concentration to below 0.2%. Biotinylated proteins (1.5 mg) were enriched using 100 μL of Thermo Scientific streptavidin agarose beads (50% slurry) at 4 °C for 4 h with gentle rotation. The beads were sequentially washed by inversion as follows: four times with 0.2% SDS in PBS, three times with 1 M NaCl, twice with HPLC-grade water, and twice with 50 mM ammonium bicarbonate to remove non-

specifically bound proteins. On-bead digestion was conducted in 50 µL of 50 mM ammonium bicarbonate containing 1 µg of sequencing-grade trypsin at 37 °C overnight. Peptides were extracted twice using 200 µL of 50% acetonitrile with 0.1% formic acid, pooled, dried under vacuum, and reconstituted in 0.1% formic acid for LC-MS/MS analysis.

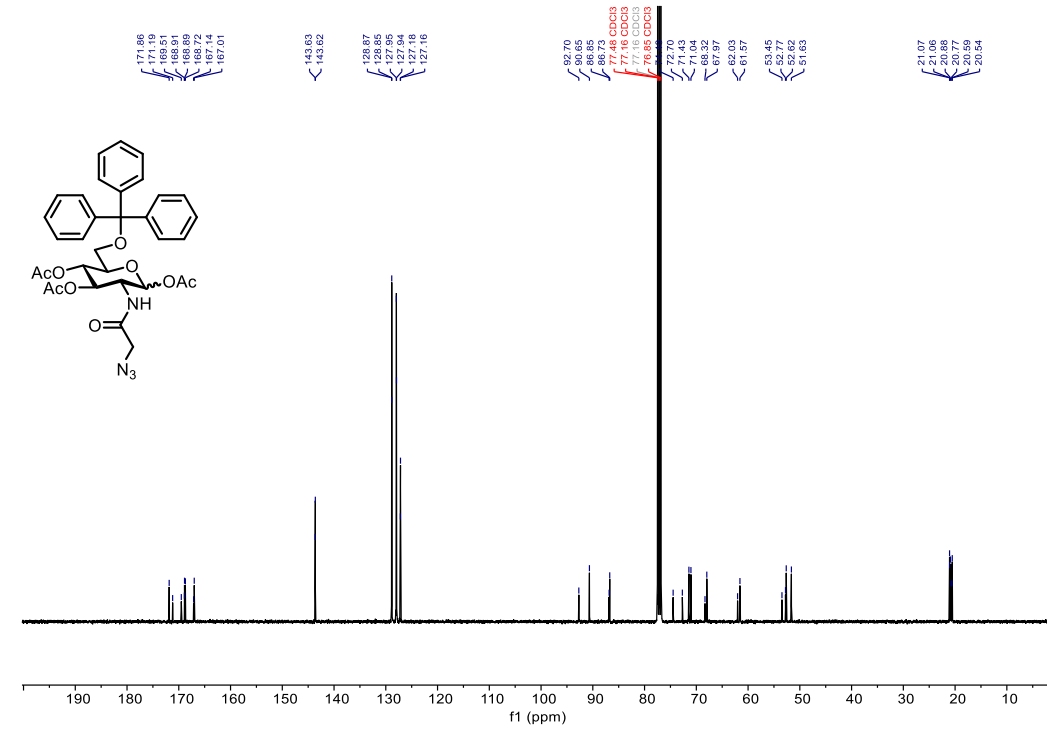
LC-MS/MS analysis was performed on a Q Exactive HF Orbitrap mass spectrometer coupled to an EASY-nLC 1200 system (Thermo Fisher Scientific). Peptides were separated on a self-packed C18 column (15 cm × 150 µm, 1.9 µm ReproSil-Pur C18-AQ, Dr. Maisch GmbH) using a 75 min gradient. Full MS scans were acquired at a resolution of 120,000 over an m/z range of 300–1400, followed by data-dependent acquisition of the 25 most intense precursors for higher-energy collisional dissociation (HCD) fragmentation at a normalized collision energy of 27. MS/MS spectra were collected at a resolution of 15,000 with a dynamic exclusion window of 15 s. Raw data were analyzed using Proteome Discoverer 2.1 against the UniProt *Saccharomyces cerevisiae* database (release 2025_11), with trypsin specified as the protease for sequence-specific cleavage and a maximum allowance of two missed cleavages. Variable modifications included methionine oxidation and protein N-terminal acetylation. Peptide and protein false discovery rates (FDRs) were controlled at 1%, and label-free quantification was performed.

NMR spectra

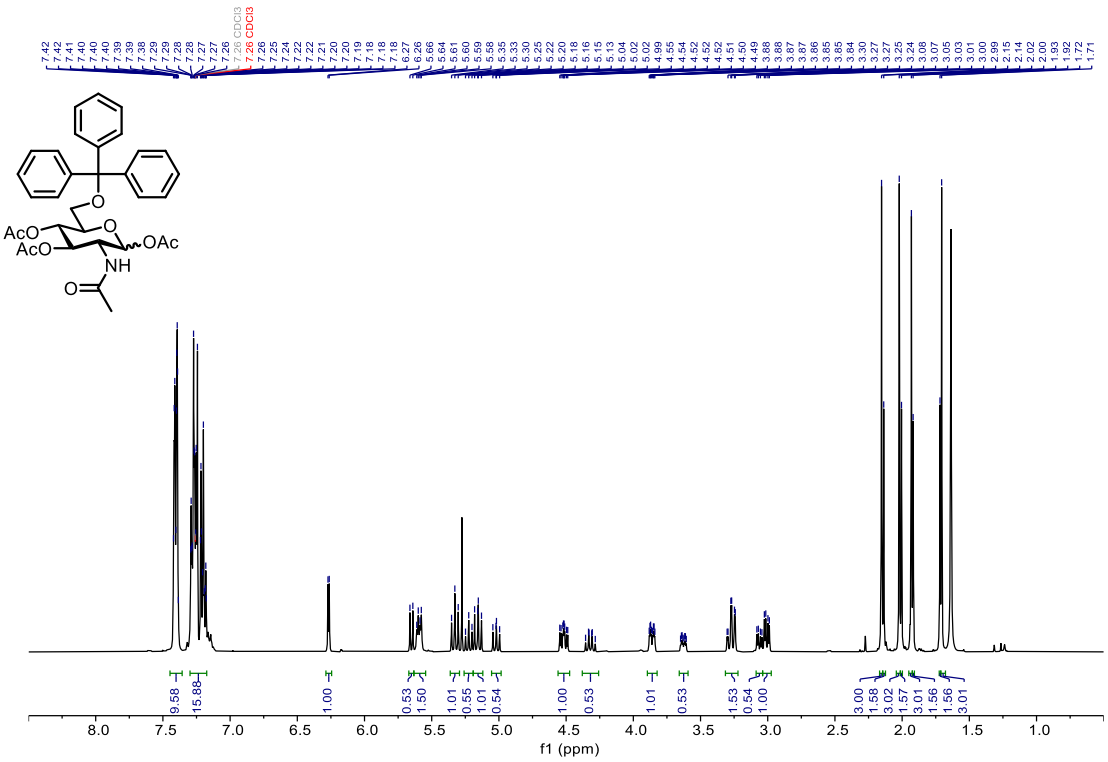
^{1}H NMR (400 MHz, $CDCl_3$) of α/β-compound 2a



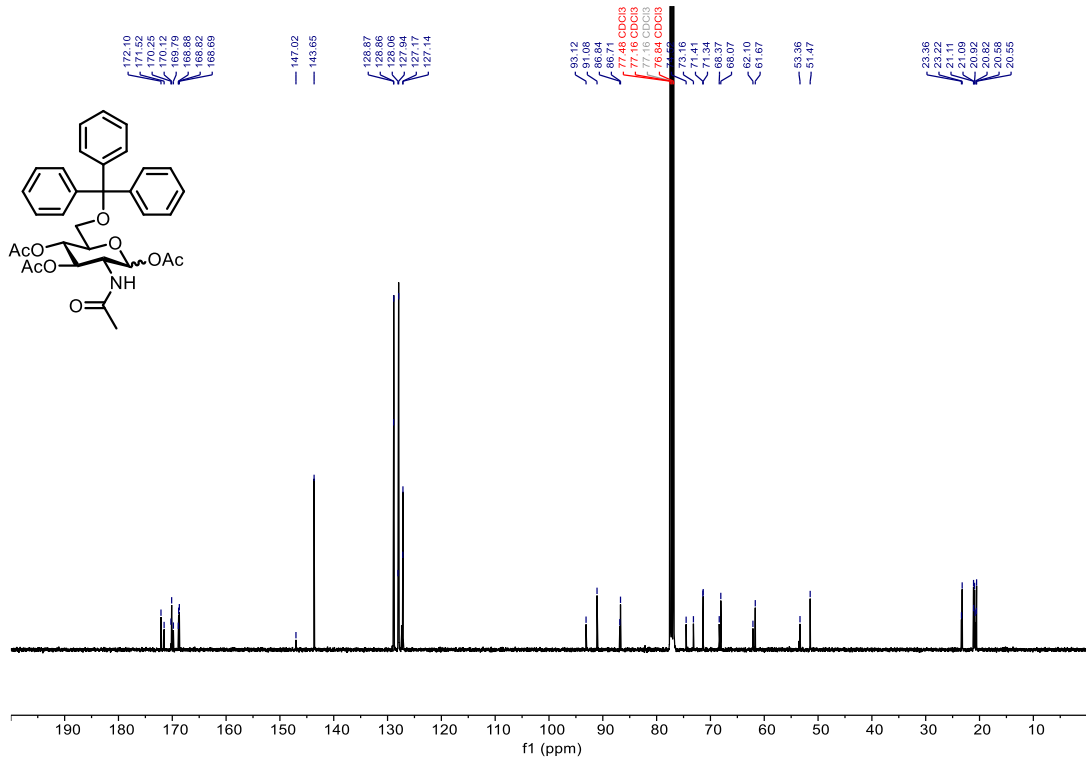
^{13}C NMR (101 MHz, $CDCl_3$) of α/β-compound 2a



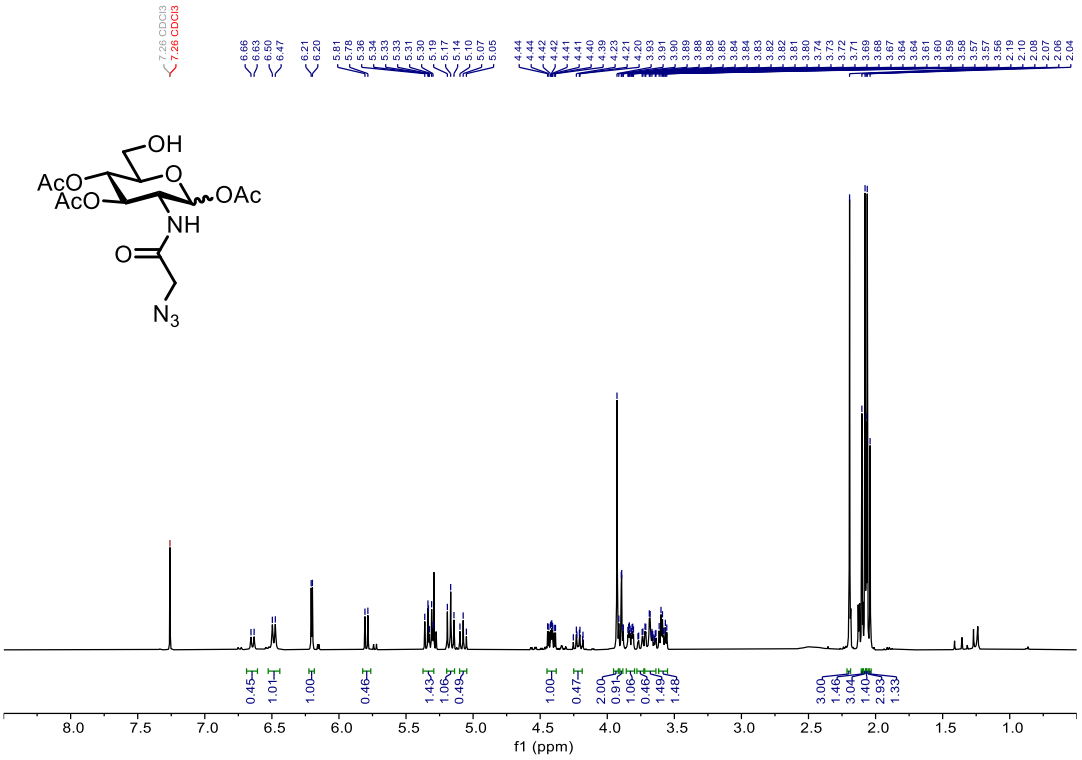
^{1}H NMR (400 MHz, $CDCl_3$) of α/β-compound 2b



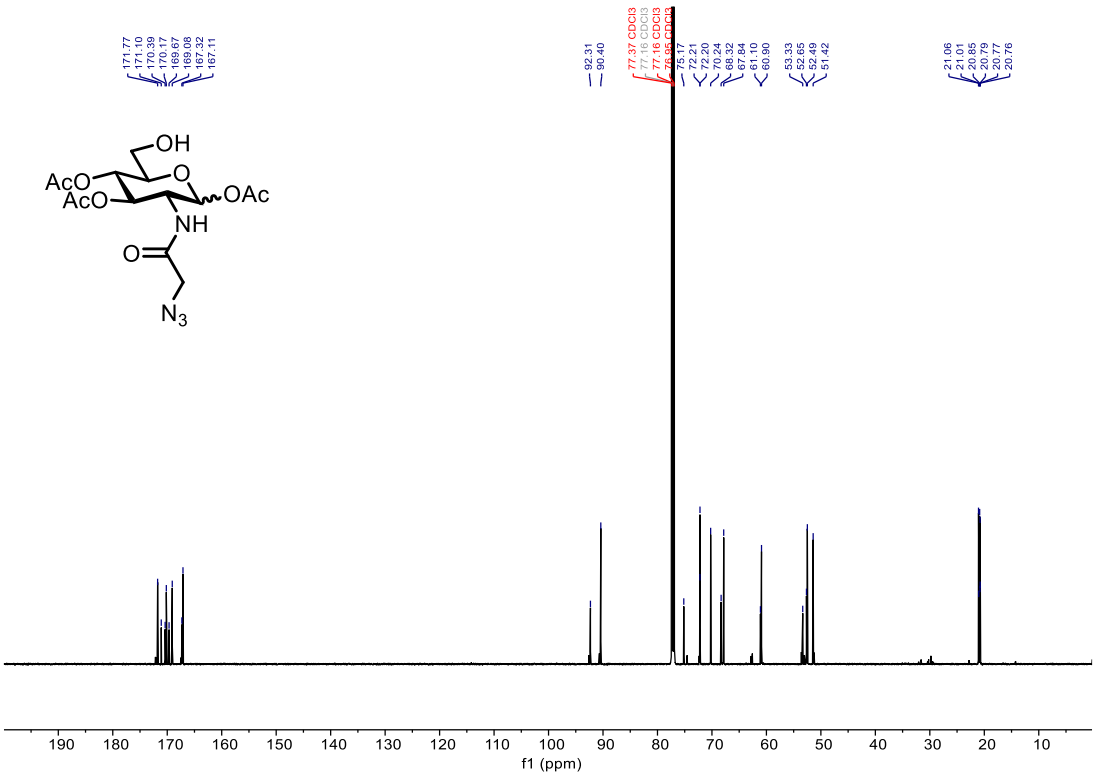
^{13}C NMR (101 MHz, $CDCl_3$) of α/β-compound 2b



