Electronic Supplementary Material (ESI) for ChemComm.

Co-factor prosthesis facilitates biosynthesis of azido-pseudaminic acid probes for use as glycosyltransferase reporters

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Supporting information

1. General experimental

All chemicals were purchased from Acros Organics, Alfa Aesar, Biosynth Carbosynth, Fisher Scientific, Fluorochem, Sigma Aldrich, VWR or TCI Chemicals and were used without further purification unless otherwise stated. NMR spectra were recorded on a Bruker Avance 400 spectrometer, JEOL ECX-400 spectrometer or a Bruker Neo 700MHz spectrometer. The chemical shift data for each signal are given as δ in units of parts per million (ppm) relative to tetramethylsilane, where δ = 0.00 ppm. The number of protons (n) for a given resonance is indicated by nH. The multiplicity of each signal is indicated by s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), p (pentet), sep (septet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dddd (doublet of doublet of doublet of doublets), dt (doublet of triplets), tt (triplet of triplets), dqd (doublet of quartets of doublets) or m (multiplet). Resonances were assigned using HH-COSY and CH-HSQC. All NMR chemical shifts (δ) were recorded in ppm and coupling constants (J) are reported in Hz. Topspin 4.0.6 and MestReNovax64 were primarily used for processing the spectral data. Thin layer chromatography was carried out on Merck silica gel 60 F254 precoated aluminium foil sheets and these were visualized using UV light (254 nm) and/or PPh3 (10% in DCM) and/or ninhydrin (1.5% ninhydrin, 3% AcOH in n-butanol), and/or H2SO4 (5% H2SO4 in MeOH). Unless otherwise indicated, flash column chromatography was performed on Supelco® silica gel (particle size 35–75 µm, pore diameter 60 Å, 220-440 mesh) and the solvent system used is recorded in parentheses.

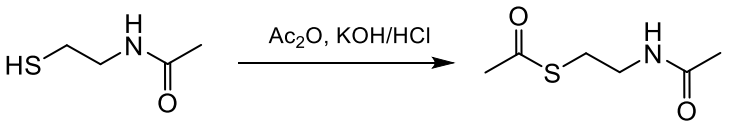
2. Mass spectrometry

Small-molecule high resolution mass spectrometry (HRMS) data were obtained at RT on a Bruker Daltonics microTOF mass spectrometer coupled to an Agilent 1200 series LC system at The University York Centre of Excellence in Mass Spectrometry (CoEMS). Nominal and exact *m/z* values are reported in Daltons.

High Performance Liquid Chromatography-Electrospray Ionisation Mass Spectrometry (ESI-LC-MS) was accomplished using a Dionex UltiMate® 3000 LC system (ThermoScientific) equipped with an UltiMate® 3000 Diode Array Detector (probing 250-400 nm) in line with a Bruker HCTultra ETD II system (Bruker Daltonics), using Chromeleon® 6.80 SR12 software (ThermoScientific), Compass 1.3 for esquire HCT Build 581.3, esquireControl version 6.2, Build 62.24 software (Bruker Daltonics), and Bruker compass HyStar 3.2-SR2, HyStar version 3.2, Build 44 software (Bruker Daltonics) at The University York Centre of Excellence in Mass Spectrometry (CoEMS). All mass spectrometry was conducted in negative ion mode. Data analysis was performed using ESI Compass 1.3 DataAnalysis, Version 4.1 software (Bruker Daltonics). For HILIC-LC-MS, samples analysed on an Accucore HILIC column (2.6 um particle size, 50 x 2.1 mm). Water, 0.1% formic acid by volume (solvent A), and acetonitrile, 0.1 % formic acid (solvent B) were used as the mobile phase at a flow rate of 0.3 ml/min at room temperature. A multi-step gradient of 15 min was programmed as follows: 95 % B for 1 min, followed by a linear gradient to 5 % B over 9.5 min, followed by 5 % B for an additional 0.5 min. Then a linear gradient to 95 % B over 1 min, followed by holding at 95 % B for 3 min.

3. Chemical synthesis

3.1 N,S-diacetylcysteamine (**9**, SNAc)



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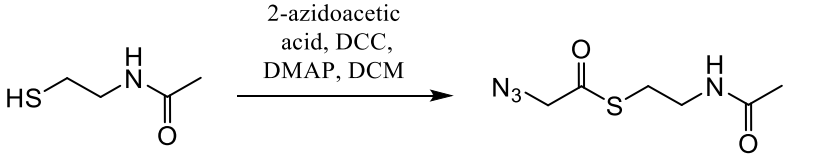
The synthesis of SNAc was adapted from the method previously reported by Wang et al.[1] Briefly, cysteamine HCl (1 g, 8.8 mmol, 1 eq.) was dissolved in 8 mL of water. The pH was adjusted to 8 on ice with 4M KOH. Acetic anhydride (2.5 mL, 24.5 mmol, 3 eq) was added dropwise, while maintaining the pH at 8 by the careful addition of 4M KOH simultaneously on ice. Following acetic anhydride addition, 1M HCl was added to adjust the pH to 7 while on ice. The reaction mixture stirred at 0 °C for 2 h. Saturated NaCl was added and the product was extracted with DCM. The organic layer dried with $MgSO_4$ and condensed in vacuo to yield a white crystal, N,S-diacetylcysteamine (**9,** 1.12 g, 85%) without further purification. HRMS (ESI-pos) for $C_6H_{11}NO_2S^+$ $[M+H]^+$: calculated 162.0589, observed 162.0582. ν_{max} cm^{-1} : 2990 (br, NH), 1691 (vs, C=O), 1132 (s, CN). ^{1}H NMR (400 MHz, $CDCl_3$): 6.02 (s, 1H, NH), 3.43 (q, J= 8 Hz, 2H, CH_2NH), 3.01 (t, J = 8 Hz, 2H, CH_2S) 2.35 (s, 3H, $NCOCH_3$), 1.96 (s, 3H, SCOH3). ^{13}C NMR (400 MHz, $CDCl_3$): 195.88 (SCO), 170.07 (NHCO), 39.09 ($NHCH_2$), 30.20 ($SCOCH_3$), 28.31(SCH_2), 22.73 ($HNCOCH_3$).

3.2 N-acetyl-S-azidocysteamine (**10**, SNAc-N_3)



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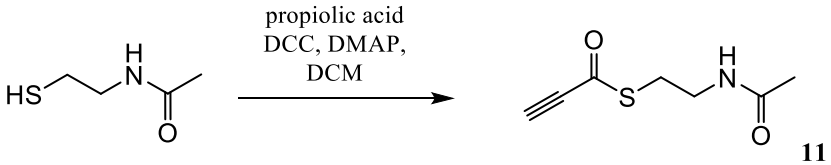
To a stirred solution of *N*-acetylcysteamine (101 mg, 0.84 mmol) and azidoacetic acid (78 mg, 0.77 mmol) in DCM (5 mL) at 0 °C was added DMAP (93 mg, 0.76 mmol). DCC (173 mg, 0.84 mmol) was then added, the resultant solution was stirred at rt overnight. DCU precipitate was then removed via filtration. The filtrate was concentrated *in vacuo*, and the resultant crude residue was redissolved in ethyl acetate, which resulted in the precipitation of additional DCU, which were removed by filtration. Concentration of the filtrate *in vacuo* yielded a crude residue which was purified via flash silica column chromatography (25 → 100% ethyl acetate in hexanes) to yield N-acetyl-S-azidocysteamine **10** as a colourless oil (113 mg, 74%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 5.91 (br s, 1H), 4.03 (s, 2H), 3.50-3.40 (m, 2H), 3.40 (s, 1H), 3.15 (t, *J* = 6.40 Hz, 1H), 1.98 (s, 3H). **^{13}C-NMR** (101 MHz, $CDCl_3$): δ_c 196.0, 170.6, 58.2, 39.3, 28.8, 24.8, 23.3. **FT-IR (ATR)** (υmax/cm^{-1}): 3265 (C-H stretch), 2101 (N-N-N stretch (strong intensity), azide), 1648 (C=O stretch). **(ESI)HRMS**: Found 225.0414, $C_6H_{10}N_4NaO_2S^+$ requires 225.0417.

3.3 N-acetyl-S-propionylcysteamine (**11**, SNAc-Alk)



11

To a stirred solution of *N*-acetylcysteamine (126 mg, 1.05 mmol) and propiolic acid (67 mg, 0.96 mmol) in DCM (5 mL) at 0 °C was added DMAP (12 mg, 0.10 mmol). DCC (218 mg, 1.06 mmol) was then added, the resultant solution was stirred at rt overnight. Hexane (4 mL) was added to the reaction mixture, and the DCU precipitate was removed via filtration. The filtrate was concentrated *in vacuo*, and the resultant crude residue was further purified via flash silica column chromatography (40% ethyl acetate in hexanes) to yield a yellow oil. Residual remnants of DCU were precipitated by dissolving the yellow oil in acetonitrile, and were removed by filtration. Concentration of the filtrate *in vacuo* yielded N-acetyl-S-propionylcysteamine **11** as a yellow oil (63 mg, 40%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 5.89 (br s, 1H), 3.52-3.43 (m, 2H), 3.40 (s, 1H), 3.15 (t, *J* = 6.33 Hz, 2H), 1.98 (s, 3H). **^{13}C-NMR** (101 MHz, $CDCl_3$): δ_c 176.2, 170.6, 80.4, 79.3, 39.2, 29.6, 23.3. **FT-IR (ATR)** (υmax/cm^{-1}): 3270 (C-H stretch), 2100 (C≡C stretch (medium intensity)), 1636 (C=O stretch). **(ESI)HRMS**: Found 194.0243, $C_7H_9NNaO_2S^+$ requires 194.0246.

4. Expression of PseB-I enzymes

PseB (WP_002869093.1) pET-30a and PseC (WP_002856503.1) pFO4 recombinant plasmids[2] were introduced into chemically competent *E. coli* BL21(DE3) cells by heatshock. pET-15b vectors containing PseH (WP_002781802.1), PseG (WP_002830499.1) or PseI (WP_002870258.1) were purchased from GenScript (restriction enzymes NdeI and BamHI) and introduced into chemically competent *E. coli* BL21(DE3) cells. Expression conditions for each enzyme are detailed in Table. S1. Starter cultures were prepared by picking a single colony into 50 mL of LB + antibiotics, and grown at 37 °C with shaking (180 rpm, 16 h). The starter cultures were diluted 1/100 into 1 L of fresh LB + antibiotics, and grown until an OD_{600} of 0.6 was reached. IPTG was added to induce recombinant protein expression and the cells were grown for 4 – 20 h, as detailed in Table. S1. Cells were harvested by centrifugation (6000 ×*g*, 20 min, 4 °C) and the pellets stored at -80 °C. For protein isolation, cell pellets were thawed on ice and suspended in ice cold lysis buffer (50 mM HEPES buffer pH 7.5, 0.15 M NaCl, protease inhibitor, benzonase). Cells were lysed on ice by sonication (30s on/30s off for 12 min) and the lysate was centrifuged (20000 x g, 50 min, 4 °C). The supernatant was purified using a HisTrap HP Ni^{2+} affinity column, pre-equilibrated with binding buffer (50 mM HEPES buffer pH 7.5, 0.15 M NaCl, 20 mM Imidazole). Protein was eluted using elution buffer (50 mM HEPES buffer pH 7.5, 0.15 M NaCl, 500 mM Imidazole). Fractions containing the desired protein were pooled and desalted using a HiPrep™ 26/10 Desalting column (GE Healthcare) in desalting buffer (50 mM HEPES buffer pH 7.5, 0.15 M NaCl).

Table. S1 Details for the protein production of PseBCHGIF enzymes

	Selection antibiotic	IPTG concentration (mM)	Post-induction temperature (°C)	Post-induction incubation time (h)	Expected molecular mass (kDa)
PseB	Kanamycin	0.1	16	20	37.4
PseC	Ampicillin	0.1	37	4	42.3
PseG	Ampicillin	0.1	16	20	18.7
PseH	Ampicillin	0.5	16	20	31.3
PseI	Ampicillin	0.5	37	20	38.6
PseF	Kanamycin	0.1	16	20	28

5. Screening SNAc as an alternative PseH co-factor

Reactions were assembled by adding UDP-GlcNAc (0.5 µl of 200 mM stock), L-glutamic acid (L-Glu, 5 µl of 200 mM stock), pyridoxal 5 phosphate (PLP, 4 µl of 50 mM stock), PseB (5 µM), PseC (20 µM) and HEPES buffer pH 7.4 (5 µl of 500 mM stock) to a 0.5 mL Eppendorf tube and incubated at 37 °C for 4 h. PseH (20 µM), SNAc (9, 5 mM) and Co-A (0.3 mM) were then added, bringing the reaction volume to 50 µL. A control reaction was assembled alongside, containing all reaction components except for SNAc 9. The samples were incubated at 37 °C for 4 h, and then diluted 1/3 in MeCN and analysed by HILIC ESI-LC-MS (Figure S1).

