α-Methylation Follows Condensation in the Gephyronic Acid Modular Polyketide Synthase

Drew T. Wagner[1,4], D. Cole Stevens[1,4], M. Rachel Mehaffey[2], Hannah R. Manion[1], Richard E. Taylor[3], Jennifer S. Brodbelt[2], and Adrian T. Keatinge-Clay[1,2,*]

[1]Department of Molecular Biosciences, The University of Texas at Austin, Austin, Texas, USA

[2]Department of Chemistry, The University of Texas at Austin, Austin, Texas, USA

[3]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana, USA

Cloning and expression of MT domains

The DNA encoding all polypeptides was amplified using primers:

Amplicon	Forward Primer (5' -> 3')	Reverse Primer (5' → 3')
GphMT1	GCGGCCTGGTGCCGCGCGGCTCTAGCGCCGCGCTGCTCGACTC	GTGGTGGTGGTGGTGGTGATGTTAAACCGGACGGGCGCGG
GphMT2	GCGGCCTGGTGCCGCGCGGCTCTAGCGACGTCTCGCTGTTCGACG	GTGGTGGTGGTGGTGGTGATGTTACGCGCGGATCCGG
GphMT3	GCGGCCTGGTGCCGCGCGGCTCTAGCACGGCTGTGCAGGAGCCAC	GTGGTGGTGGTGGTGGTGATGTTATTGCGGGAGGCCACCCA
GphMT4	GCGGCCTGGTGCCGCGCGGCTCTAGCGCCGAGGGGCTCCAAGG	GTGGTGGTGGTGGTGGTGATGTTACGATCCGCGCGACCAG
GphMT5	GCGGCCTGGTGCCGCGCGGCTCTAGCGCCCCCGGCACTTTCG	GTGGTGGTGGTGGTGGTGATGTTACTCGAGCGTGGCGAGCC
GphHmod	GCGGCCTGGTGCCGCGCGGCTCTAGCATGAGCAACGGGCAGTCGG	GTGGTGGTGGTGGTGGTGATGTTATCACGTGCCCAGGAGCG
GphMT6	GCGGCCTGGTGCCGCGCGGCTCTAGCGCCGTTGGCGCCGTC	GTGGTGGTGGTGGTGGTGATGTTACGCGGCGGGACTCCC
GphACP1	GCGGCCTGGTGCCGCGCGGCTCTAGCACGGCGGCTGAGCCC	GTGGTGGTGGTGGTGGTGATGTTACACCTCGTCCATCAGCGC
GphACP6	GCGGCCTGGTGCCGCGCGGCTCTAGCGCCAACCCGTCCGTGC	GTGGTGGTGGTGGTGGTGATGTTACACCGGGACCTCGGG
Pfs	GCGGCCTGGTGCCGCGCGGCTCTAGCATGAAAATCGGCATCATTGGTG	GTGGTGGTGGTGGTGGTGATGTTAGCCATGTGCAAGTTTCTGCAC

(ligation independent cloning regions highlighted red)

from *Cystobacter violaceus* or *E. coli* BL21(DE3) genomic DNA and inserted into pGAY28b, a ligation independent cloning vector constructed from pET28b.[1] *E. coli* BL21(DE3) transformed with the expression plasmid was inoculated into LB media containing 50 mg/L kanamycin at 37 °C, grown to OD_{600} = 0.4, and induced with 0.5 mM IPTG. After 18 h at 15 °C, cells were collected by centrifugation and resuspended in lysis buffer (0.5 M NaCl, 10% (v/v) glycerol, 0.1 M HEPES, pH 7.5). Following sonication, cell debris was removed by centrifugation (30,000 x g, 30 min). The supernatant was poured over a column of nickel-NTA resin (Thermoscientific), which was then washed with 40 mL lysis buffer containing 15 mM imidazole and eluted with 5 mL lysis buffer containing 150 mM imidazole. The eluted protein was concentrated to ~10 mg/mL in the equilibration buffer and stored at -80 ˚C until needed.

Substrate synthesis

3-oxopentanoyl-*S*-NAC (**1**), 2-methyl-3-oxopentanoyl-*S*-NAC (**2**), 3-oxohexanoyl-*S*-NAC (**4**), and 3-oxobutanoyl-*S*-NAC (**6**) were synthesized as previously reported.[2]

In vitro MT reaction conditions

Reaction conditions for 200 µL monomethylating MT reactions: either **1** (10 mM), **2** (10 mM) or malonyl-*S*-NAC (10 mM); Tris-HCl (150 mM); NaCl (100 mM); SAM (15 mM); GphMT (50 µM); and glycerol (10% v/v).

Reactions conditions for 200 µL dimethylating MT reactions: either **1** (10 mM), **2** (10 mM) or malonyl-*S*-NAC (10 mM); Tris-HCl (150 mM); NaCl (100 mM); SAM (30 mM); GphMT (50 µM); and glycerol (10% v/v).

Reaction conditions for 200 µL monomethylating MT reactions containing Pfs: either **1** (10 mM), **2** (10 mM) or malonyl-*S*-NAC (10 mM); Tris-HCl (150 mM); NaCl (100 mM); SAM (15 mM); Pfs (25 µM); GphMT (50 µM); and glycerol (10% v/v). High conversion

of **1** to **2** was observed at 25-50 μM Pfs; Pfs concentrations below 25 μM provided lower conversions.

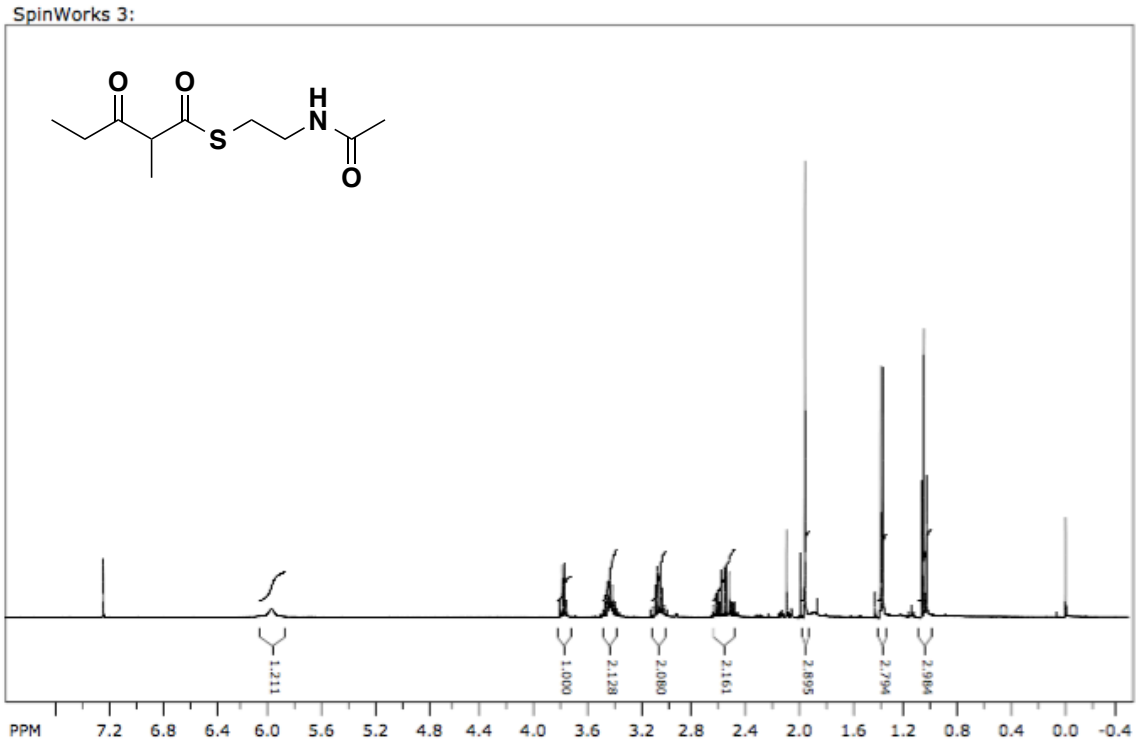
Reactions conditions for 200 μL dimethylating MT reactions containing Pfs were as follows, either **1** (10 mM), **2** (10 mM) or malonyl-*S*-NAC (10 mM); Tris-HCl (150 mM); NaCl (100 mM); SAM (30 mM); Pfs (25 μM); GphMT (50 μM); and glycerol (10% v/v).

Reactions were run at ~25 °C for 24-72 h. After completion, reactions with diketide **1** were extracted with 3 reaction volumes of EtOAc. The organic layer was dried *in vacuo* and resuspended in MeOH for HPLC analysis. After completion, reactions with malonyl-*S*-NAC were heated to precipitate enzyme and centrifuged to clarify the supernatant for HPLC analysis.

Scaled 20 mL GphMT1 reactions: **1** (10 mM), Tris-HCl (150 mM); NaCl (100 mM); SAM (30 mM); Pfs (50 μM); GphMT (50 μM); and glycerol (10% v/v). Reaction was stirred at room temperature for 60 h and extracted with 3 volumes of EtOAc (3 x 20 mL). The EtOAc layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* to provide a yellow oil that was purified *via* flash chromatography (75% EtOAc:hexanes) to provide **2** as a yellow oil (36 mg, 78%).

Methylation of excised acyl-ACPs: Thiol reactions to produce acyl-ACPs were **1** (5 mM), $NaHCO_3$ pH 8.1(150mM), and GphACP(1/6) (50 μM). Thiol reactions to produce malonyl-ACPs were malonyl-CoA (5 mM), $NaHCO_3$ pH 8.1 (300mM), and GphACP(1/6) (50 μM). Thiol exchange were run at room temperature for ~30m. Following thiol exchange, Acyl-/malonyl-ACPs were collected with a 10 kDa spin filter. Methylation reactions to monitor methylation of excised ACPs were malonyl-/acyl-ACP (~10 μM), Tris-HCl (150 mM); NaCl (100 mM); SAM (10 mM); Pfs (20 μM); GphMT (20 μM) at room temperature for ~16h. Methylation reactions were first filtered with a 30kDa spin filter to remove GphMTs and Pfs followed by a 10 kDa spin filter to collect malonyl-/acyl-ACPs for MS analysis.

1H-NMR (400 MHz, $CDCl_3$): 6.0 (s, broad, 1H), 3.8 (q, 1H), 3.4 (m, 2H), 3.1 (m, 2H), 2.6 (m, 2H), 1.9 (s, 3H), 1.4 (d, J = 7.2 Hz, 3H), 1.1 (t, J = 7.1 Hz, 3H).



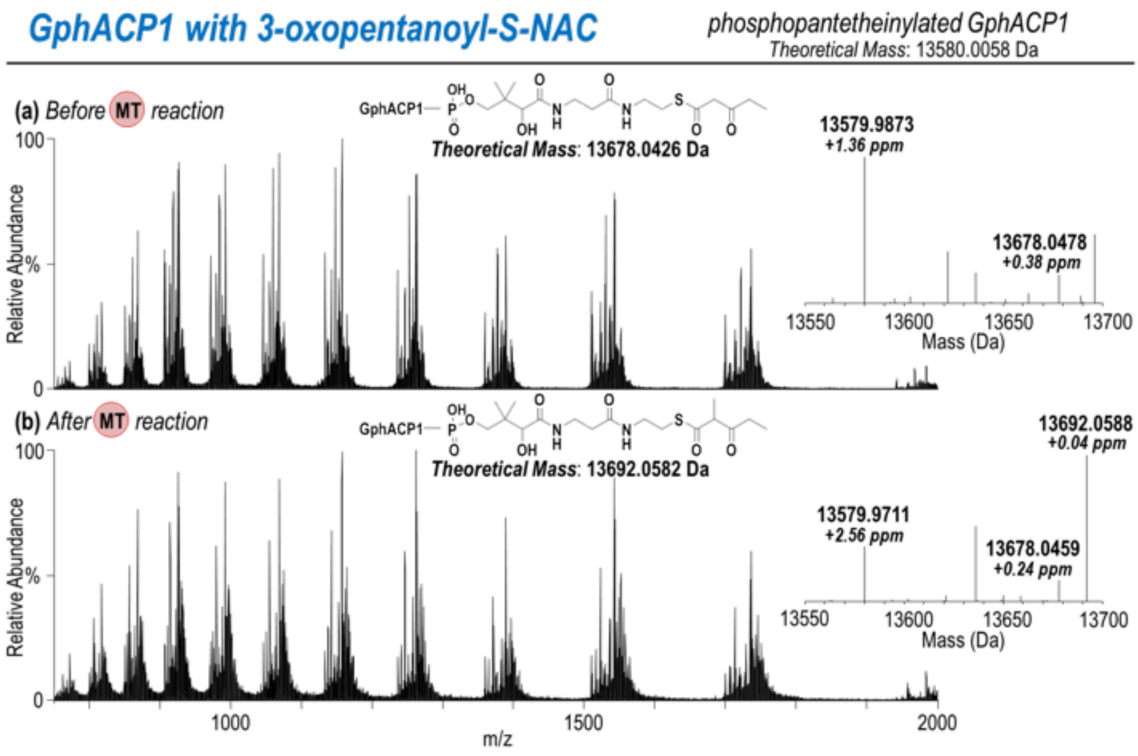
HPLC and HRMS analysis

HPLC conditions: All HPLC monitoring was performed on a tandem Waters 2707 autosampler and Waters 1525 binary HPLC pump connected to a Waters 2998 photodiode array detector using a Varian Microsorb-MV C18 column (250 x 4.6 mm, 5 μm particle size, 100 Å pore size) and mobile phases consisting of water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B) with a solvent gradient of 5%-100% B over 30 min at a flow rate of 1 mL/min.