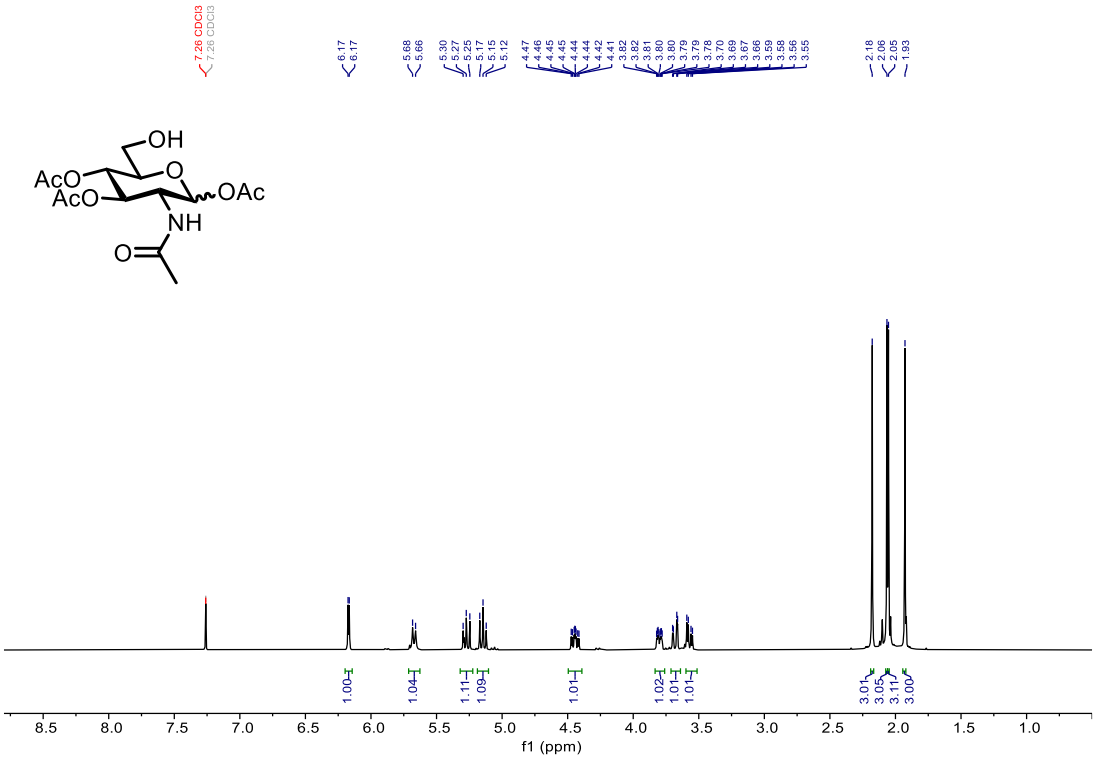
^{1}H NMR (400 MHz, $CDCl_3$) of α/β-compound 3a



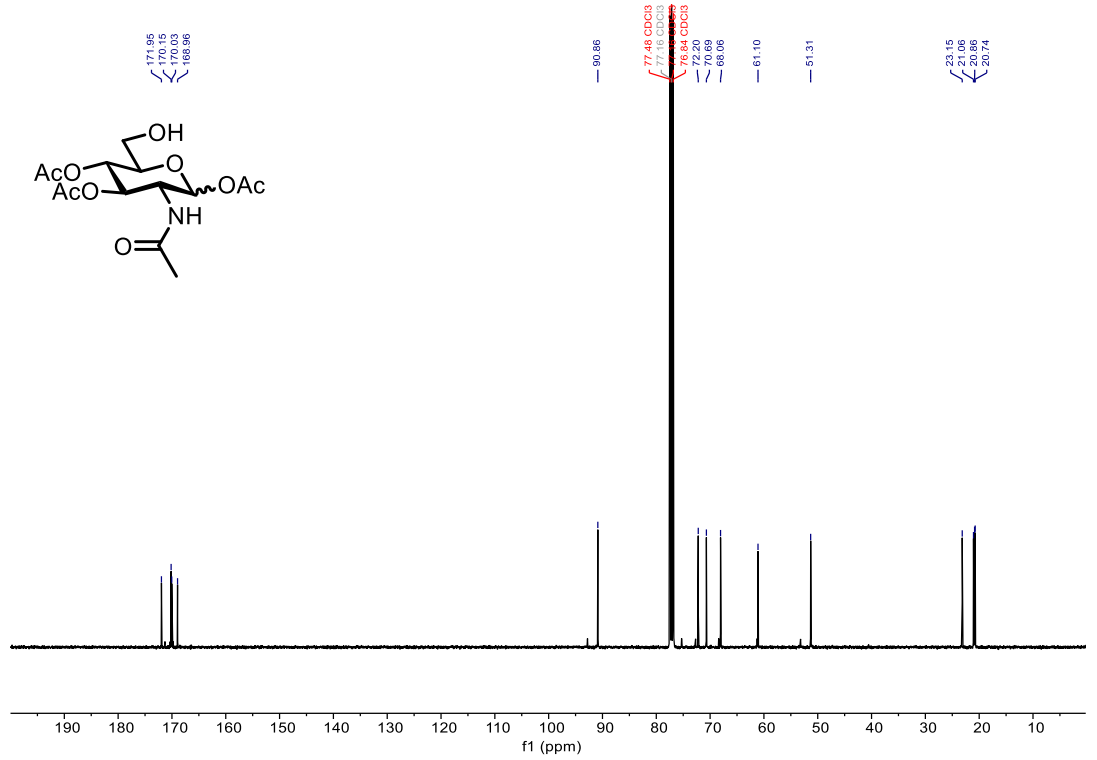
^{13}C NMR (101 MHz, $CDCl_3$) of α/β-compound 3a



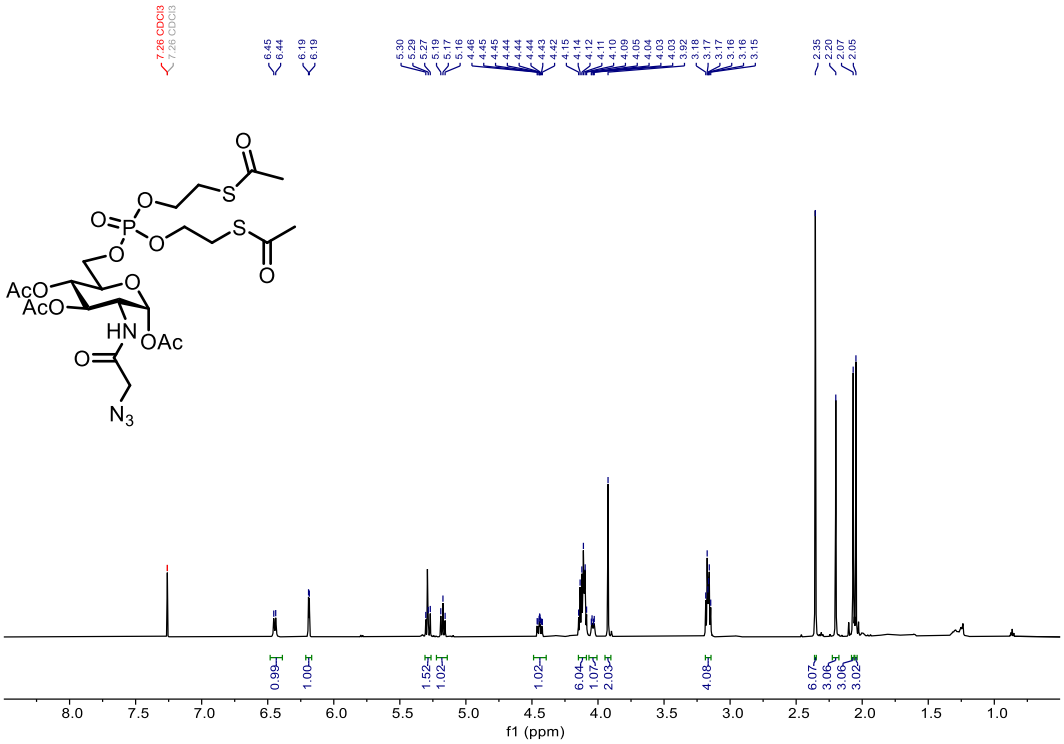
^{1}H NMR (400 MHz, $CDCl_3$) of α-compound 3b



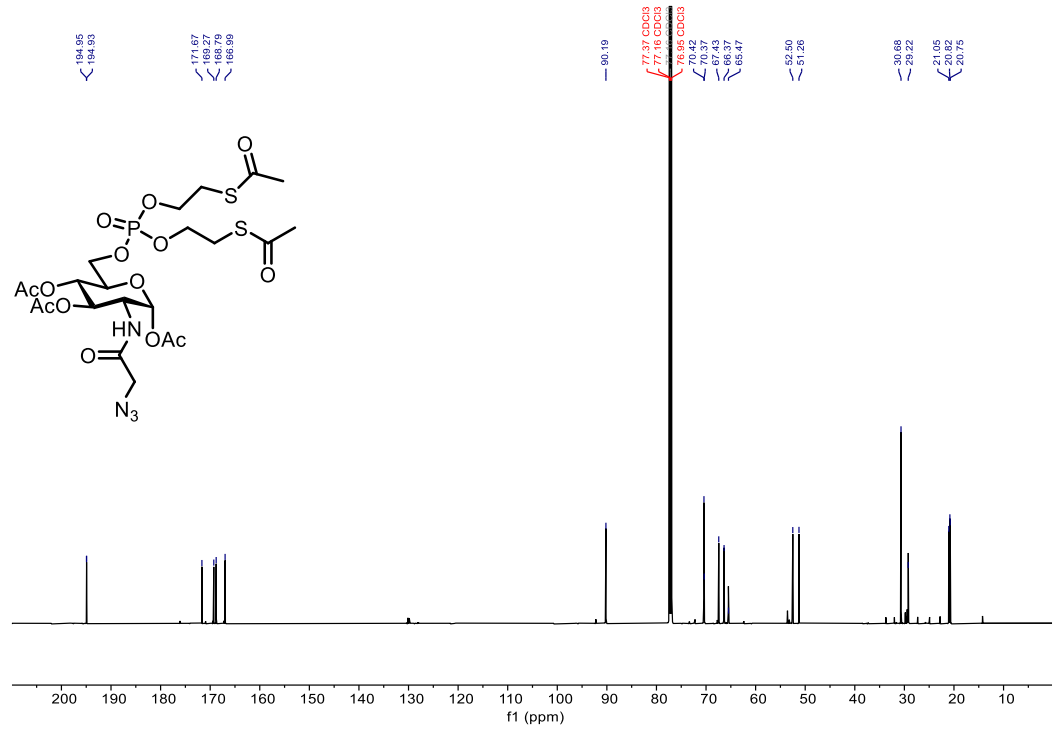
^{13}C NMR (101 MHz, $CDCl_3$) of α-compound 3b



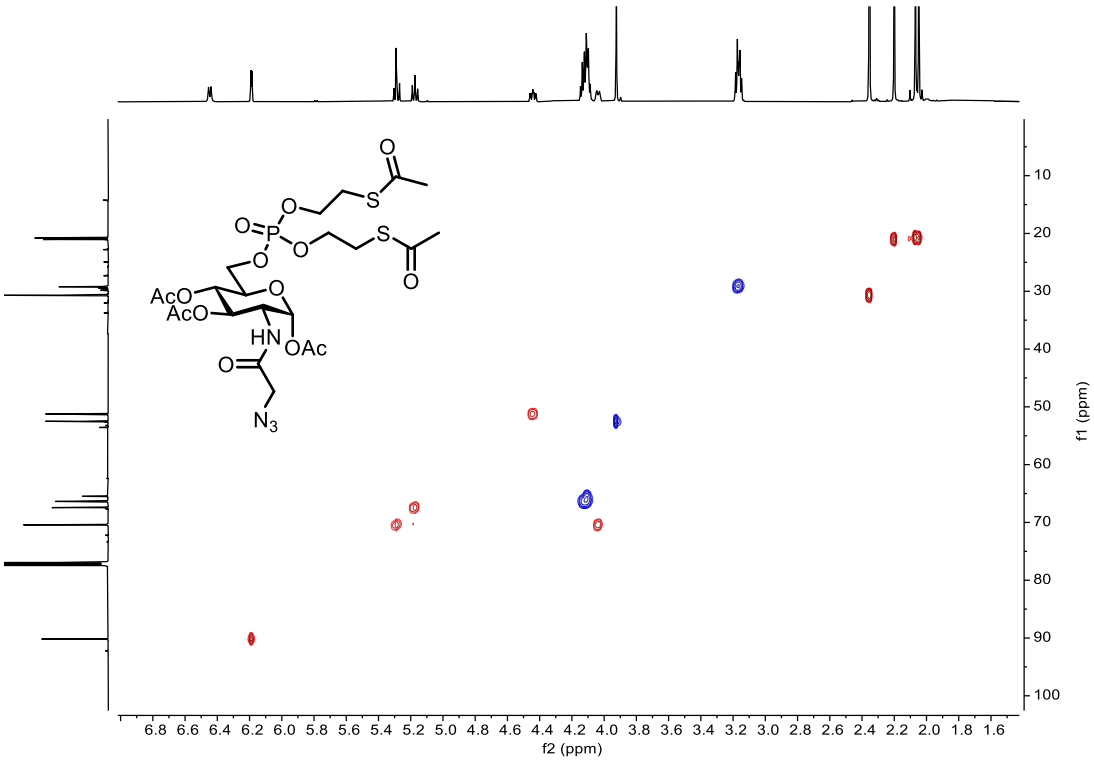
^{1}H NMR (600 MHz, $CDCl_3$) of α-compound 4a



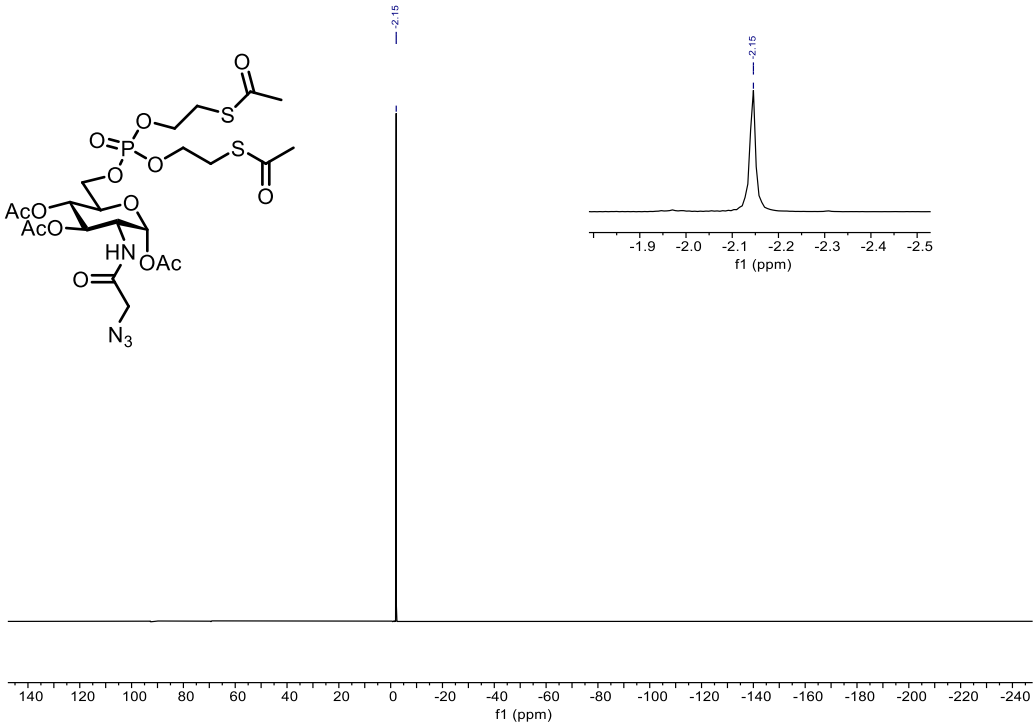
^{13}C NMR (151 MHz, $CDCl_3$) of α-compound 4a