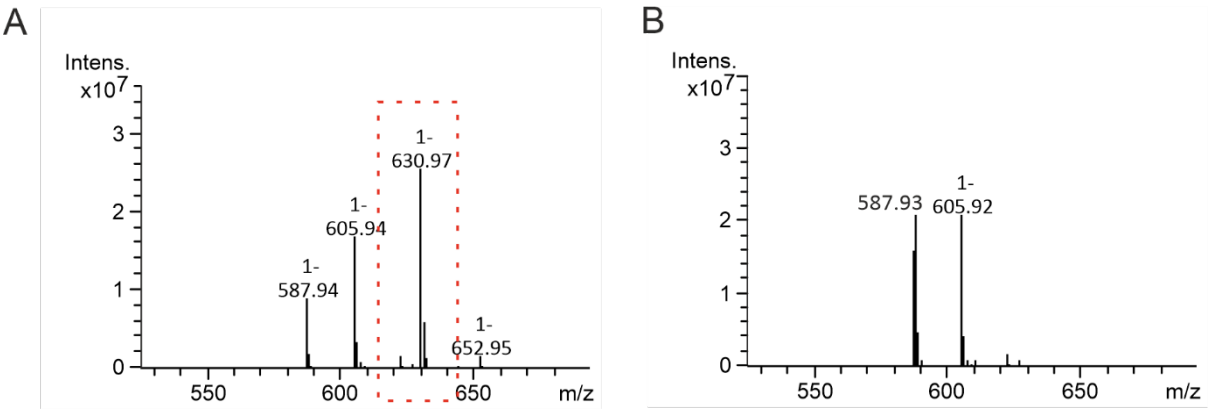


Figure S1. Mass spectrum showing the formation of the PseH product ($[M-H]^-$ = *m/z* 631) in reactions containing PseH, SNAc 9 and Co-A (A), and the absence of product formation in the no SNAc control reaction (B).

6. Screening for activity towards functionalized SNAcs

Reactions were assembled by adding UDP-GlcNAc (0.5 µl of 200 mM stock), L-glutamic acid (L-Glu, 5 µl of 200 mM stock), pyridoxal 5 phosphate (PLP, 4 µl of 50 mM stock), coenzyme A (coA, 15 µl of 1 mM stock), phosphoenolpyruvate (PEP, 0.5 µl of 300 mM stock), PseB (6.25 µl of 1.6 mg/mL stock), PseC (12.5 µl of 1.6 mg/mL stock) and HEPES buffer pH 7.4 (5 µl of 500 mM stock). The reactions were incubated at 37 °C for 4 h. Then SNAc-N_3 **10** or SNAc-Alk **11** (1 µl of a 1M stock in DMSO), PseH (3.8 µl of 2.6 mg/mL stock), PseG (6.25 µl of 1.6 mg/mL stock) and PseI (2.5 µl of 4 mg/mL stock) were added to the reactions and further incubated at 30 °C for 16 h. Alongside the reactions, no enzyme and no SNAc-N_3/SNAc-Alk controls were assembled. Reactions were then diluted with 1 eq. volume of MeCN, centrifuged at 10 000 x g for 1 min and the supernatant analysed by negative HILIC ESI-LC-MS (see section 2). The LC-MS results are shown in Figure S2.

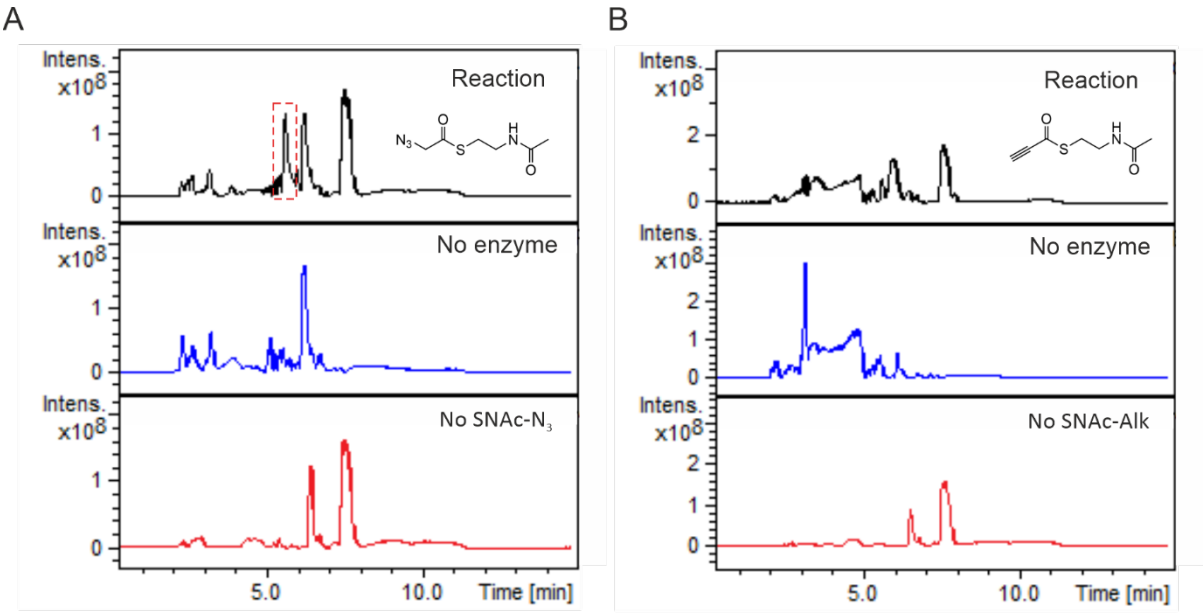
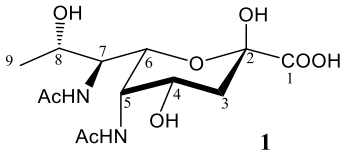


Figure S2. Screening of SNAc-N_3 **10** (A) and SNAc-Alk **11** (B) for the biosynthesis of C7 functionalised pseudaminic acid analogues. Shown are the base peak chromatograms (BPCs) for the reactions (black), no enzyme controls (blue) and no SNAc controls (red) respectively, following analysis by HILIC LC-MS. The peak indicative of Pse5Ac7N_3 **12** formation is highlighted in the red box (A).

7. Chemoenzymatic synthesis on semi-preparative scale.

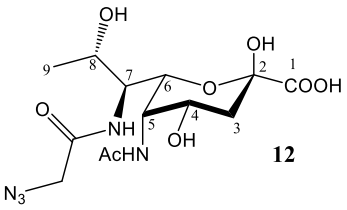
7.1 Chemoenzymatic synthesis of Pse5Ac7Ac **1**



1

Reactions were assembled by adding UDP-GlcNAc **2** (2 mM, 65.4 mg), L-glutamic acid (20 mM, 147 mg), pyridoxal 5'-phosphate (4 mM, 49.4 mg), SNAc **9** (20 mM, 121 mg), coenzyme A (300 µM, 11.51 mg), phosphoenolpyruvate (3 mM, 25.2 mg), 5 mL of 500 mM HEPES buffer pH 7.5 (50 mM final concentration) and H_2O to a final volume of 30.15 mL in a 50 mL falcon tube. PseB (0.2 mg. mL^{-1}), PseC (0.4 mg. mL^{-1}), PseH (0.2 mg. mL^{-1}), PseG (0.2 mg. mL^{-1}) and PseI (0.2 mg. mL^{-1}) were then added, making the final reaction volume up to 50 mL. After gently mixing, the reaction was divided into 1 mL aliquots and incubated at 30 °C for 18 h. The aliquots were then pooled back together and the enzymes were removed by ultrafiltration (Vivaspin Protein Concentrator Spin Column 10000 MWCO). The reaction was then lyophilised, split into four batches and each was purified by BioGel P2 column chromatography in HPLC-grade H_2O. Fractions containing the desired product **1**, were then pooled and lyophilised (27 mg, 76%). Appearance: yellow solid. Characterisation was in agreement with previously published spectra.[3] ^{1}H NMR (700 MHz, D_2O): δ = 1.10 (d, *J* = 6.5, 3H, H9), 1.78 (t, *J* = 12.8 Hz, 1H, H3-axial), 1.94 – 1.90 (m, 1H, H3-equatorial), 1.97 (s, 3H, NHAc), 2.01 (s, 3H, NHAc), 4.02 (dd, *J* = 10.3, 1.9 Hz, 1H, H6), 4.20 – 4.09 (m, 3H, H4, H7, H8), 4.24 (t, *J* = 2.8 Hz, 1H, H5). HRMS (ESI-neg) for $C_{13}H_{21}N_2O_8$ $[M-H]^-$: calculated 333.1303, observed 333.1292.

7.2 Chemoenzymatic synthesis of Pse5Ac7N_3 **12**



12

Reactions were assembled by adding UDP-GlcNAc **2** (4 mM, 21 mg), L-glutamic acid (40 mM, 47.3 mg), pyridoxal 5'-phosphate (8 mM, 15.9 mg), HEPES buffer pH 7.5 (to a final concentration of 50 mM), PseB (10 mg) and PseC (20 mg) in a final reaction volume of 8.05 mL (made up in HPLC-grade H_2O). The reaction was divided into 0.5 mL aliquots and incubated at 30 °C for 20 h. The aliquots were then pooled back together and a 5 µL sample of the reaction was added to 20 µL of MeCN, and analysed by negative ESI-LC-MS to validate PseBC product 3 formation ($[M-H]^-$ m/z 589). The protein was removed by using a Vivaspin 20 spin concentrator (Cytivia, MWCO 30000) and the sample was lyophilised. The sample was then purified by using Biogel P2 column chromatography in HPLC-grade H_2O. All fractions containing PseBC product **3** as shown by negative ESI-LC-MS (Figure S3) were pooled and lyophilized. For the biosynthesis of Pse5Ac7N_3 **12**, reactions were assembled by adding semi-crude PseBC product

3 (16 mg), SNAc-N_3 **10** (20 mM, 54.9 mg), coenzyme A (300 µM, 3.2 mg), phosphoenolpyruvate (3 mM, 8.4 mg), PseG (2.7 mg), PseH (2.9 mg), PseI (2.7 mg), HEPES buffer pH 7.5 (to a final concentration of 50 mM) in a final reaction volume of 13.6 mL (made up in HPLC-grade H_2O). The reaction was divided into 0.75 mL aliquots and incubated at 30 °C for 20 h. The aliquots were then pooled back together and more PseG (1.7 mg), PseH (1.6 mg), PseI (2 mg) were added. Following another incubation at 30 °C for 20 h, 1 eq. volume of ice-cold EtOH was added and the sample was incubated at -20 °C for 30 min to precipitate the enzyme. The sample was centrifuged at 4000 x g for 5 min, and the supernatant retained and lyophilized. The sample was purified by using Biogel P2 column chromatography in HPLC-grade H_2O. Fractions containing the desired product **12** were pooled. The purest fractions containing the desired product Pse5Ac7N_3 **12** (3.9 mg, 33%) were pooled and lyophilized.

^{1}H NMR (700 MHz, D_2O) δ 4.25 (m, 1H, H5), 4.21 (dd, *J* = 10.5, 3.5 Hz, 1H, H7), 4.20 – 4.12 (m, 2H, H4, H8), 4.10 (dd, *J* = 10.5, 1.8 Hz, 1H, H6), 4.04 – 3.94 (m, 2H, Az-CH_2), 2.01 (s, 3H, NHCOCH_3), 1.96 – 1.90 (m, 1H, H3-eq.), 1.78 (t, *J* = 12.8 Hz, 1H, H3-ax,), 1.11 (d, *J* = 6.6 Hz, 3H, CH_3). ^{13}C NMR (176 MHz, D_2O) δ 176.34 (C=O), 174.71 (C=O), 170.24 (C=O), 96.51 (C2), 69.84 (C6), 66.77 & 65.14 (C4, C8), 53.11 (C7), 51.90 (Az-CH_2), 48.73 (C5), 34.78 (C3), 21.88 (NHCOCH_3), 15.30 (C9, CH_3). HRMS (ESI-neg) for $C_{13}H_{20}N_5O_8$ $[M-H]^-$: calculated 374.1317, observed 374.1300.