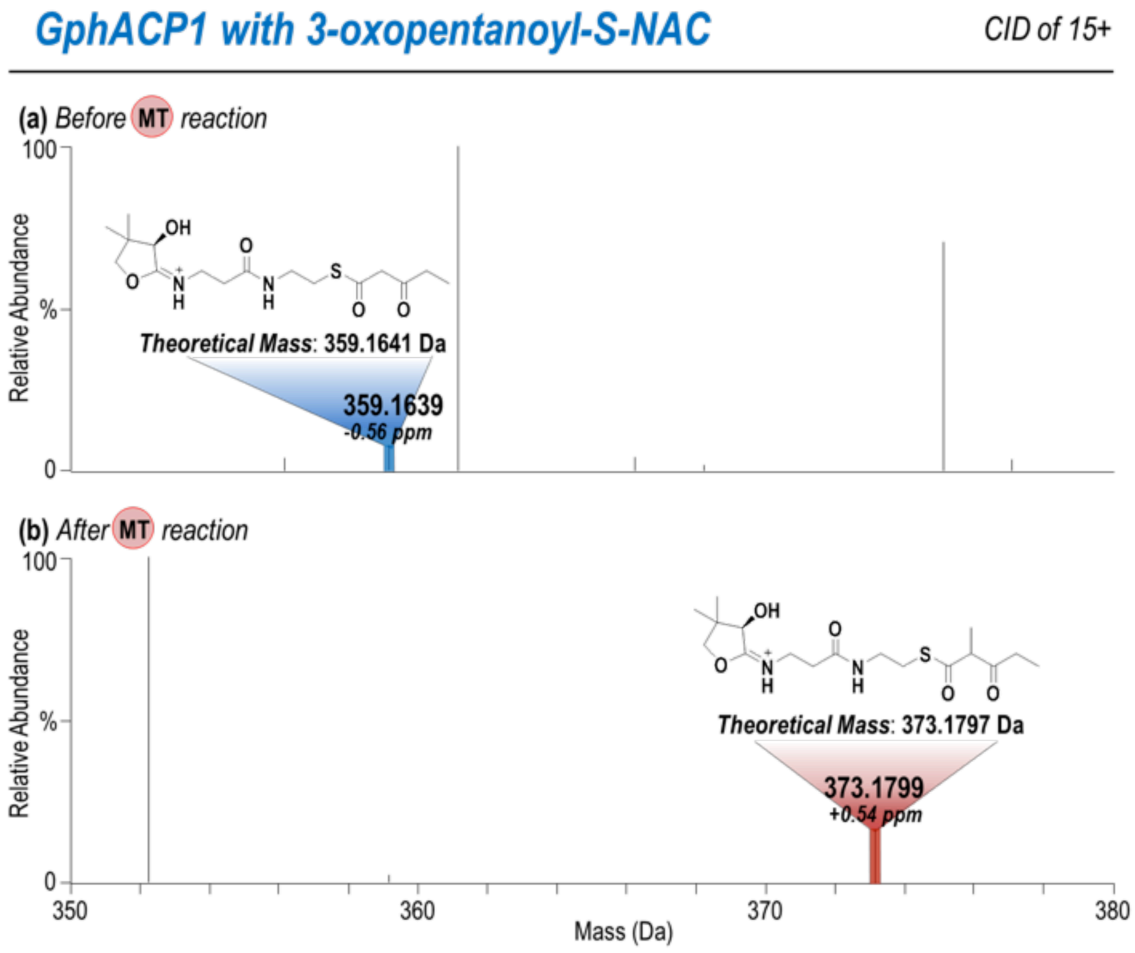
HRMS conditions: High-resolution mass spectrometry measurements were obtained by chemical ionization (ESI) with a VG analytical ZAB2-E instrument.

HRMS & MS/MS conditions: All experiments were performed on a Thermo Fisher Scientific Orbitrap Elite mass spectrometer (San Jose, CA) modified with a 193 nm ArF excimer laser (Coherent ExciStar XS) to perform ultraviolet photodissociation (UVPD) experiments in the HCD cell. Protein solutions were loaded into Au-coated borosilicate emitters (10 μM in 50% (v/v) acetonitrile) and ionized offline using a nano-ESI source or by separation using a Dionex UltiMate 3000 RPLC nanoLC system integrated with an ESI source. Chromatographic separations were performed using water (mobile phase A) and acetonitrile (mobile phase B), each containing 0.1% formic acid with a solvent gradient of 15% to 55% B over 45 min at a flow rate of 300 nL/min. The trap (30 mm × 0.1 mm) and analytical columns (with integrated emitter) (20 cm × 0.075 cm) were packed in-house using 5 μm Agilent PLRP-S resin (1000 Å pore size). CID mass spectra were collected using normalized collision energy 25%. UV photoactivation of the modified proteins was achieved using one pulse with energy 2 mJ/pulse. MS1 and MS/MS spectra (50-250 scans) were acquired at 240 K resolving power (at m/z 400). Processing of MS/MS spectra was performed using Xtract and ProSight 3.0 to identify fragment ions.

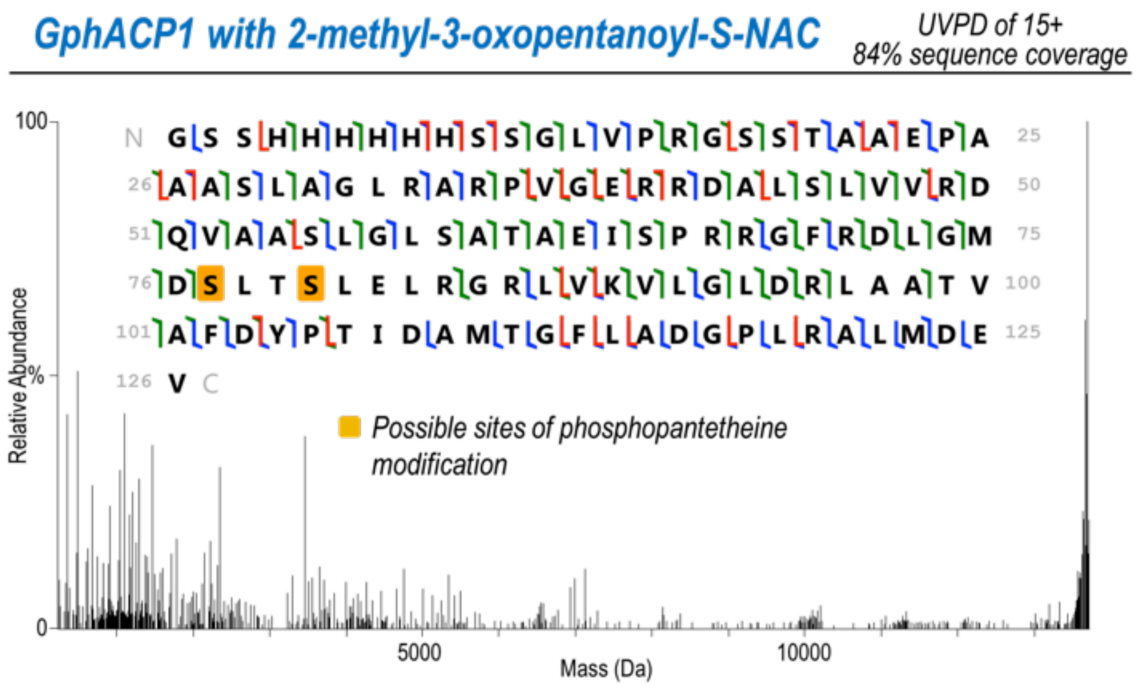
ESI-MS of phosphopantetheinylated GphACP1 thioesterified with 3-oxopentanoyl-*S*-NAC (a) before and (b) after MT reaction. Insets give expanded views of the deconvoluted spectra (mass range 13550-13700 Da) labeled with the monoisotopic masses and mass accuracies relative to the theoretical mass of the modified proteins.



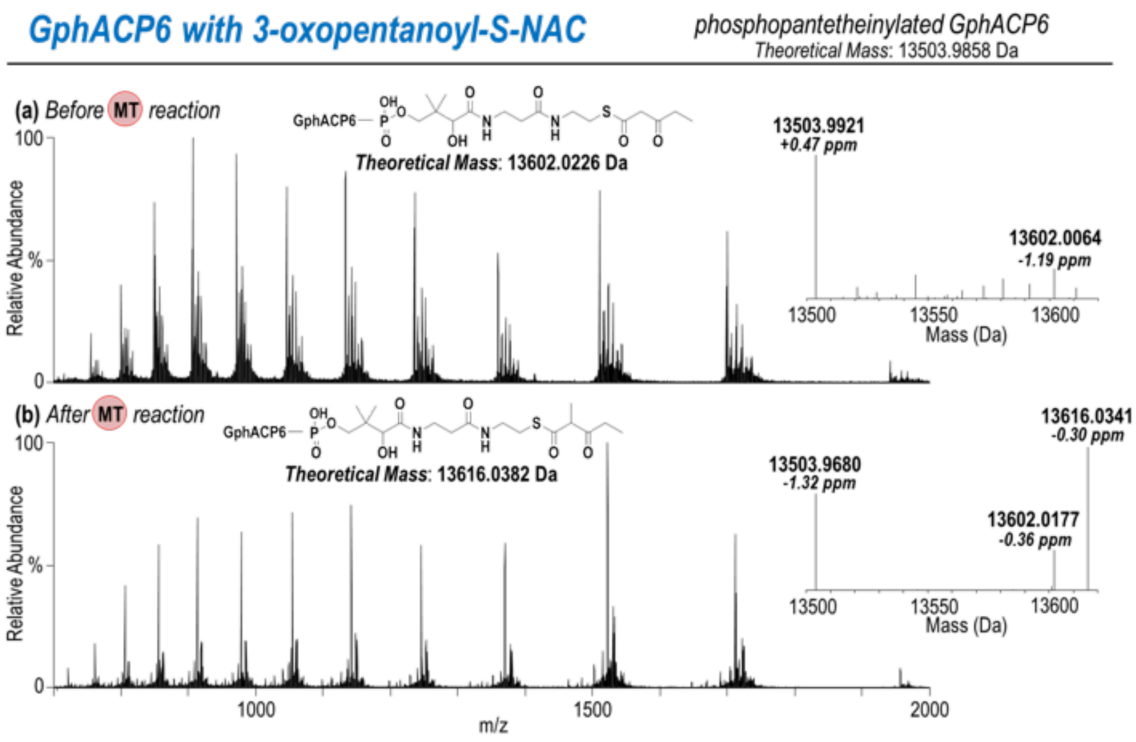
Deconvoluted MS/MS spectrum (mass range 350-380 Da) resulting from CID of the 15+ charge state of phosphopantetheinylated GphACP1 with 3-oxopentanoyl-*S*-NAC (a) before and (b) after MT reaction. Highlighted peaks correspond to the thioesterified phosphopantetheine moiety cleaved from the protein upon collisional activation labeled with the monoisotopic mass and mass accuracy relative to the theoretical mass.



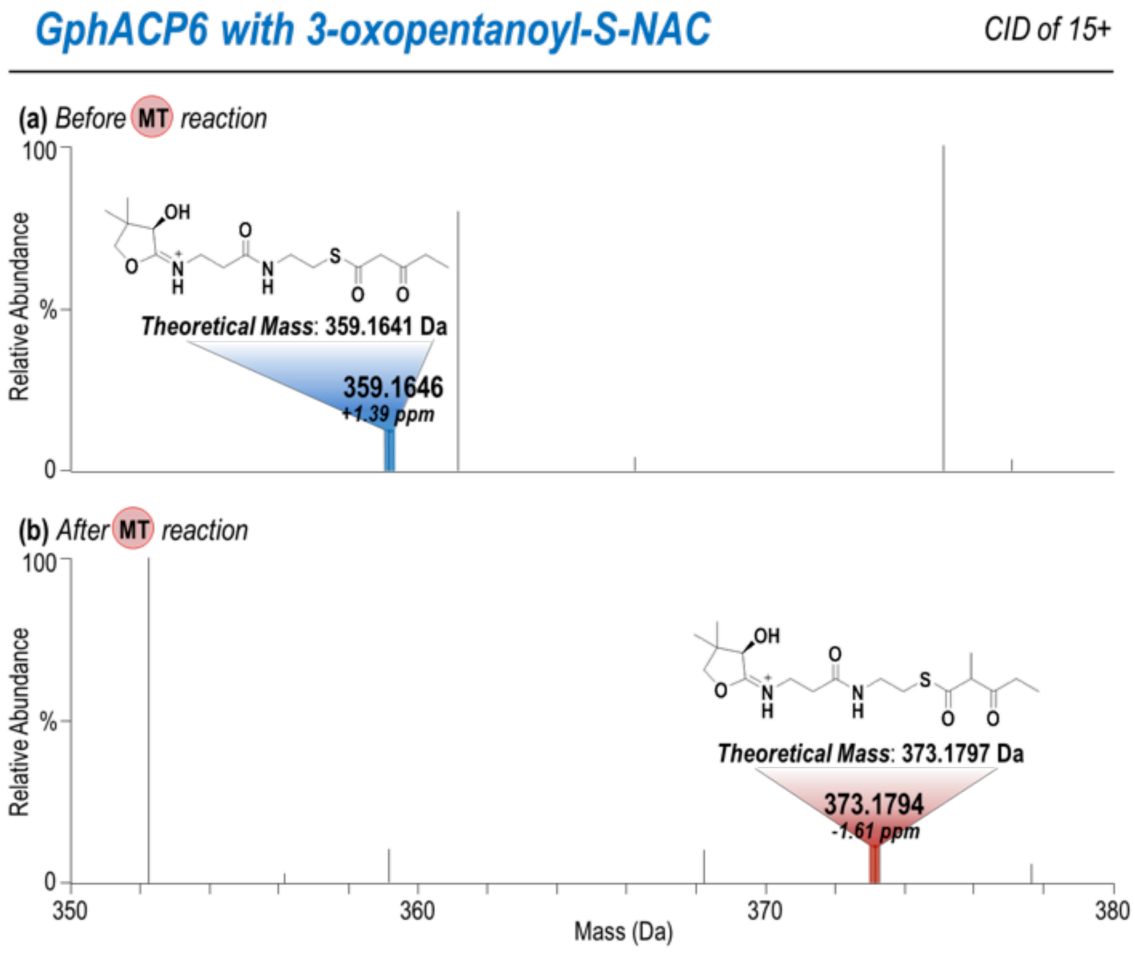
Deconvoluted MS/MS spectrum resulting from UVPD of the 15+ charge state of phosphopantetheinylated GphACP1 with 2-methyl-3-oxopentanoyl-*S*-NAC. Two possible modification sites were identified (Ser77 or Ser80) based on mass shifts in the observed fragment ions.