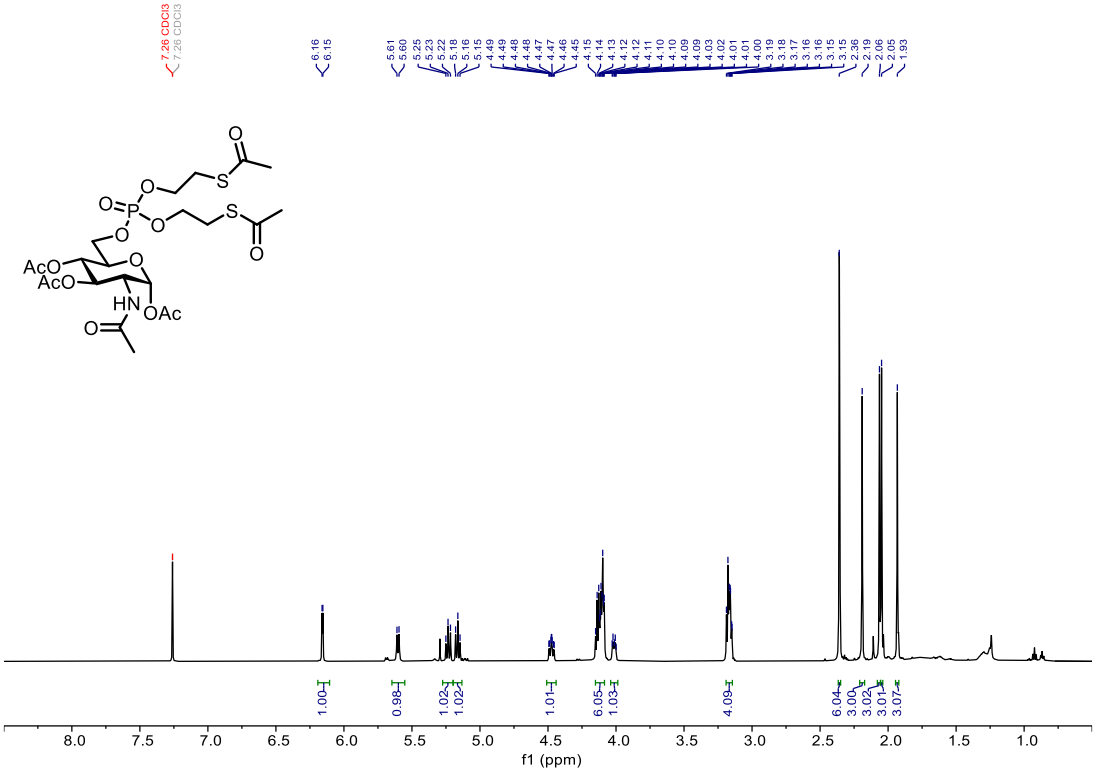
^{1}H-^{13}C HSQC ($CDCl_3$) of α-compound 4a



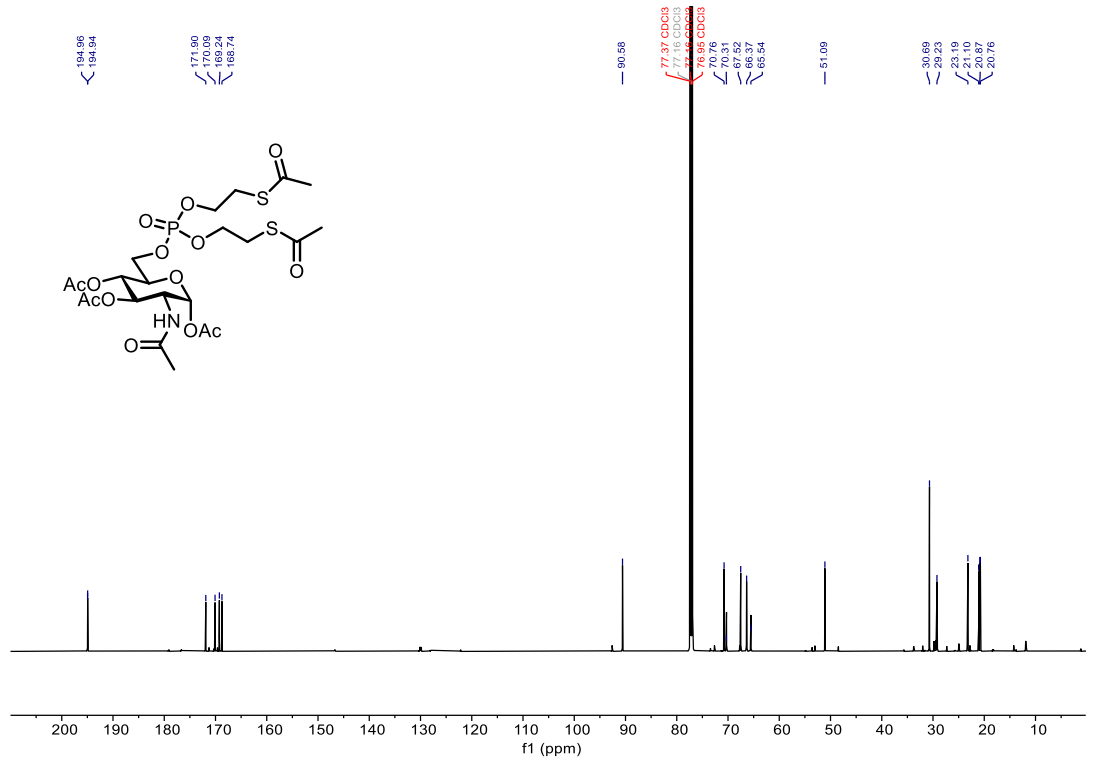
^{31}P NMR (243 MHz, $CDCl_3$) of α-compound 4a



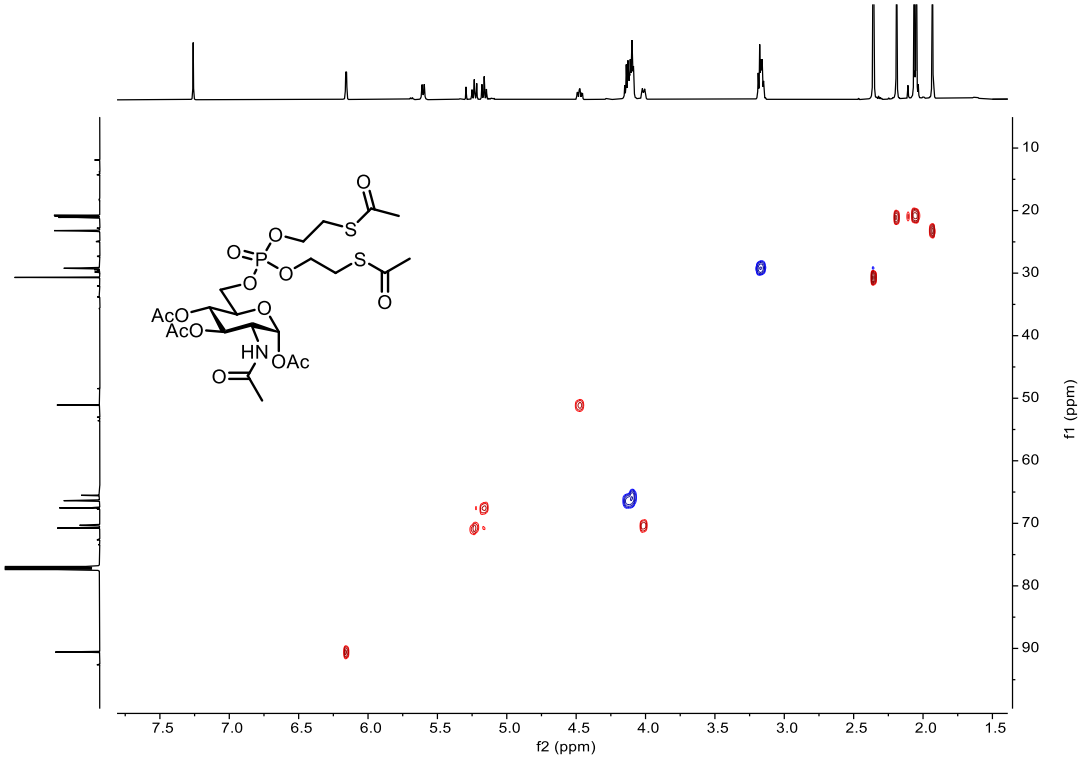
^{1}H NMR (600 MHz, $CDCl_3$) of α-compound 4b



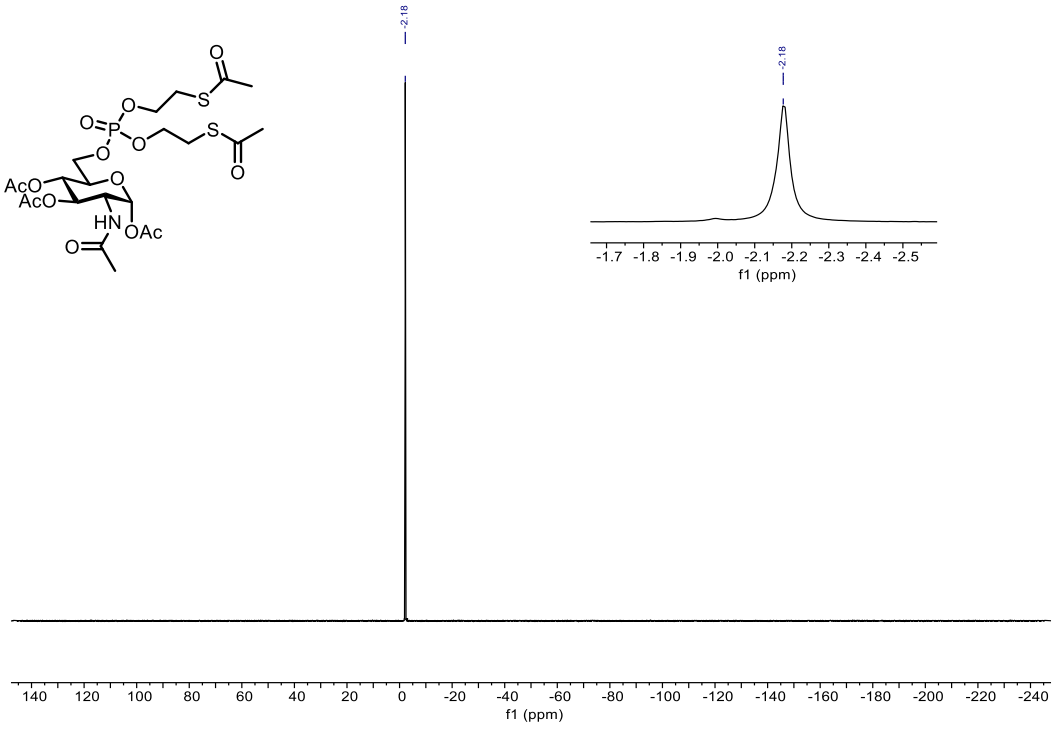
^{13}C NMR (151 MHz, $CDCl_3$) of α-compound 4b



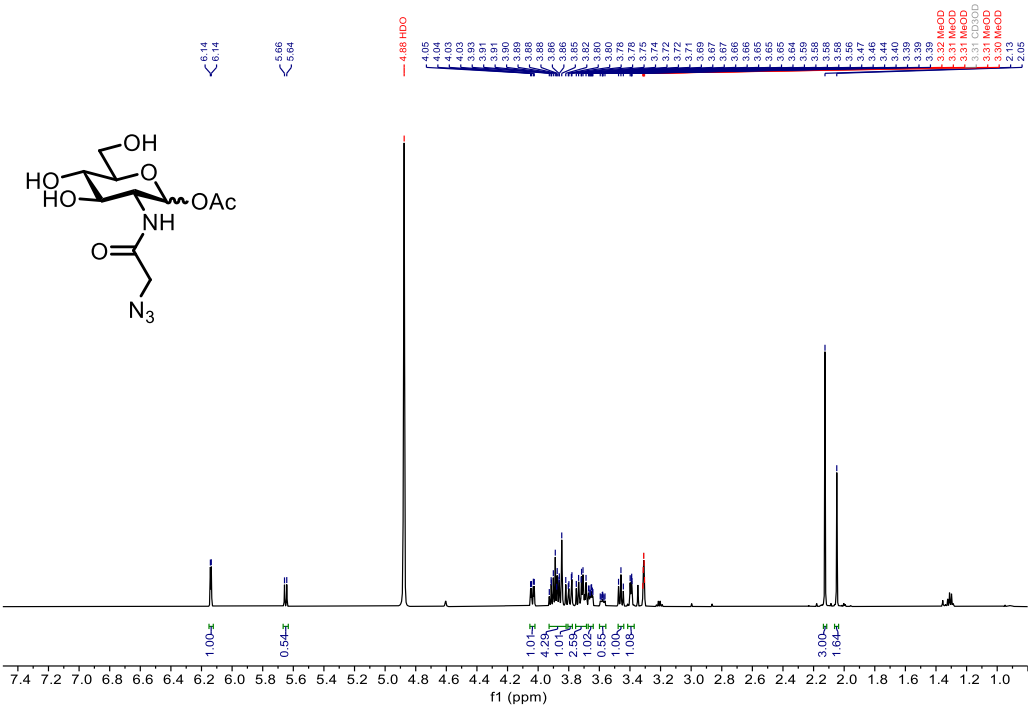
^{1}H-^{13}C HSQC ($CDCl_3$) of α-compound 4b



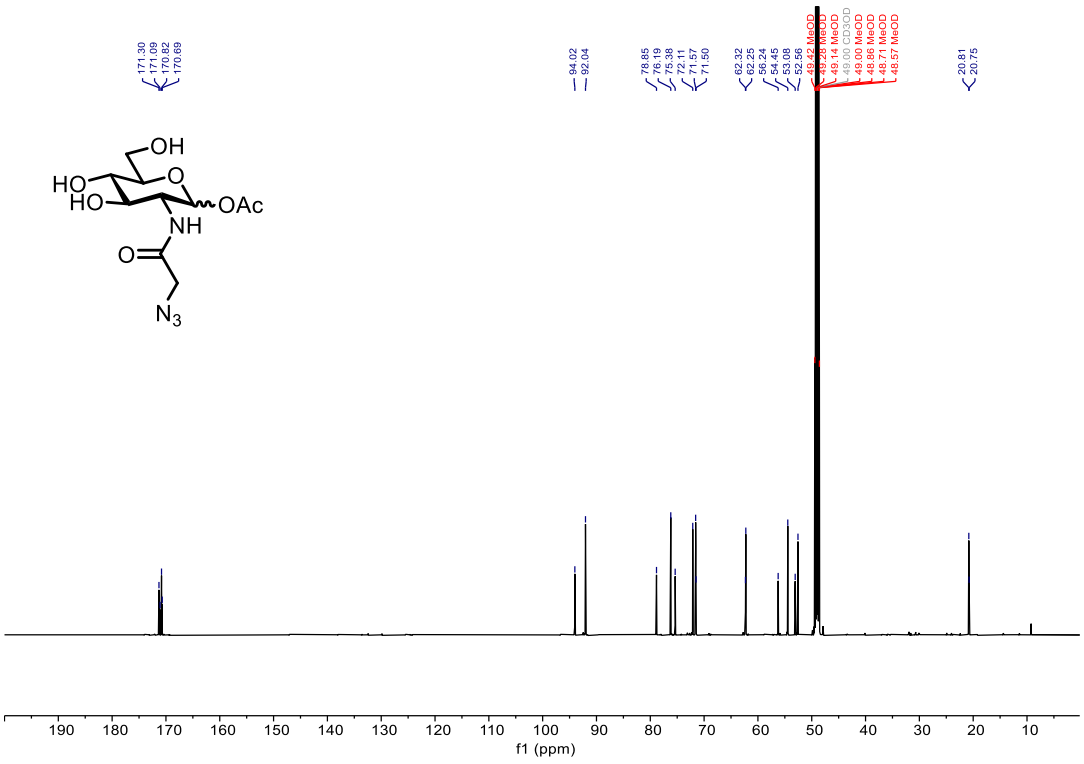
^{31}P NMR (243 MHz, $CDCl_3$) of α-compound 4b