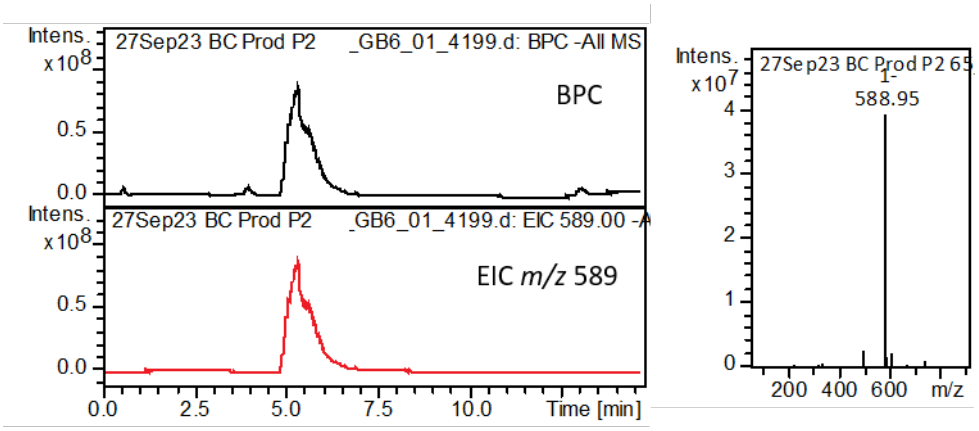
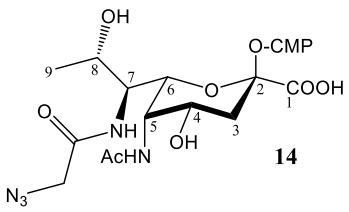


Figure S3. Negative ESI-LC-MS spectrum of PseBC product **3** following purification by Biogel P2 column chromatography. The BPC (black) and the extracted ion chromatogram (EIC) for PseBC product $[M-H]^-$ *m/z* 589 (red) are shown in the left-hand panel. The average spectrum from 0 -15 min is shown in the right-hand panel.

7.3 Chemoenzymatic synthesis of CMP-Pse5Ac7N_3 **14**



14

Reactions were assembled by adding Pse5Ac7N$_3$ **12** (2 mg), cytidine triphosphate (CTP, 4 mM, 10.5 mg in 210 µL of 500 mM sodium phosphate buffer pH 7.4), $MgCl_2$ (20 mM, 1050 µl of a 100 mM stock solution in H_2O), sodium phosphate buffer pH 7.4 (50 mM, 525 µl of a 500 mM stock solution) and PseF (1 mg/mL, 872 µL of a 6 mg/mL stock solution) to H_2O in a final reaction volume of 2488 µL. After gently mixing, the reaction was split into 200 µL aliquots and incubated at 37 °C for 18 h. The aliquots were then pooled back together, 5 mL of ice cold EtOH was added and the sample was incubated at -20 °C for 30 min to precipitate the enzyme. The sample was centrifuged at 4000 x g for 5 min, and the supernatant retained and lyophilized. The sample was purified by using Biogel P2 column chromatography in HPLC-grade H_2O. Fractions containing the desired product CMP-Pse5Ac7N$_3$ **14** were pooled and lyophilized. Analysis of the product by negative ESI-LC-MS suggested the presence of a cytidine diphosphate (CDP) impurity (Figure S4). To remove the cytidine diphosphate (CDP) impurity, the CMP-Pse5Ac7N$_3$ 14 sample was suspended in 870 µL of H_2O and 100 µL Fast AP buffer, and 30 µL of Fast AP Thermosensitive alkaline phosphatase (Thermo Fisher Scientific, 1U/µL) were added. Following an incubation at 37 °C for 30 min, 1 mL of EtOH was added to precipitate the enzyme and the sample was incubated at -20 °C for 30 min. The sample was then centrifuged at 12 000 x g for 5 min, and the supernatant was retained and lyophilised. The sample was then purified again by using Biogel P2 column chromatography in HPLC-grade H_2O. Fractions containing the desired product CMP-Pse5Ac7N$_3$ **14** were pooled and lyophilized. NMR characterization was performed pre- and post-AP treatment (Figure S5-S6a). Additionally, HRMS was performed post-AP treatment (Figure S6b).

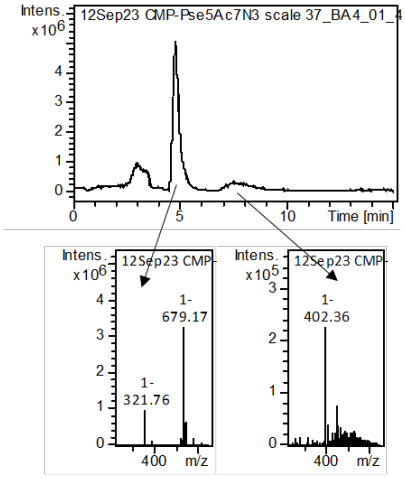


Figure S4. Negative ESI-LC-MS analysis of pooled CMP-Pse5Ac7N$_3$ **14** ([M-H]$^-$ *m/z* 679) fractions show that a CDP ([M-H]$^-$ *m/z* 402) impurity is present.

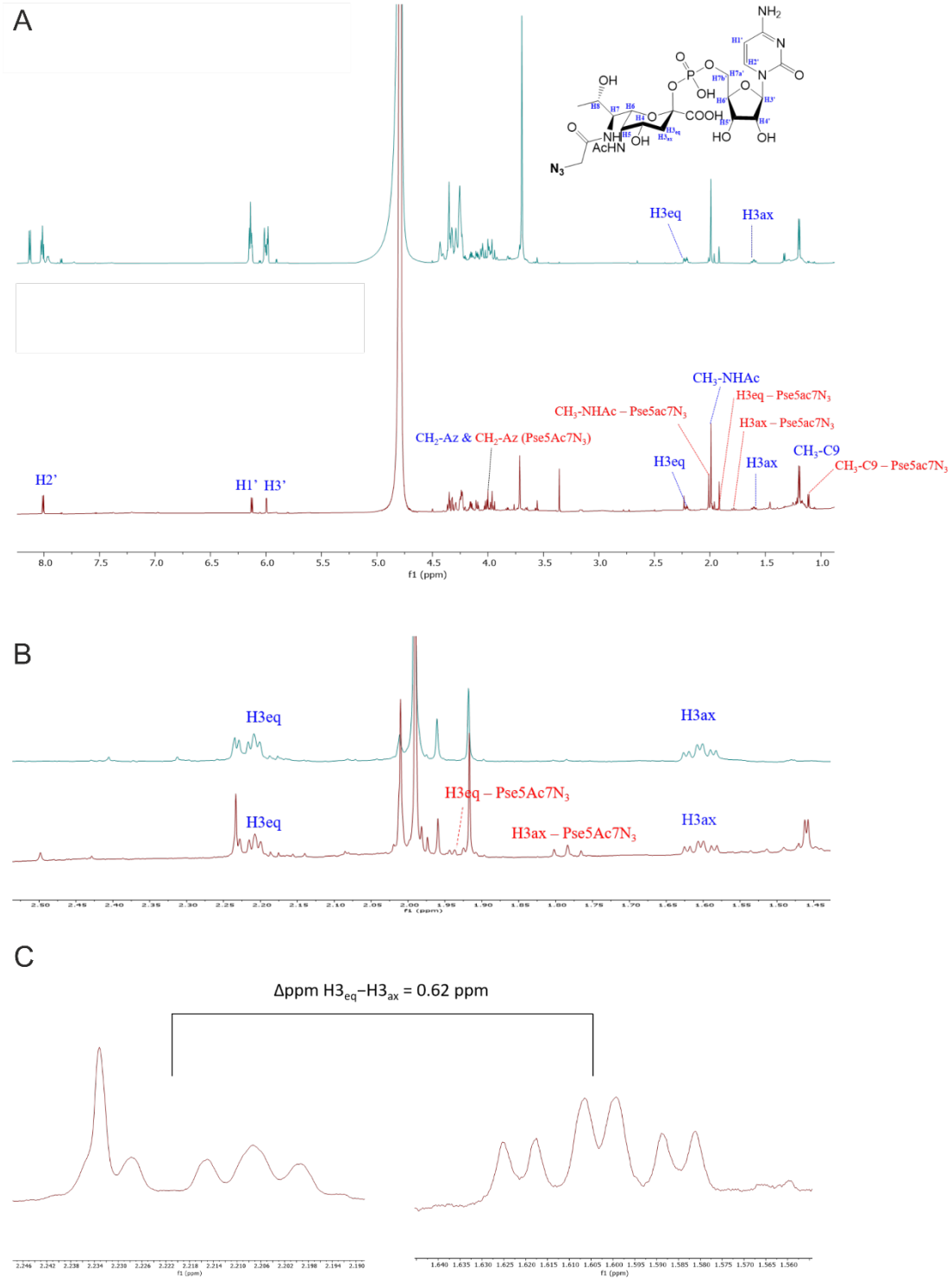


Figure S5. A. Overlayed ^{1}H NMR spectra of Biogel P2-purified CMP-Pse5Ac7N$_3$ **14** both pre-(turquoise) and post-AP (red) treatment. Peak assignment was aided by ^{1}H-^{1}H COSY (Figure S6a). Extra signals consistent with CDP impurity are absent post-AP treatment. Extra signals, particularly apparent in the 2.0 – 1.0 ppm region, are suggestive of some donor hydrolysis to Pse5Ac7N$_3$. This is further validated by HRMS (Figure S6b) B. Enlarged overlayed spectrum in the 2.50 – 1.45 ppm region, highlighting the H3ax and H3eq peaks that are diagnostic of Pse5Ac7N$_3$ formed by donor hydrolysis. C. The chemical shift of the H3$_{eq}$ (2.22 ppm) and H3$_{ax}$ (1.60 ppm) proton NMR peaks used to assign the PseF enzymatic product as αCMP-Pse5Ac7N$_3$ **14**.