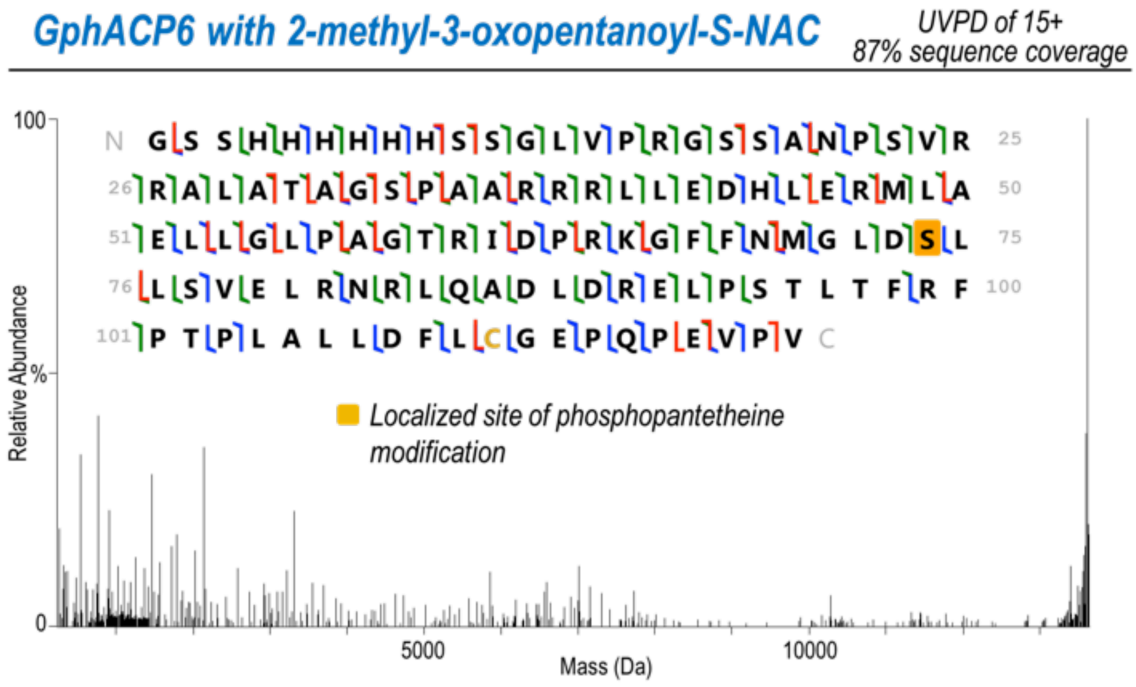
ESI-MS of phosphopantetheinylated GphACP6 thioesterified with 3-oxopentanoyl-*S*-NAC (a) before and (b) after MT reaction. Insets give expanded views of the deconvoluted spectra (mass range 13500-13620 Da) labeled with the monoisotopic masses and mass accuracies relative to the theoretical mass of the modified proteins.



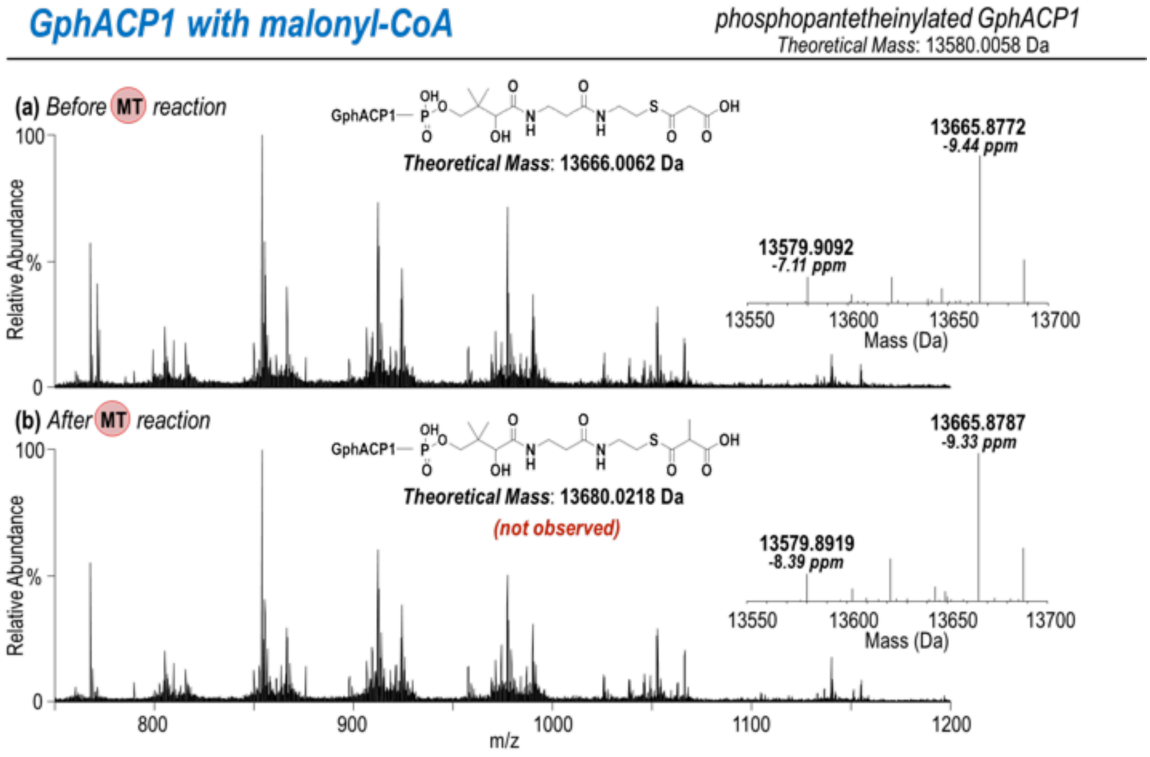
Deconvoluted MS/MS spectrum (mass range 350-380 Da) resulting from CID of the 15+ charge state of phosphopantetheinylated GphACP6 with 3-oxopentanoyl-*S*-NAC (a) before and (b) after MT reaction. Highlighted peaks correspond to the thioesterified phosphopantetheine moiety cleaved from the protein upon collisional activation labeled with the monoisotopic mass and mass accuracy relative to the theoretical mass.



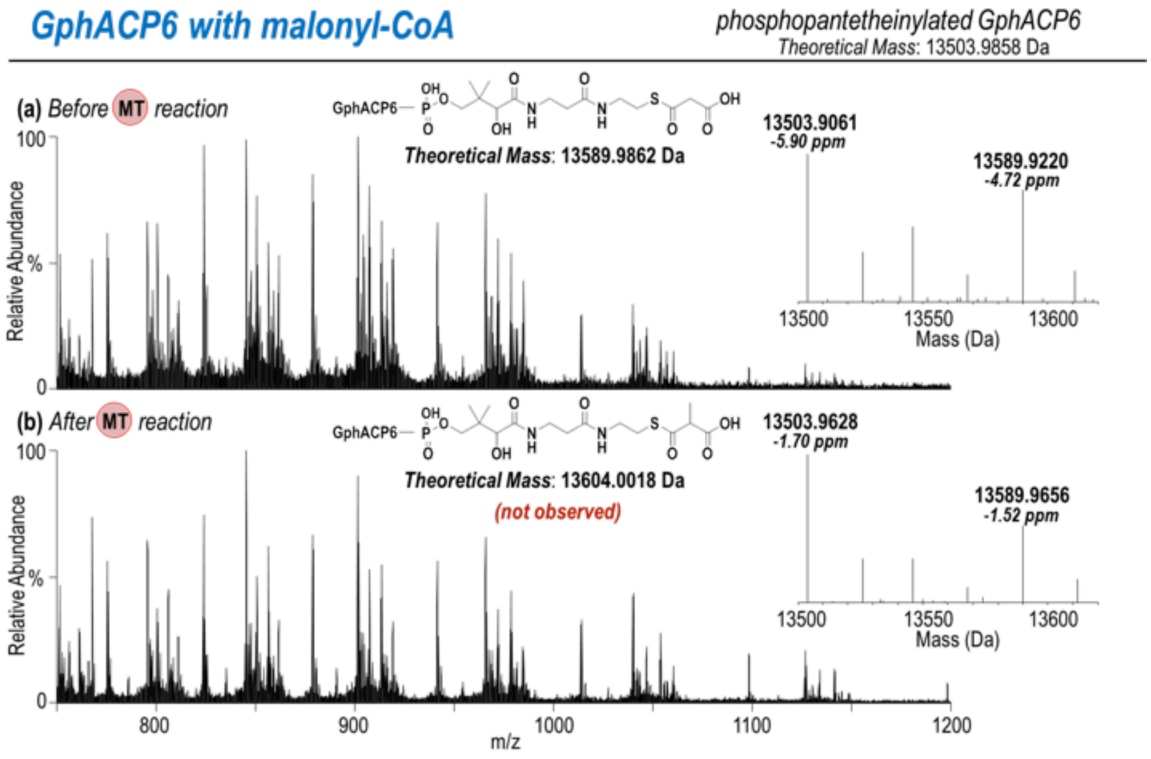
Deconvoluted MS/MS spectrum resulting from UVPD of the 15+ charge state of phosphopantetheinylated GphACP6 with 2-methyl-3-oxopentanoyl-*S*-NAC. The modification was localized to Se74 based on mass shifts in the observed fragment ions.



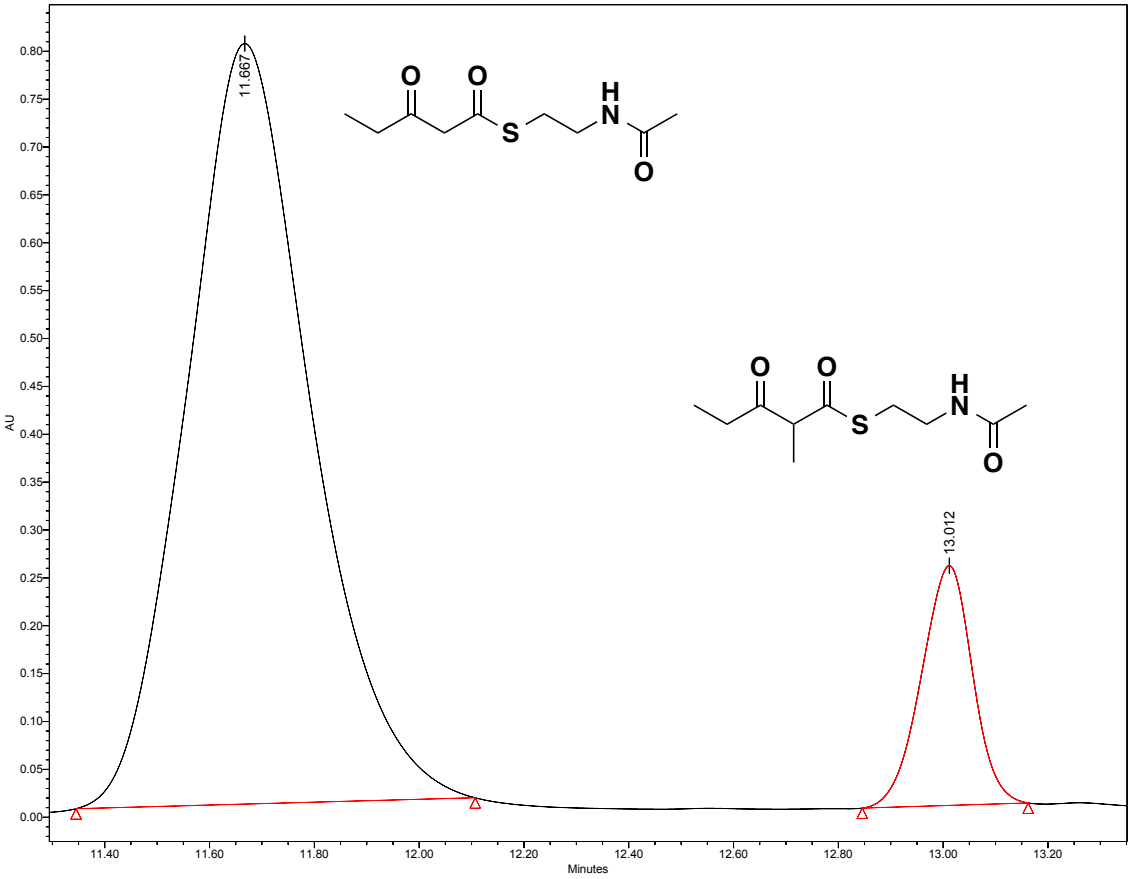
ESI-MS of phosphopantetheinylated GphACP1 thioesterified with malonyl-CoA (a) before and (b) after MT reaction. The methylated form was not observed after the reaction. Insets give expanded views of the deconvoluted spectra (mass range 13550-13700 Da) labeled with the monoisotopic masses and mass accuracies relative to the theoretical mass of the modified proteins.