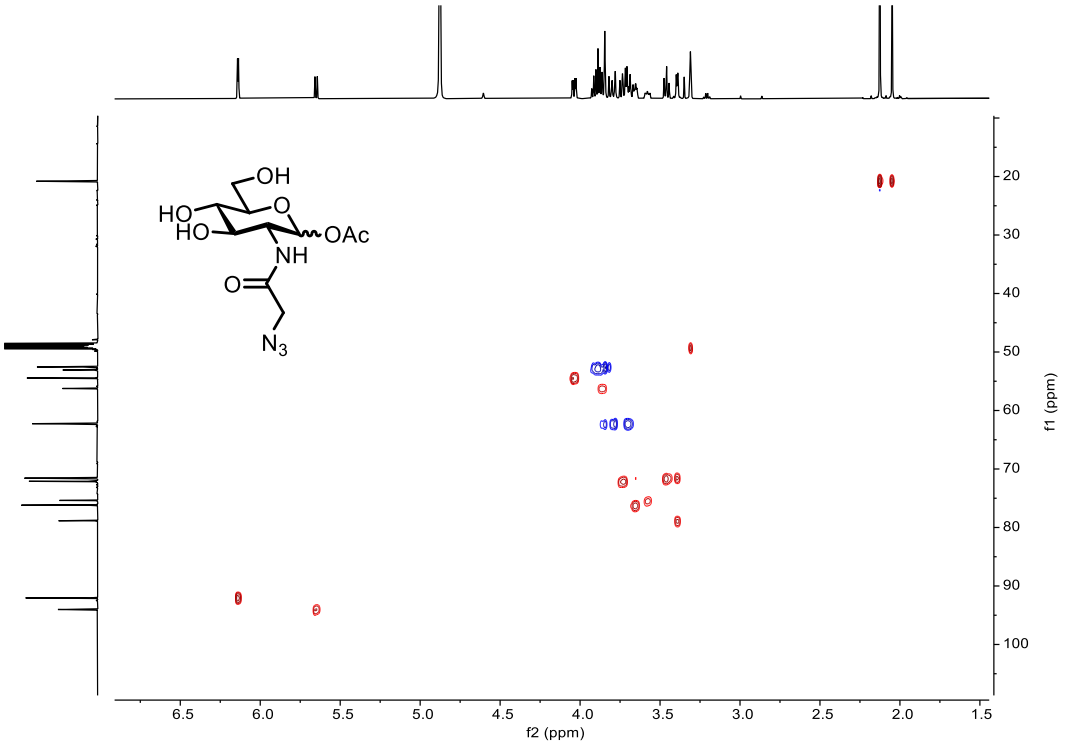
^{1}H NMR (600 MHz, CD_3OD) of α/β-compound 5a



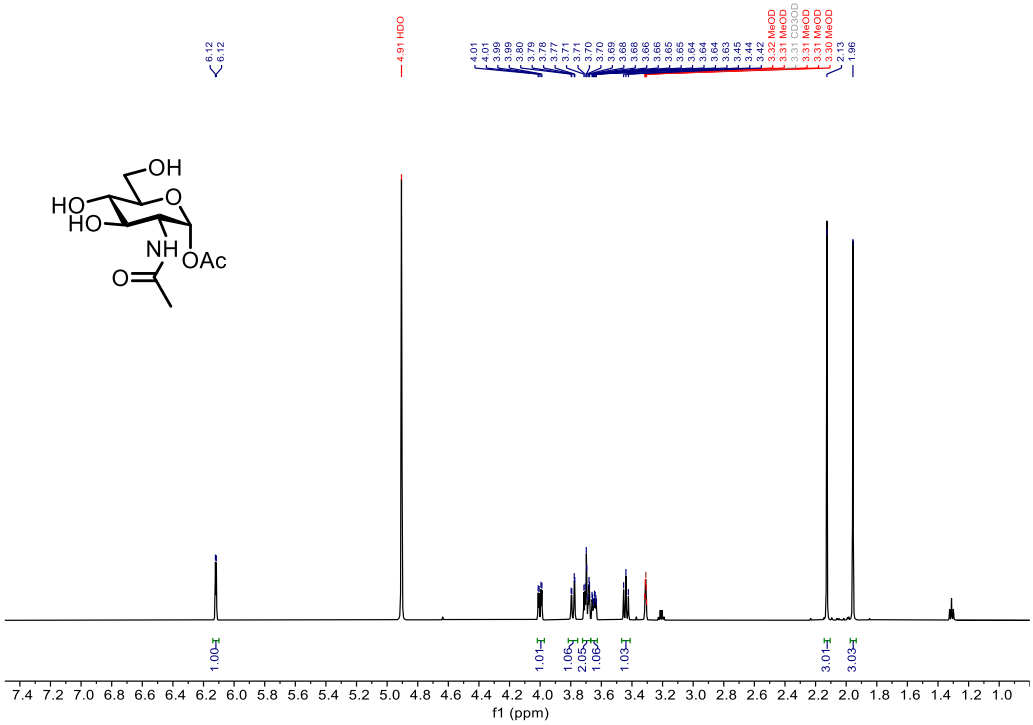
^{13}C NMR (151 MHz, CD_3OD) of *α*/*β*-compound 5a



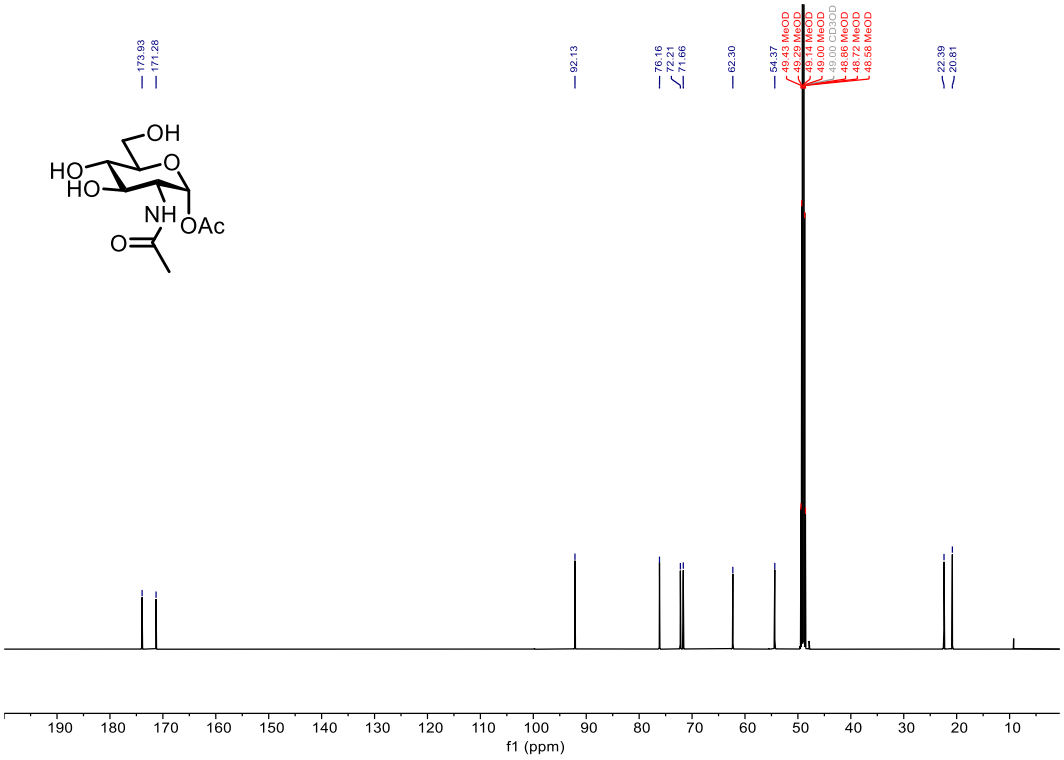
^{1}H-^{13}C HSQC (CD_3OD) of *α*/*β*-compound 5a



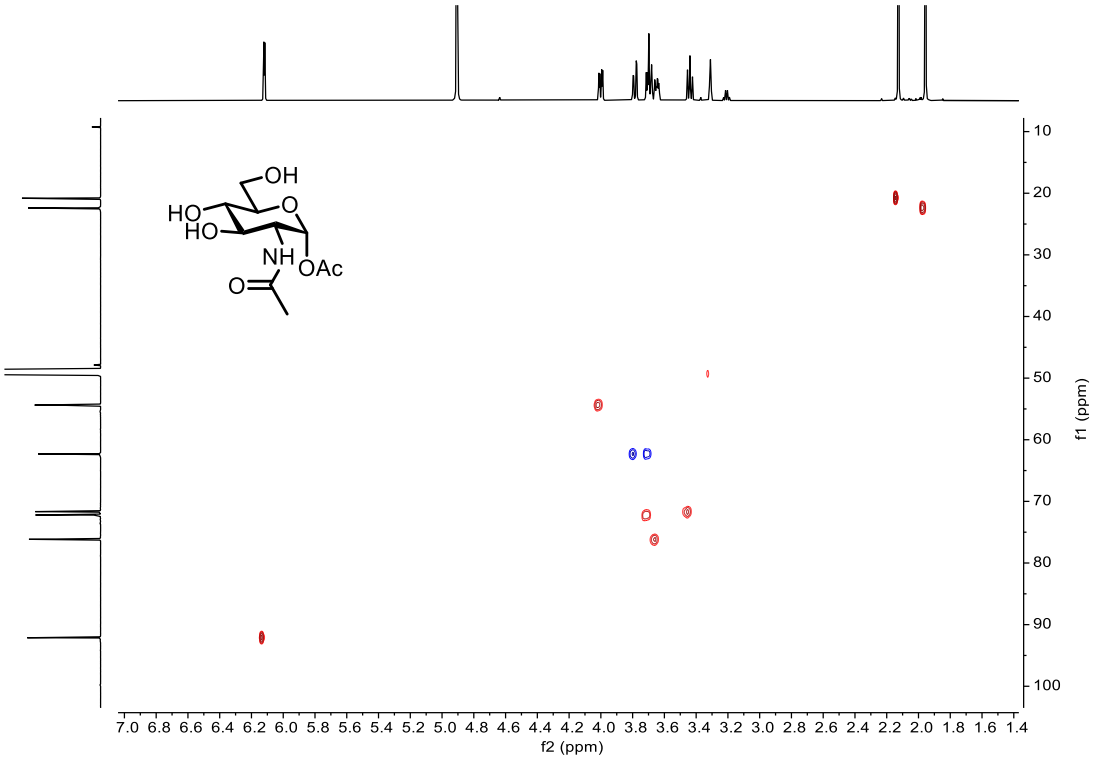
^{1}H NMR (600 MHz, CD_3OD) of *α*-compound 5b



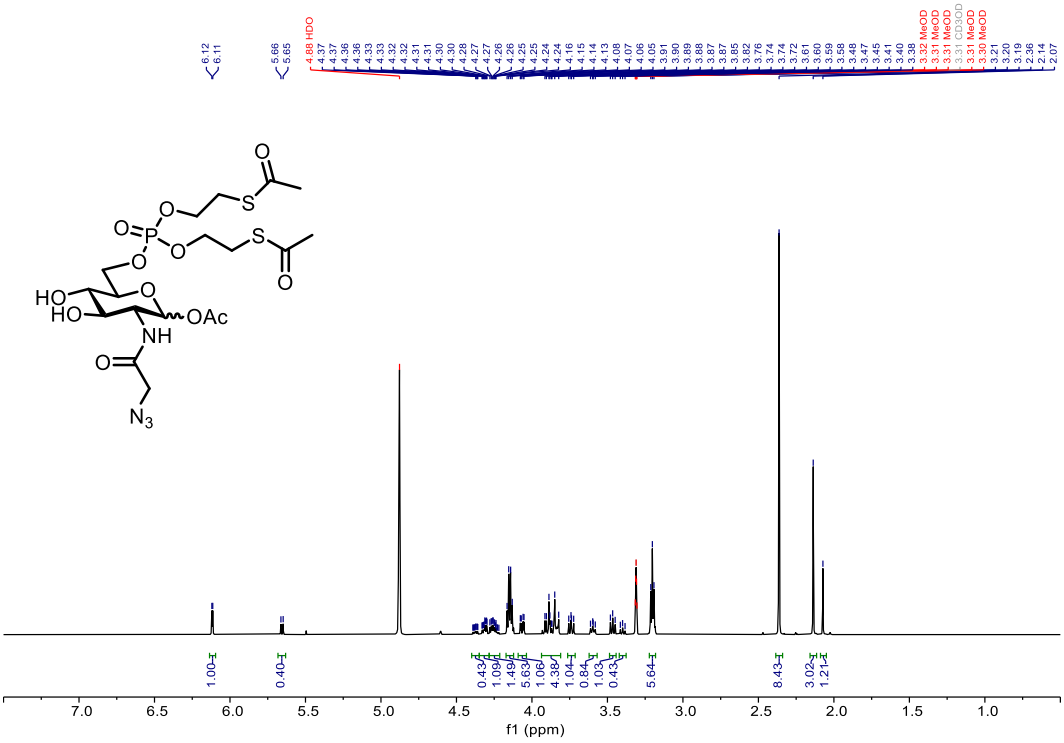
^{13}C NMR (151 MHz, CD_3OD) of α-compound 5b