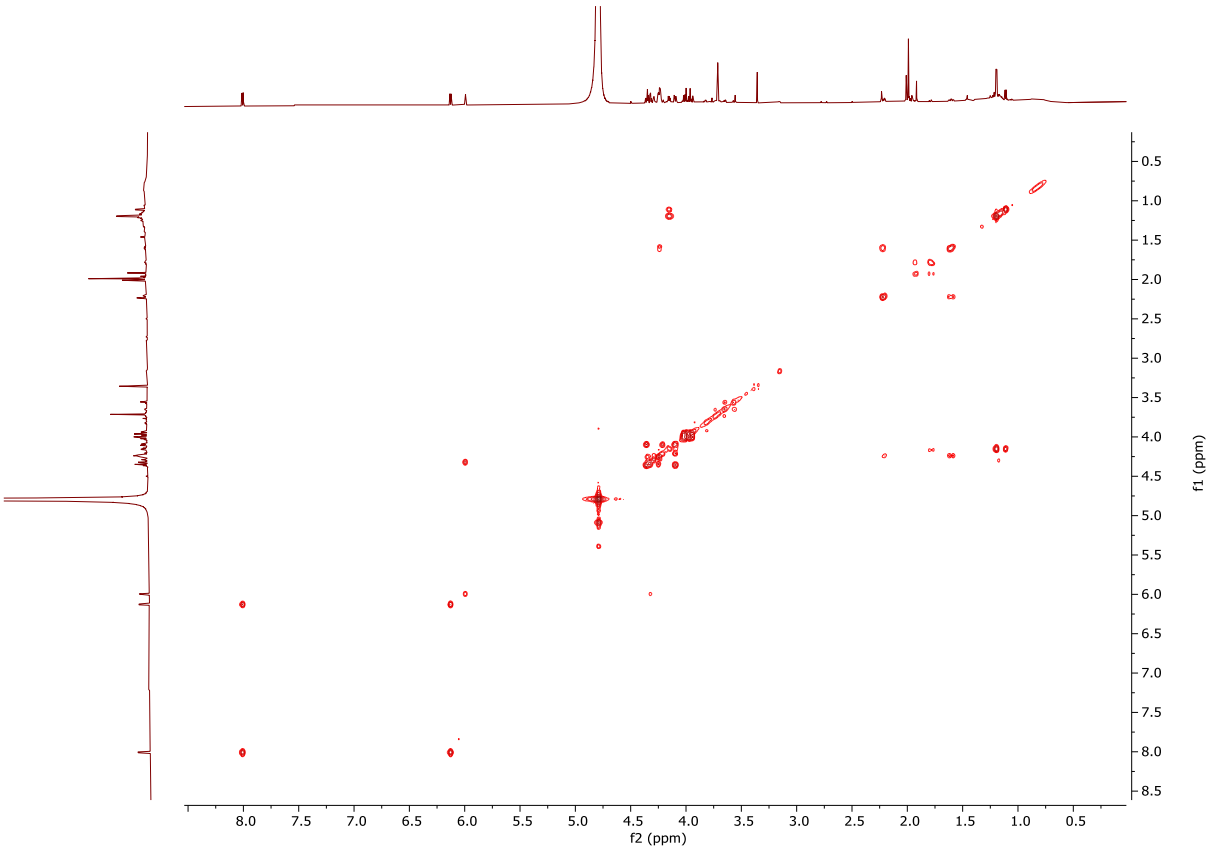
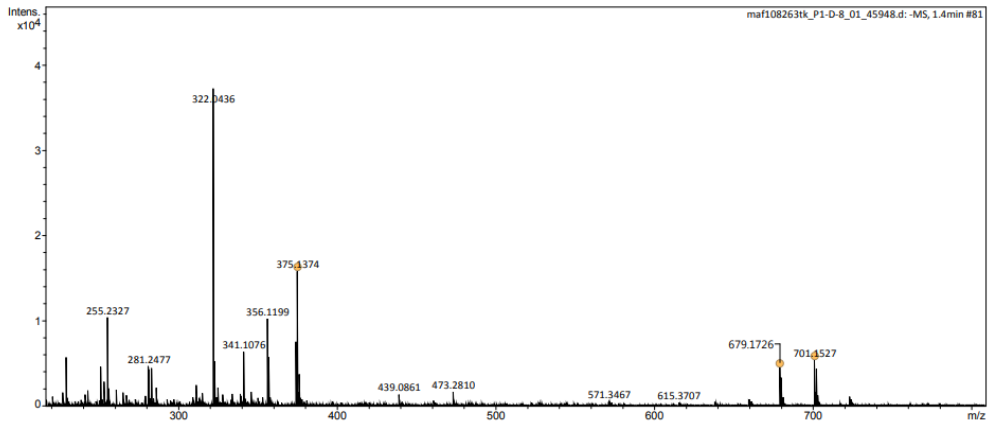


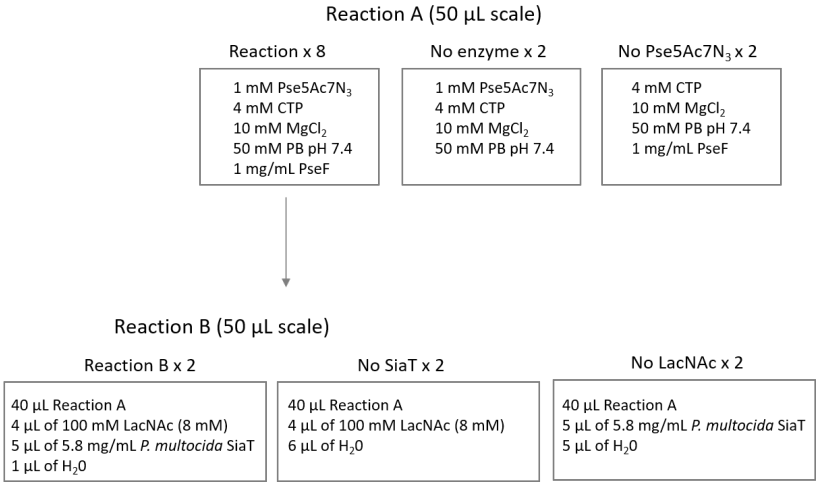
Figure S6a. ^{1}H-^{1}H COSY NMR spectrum of CMP-Pse5Ac7N$_3$ **14** post alkaline phosphatase treatment.



Meas. m/z	#	Ion Formula	m/z	err [ppm]	err [mDa]	mSigma	Mean err [ppm]
375.1374	1	C13H21N5O8	375.1396	5.8	2.2	46.7	1.9
679.1726	1	C22H32N8O15P	679.1730	0.6	0.4	231.8	-2.4
701.1527	1	C22H31N8NaO15P	701.1550	3.3	2.3	284.5	2.1

Figure S6b. HRMS of CMP-Pse5Ac7N$_3$ **14** post alkaline phosphatase treatment validates the mixture of CMP-Pse5Ac7N$_3$ **14** (HRMS (ESI-neg) for $C_{22}H_{32}N_8O_{15}P$ $[M-H]^-$: calculated 679.1730, observed 679.1726) and Pse5Ac7N$_3$ **12** (HRMS (ESI-neg) for $C_{13}H_{21}N_5O_8$ $[M-H]^-$: calculated 375.1396, observed 375.1374)

8. Using Pse5Ac7N_3 as a reporter of pseudaminyltransferase activity.



Reaction A was assembled on a 50 µL scale containing 1 mM Pse5Ac7N_3 12, 4 mM CTP (dissolved in 500 mM sodium phosphate buffer pH 7.4), 10 mM $MgCl_2$, 50 mM sodium phosphate buffer pH 7.4 and PseF (1 mg/mL). 8 x Reaction A were assembled, along with no enzyme and no Pse5Ac7N_3 controls assembled in duplicate. Reaction A and controls were incubated at 37 °C for 6 h. To validate the formation of CMP-Pse5Ac7N_3 14, 10 µL of reaction was diluted in 20 µL of MeCN and analysed by negative ESI-LC-MS using a HILIC column (Figure S7).

Reaction B was assembled in duplicate by taking 40 µL of Reaction A and adding LacNAc (4 µL of 100 mM stock in H_2O) and *Pasteurella multocida* SiaT (tPm0188Ph, 30 µg).[4] Additionally, no SiaT enzyme and no LacNAc controls were assembled in duplicate. Reaction B and controls were incubated at 37 °C for 18 h. 10 µL of each sample was then diluted in 20 µL of MeCN and analysed by negative ESI-LC-MS using a HILIC column (Figure S8). Reaction B and controls were then labelled with Cy3 as follows: To each 40 µL sample, 1 uL of 10 mM sulfo-Cyanine 3 alkyne (https://www.aatbio.com/products/cyanine-3-alkyne-cy3-alkyne , AAT Bioquest) was added and the samples were briefly vortexed to mix. Then 5 µL of a solution containing $CuSO_4$ and Tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine (THPTA) (25 mM each) was added and the samples were briefly vortexed to mix again. Then 5 µL of 50 mM sodium ascorbate solution was added and the samples were briefly vortexed and then incubated at 22 °C, 300 rpm for 2 h. 500 µL of H_2O was added to each sample to stop the reaction and they were lyophilized. Samples were suspended in 25 µL of H_2O, diluted 1/5 in MeCN and analysed by negative ESI-LC-MS (Figure S9).

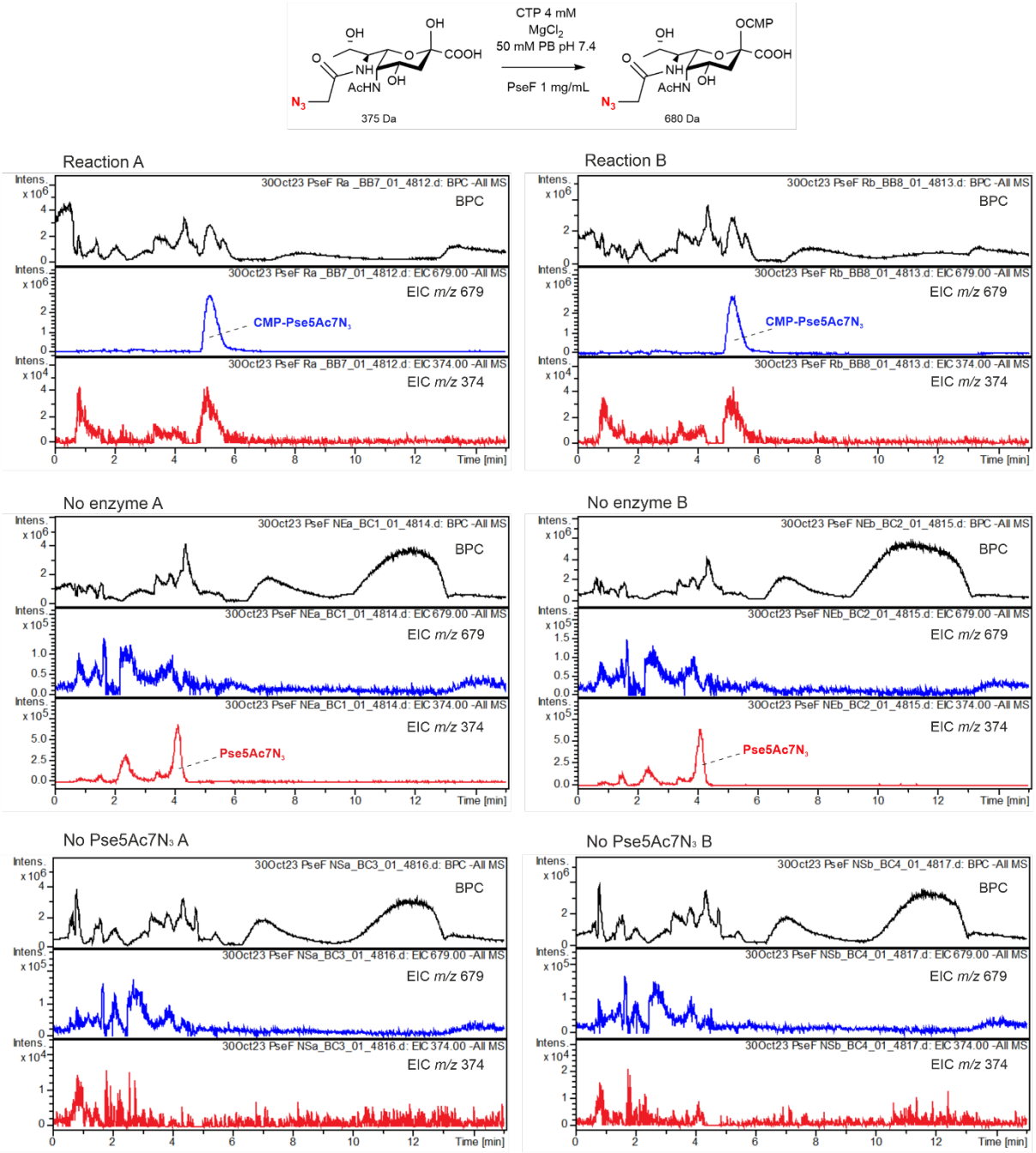


Figure S7. Negative ESI-LC-MS spectra of PseF reaction (Reaction A), no enzyme and no Pse5Ac7N_3 controls. A and B denote duplicate samples. BPC (black), EIC for CMP-Pse5Ac7N_3 14 $[M-H]^-$ *m/z* 679 (blue) and EIC for Pse5Ac7N_3 12 $[M-H]^-$ *m/z* 374 (red) are shown.