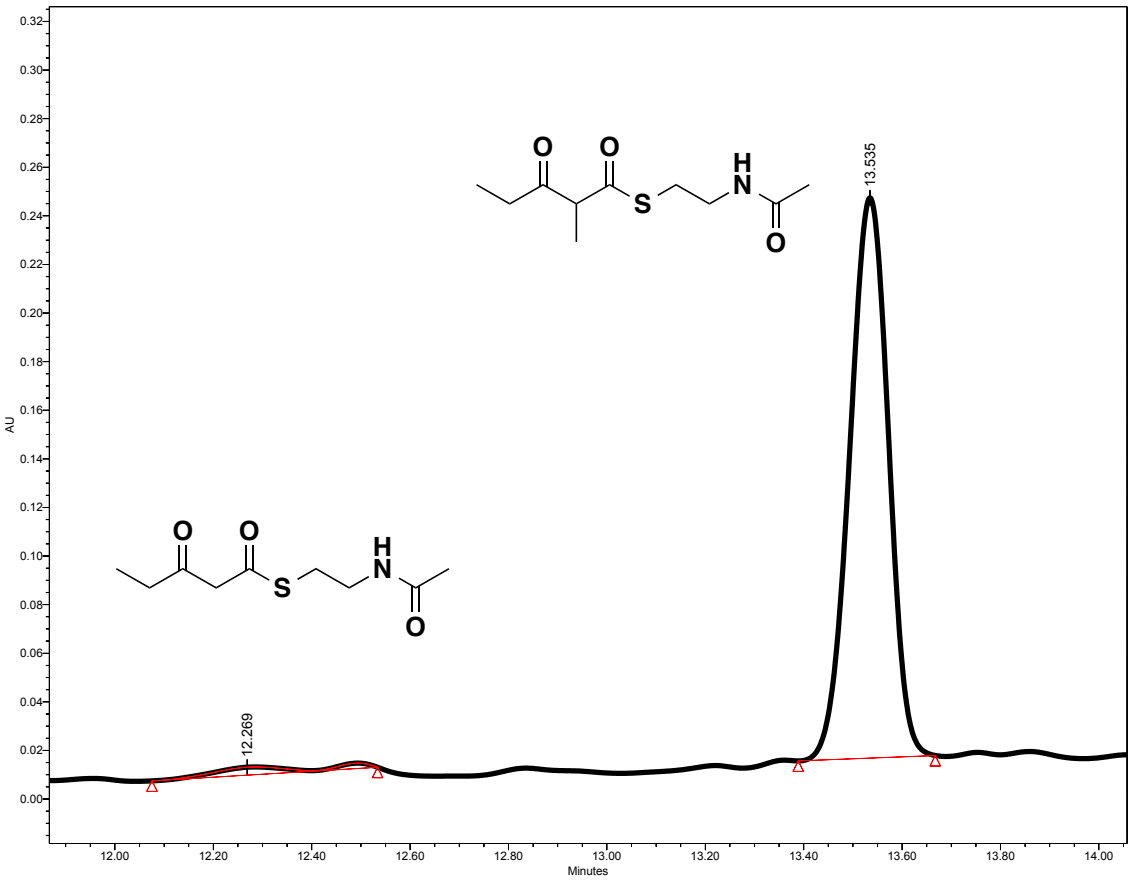
ESI-MS of phosphopantetheinylated GphACP6 thioesterified with malonyl-CoA (a) before and (b) after MT reaction. The methylated form was not observed after the reaction. Insets give expanded views of the deconvoluted spectra (mass range 13500-13620 Da) labeled with the monoisotopic masses and mass accuracies relative to the theoretical mass of the modified proteins.



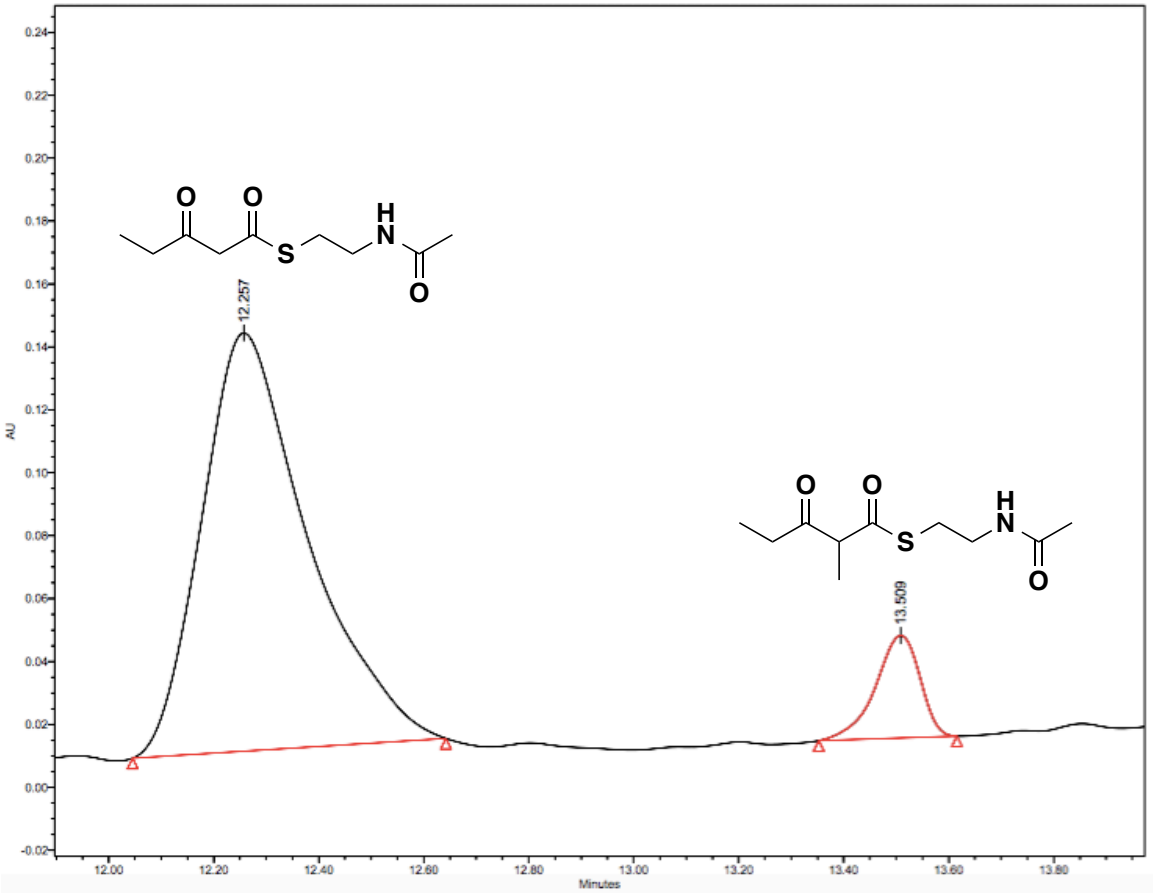
GphMT1 HPLC trace (monitoring 235 nm) 72 h without Pfs



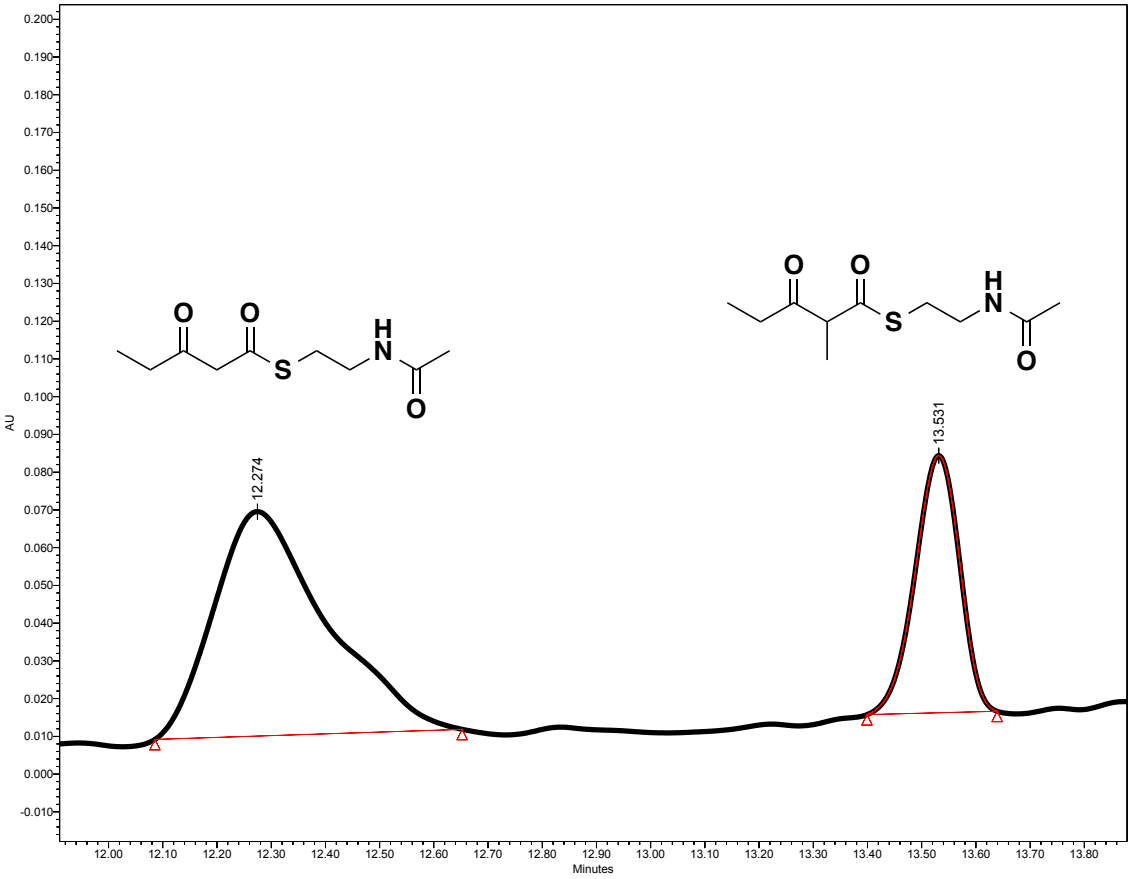
GphMT1 HPLC trace (monitoring 235 nm) 72 h with Pfs



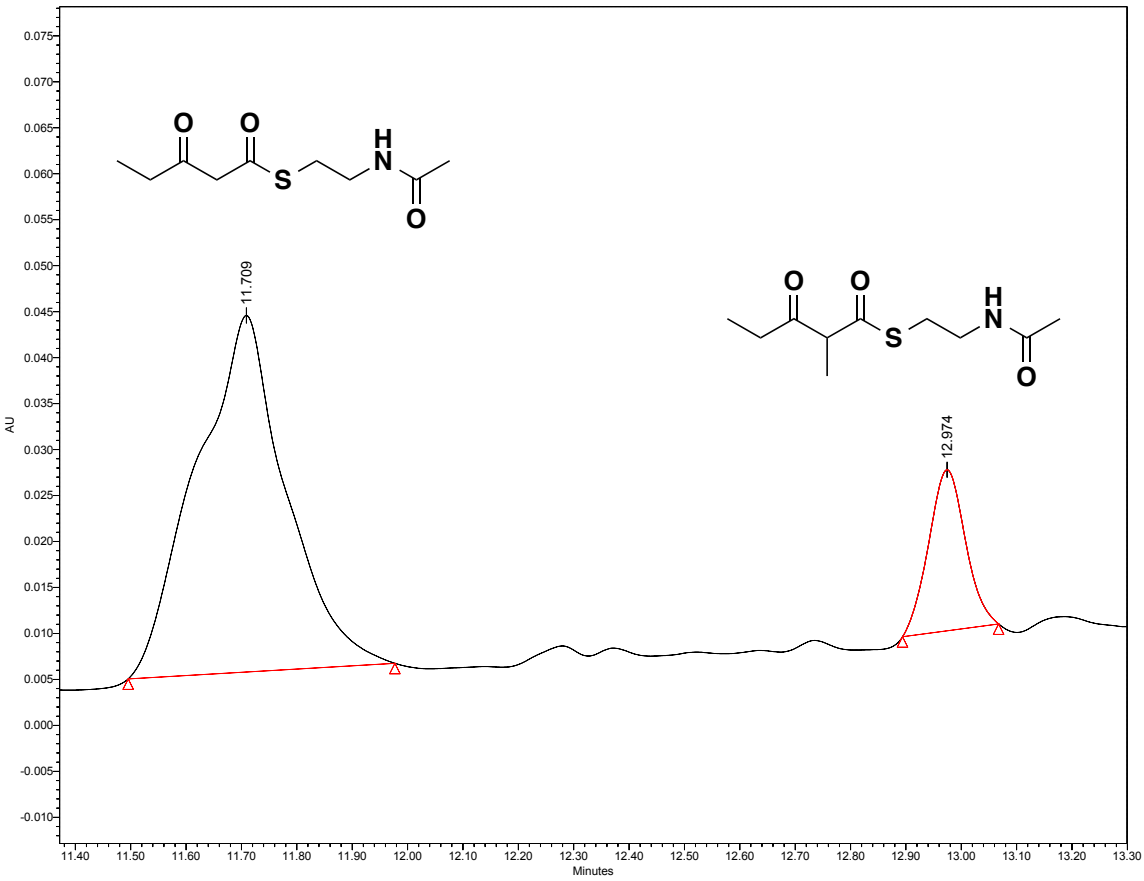
GphMT2 HPLC trace (monitoring 235 nm) 72 h without Pfs