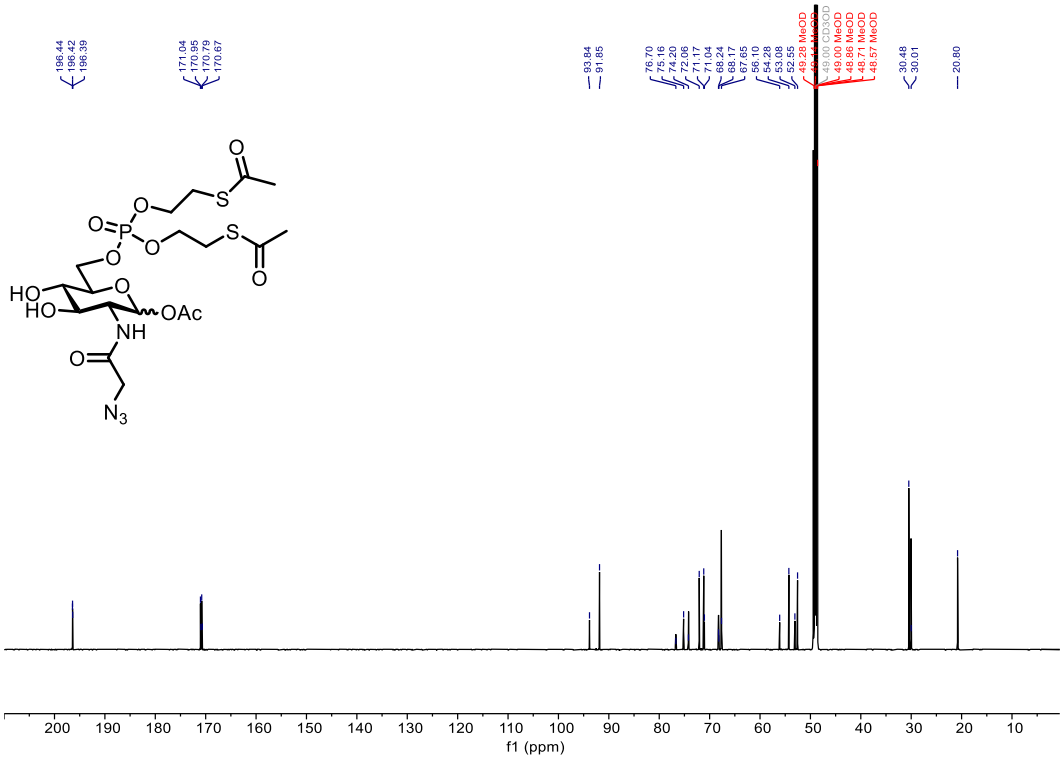
^{1}H-^{13}C HSQC (CD_3OD) of α-compound 5b



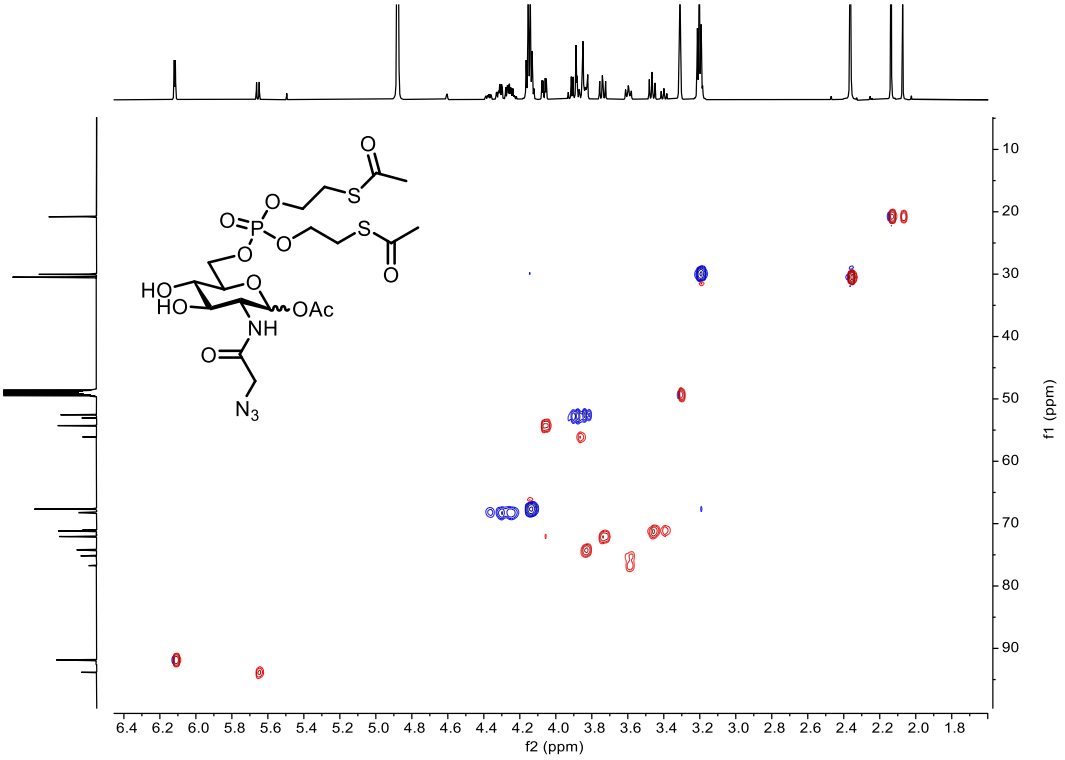
^{1}H NMR (600 MHz, CD_3OD) of α/β-compound 6a



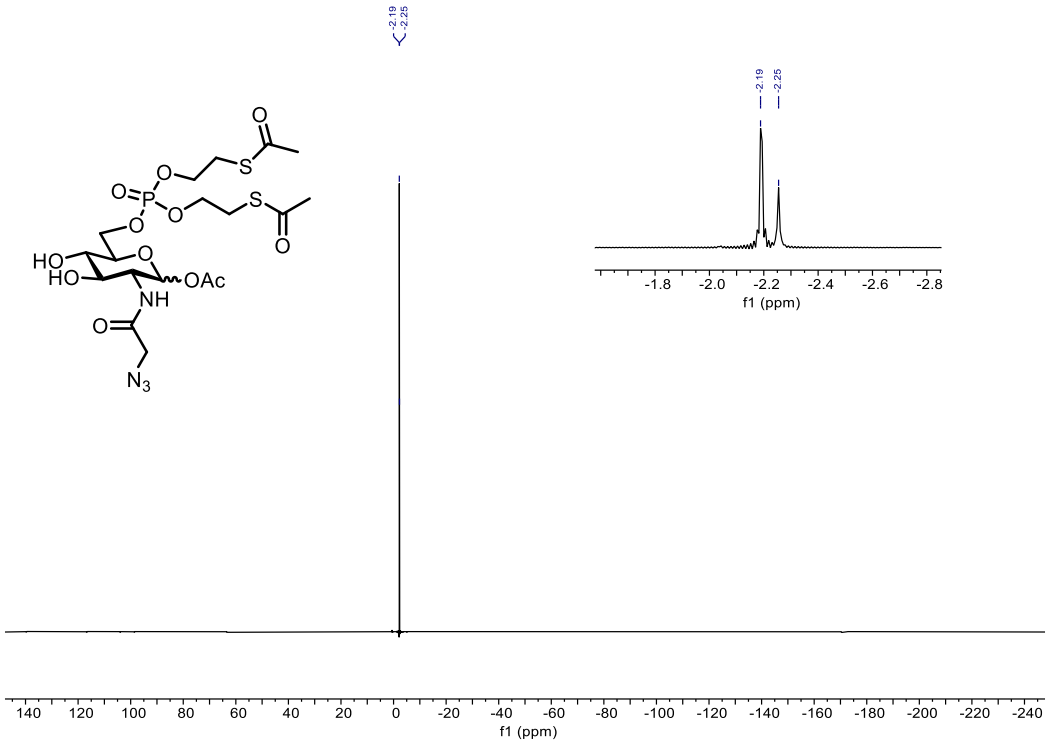
^{13}C NMR (151 MHz, CD_3OD) of α/β-compound 6a



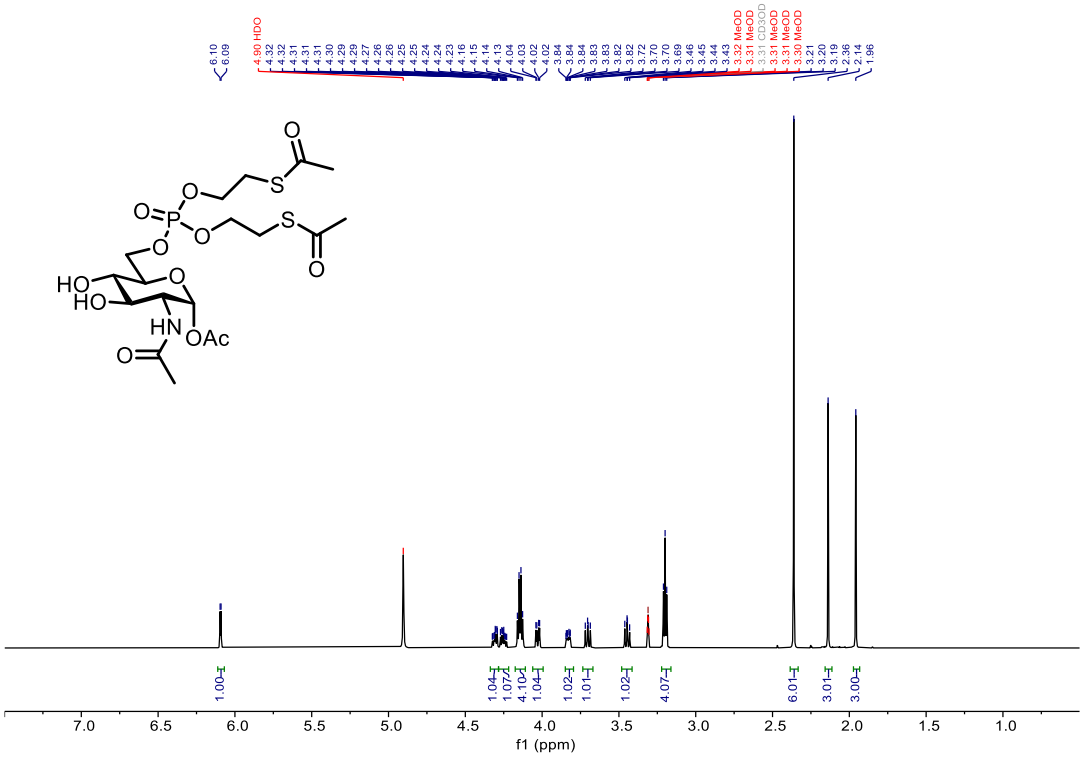
^{1}H-^{13}C HSQC (CD_3OD) of α/β-compound 6a



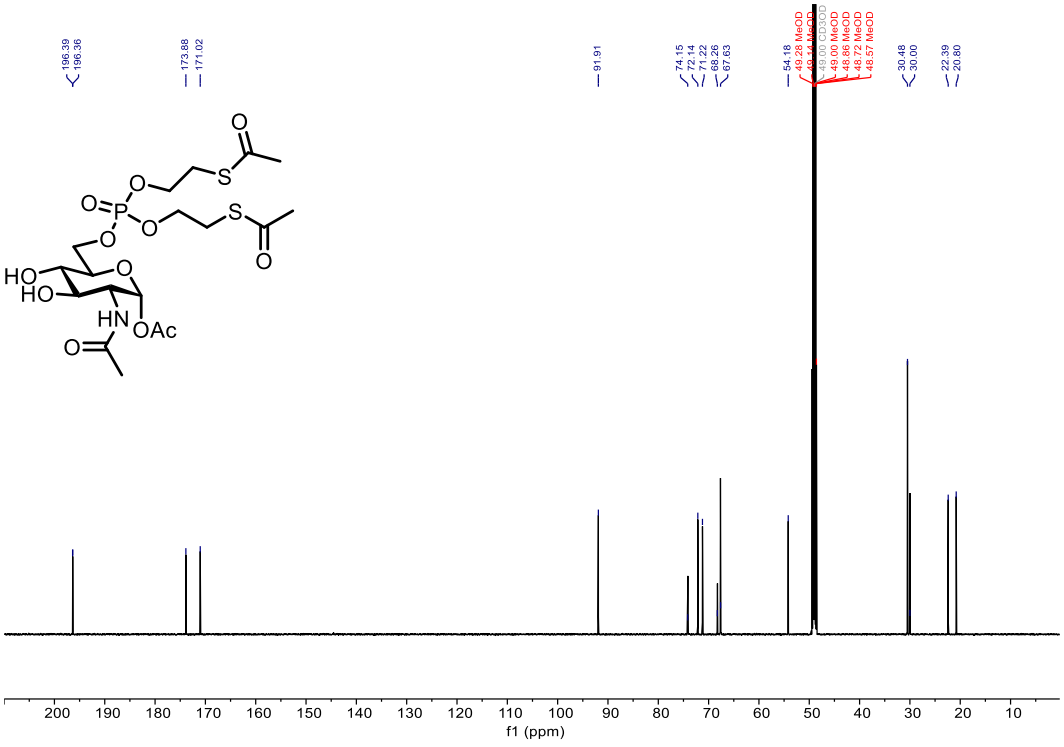
^{31}P NMR (243 MHz, CD_3OD) of α/β-compound 6a



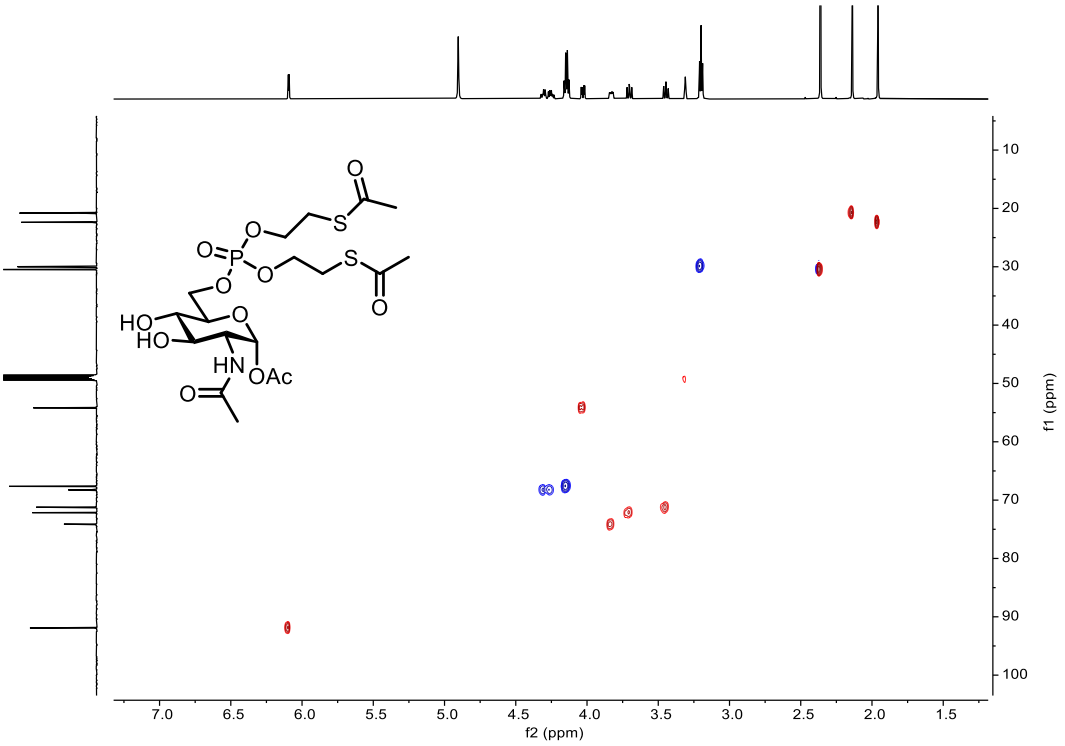
^{1}H NMR (600 MHz, CD_3OD) of α-compound 6b