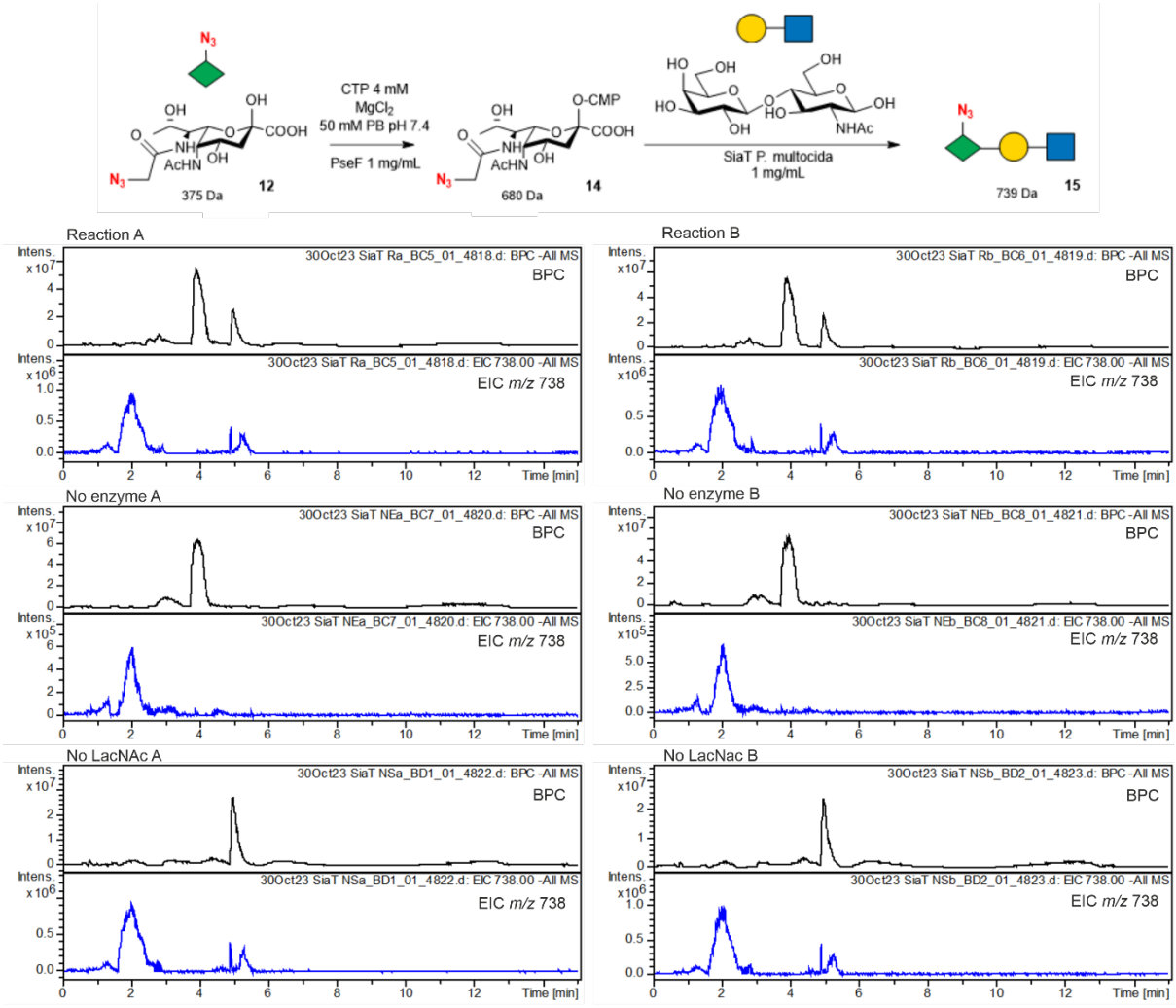


Figure S8. Negative ESI-LC-MS spectra of SiaT reaction (Reaction B), no enzyme and no LacNAc controls. A and B denote duplicate samples. BPC (black) and EIC for Pse5Ac7N_3-LacNAc **15** $[M-H]^-$ *m/z* 738 (blue) are shown.

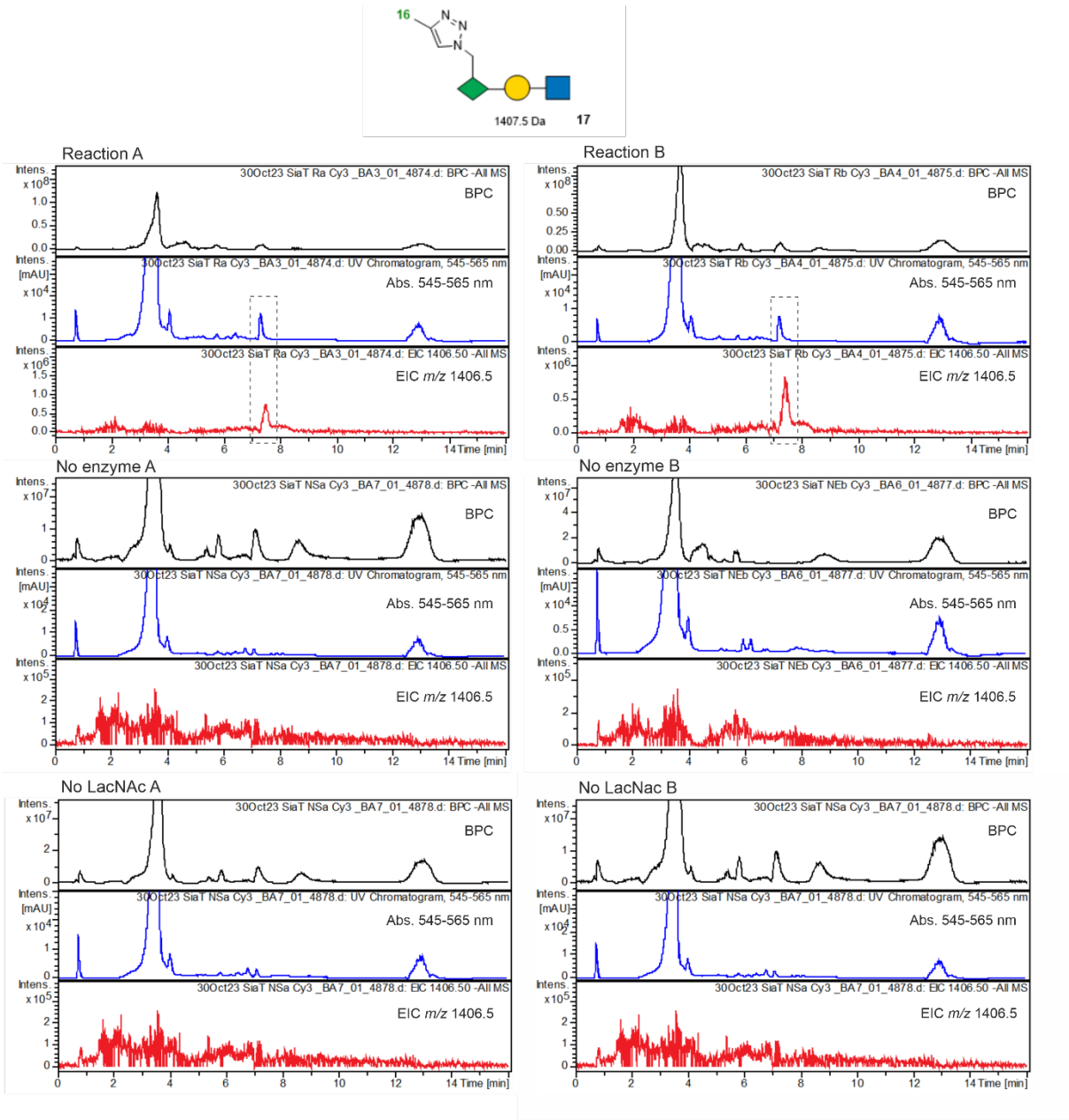


Figure S9. Negative ESI-LC-MS spectra of SiaT reaction (Reaction B), no enzyme and no LacNAc controls. A and B denote duplicate samples. BPC (black), UV/Vis $_{545\text{-}565\ nm}$ spectrum (blue) and EIC for Cy3-conjugated Pse5Ac7N$_3$-LacNAc **17** $[M\text{-}H]^-$ *m/z* 1406.5 (red) are shown.

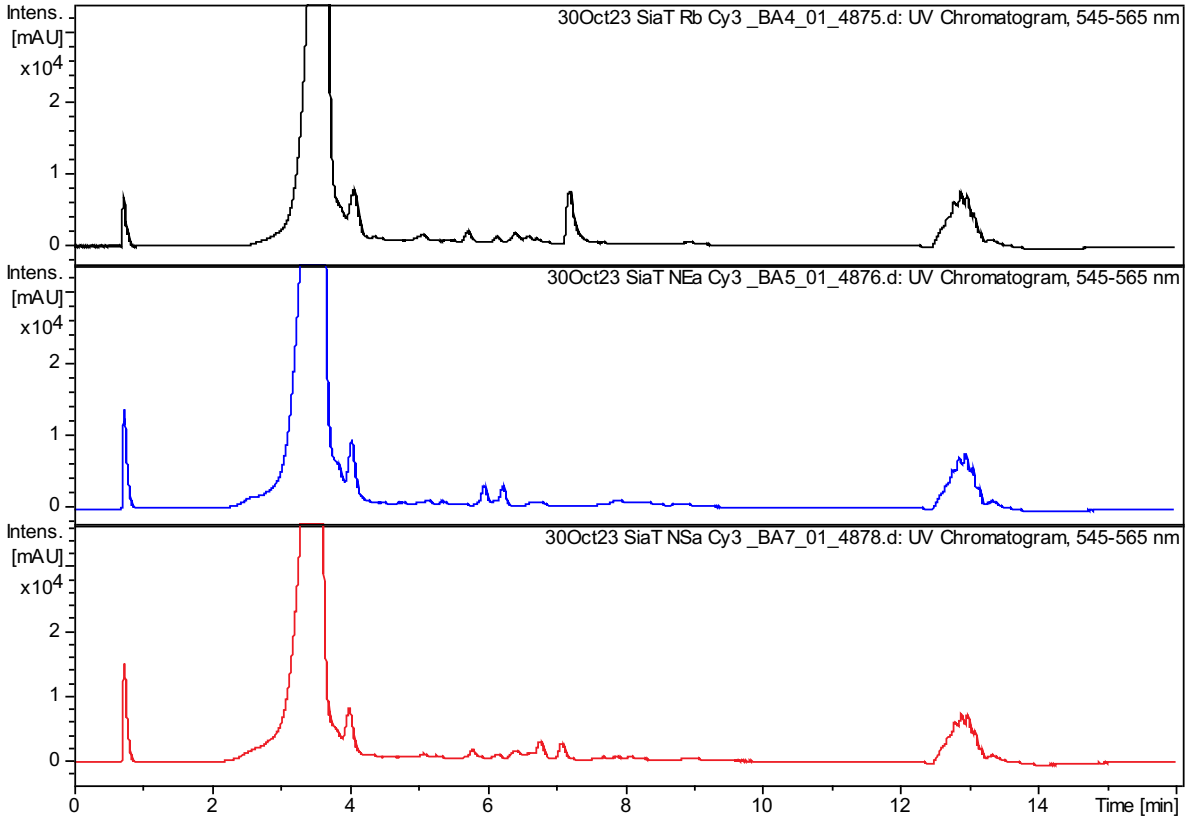
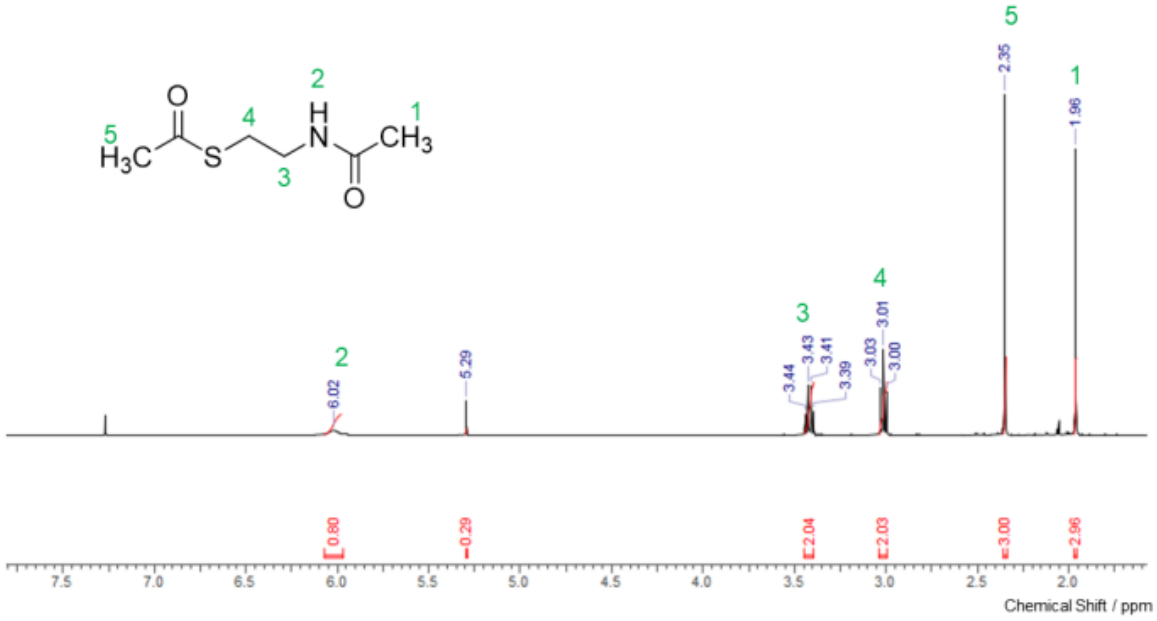


Figure S10. Full stacked UV/Vis $_{545\text{-}565\ nm}$ spectra for SiaT reaction (black), no enzyme (blue) and no LacNAc control (red), following ESI-LC-MS analysis.