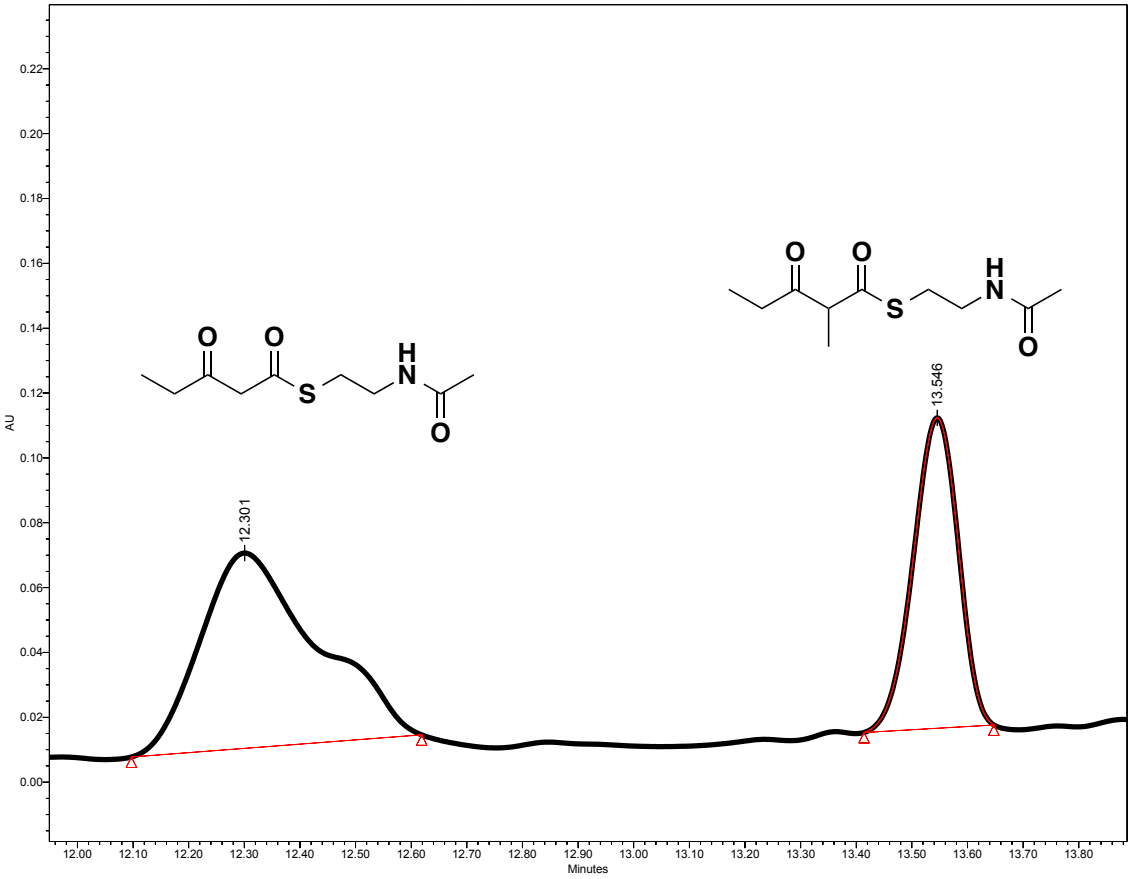
GphMT2 HPLC trace (monitoring 235 nm) 72 h with Pfs



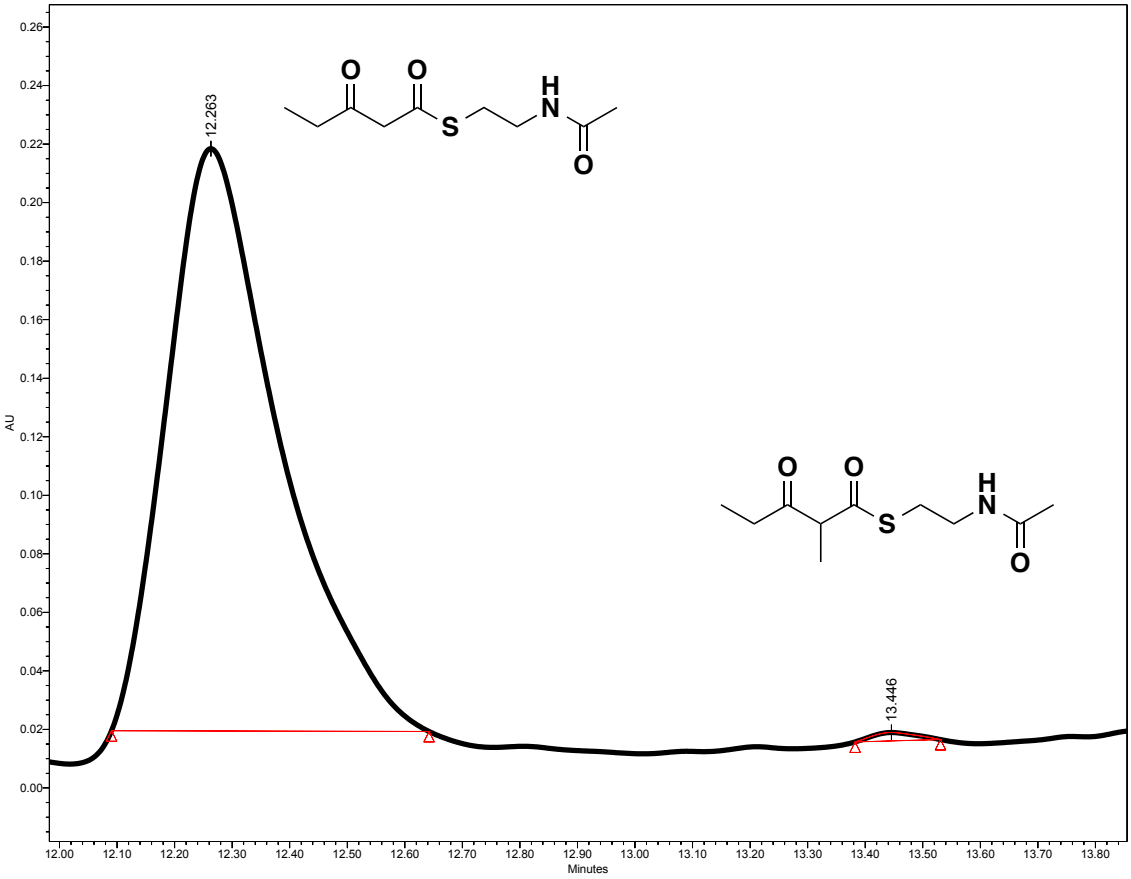
GphMT3 HPLC trace (monitoring 235 nm) 72 h without Pfs



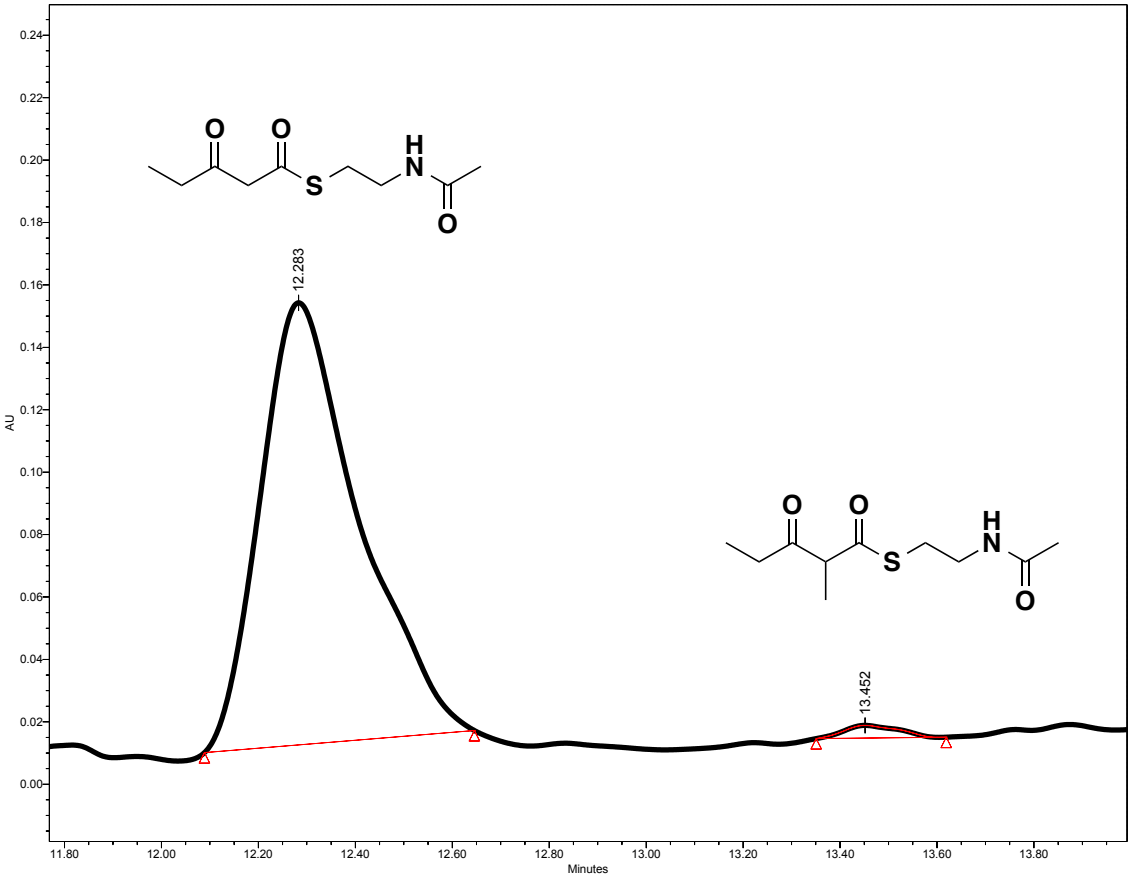
GphMT3 HPLC trace (monitoring 235 nm) 72 h with Pfs



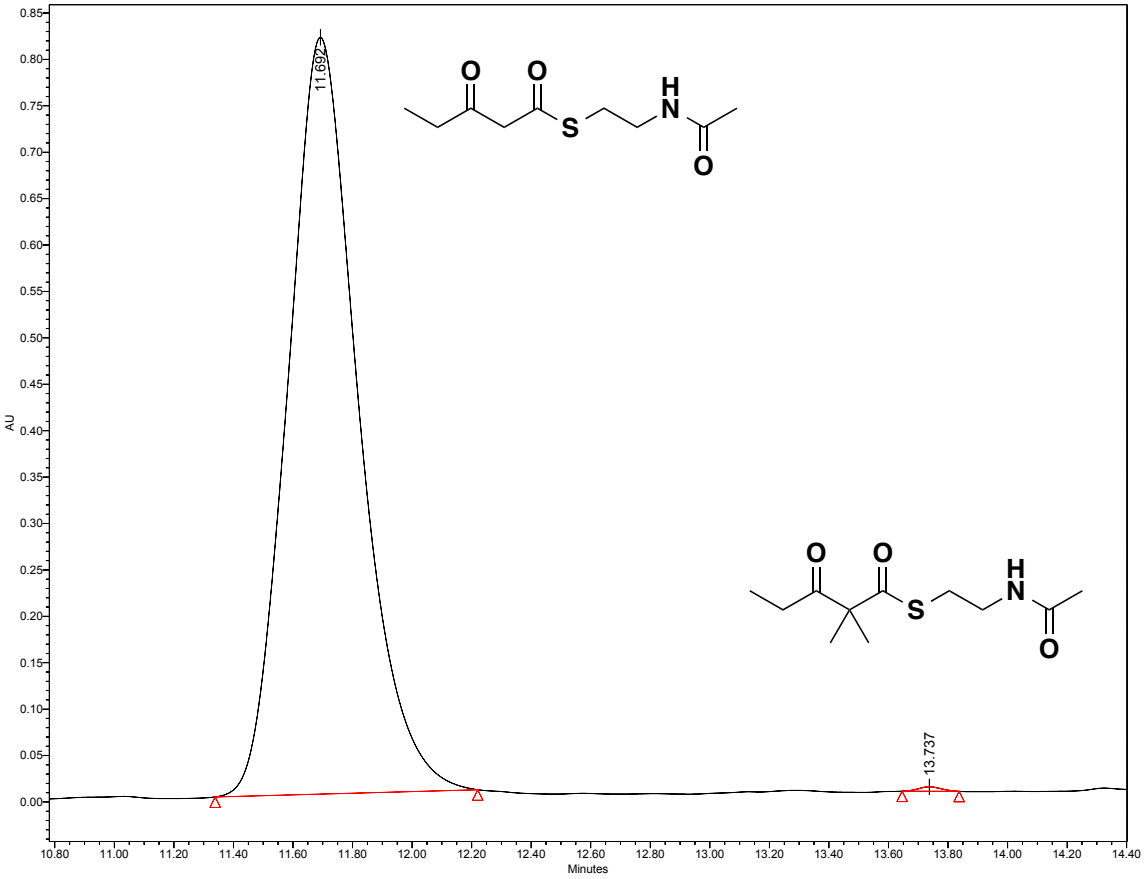
GphMT4 HPLC trace (monitoring 235 nm) 72 h without Pfs