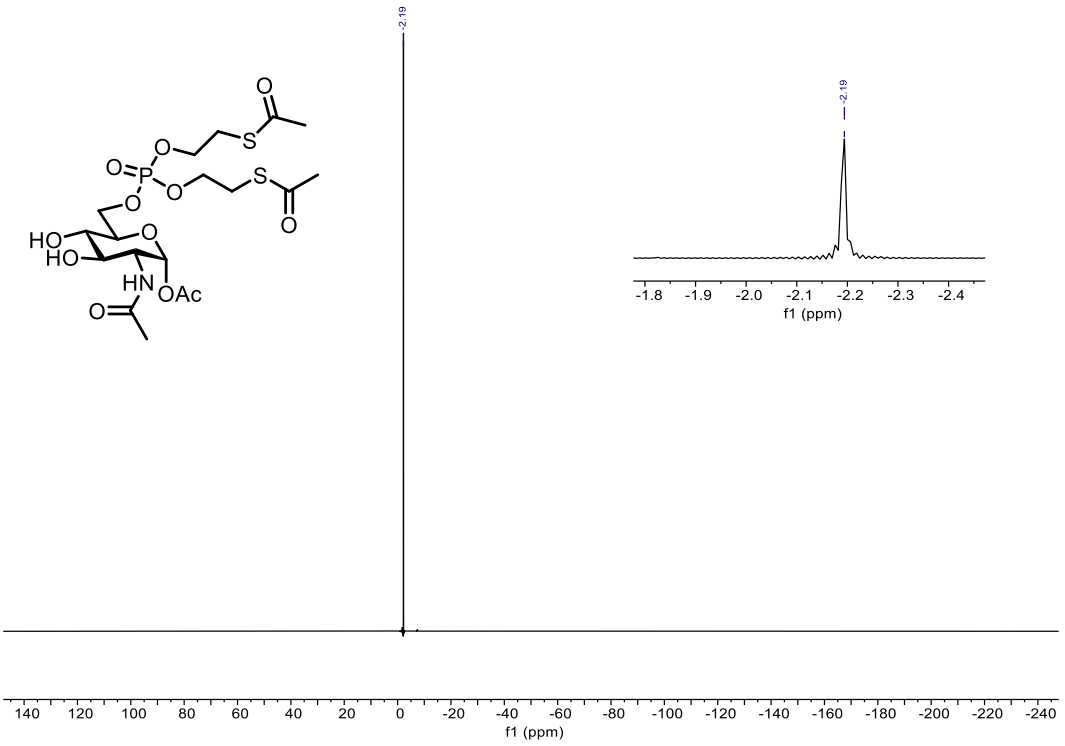
^{13}C NMR (151 MHz, CD_3OD) of *α*-compound 6b



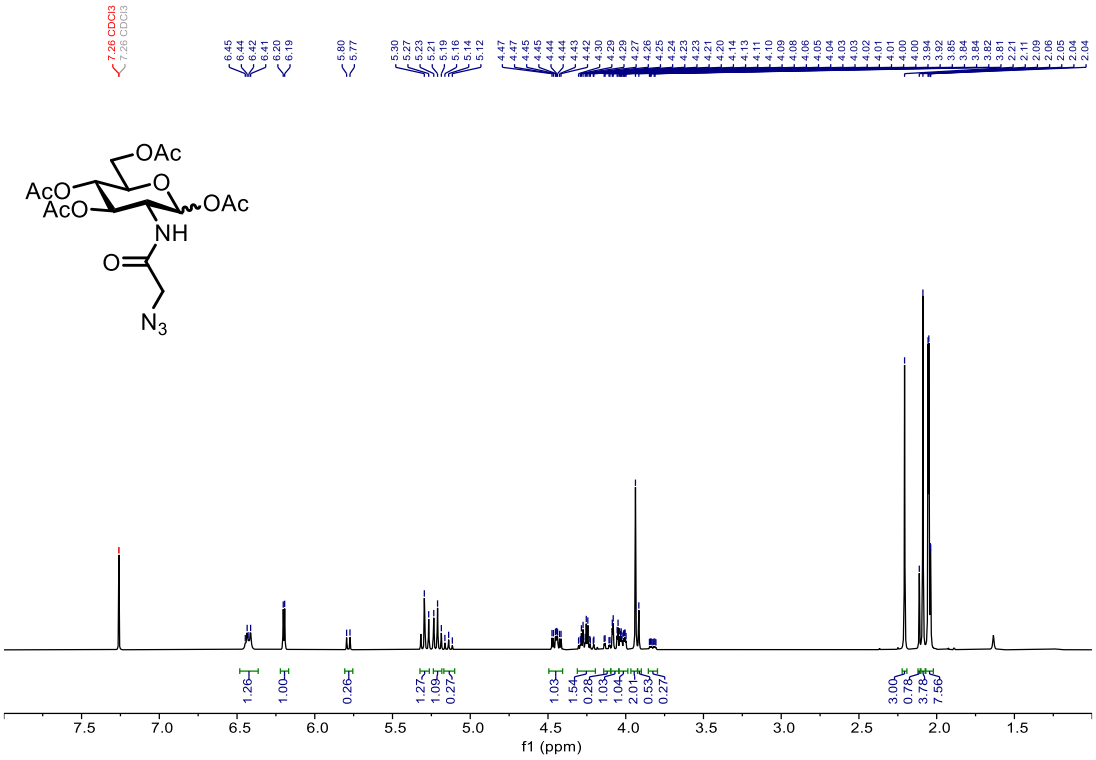
^{1}H-^{13}C HSQC (CD_3OD) of *α*-compound 6b



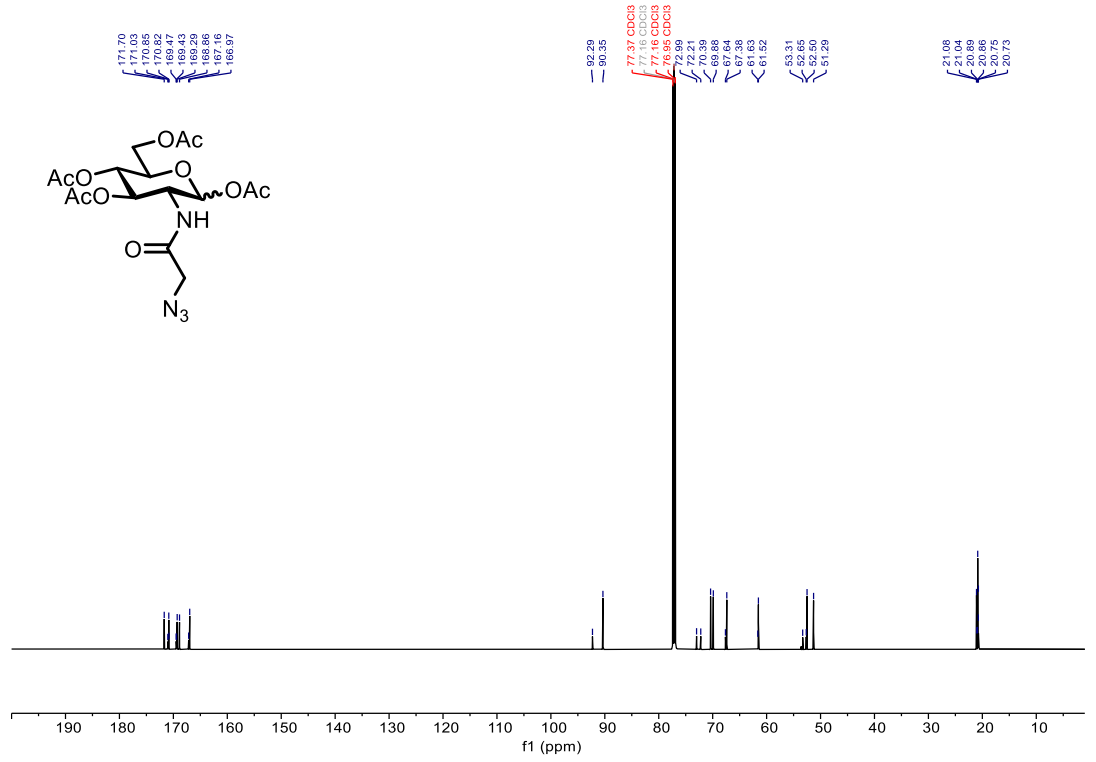
^{31}P NMR (243 MHz, CD_3OD) of *α*-compound 6b



^{1}H NMR (400 MHz, $CDCl_3$) of $Ac_4GlcNAz$



^{13}C NMR (151 MHz, $CDCl_3$) of $Ac_4GlcNAz$



Original MS Data of Protein with High Confidence

Original Data Table of Experimental Group 1： Proteins identified with high confidence by mass spectrometry from Ac_3SATE-GlcNAz-P-treated *S. cerevisiae.*

Checked	Protein FDR Confidence	Master	Accession	Description	Exp. q-value	Sum PEP Score	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI	Area: F1750: Sample	emPAI	Score Mascot	# Peptides Mascot
FALSE	High	Master Protein	P38109	Vacuolar serine-type carboxypeptidase ATG42 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATG42 PE=1 SV=1	0	6.31	4.92126	3	3	3	1	508	57.6	5.31	17079344.79	0.369	74	3
FALSE	High	Master Protein	P33754	Translocation protein SEC66 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SEC66 PE=1 SV=1	0	11.474	16.01942	4	4	4	1	206	24.2	7.24	45665180.42	0.778	83	4
FALSE	High	Master Protein	P37302	Aminopeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=APE3 PE=1 SV=1	0	112.715	27.56052	9	15	9	1	537	60.1	5.31	217540452	1.593	455	9
FALSE	High	Master Protein	P17967	Protein disulfide-isomerase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDI1 PE=1 SV=2	0	134.03	29.50192	14	18	14	1	522	58.2	4.53	115461766.5	2.687	624	14
FALSE	High	Master Protein	P25574	ER membrane protein complex subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=EMC1 PE=1 SV=1	0	58.987	14.86842	10	10	9	1	760	87.1	5.35	33077093.85	0.65	229	10
FALSE	High	Master Protein	P25371	Probable ATP-dependent permease OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ADP1 PE=1 SV=2	0	25.206	6.101049	7	7	7	1	1049	117.2	5.16	34368317.38	0.38	164	7
FALSE	High	Master Protein	P33775	Dolichyl-phosphate-mannose--protein mannosyltransferase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PMT1 PE=1 SV=1	0	24.923	7.955936	6	6	6	1	817	92.6	6.68	28937171.13	0.425	84	6
FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	15.336	8.052434	3	3	3	1	534	60.8	4.83	24772142	0.413	51	3
FALSE	High	Master Protein	P33767	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit WBP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=WBP1 PE=1 SV=1	0	90.98	31.16279	9	12	9	1	430	49.4	5.9	82576635.96	1.721	404	9
FALSE	High	Master Protein	P09232	Cerevisin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRB1 PE=1 SV=1	0	50.144	13.54331	8	10	8	1	635	69.6	6.39	127223831.1	0.812	265	8
FALSE	High	Master Protein	P39007	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=STT3 PE=1 SV=2	0	41.464	9.749304	6	6	6	1	718	81.5	8	37909569.86	0.389	229	6
FALSE	High	Master Protein	P41543	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=OST1 PE=1 SV=1	0	22.458	13.44538	5	5	5	1	476	54	5.12	24371919.92	0.73	91	5
FALSE	High	Master Protein	P27614	Carboxypeptidase S OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=CPS1 PE=1 SV=2	0	114.386	25	11	14	11	1	576	64.6	5.53	60046491.44	1.626	350	11
FALSE	High	Master Protein	P12684	3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=HMG2 PE=1 SV=1	0	35.787	7.177033	6	6	6	1	1045	115.6	7.58	18453982.52	0.274	141	6
FALSE	High	Master Protein	P00729	Carboxypeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRC1 PE=1 SV=1	0	12.298	5.639098	4	6	4	1	532	59.8	4.73	169186710.4	0.492	112	4
FALSE	High	Master Protein	P38616	Protein YGP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGP1 PE=1 SV=2	0	41.206	11.86441	3	4	3	1	354	37.3	5.44	64511600.94	1.31	172	3
FALSE	High	Master Protein	P07267	Saccharopepsin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PEP4 PE=1 SV=1	0	24.266	8.148148	2	2	2	1	405	44.5	4.84	61720777.13	0.274	105	2

FALSE	High	Master Protein	P20107	Vacuolar zinc transporter ZRC1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ZRC1 PE=1 SV=2	0	53.517	18.32579	5	7	5	1	442	48.3	6.35	47759242.54	2.455	164	5
FALSE	High	Master Protein	P38247	Protein SLM4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SLM4 PE=1 SV=1	0	9.185	13.58025	2	2	2	1	162	18.3	5.05	14985387.44	0.778	47	2
FALSE	High	Master Protein	P39923	Nitrogen permease regulator 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=NPR2 PE=1 SV=2	0.007	2.846	2.764228	1	1	1	1	615	69.8	8.53	3218220.313	0.059	28	1
FALSE	High	Master Protein	Q99385	Vacuolar calcium ion transporter OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VCX1 PE=1 SV=1	0	22.49	6.812652	2	2	2	1	411	44.6	5.69	32120288.28	0.425	110	2
FALSE	High	Master Protein	P48016	Periplasmic acid trehalase ATH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATH1 PE=1 SV=1	0	11.843	2.807597	2	2	2	1	1211	136.8	5.43	19178847	0.076	63	2
FALSE	High	Master Protein	P51533	ATP-dependent permease PDR10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDR10 PE=1 SV=1	0	15.659	3.196931	3	3	3	1	1564	176.3	7.93	14713595.35	0.094	61	3
FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	15.336	8.052434	3	3	3	1	534	60.8	4.83	24772142	0.413	51	3
FALSE	High	Master Protein	P23642	Mannan polymerase I complex VAN1 subunit OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VAN1 PE=1 SV=3	0	20.799	6.35514	2	2	2	1	535	61.1	5.4	23350830.47	0.194	100	2
FALSE	High	Master Protein	P36057	Signal recognition particle receptor subunit beta OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SRP102 PE=1 SV=1	0	4.748	6.967213	1	1	1	1	244	27	7.8	11583508.31	0.212	40	1
FALSE	Medium	Master Protein	P40207	Ergosterol biosynthesis protein 29 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG29 PE=1 SV=1	0.013	2.41	3.375527	1	1	1	1	237	27.9	5.44	6398490	0.145	26	1
FALSE	High	Master Protein	P53045	C-4 methylsterol oxidase ERG25 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG25 PE=1 SV=1	0	17.698	13.26861	3	3	3	1	309	36.5	8.1	41867804.08	0.638	41	3
FALSE	High	Master Protein	P53217	Uncharacterized membrane protein YGR026W OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGR026W PE=1 SV=1	0	12.908	14.38849	4	5	4	1	278	33.2	9.89	32672253.6	1.154	92	4
FALSE	High	Master Protein	P53739	Flippase kinase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=FPK1 PE=1 SV=1	0	6.015	2.68757	2	2	2	1	893	100.5	7.97	5826152.758	0.113	27	2
FALSE	Low	Master Protein	P38146	GTP-binding protein YPT10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YPT10 PE=1 SV=2	0.131	0.835	5.527638	1	1	1	1	199	21.8	7.3	10243644.75	0.166	24	1
FALSE	High	Master Protein	Q00246	GTP-binding protein RHO4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RHO4 PE=1 SV=2	0.002	3.558	3.780069	1	1	1	1	291	32.2	6.68	16250551.5	0.179	36	1
FALSE	High	Master Protein	Q12100	Probable serine/threonine-protein kinase RTK1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RTK1 PE=1 SV=1	0	17.845	7.580645	3	3	3	1	620	69.6	8.28	16784706.38	0.241	66	3

Original Data Table of Experimental Group 2： Proteins identified with high confidence by mass spectrometry from Ac_3SATE-GlcNAz-P-treated *S. cerevisiae*.