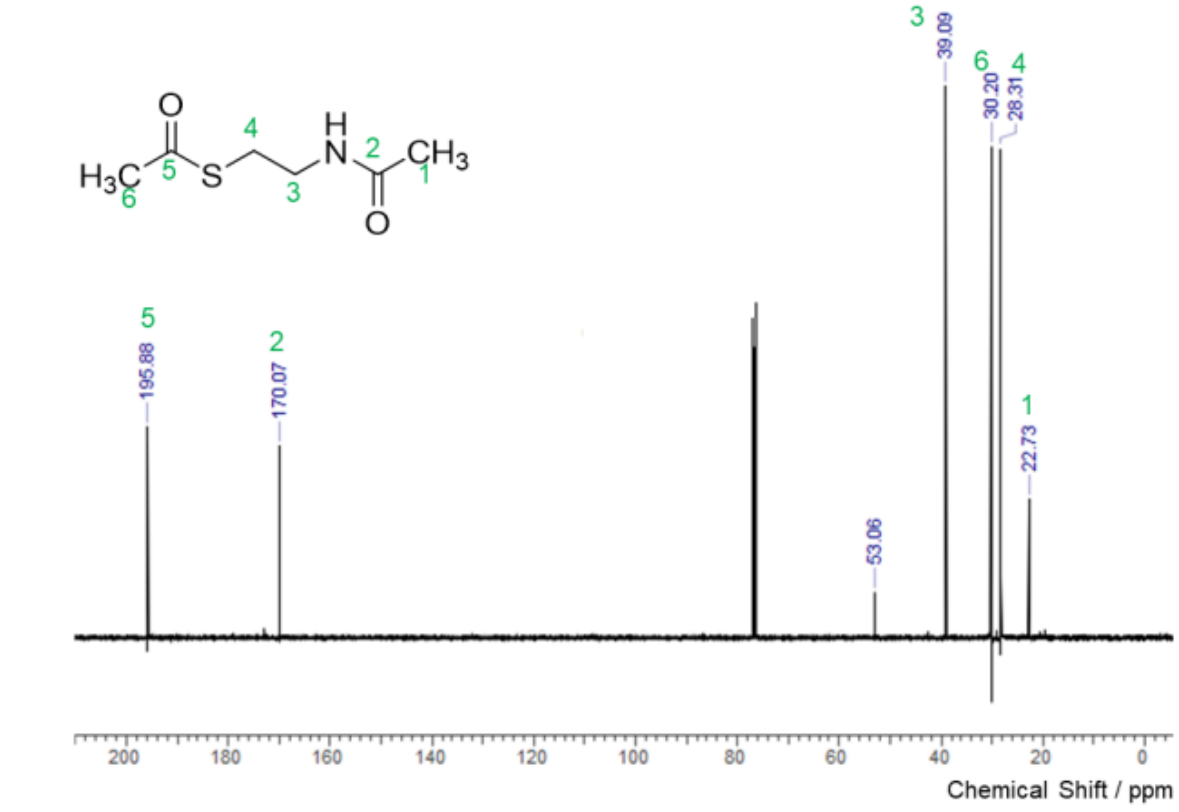
8. NMR Spectra

N,S-diacetylcysteamine (**9**, SNAc)

^{1}H

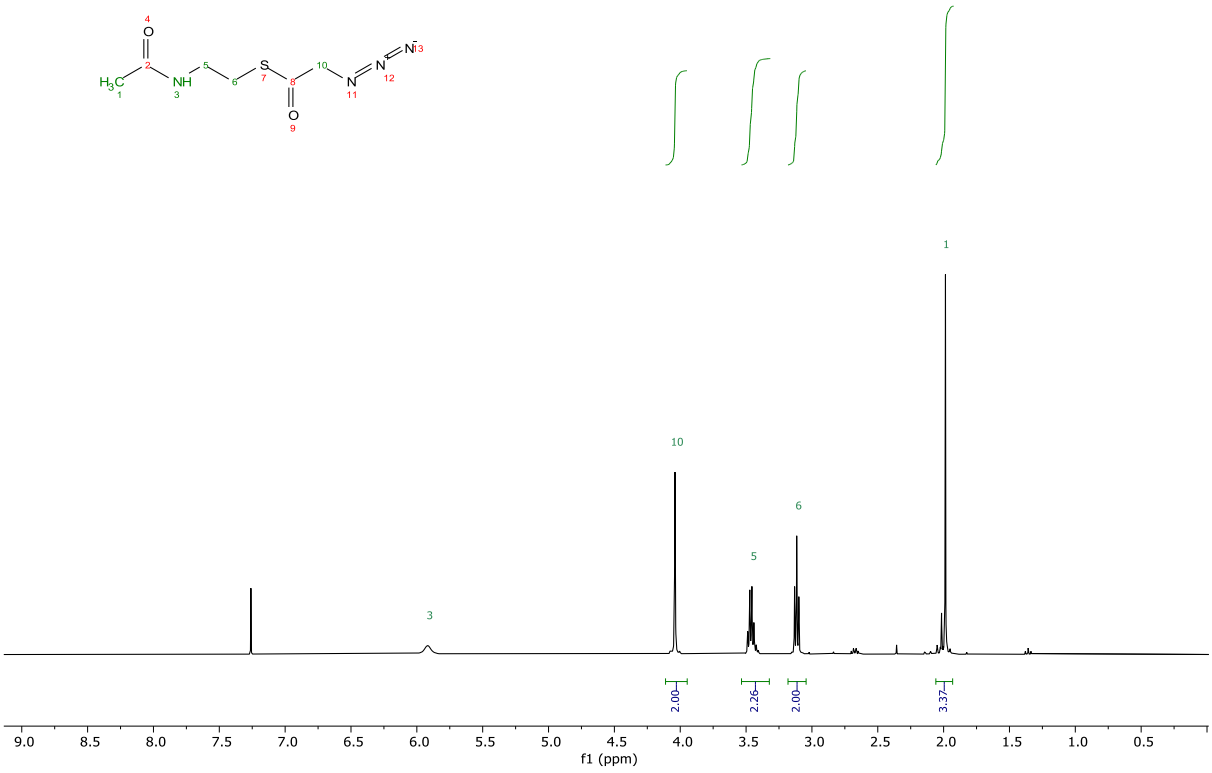


^{13}C

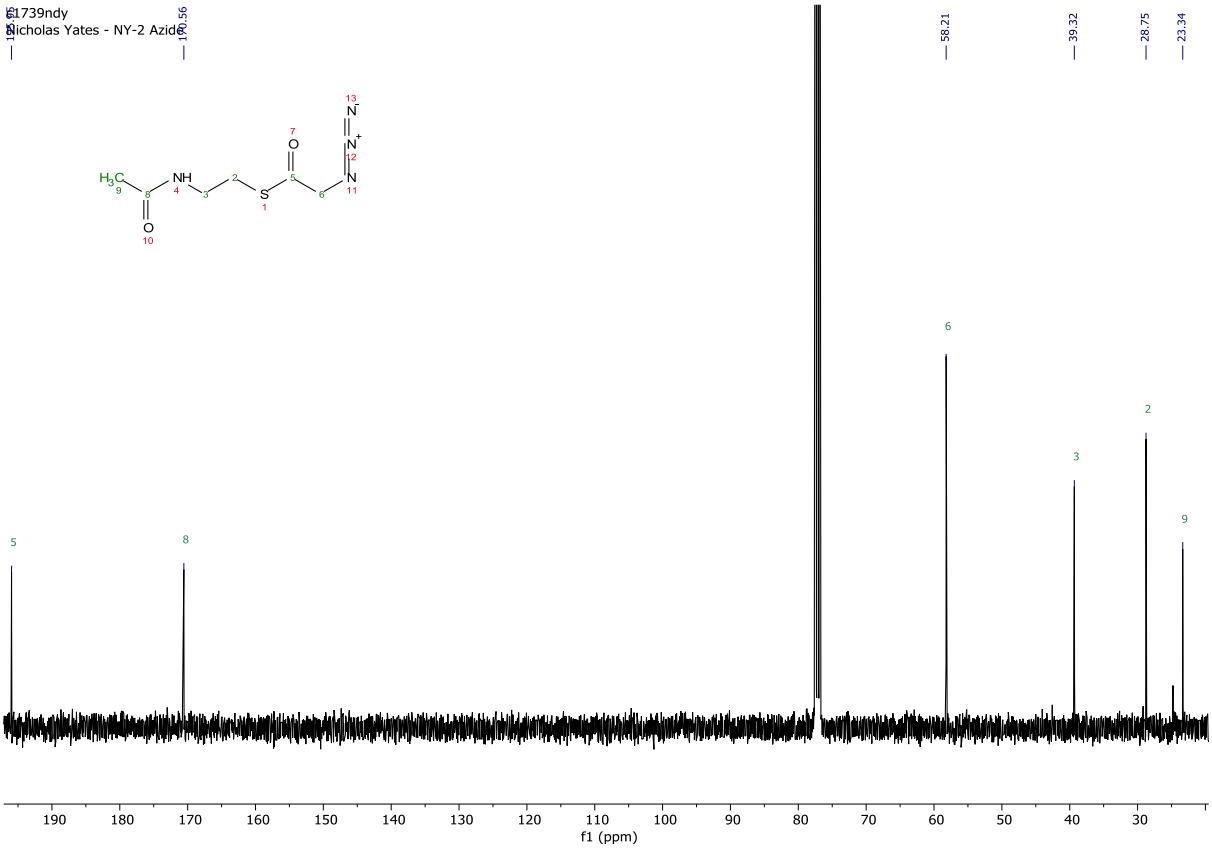


N-acetyl-S-azidocysteamine (**10**, SNAc-N_3)