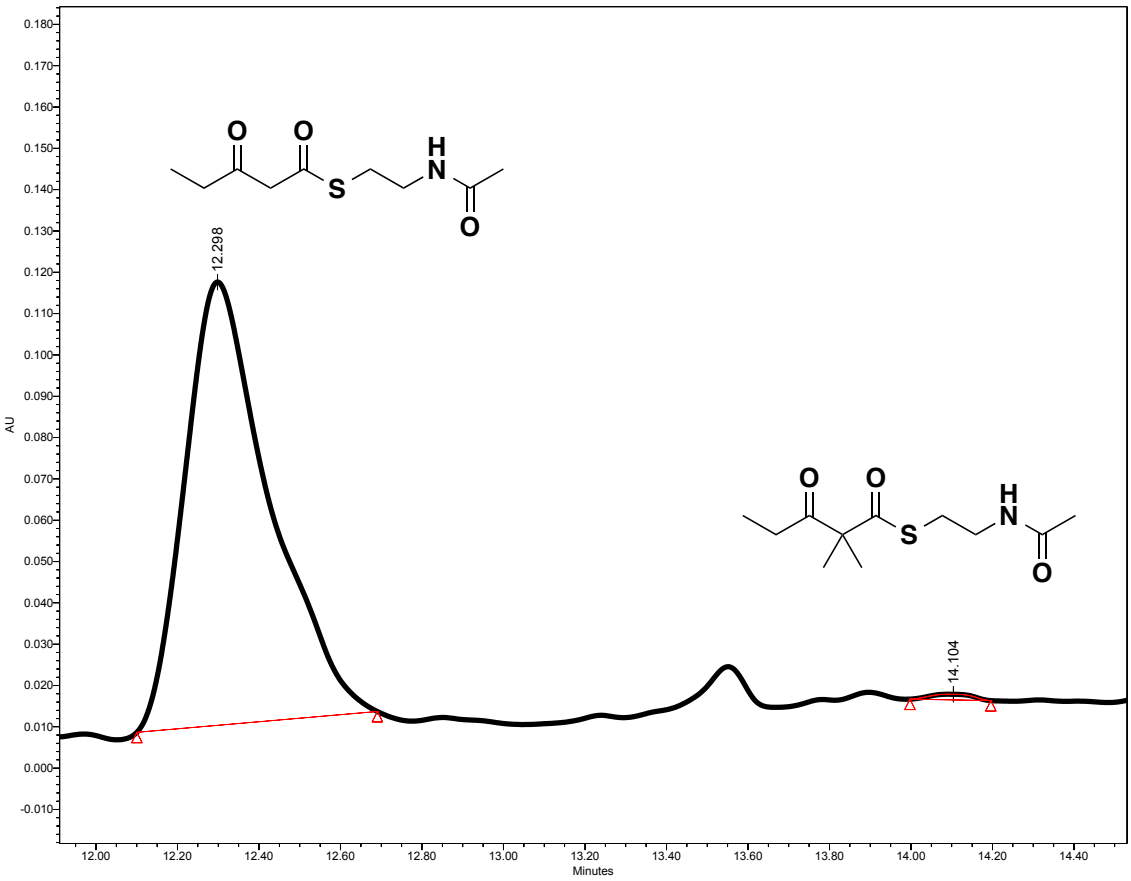
GphMT4 HPLC trace (monitoring 235 nm) 72 h with Pfs



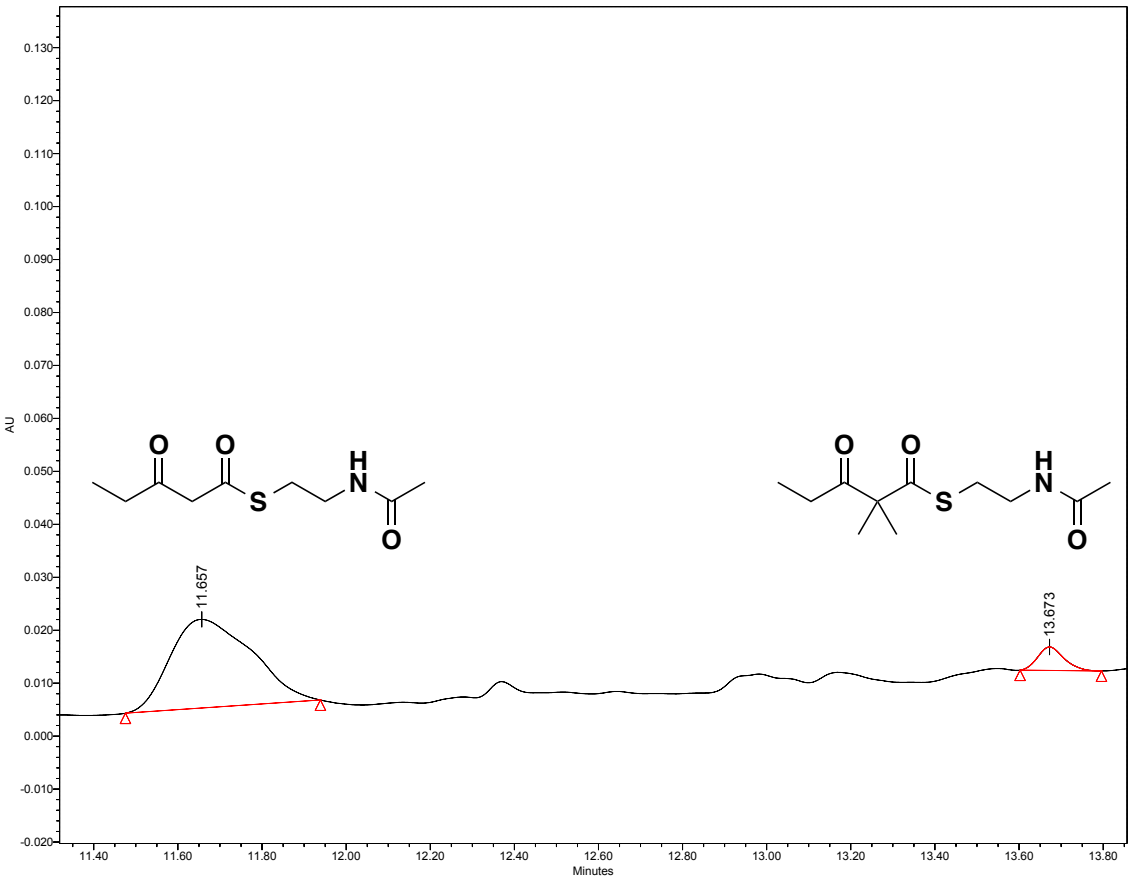
GphH module HPLC trace (monitoring 235 nm) 72 h without Pfs



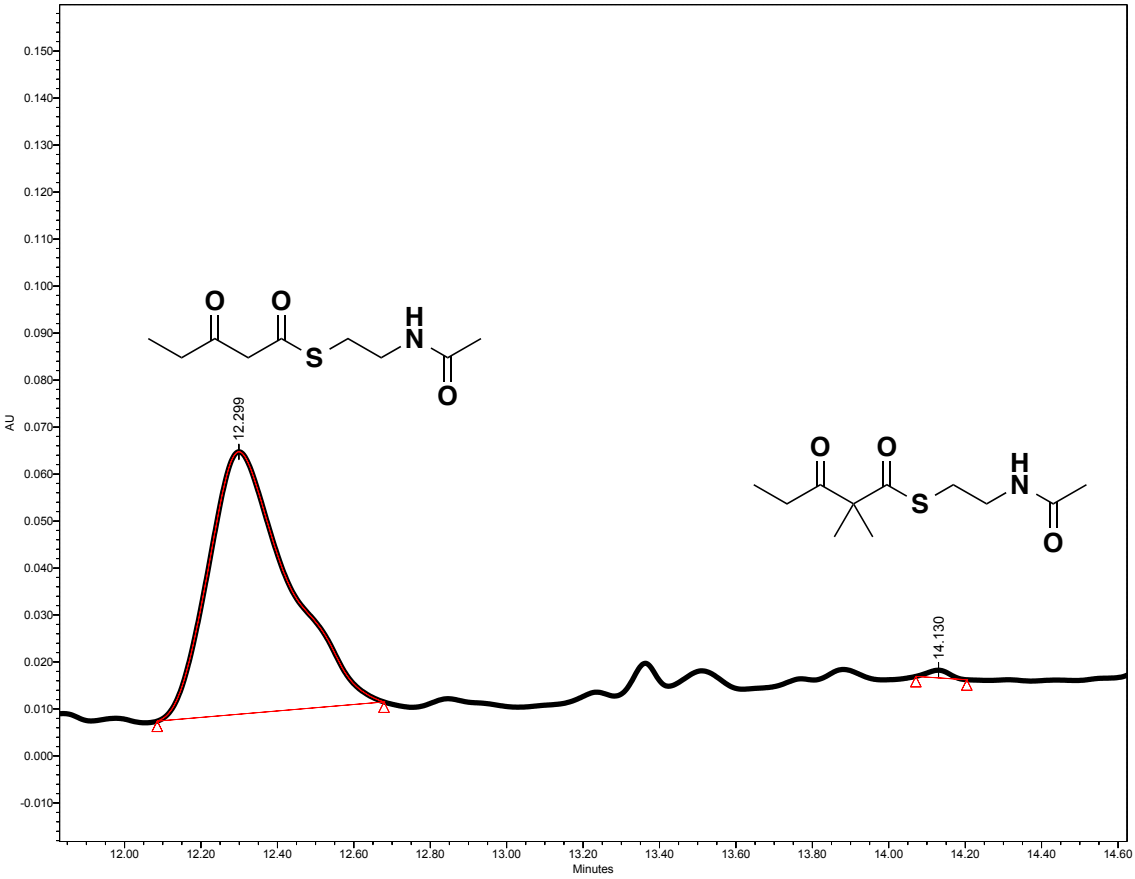
GphH module HPLC trace (monitoring 235 nm) 72 h with Pfs



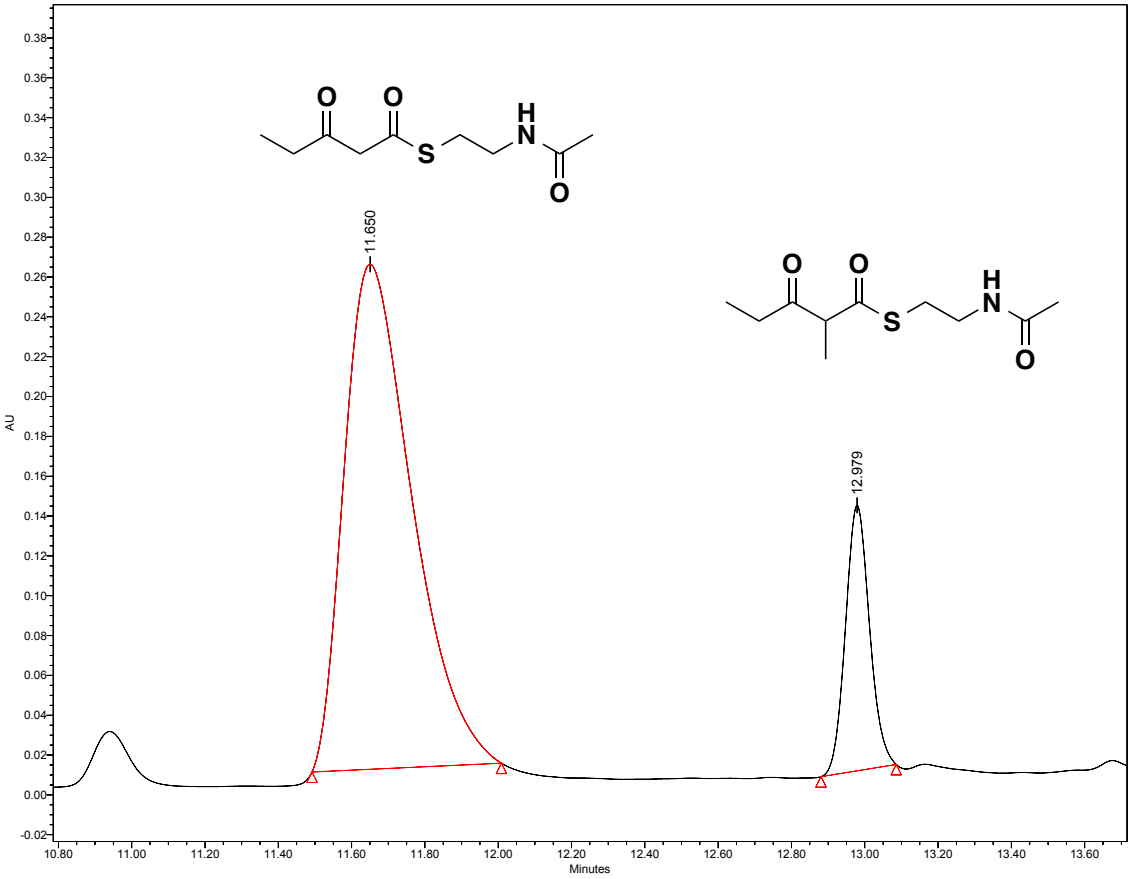
GphMT5 HPLC trace (monitoring 235 nm) 72 h without Pfs