Checked	Protein FDR Confidence	Master	Accession	Description	Exp. q-value	Sum PEP Score	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI	Area: F1751: Sample	emPAI	Score Mascot	# Peptides Mascot
FALSE	High	Master Protein	P38109	Vacuolar serine-type carboxypeptidase ATG42 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATG42 PE=1 SV=1	0	5.838	3.149606	2	2	2	1	508	57.6	5.31	23196132.44	0.233	62	2
FALSE	High	Master Protein	P33754	Translocation protein SEC66 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SEC66 PE=1 SV=1	0	19.209	30.58252	6	6	6	1	206	24.2	7.24	55727776.29	1.371	85	6
FALSE	High	Master Protein	P37302	Aminopeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=APE3 PE=1 SV=1	0	53.721	17.87709	7	10	7	1	537	60.1	5.31	215988183.7	1.043	246	7
FALSE	High	Master Protein	P17967	Protein disulfide-isomerase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDI1 PE=1 SV=2	0	102.071	28.9272	14	20	14	1	522	58.2	4.53	1135158955.7	2.981	616	14
FALSE	High	Master Protein	P25574	ER membrane protein complex subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=EMC1 PE=1 SV=1	0	40.079	15.13158	10	10	9	1	760	87.1	5.35	31410057.81	0.65	223	10
FALSE	High	Master Protein	P25371	Probable ATP-dependent permease OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ADP1 PE=1 SV=2	0	21.435	6.101049	7	7	7	1	1049	117.2	5.16	39046317.11	0.38	165	7
FALSE	High	Master Protein	P33775	Dolichyl-phosphate-mannose--protein mannosyltransferase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PMT1 PE=1 SV=1	0	27.89	11.50551	8	9	8	1	817	92.6	6.68	32713110.71	0.701	98	8
FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	8.714	3.932584	2	2	2	1	534	60.8	4.83	22638189.31	0.259	44	2
FALSE	High	Master Protein	P33767	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit WBP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=WBP1 PE=1 SV=1	0	68.007	32.32558	10	11	10	1	430	49.4	5.9	84467403.58	2.008	389	10
FALSE	High	Master Protein	P09232	Cerevisin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRB1 PE=1 SV=1	0	31.628	11.65354	7	11	7	1	635	69.6	6.39	130166757.3	0.682	276	7
FALSE	High	Master Protein	P39007	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=STT3 PE=1 SV=2	0	22.598	8.913649	6	6	6	1	718	81.5	8	51525948.05	0.389	167	6
FALSE	High	Master Protein	P41543	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=OST1 PE=1 SV=1	0	19.36	15.33613	6	6	6	1	476	54	5.12	23089842.58	0.931	86	6
FALSE	High	Master Protein	P27614	Carboxypeptidase S OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=CPS1 PE=1 SV=2	0	83.376	26.5625	12	17	12	1	576	64.6	5.53	128225528.9	2.047	314	12
FALSE	High	Master Protein	P12684	3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=HMG2 PE=1 SV=1	0	24.208	9.473684	7	7	3	1	1045	115.6	7.58	11935623.78	0.327	127	7
FALSE	High	Master Protein	P00729	Carboxypeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRC1 PE=1 SV=1	0	10.858	5.639098	4	4	4	1	532	59.8	4.73	191786748.7	0.492	70	4
FALSE	High	Master Protein	P38616	Protein YGP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGP1 PE=1 SV=2	0	26.158	11.86441	3	5	3	1	354	37.3	5.44	61620727.41	1.311	203	3
FALSE	High	Master Protein	P07267	Saccharopepsin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PEP4 PE=1 SV=1	0	15.147	9.62963	3	4	3	1	405	44.5	4.84	62132757.44	0.438	77	3
FALSE	High	Master Protein	P20107	Vacuolar zinc transporter ZRC1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ZRC1 PE=1 SV=2	0	36.366	13.57466	4	5	4	1	442	48.3	6.35	54140468.56	1.424	194	4
FALSE	High	Master Protein	P38247	Protein SLM4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SLM4 PE=1 SV=1	0	11.4	17.90123	3	3	3	1	162	18.3	5.05	13871533.04	1.371	61	3
FALSE	High	Master Protein	P39923	Nitrogen permease regulator 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=NPR2 PE=1 SV=2	0	8.471	5.853659	3	3	3	1	615	69.8	8.53	6476145.667	0.189	52	3

FALSE	High	Master Protein	Q99385	Vacuolar calcium ion transporter OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VCX1 PE=1 SV=1	0	14.993	6.812652	2	2	2	1	411	44.6	5.69	28839759.25	0.425	92	2
FALSE	High	Master Protein	Q06490	Thiamine biosynthesis protein THI22 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=THI22 PE=2 SV=2	0	11.234	5.41958	3	4	1	1	572	63.3	5.88		0.283	85	3
FALSE	High	Master Protein	P48016	Periplasmic acid trehalase ATH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATH1 PE=1 SV=1	0	11.136	2.807597	2	2	2	1	1211	136.8	5.43	17946025.06	0.076	62	2
FALSE	High	Master Protein	P51533	ATP-dependent permease PDR10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDR10 PE=1 SV=1	0	12.669	1.982097	2	2	1	1	1564	176.3	7.93	18655219.66	0.062	69	2
FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	8.714	3.932584	2	2	2	1	534	60.8	4.83	22638189.31	0.259	44	2
FALSE	High	Master Protein	P23642	Mannan polymerase I complex VAN1 subunit OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VAN1 PE=1 SV=3	0	12.221	5.981308	2	2	2	1	535	61.1	5.4	21197048.59	0.194	86	2
FALSE	High	Master Protein	P36057	Signal recognition particle receptor subunit beta OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SRP102 PE=1 SV=1	0.001	3.912	6.967213	1	1	1	1	244	27	7.8	11025238.38	0.212	24	1
FALSE	High	Master Protein	P40207	Ergosterol biosynthesis protein 29 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG29 PE=1 SV=1	0.003	3.28	3.375527	1	1	1	1	237	27.9	5.44	4881203.125	0.145	46	1
FALSE	High	Master Protein	P53045	C-4 methylsterol oxidase ERG25 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG25 PE=1 SV=1	0	10.214	7.119741	2	2	2	1	309	36.5	8.1	48502265.63	0.389	42	2
FALSE	High	Master Protein	P53217	Uncharacterized membrane protein YGR026W OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGR026W PE=1 SV=1	0	11.816	11.51079	4	5	4	1	278	33.2	9.89	40341780.56	1.154	69	4
FALSE	High	Master Protein	P53739	Flippase kinase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=FPK1 PE=1 SV=1	0	10.45	6.159015	4	4	4	1	893	100.5	7.97	5414061.51	0.239	52	4
FALSE	High	Master Protein	P38146	GTP-binding protein YPT10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YPT10 PE=1 SV=2	0.008	2.768	5.527638	1	1	1	1	199	21.8	7.3	10255104.55	0.166	0	1
FALSE	High	Master Protein	Q00246	GTP-binding protein RHO4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RHO4 PE=1 SV=2	0	5.34	7.560137	2	2	2	1	291	32.2	6.68	16341201	0.389	27	2
FALSE	High	Master Protein	Q12675	Phospholipid-transporting ATPase DNF2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=DNF2 PE=1 SV=1	0	9.461	1.861042	3	3	1	1	1612	182.5	6.18	18396366.25	0.085	35	3
FALSE	High	Master Protein	Q12100	Probable serine/threonine-protein kinase RTK1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RTK1 PE=1 SV=1	0	22.003	13.54839	6	6	6	1	620	69.6	8.28	18909185.1	0.54	109	6

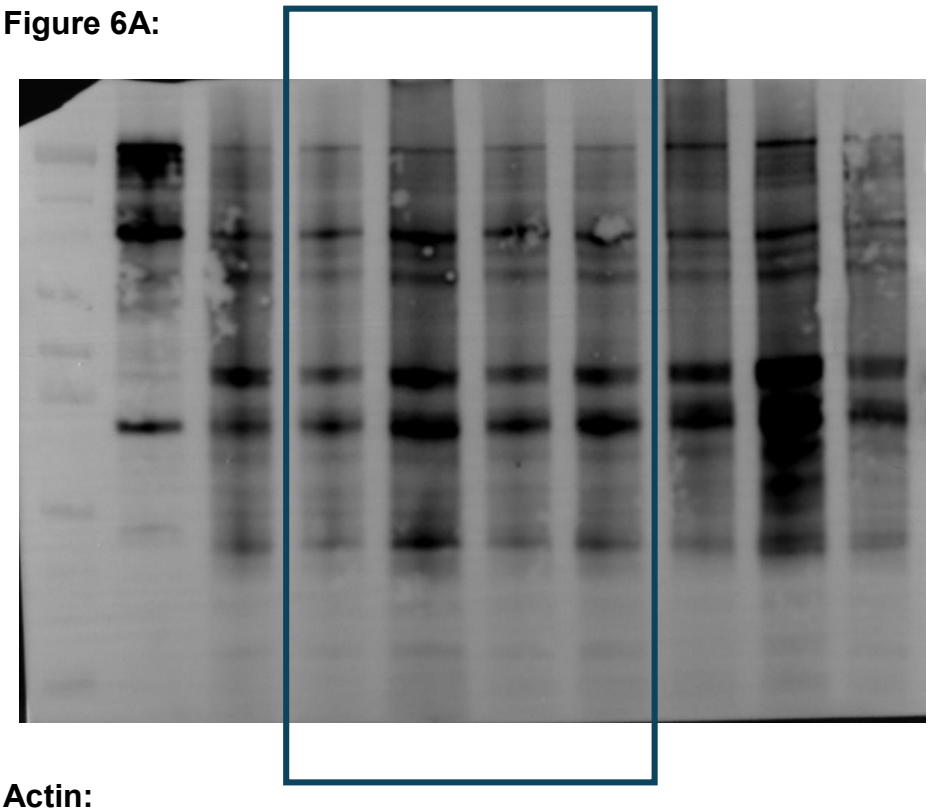
Original Data Table of Experimental Group 3： Proteins identified with high confidence by mass spectrometry from Ac_3SATE-GlcNAz-P-treated *S. cerevisiae*.

Checked	Protein FDR Confidence	Master	Accession	Description	Exp. q-value	Sum PEP Score	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI	Area: F1752: Sample	emPAI	Score Mascot	# Peptides Mascot
FALSE	High	Master Protein	P38109	Vacuolar serine-type carboxypeptidase ATG42 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATG42 PE=1 SV=1	0.005	2.903	3.346457	2	2	2	1	508	57.6	5.31	34483649.5	0.233	35	2
FALSE	High	Master Protein	P33754	Translocation protein SEC66 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SEC66 PE=1 SV=1	0	27.359	33.98058	7	7	7	1	206	24.2	7.24	53491436.42	1.738	109	7
FALSE	High	Master Protein	P37302	Aminopeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=APE3 PE=1 SV=1	0	104.688	30.91248	10	13	10	1	537	60.1	5.31	205201637.2	1.807	413	10
FALSE	High	Master Protein	P17967	Protein disulfide-isomerase OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDI1 PE=1 SV=2	0	114.112	35.44061	15	21	15	1	522	58.2	4.53	115765594.3	2.687	640	15
FALSE	High	Master Protein	P25574	ER membrane protein complex subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=EMC1 PE=1 SV=1	0	30.24	10.78947	8	9	7	1	760	87.1	5.35	30033767.52	0.492	155	8
FALSE	High	Master Protein	P25371	Probable ATP-dependent permease OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ADP1 PE=1 SV=2	0	27.582	6.86368	8	8	8	1	1049	117.2	5.16	34942694.3	0.445	199	8
FALSE	High	Master Protein	P33775	Dolichyl-phosphate-mannose--protein mannosyltransferase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PMT1 PE=1 SV=1	0	30.342	10.28152	7	9	7	1	817	92.6	6.68	32157018.13	0.604	136	7
FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	17.549	6.554307	3	3	3	1	534	60.8	4.83	16136896.08	0.413	98	3
FALSE	High	Master Protein	P33767	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit WBP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=WBP1 PE=1 SV=1	0	91.093	41.39535	14	15	14	1	430	49.4	5.9	89928753.9	3.489	392	14
FALSE	High	Master Protein	P09232	Cerevisin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRB1 PE=1 SV=1	0	35.268	11.65354	7	9	7	1	635	69.6	6.39	120521654.8	0.682	230	7
FALSE	High	Master Protein	P39007	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=STT3 PE=1 SV=2	0	40.051	12.11699	8	9	8	1	718	81.5	8	45540265.49	0.551	255	8
FALSE	High	Master Protein	P41543	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=OST1 PE=1 SV=1	0	17.973	13.02521	4	4	4	1	476	54	5.12	17864804.79	0.551	101	4
FALSE	High	Master Protein	P27614	Carboxypeptidase S OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=CPS1 PE=1 SV=2	0	71.05	23.09028	9	12	9	1	576	64.6	5.53	118652651.4	1.438	240	9
FALSE	High	Master Protein	P12684	3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=HMG2 PE=1 SV=1	0	32.91	10.7177	7	7	4	1	1045	115.6	7.58	13940960.23	0.327	176	7
FALSE	High	Master Protein	P00729	Carboxypeptidase Y OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PRC1 PE=1 SV=1	0	10.698	5.639098	4	5	4	1	532	59.8	4.73	183706798.2	0.492	110	4
FALSE	High	Master Protein	P38616	Protein YGP1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGP1 PE=1 SV=2	0	33.283	11.86441	3	5	3	1	354	37.3	5.44	68528689.46	1.311	230	3
FALSE	High	Master Protein	P07267	Saccharopepsin OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PEP4 PE=1 SV=1	0	22.261	12.34568	3	3	3	1	405	44.5	4.84	43750051.56	0.438	123	3
FALSE	High	Master Protein	P20107	Vacuolar zinc transporter ZRC1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ZRC1 PE=1 SV=2	0	39.106	13.57466	4	5	4	1	442	48.3	6.35	40851121.96	1.424	186	4
FALSE	High	Master Protein	P38247	Protein SLM4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SLM4 PE=1 SV=1	0	11.021	17.90123	3	3	3	1	162	18.3	5.05	14127093.04	1.371	68	3
FALSE	High	Master Protein	P39923	Nitrogen permease regulator 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=NPR2 PE=1 SV=2	0.004	3.072	1.788618	1	1	1	1	615	69.8	8.53	6316143	0.059	0	1
FALSE	High	Master Protein	Q99385	Vacuolar calcium ion transporter OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VCX1 PE=1 SV=1	0	18.379	8.272506	3	3	3	1	411	44.6	5.69	27308538.58	0.425	112	3
FALSE	High	Master Protein	P48016	Periplasmic acid trehalase ATH1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ATH1 PE=1 SV=1	0	16.991	3.715937	3	3	3	1	1211	136.8	5.43	15861365.31	0.116	71	3
FALSE	High	Master Protein	P51533	ATP-dependent permease PDR10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=PDR10 PE=1 SV=1	0	7.193	2.493606	3	3	3	1	1564	176.3	7.93	8945254.708	0.094	54	3