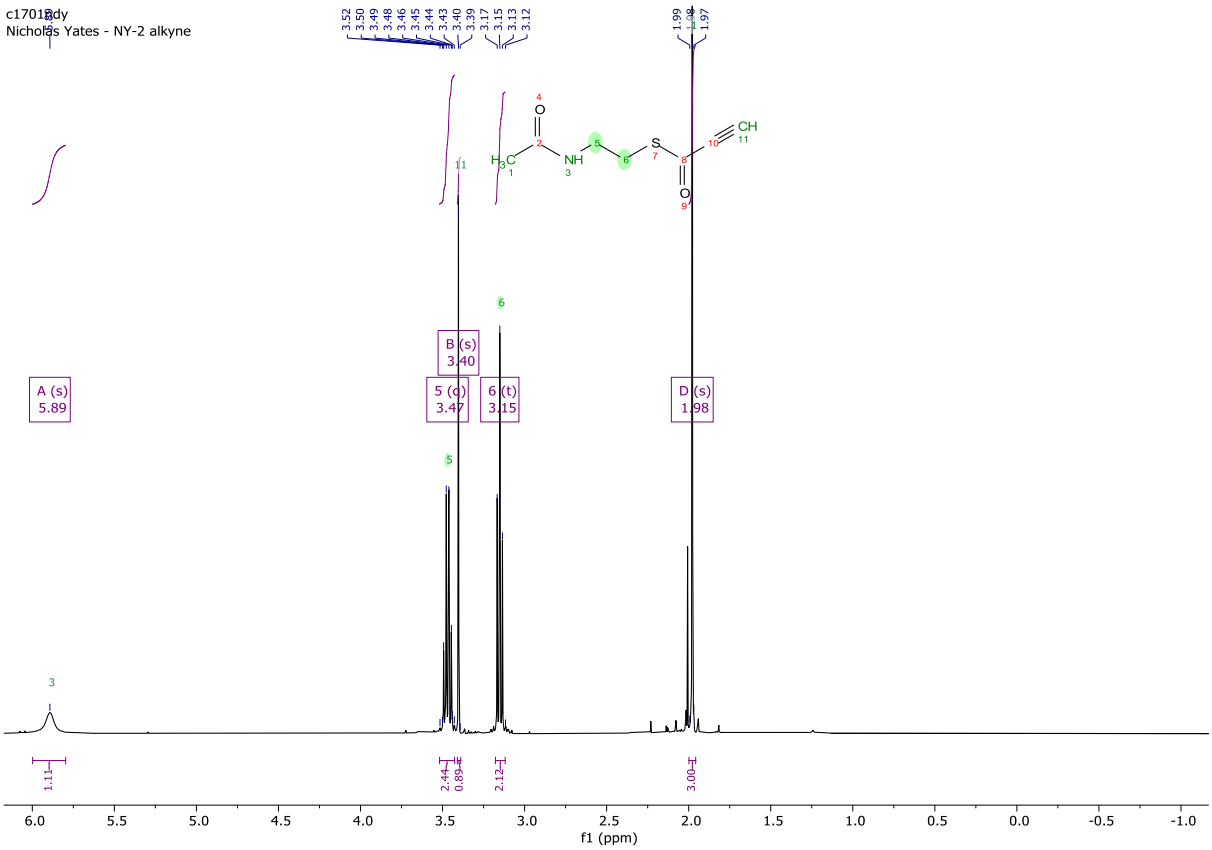
c1700ndy
Nicholas Yates - NY-2 azide

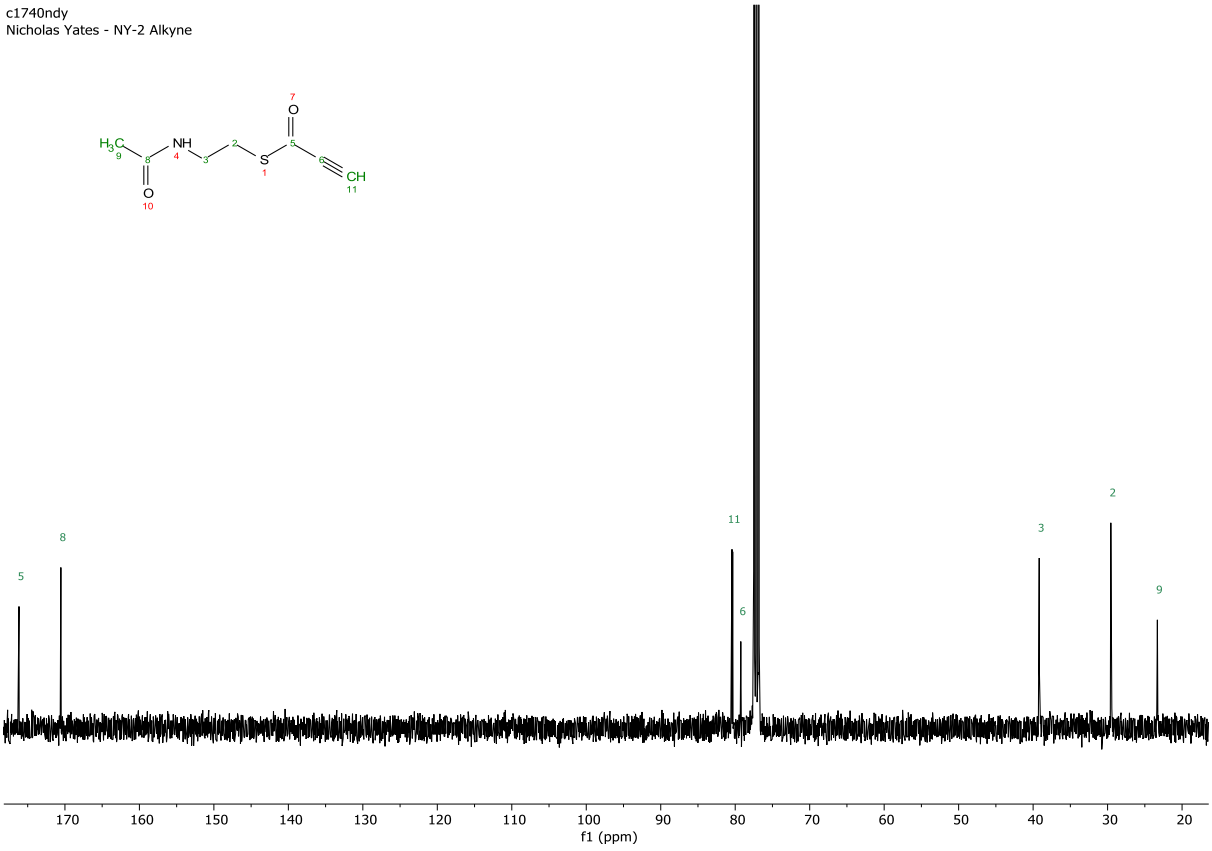


c1739ndy
Nicholas Yates - NY-2 Azide



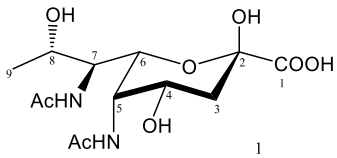
N-acetyl-S-propionylcysteamine (**11**, SNAc-Alk)

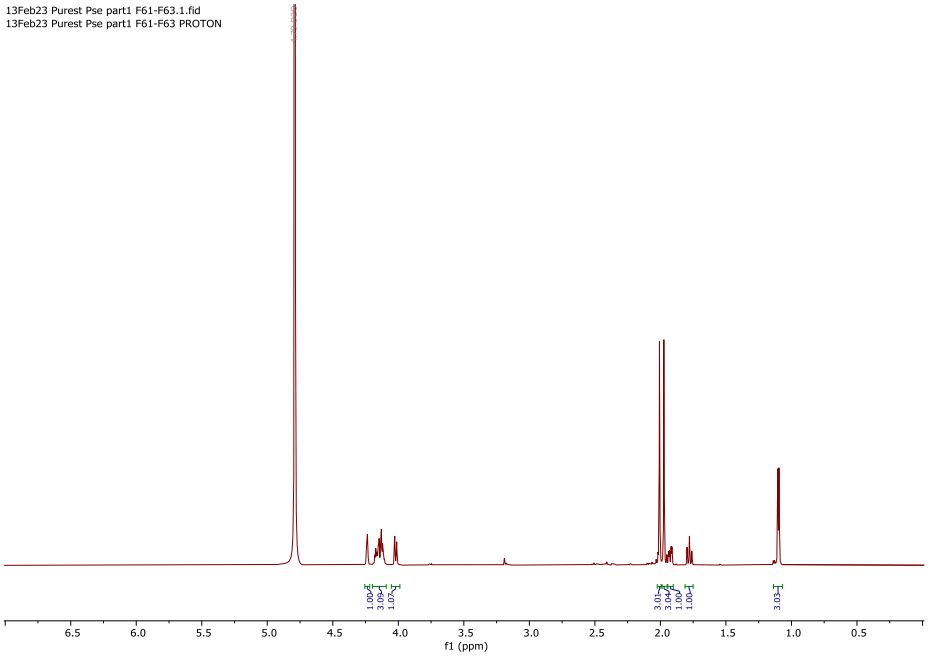




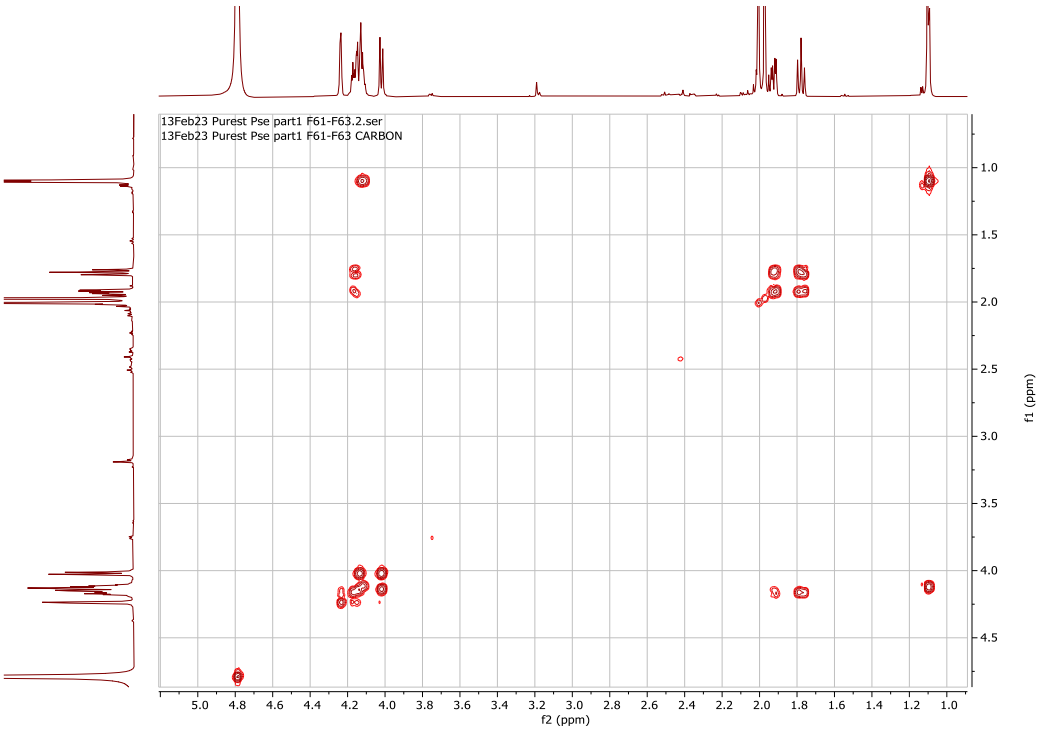
Pse5Ac7Ac (**1**)