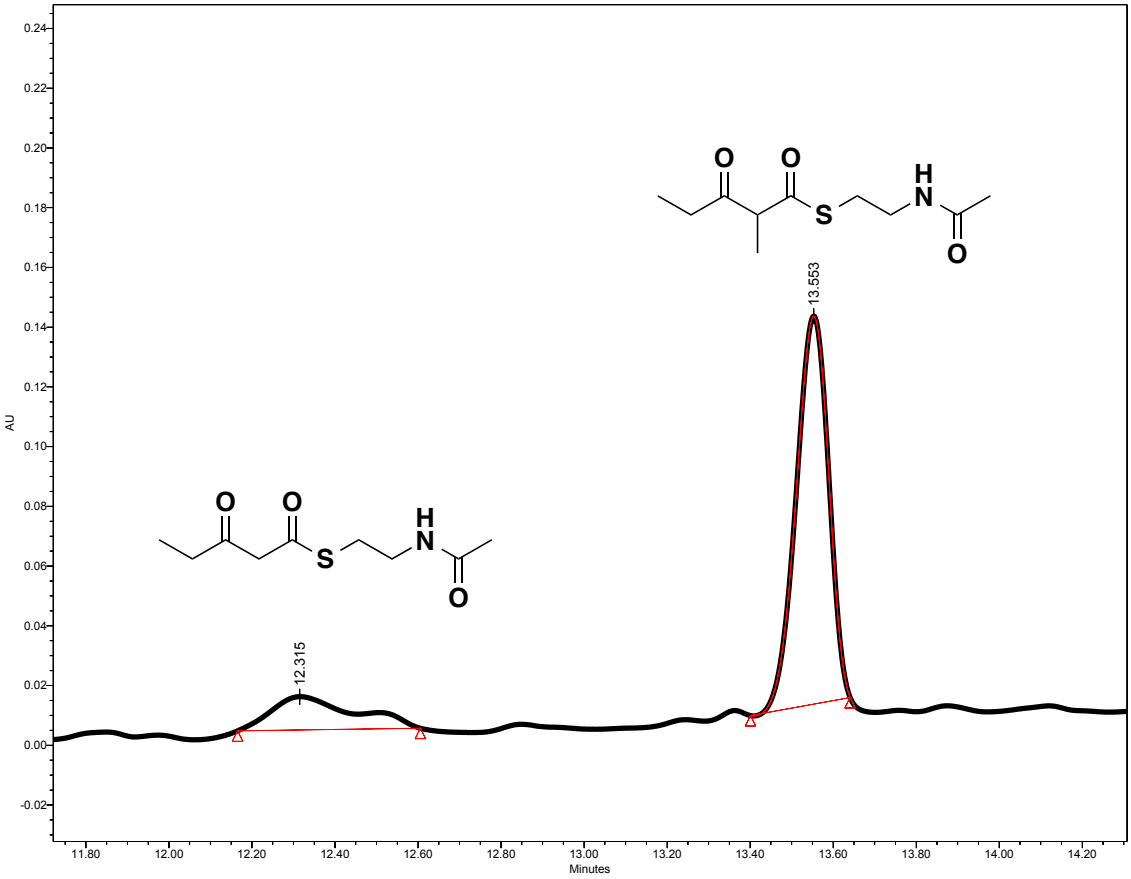
GphMT5 HPLC trace (monitoring 235 nm) 72 h with Pfs



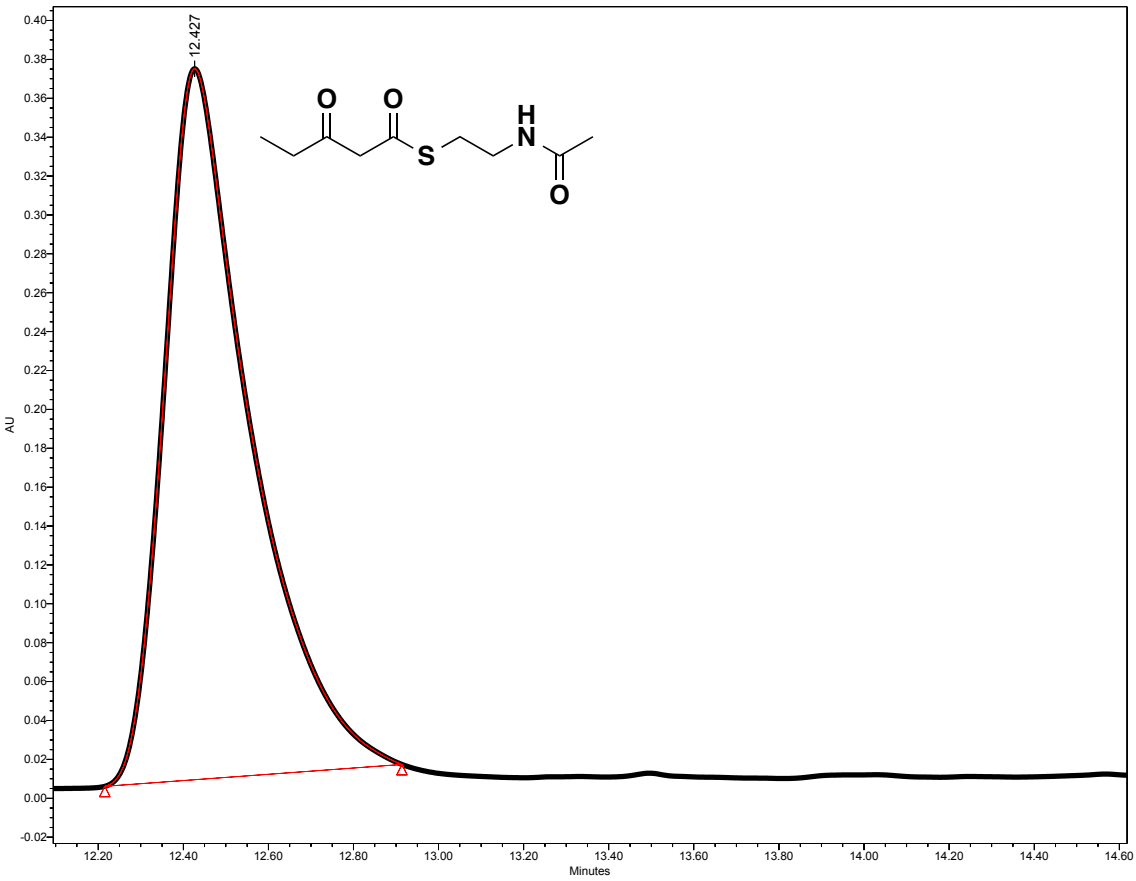
GphMT6 HPLC trace (monitoring 235 nm) 72 h without Pfs



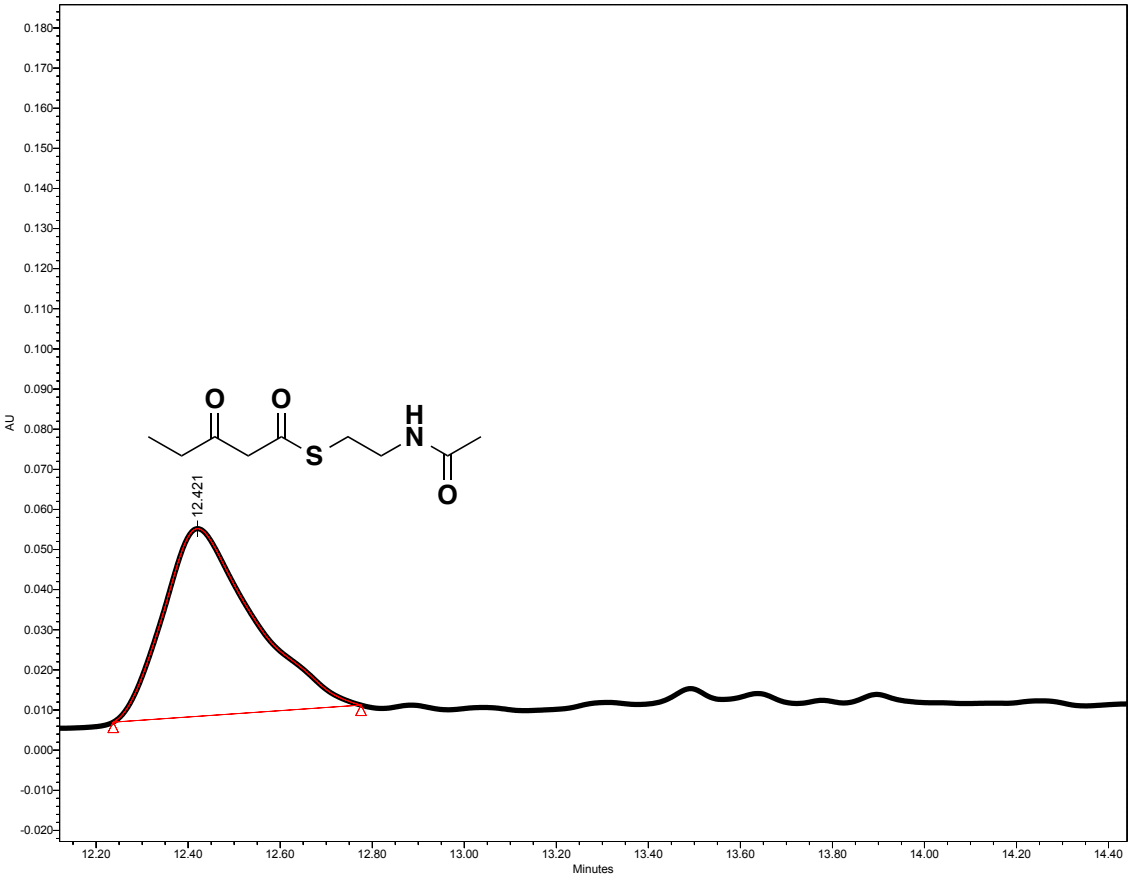
GphMT6 HPLC trace (monitoring 235 nm) 72 h with Pfs



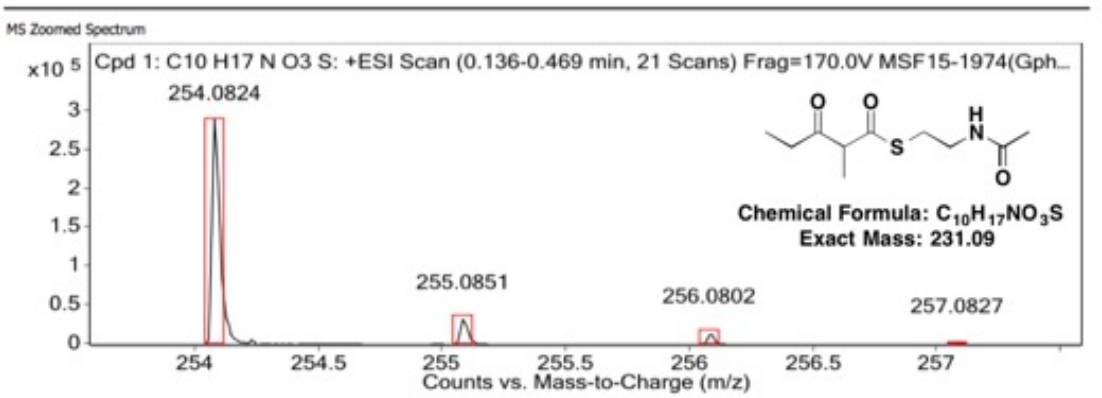
No Enzyme negative control HPLC trace (monitoring 235 nm) 72 h without Pfs



No Enzyme negative control HPLC trace (monitoring 235 nm) 72 h with Pfs



HRMS of HPLC purified **2** generated from **1** via GphMT1

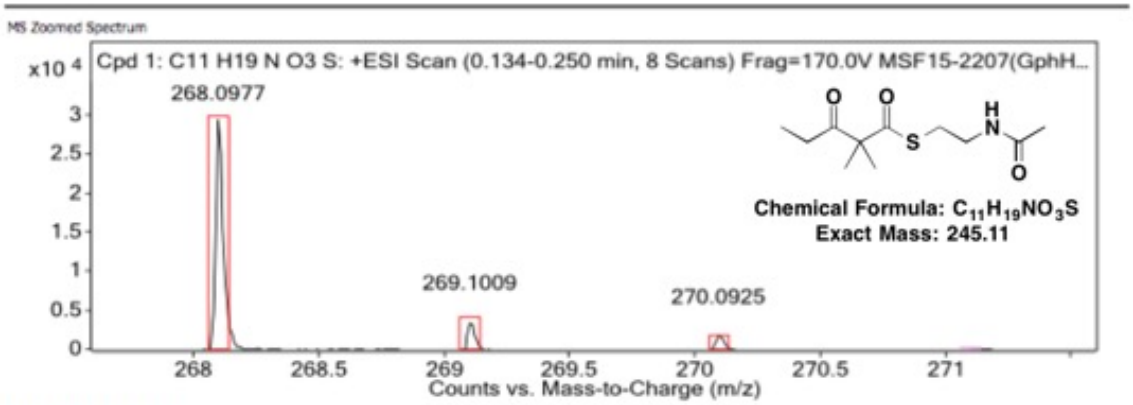


MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
254.08240	254.08210	1	289582.75	C10H17NO3S	(M+Na)+	-0.99
255.08510	255.08510	1	32091.79	C10H17NO3S	(M+Na)+	0
256.08020	256.08000	1	14219.6	C10H17NO3S	(M+Na)+	-0.57
257.08270	257.08250	1	1513.8	C10H17NO3S	(M+Na)+	-0.52
258.07510	258.08310	1	164.87	C10H17NO3S	(M+Na)+	30.9