FALSE	High	Master Protein	Q04080	GPI transamidase component GPI17 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=GPI17 PE=1 SV=1	0	17.549	6.554307	3	3	3	1	534	60.8	4.83	16136896.08	0.413	98	3
FALSE	High	Master Protein	P23642	Mannan polymerase I complex VAN1 subunit OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=VAN1 PE=1 SV=3	0	17.659	8.411215	3	3	3	1	535	61.1	5.4	15502468.71	0.304	94	3
FALSE	High	Master Protein	P36057	Signal recognition particle receptor subunit beta OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=SRP102 PE=1 SV=1	0	18.154	30.7377	3	3	3	1	244	27	7.8	10869950.81	0.778	102	3
FALSE	High	Master Protein	P40207	Ergosterol biosynthesis protein 29 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG29 PE=1 SV=1	0	11.352	12.23629	3	3	3	1	237	27.9	5.44	12438931.63	0.501	85	3
FALSE	High	Master Protein	P53045	C-4 methylsterol oxidase ERG25 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=ERG25 PE=1 SV=1	0	9.992	7.119741	2	2	2	1	309	36.5	8.1	48034773.13	0.389	50	2
FALSE	High	Master Protein	P53217	Uncharacterized membrane protein YGR026W OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YGR026W PE=1 SV=1	0	6.25	9.352518	3	3	3	1	278	33.2	9.89	38376554.23	0.778	50	3
FALSE	High	Master Protein	P53739	Flippase kinase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=FPK1 PE=1 SV=1	0	6.735	3.919373	3	3	3	1	893	100.5	7.97	6134342.344	0.174	37	3
FALSE	High	Master Protein	P38146	GTP-binding protein YPT10 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=YPT10 PE=1 SV=2	0	5.402	16.58291	3	3	3	1	199	21.8	7.3	7630714.813	0.585	24	3
FALSE	High	Master Protein	Q00246	GTP-binding protein RHO4 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RHO4 PE=1 SV=2	0	11.006	13.7457	3	3	3	1	291	32.2	6.68	50701334.75	0.638	64	3
FALSE	High	Master Protein	Q12100	Probable serine/threonine-protein kinase RTK1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=RTK1 PE=1 SV=1	0	26.609	10.80645	4	4	4	1	620	69.6	8.28	19120708.46	0.334	75	4

Raw Data for the Western Blot Experiments

Figure 6A:



Actin:

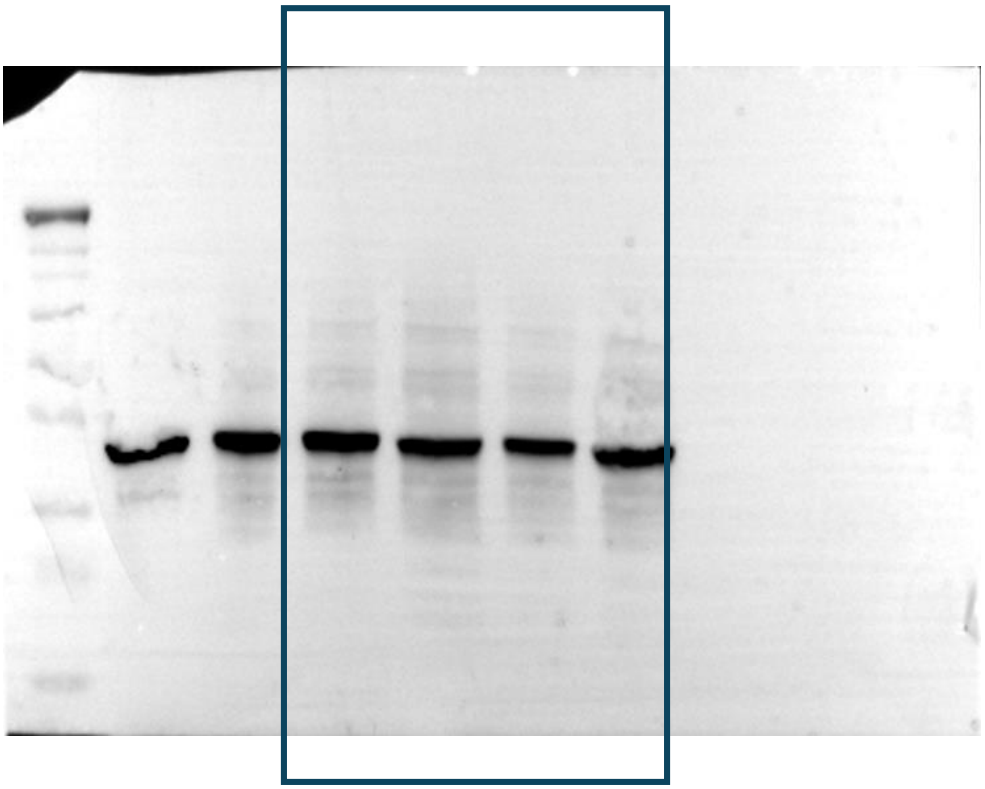


Figure 6D:

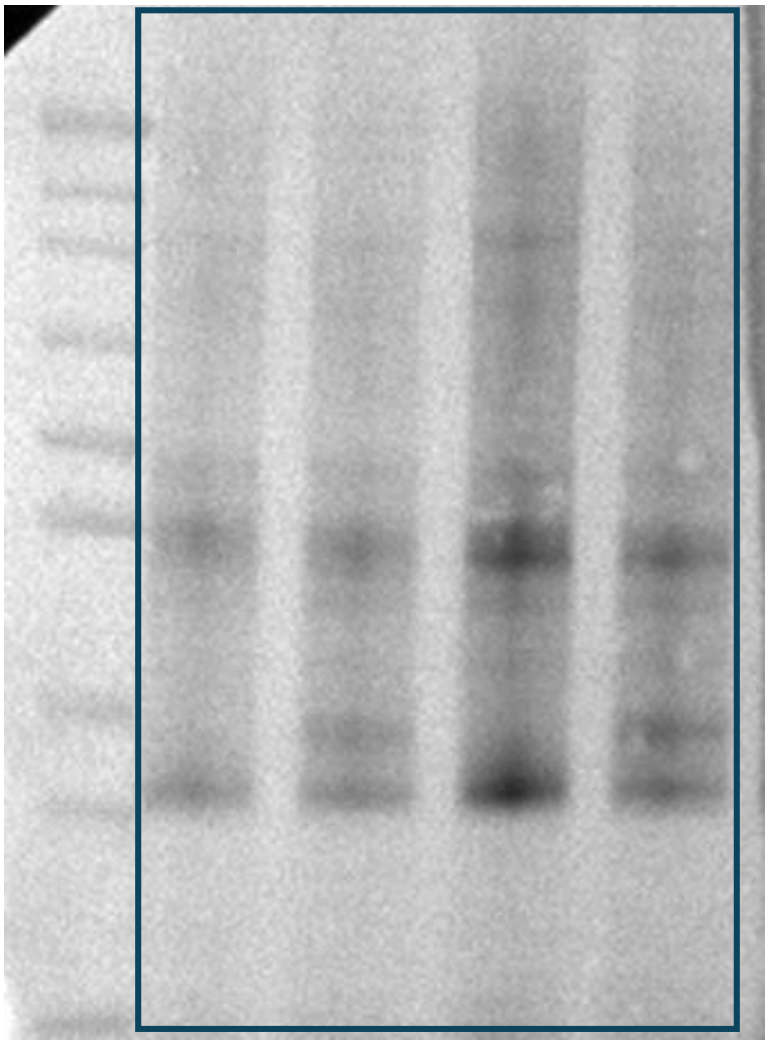
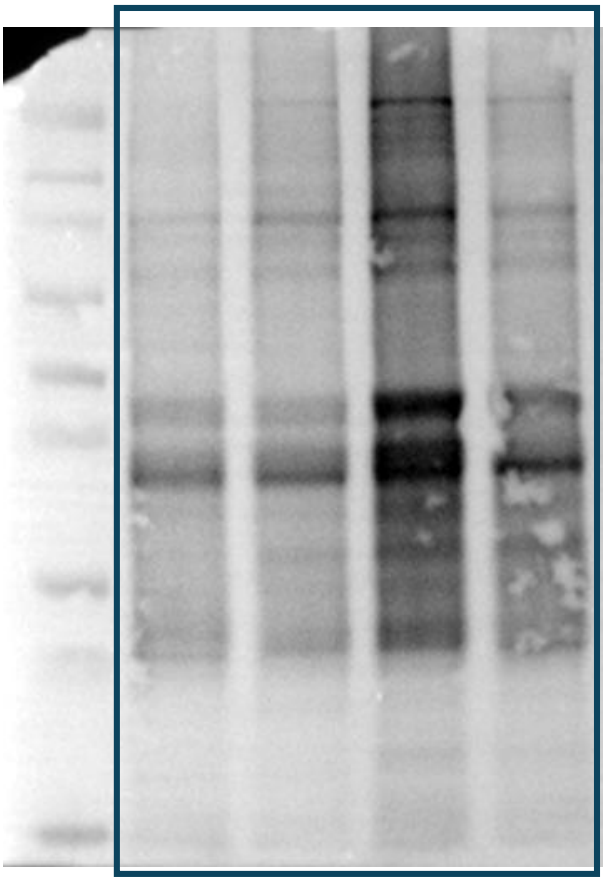


Figure S4A:



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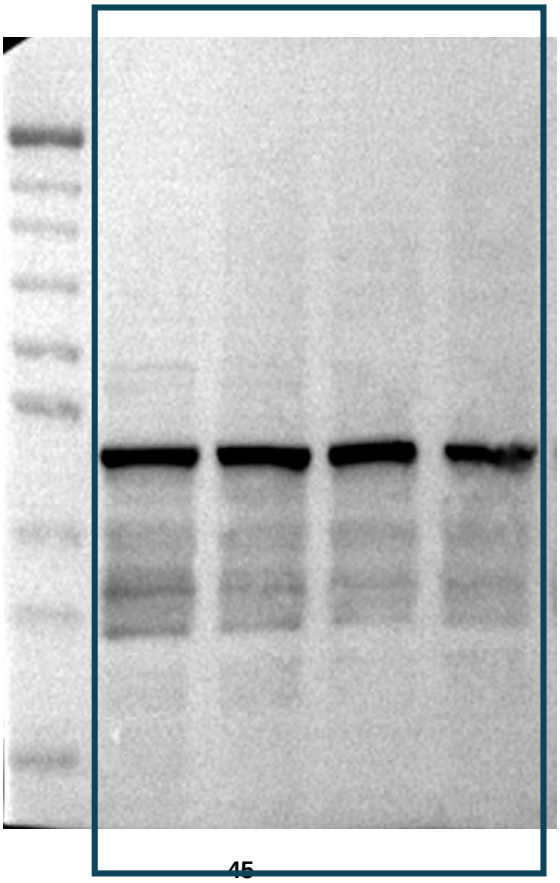
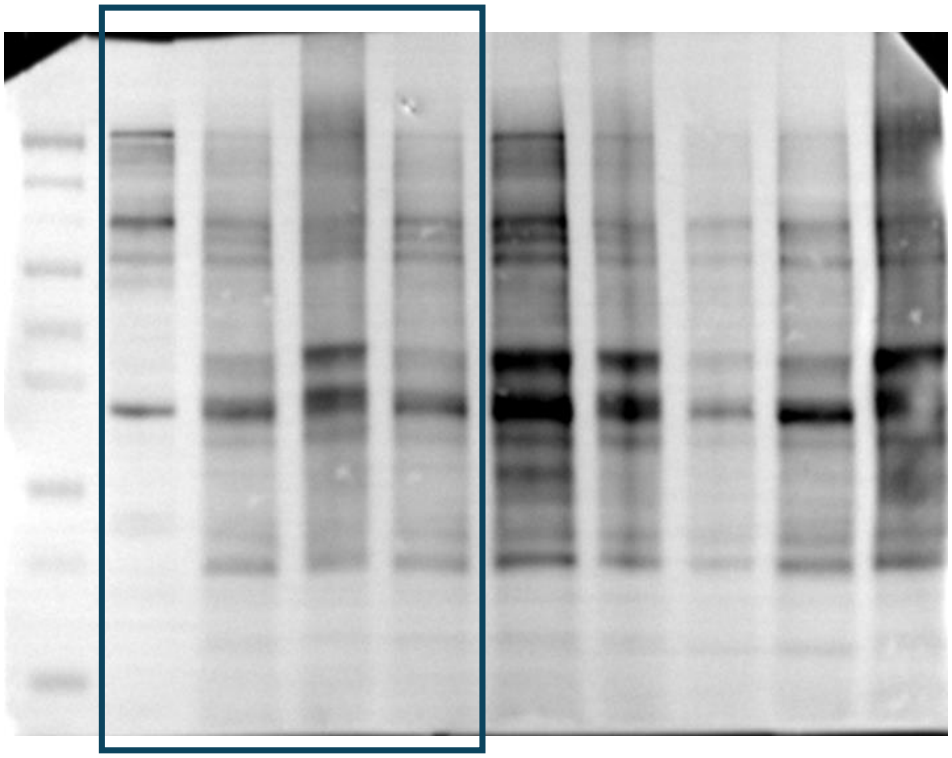


Figure S4B:



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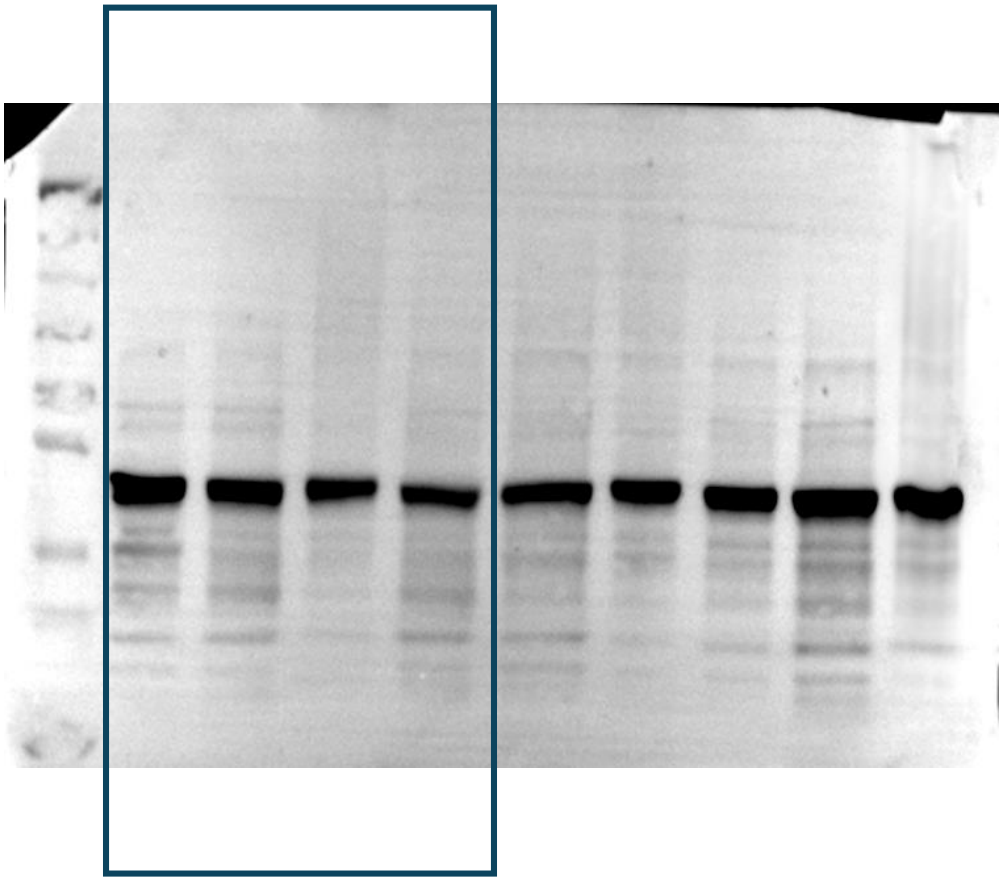

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