^{1}H

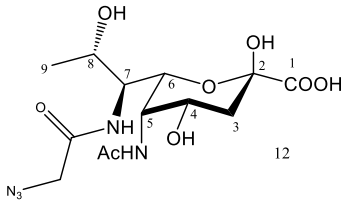




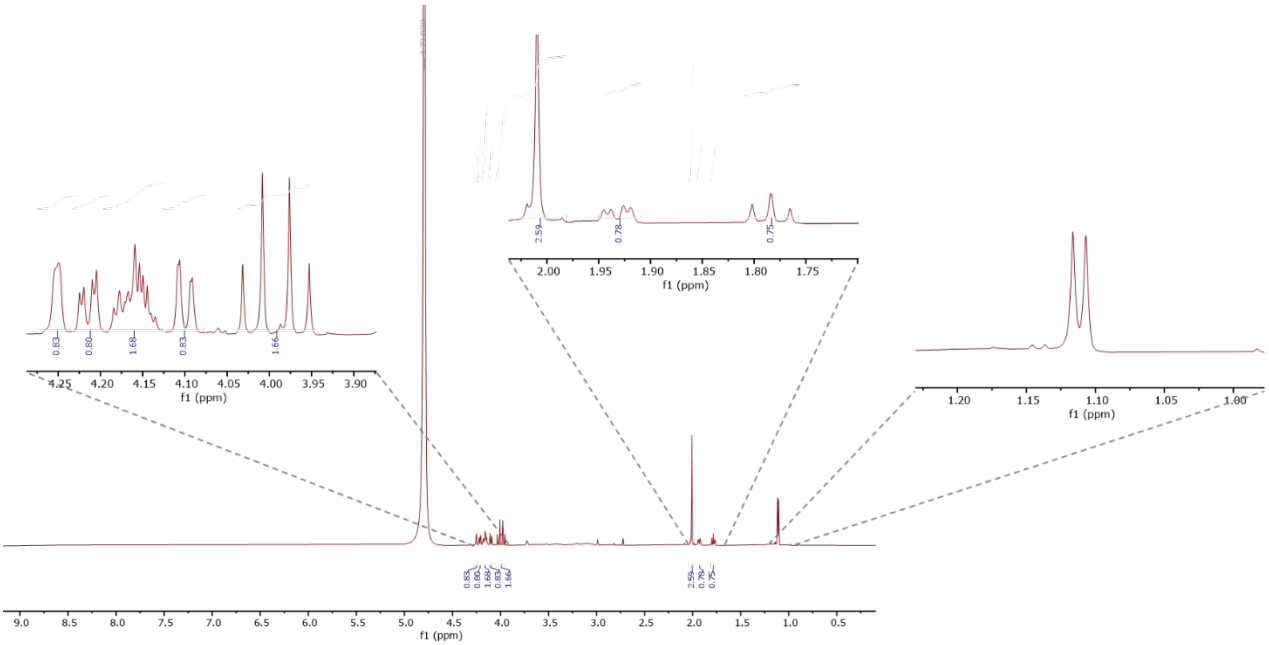
COSY



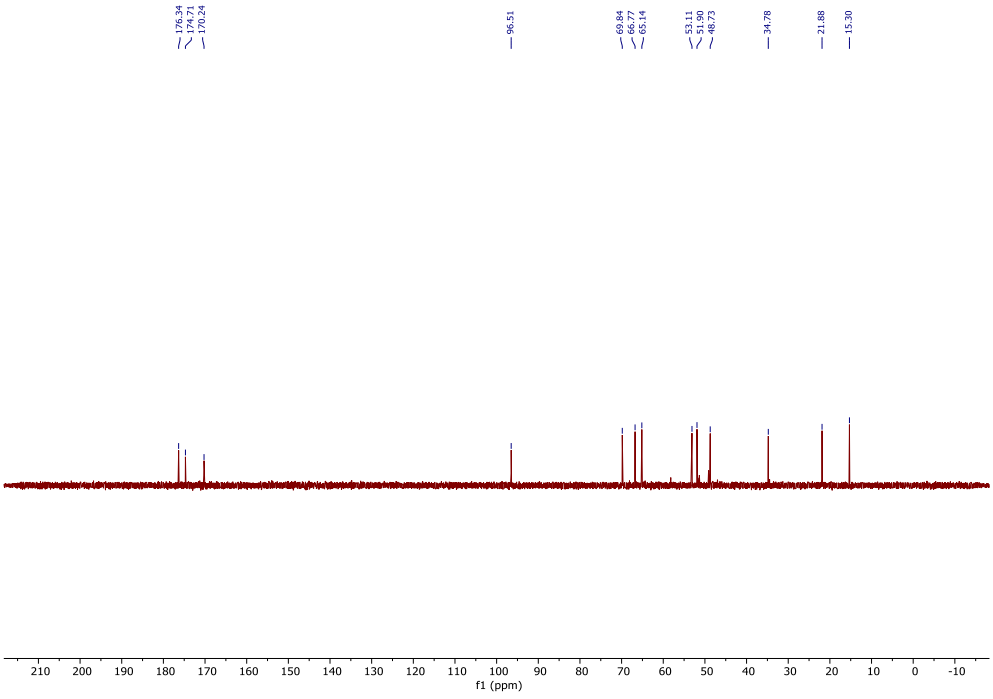
Pse5Ac7N$_3$ (**12**)



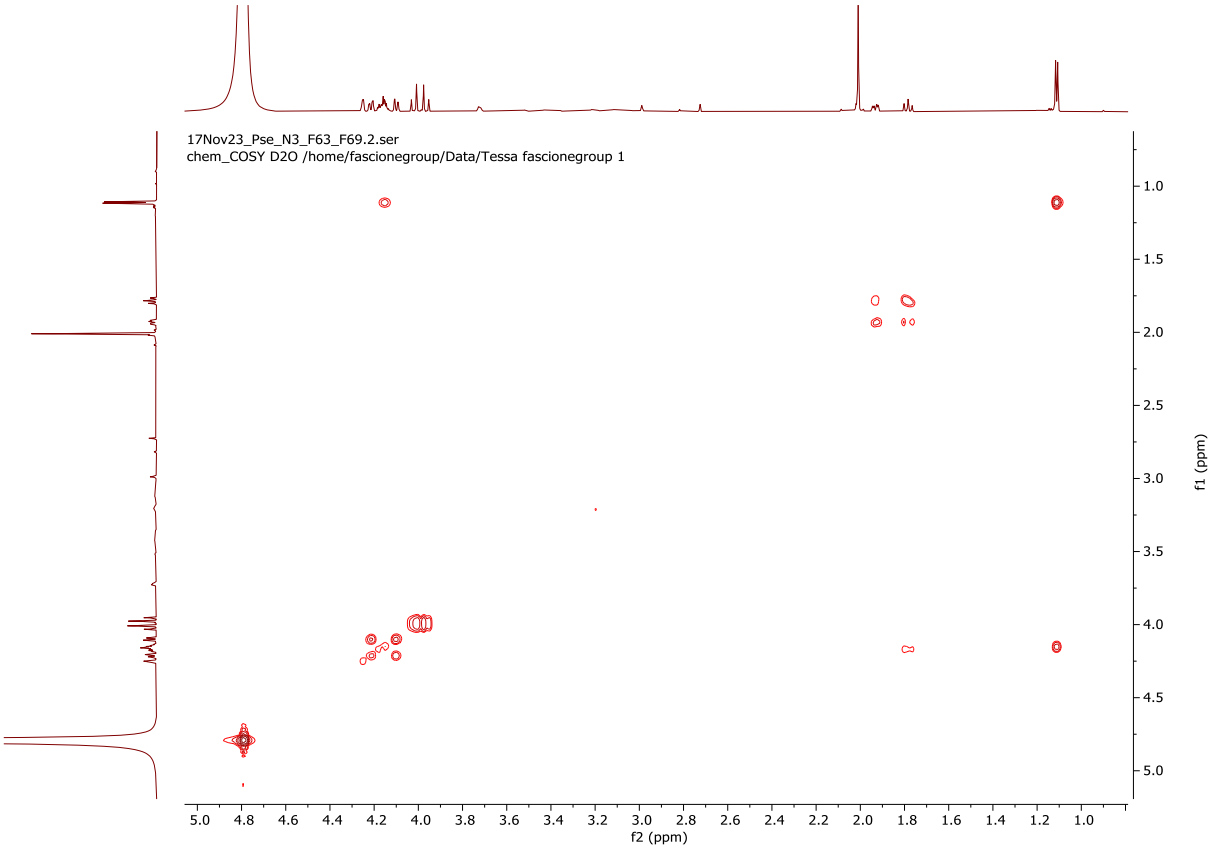
^{1}H



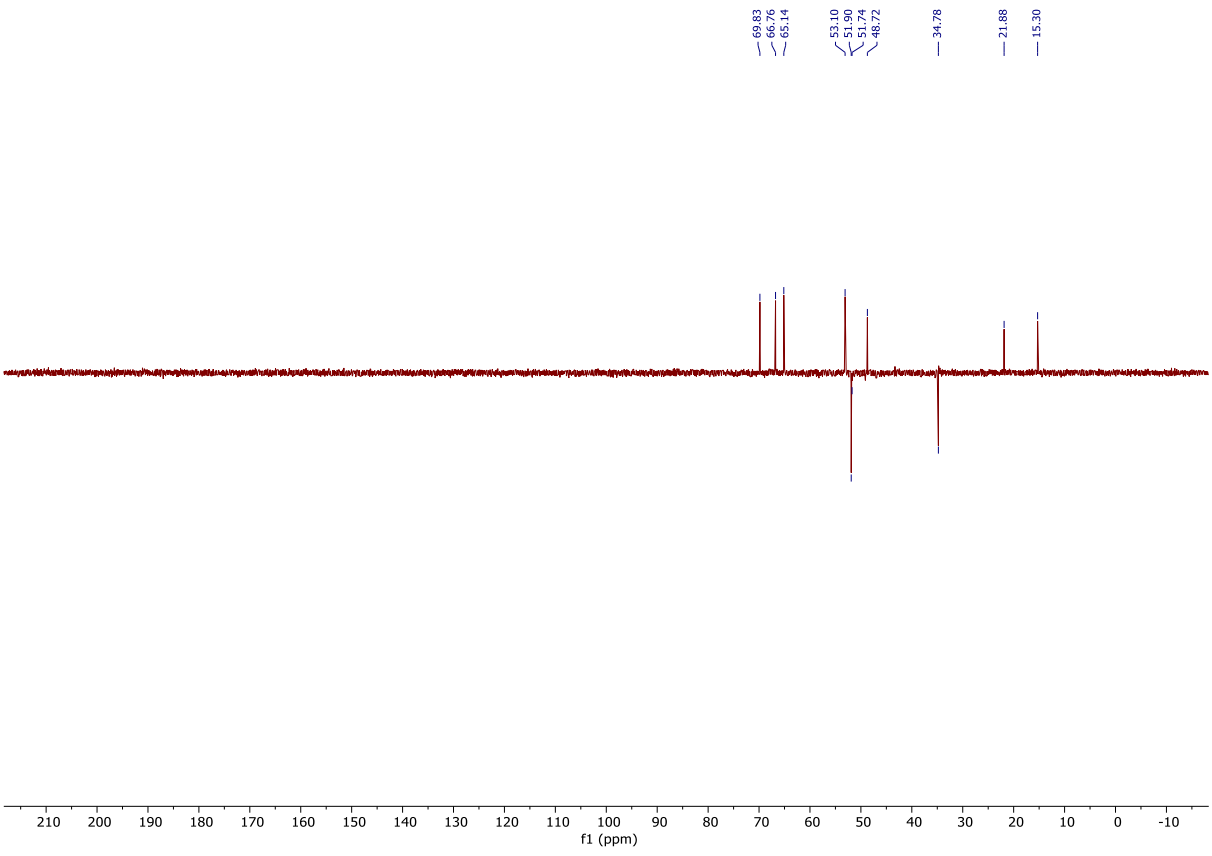
^{13}C



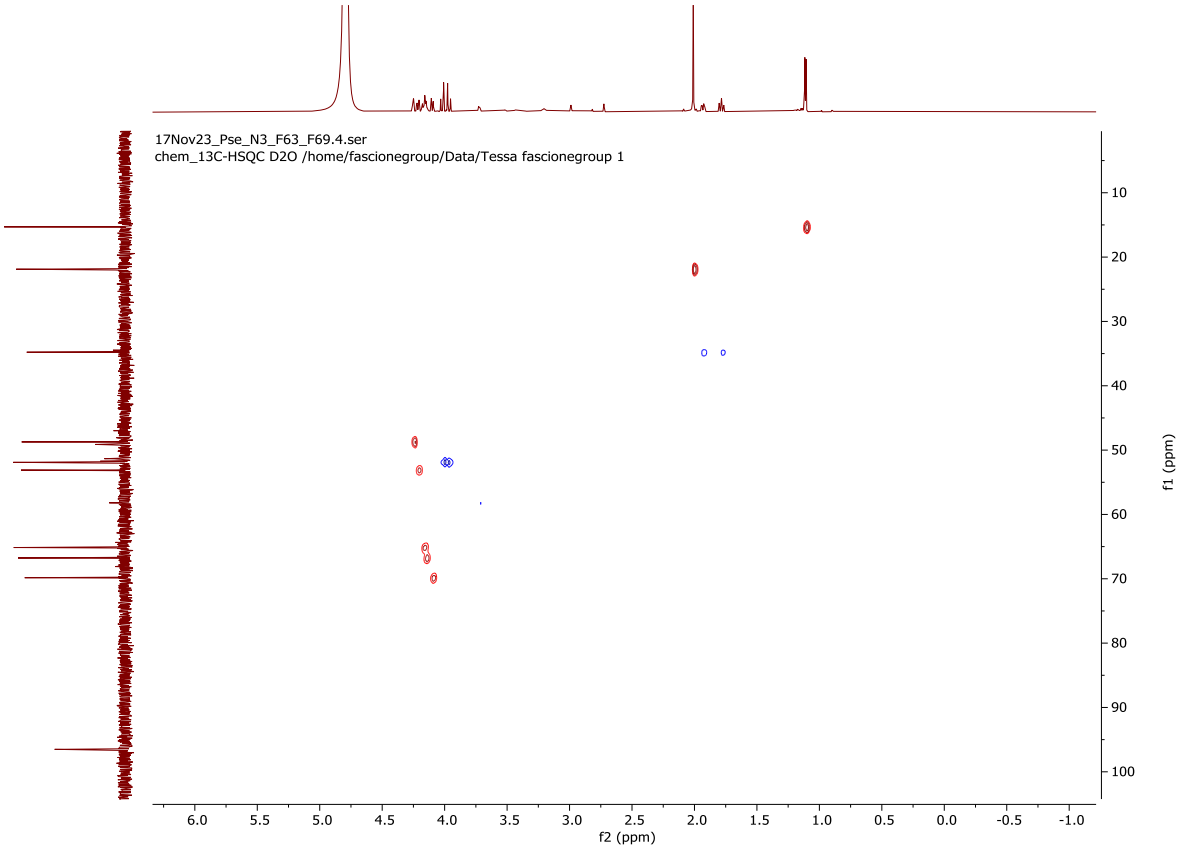
COSY


17Nov23_Pse_N3_F63_F69.2.ser
chem_COSY D2O /home/fascionegroup/Data/Tessa fascionegroup 1
f2 (ppm)
f1 (ppm)

DEPT


69.83
66.76
65.14
53.10
51.90
51.74
48.72
34.78
21.88
15.30
f1 (ppm)

HSQC


17Nov23_Pse_N3_F63_F69.4.ser
chem_13C-HSQC D2O /home/fascionegroup/Data/Tessa fascionegroup 1
f2 (ppm)
f1 (ppm)

References

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