--- End Of Report ---

HRMS of HPLC purified **3** generated from **1** via GphMT5

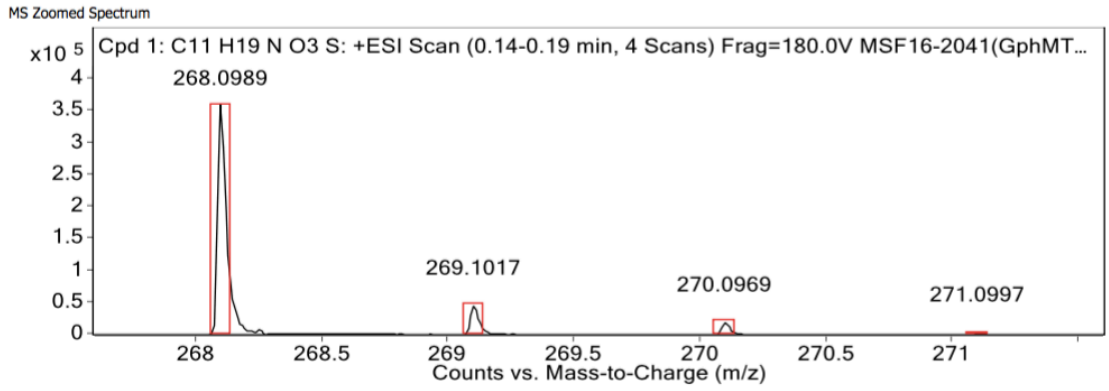


MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
268.09770	268.09780	1	29836.34	C11H19NO3S	(M+Na)+	0.43
269.10090	269.10080	1	3750.72	C11H19NO3S	(M+Na)+	-0.57
270.09250	270.09590	1	1969.95	C11H19NO3S	(M+Na)+	12.57
430.91370			93328.2			

--- End Of Report ---

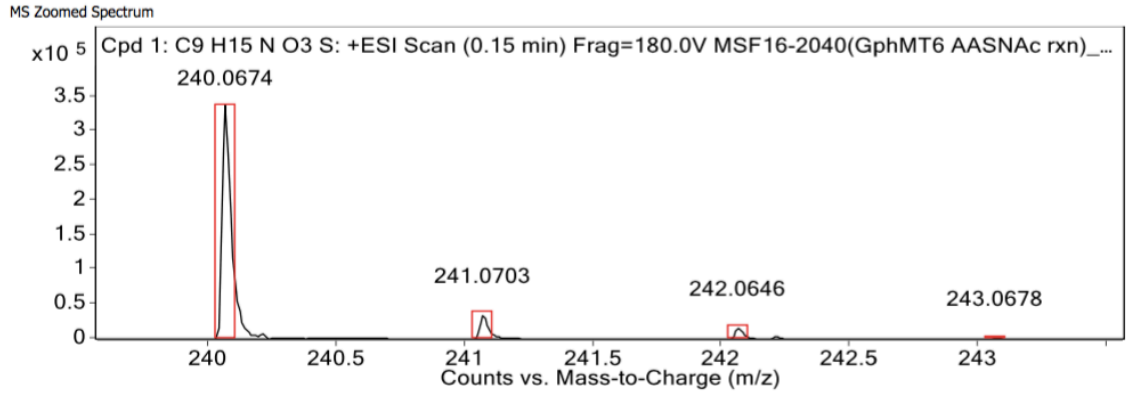
HRMS of HPLC purified **5** generated from **4** via GphMT6



MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
268.09890	268.09780	1	360081.49	C11H19NO3S	(M+Na)+	-4.24
269.10170	269.10080	1	45052.04	C11H19NO3S	(M+Na)+	-3.53
270.09690	270.09590	1	18776.23	C11H19NO3S	(M+Na)+	-3.78
271.09970	271.09830	1	3095.82	C11H19NO3S	(M+Na)+	-5.28
272.09880	272.09900	1	471.19	C11H19NO3S	(M+Na)+	0.59

HRMS of HPLC purified **7** generated from **6** via GphMT6



MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
240.06740	240.06650	1	337391.31	C9H15NO3S	(M+Na)+	-3.66
241.07030	241.06940	1	35095.04	C9H15NO3S	(M+Na)+	-3.77
242.06460	242.06420	1	16616.93	C9H15NO3S	(M+Na)+	-1.58
243.06780	243.06680	1	2126.64	C9H15NO3S	(M+Na)+	-4.29
244.06610	244.06720	1	494.45	C9H15NO3S	(M+Na)+	4.63

SUPPLEMENTARY FIGURES

Figure S1. SDS-PAGE gel of purified MT-containing PKS fragments: 1) MW marker, 2) GphMT1, 3) GphMT2, 4) GphMT3, 5) GphMT4, 6) GphMT5, 7) GphH (phosphopantetheinylated), 8) GphH, 9) GphMT6, 10) Pfs

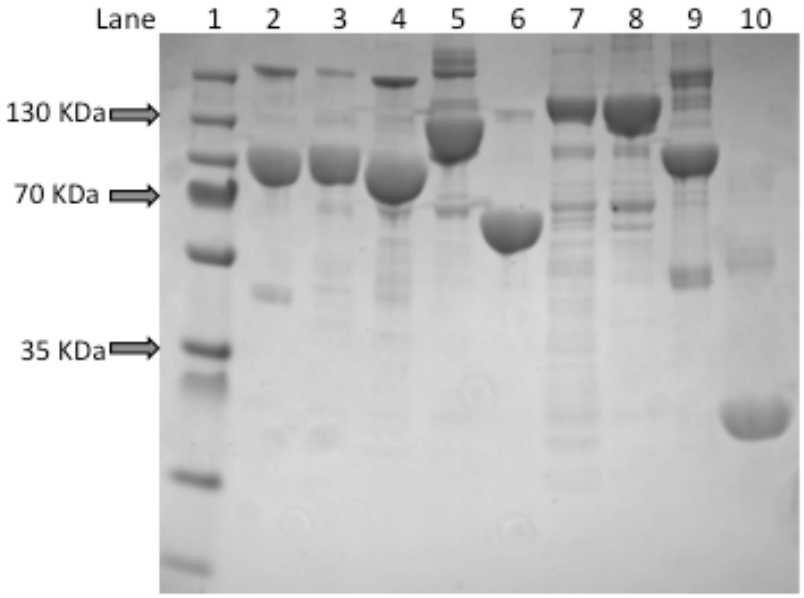


Figure S2. MAFFT multiple sequence alignment of MT domains from several *cis*-AT PKS systems.[3] Myc = microcystin, Nda = nodularin, Gph = gephyronic acid, Cur = curacin, Epo = epothilone, and Ybt = yersiniabactin. 'β1' indicates first β-strand of KR structural subdomain. The two subsequent black lines indicate SAM binding motif of MT domain and NADPH binding motif that begins the KR catalytic subdomain, respectively.

Figure S3. Conversion of **1** to **3** via GphH and HMT catalyzed dimethylation in the presence of Pfs.

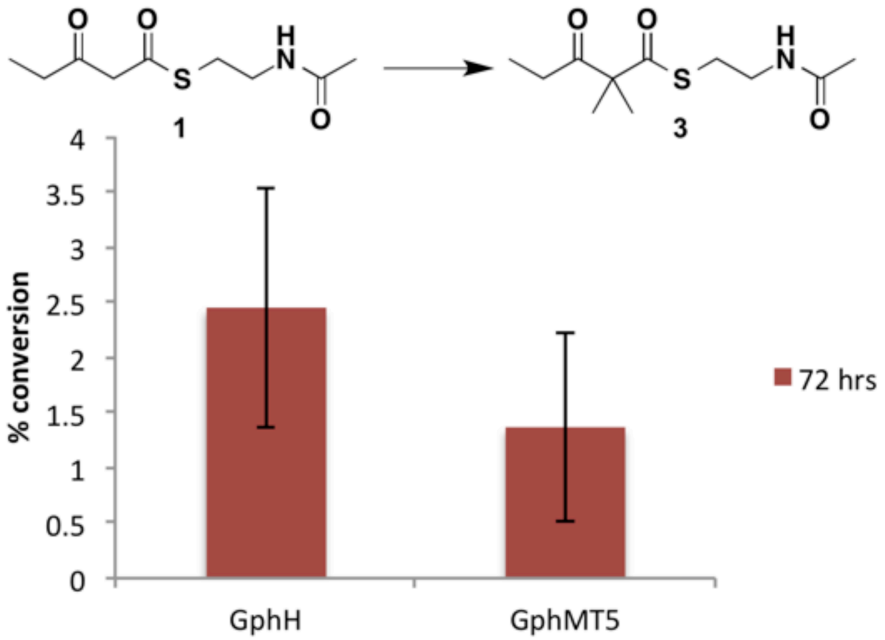


Figure S4. Reduction of **1** by GphMT constructs with relative conversions. *In vitro* reductions were run as previously reported.[2]

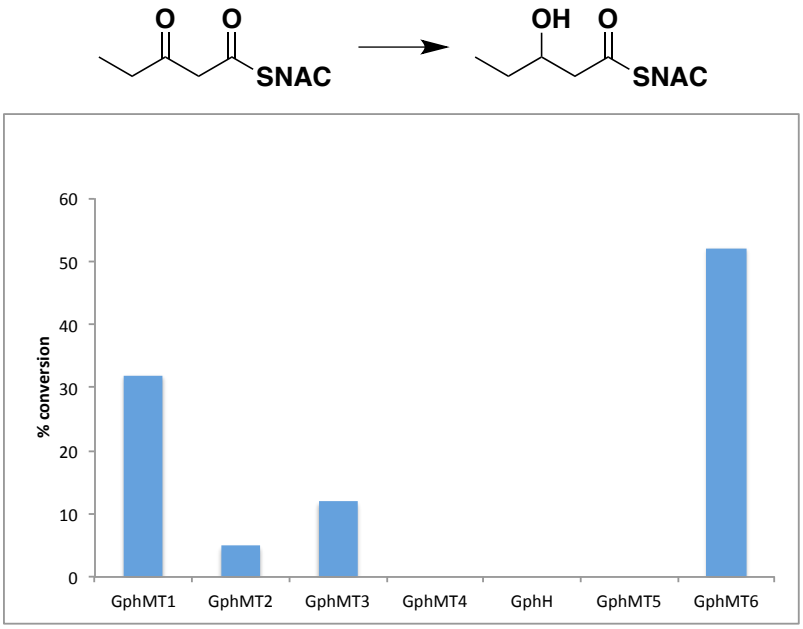
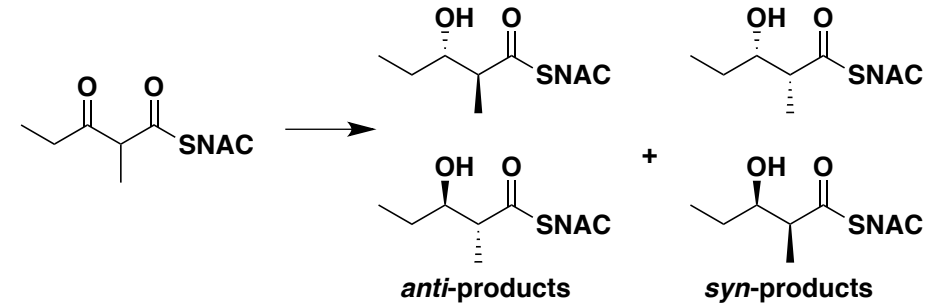
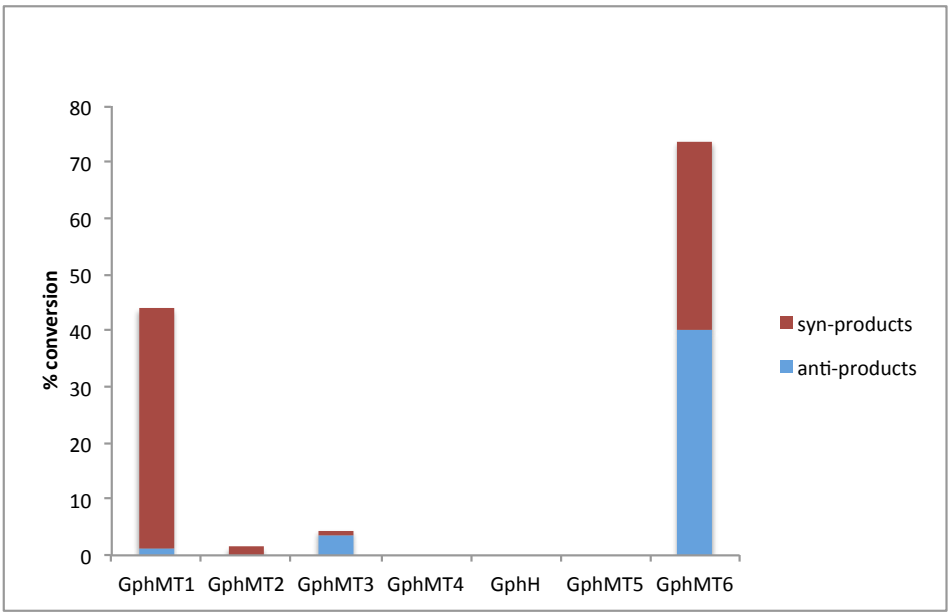


Figure S5. Reduction of **2** by KR-containing GphMT constructs with relative conversions. *In vitro* reductions were run as previously reported.[2]





SUPPLEMENTARY REFERENCES

1) Gay, G.; Wagner, D.T., Keatinge-Clay, A.T., Gay, D.C. *Plasmid* **2014**, *76C*, 66.
2) Piasecki, S.K.; Taylor, C.A.; Detelich, J.F.; Liu, J.; Zheng, J.; Komsoukaniants, A.; Siegel, D.R.; Keatinge-Clay, A.T. *Chem. Biol.* **2011**, *18*, 1331.
3) Katoh, K.; Standley, D.M. *Mol. Biol. Evol.* **2013**, *4*, 772.