Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Investigating the Reactivities of a Polyketide Synthase Module through Fluorescent Click Chemistry

Amanda J. Hughes, Matthew R. Tibby, Drew T. Wagner, Johnathan N. Brantley and Adrian T. Keatinge-Clay*

Department of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station A5300, Austin, TX 78712, United States

**corresponding author:* adriankc@utexas.edu

Table of Contents

EXPERIMENTAL PROCEDURES

Synthesis of methylmalonyl-*S*-*N*-acetylcysteamine thioester (mm-*S*-NAC, 1)

Mono-tert-butyl methylmalonic acid. To methyl meldrum's acid (8.5 g, 138 mmol) was added *tert*-butanol (40 mL) and pyridine (2 mL). The mixture was heated at 80 °C for 24 hrs. The reaction was then cooled to 23 °C and concentrated *in vacuo* to furnish mono-*tert*-butyl methylmalonic acid.

Tert-butyl-methylmalonyl-S-N-acetylcysteamine. To a stirred solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI; 3.9 g, 21 mmol, 1.1 eq) in dichloromethane (DCM, 60 mL) at 23 °C was added triethylamine (TEA; 2.9 mL, 21 mmol, 1.1 eq). To the yellow solution was added the mono-*tert*-butyl methylmalonic acid (3.0 g, 19 mmol, 1.0 eq) followed by *N*-acetylcysteamine (NAC; 2.0 mL, 21 mmol, 1.1 eq). The resulting solution was stirred for 48 hrs, then diluted with ethyl acetate (50 mL) and washed with 0.1 M HCl (4 x 10mL). The organic layer was dried over sodium sulfate and concentrated *in vacuo*.

Methylmalonyl-S-N-acetylcysteamine. To a stirred solution of *tert*-butyl-methylmalonyl-*S*-NAC (500 mg, 1.8 mmol, 1.0 eq) in DCM (9 mL) at 0 °C under argon was added 4.0 M HCl in dioxane (4.5 mL, 18 mmol, 10.0 eq). After 18 hrs, the reaction was concentrated *in vacuo* and purified by dry flash column chromatography (silica, eluting with 10:1 DCM:methanol) to afford compound **1**. ^{1}H NMR characterization was consistent with a previously reported synthesis (1).

Methylmalonyl-*S*-*N*-acetylcysteamine thioester (mm-*S*-NAC, **1**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.71 (q, 1H, *J* = 7.2), 3.48 (q, 2H, *J* = 6.0), 3.10 (dtd, 2H, *J* = 47.6, 14.2, 6.4), 1.98 (s, 3H), 1.45 (d, 3H, *J* = 7.2)

Synthesis of methylmalonyl-ethanethiol thioester (mm-*S*-Et, 2)

To a round-bottom flask charged with methylmalonic acid (10 g, 5 mmol, 1.0 eq), was added 4-dimethylaminopyridine (DMAP; 1 g, 8.5 mmol, 0.1 eq) followed by toluene (250 mL). Upon stirring, phosphoryl chloride ($POCl_3$; 8.8 mL, 93 mmol, 1.1 eq) was added followed by the dropwise addition of ethanethiol (6.9 mL, 93 mmol, 1.1 eq). The reaction was heated to 60 °C under an argon atmosphere. After 18 hrs, the reaction was cooled to 23 °C and concentrated *in vacuo*. The residue was suspended in DCM, and purified via dry flash column chromatography (silica, eluting with an ethyl acetate:hexanes gradient from 1:9 to 1:3) to furnish compound **2**.

Methylmalonyl ethanethiol thioester (**2**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67 (q, 1H, *J* = 7.1), 2.94 (q, 2H, *J* = 7.4), 1.47 (d, 3H, *J* = 7.2), 1.28 (t, 3H, *J* = 7.4)

Synthesis of 3, 6-10

To a round-bottom flask charged with 75 mL of anhydrous DCM cooled to 0 °C under an atmosphere of argon was dissolved EDCI (1.054 g, 5.5 mmol, 1.1 eq), TEA (773 μL, 5.5 mmol, 1.1 eq), 4-pentynoic acid (**6** and **8**, 490 μL, 5.0 mmol, 1.0 eq) or 5-hexynoic acid (**3** and **9**, 562 μL, 5.0 mmol, 1.0 eq) or 6-heptynoic acid (**7** and **10**, 633 μL, 5.0 mmol, 1.0 eq), and catalytic DMAP (307 mg, 2.5 mmol, 0.5 eq). The reaction was stirred on ice for 10 min before the addition of NAC (**3**, **6-7**, 504 μL, 4.75 mmol, 0.95 eq) or ethanethiol (**8-10**, 342.3 μL, 4.75 mmol, 0.95 eq) and was allowed to warm to room temperature. After 16 hrs, the reaction was concentrated *in vacuo* and the remaining oil was resuspended in 50 mL of water. The product was extracted with ethyl acetate (1 x 150 mL) and the organic layer was washed with 0.1 M HCl (2 x 50 mL) and saturated sodium bicarbonate (1 x 50 mL). The organic layer was dried over anhydrous sodium sulfate, and product was concentrated *in vacuo*. Products were purified by dry flash column chromatography (silica, eluting with 10:90 ethyl acetate:hexanes) to afford title compounds **3, 6–10**. Products were characterized by ^{1}H NMR.

5-Hexynoyl-*S*-*N*-acetylcysteamine (**3**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (bs, 1H), 3.44 (q, 2H, *J* = 6.0), 3.04 (t, 2H, *J* = 6.6), 2.74 (t, 2H, *J* = 7.6), 2.26 (dt, 2H, *J* = 6.9, 2.6), 1.99 (t, 1H, *J* = 2.6), 1.97 (s, 3H), 1.89 (m, 2H, *J* = 7.7)

4-Pentynoyl-*S*-*N*-acetylcysteamine (**6**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (bs, 1H), 3.44 (q, 2H, *J* = 6.0), 3.06 (t, 2H, *J* = 6.6), 2.81 (t, 2H, *J* = 7.3), 2.54 (dt, 2H, *J* = 7.2 and 2.6), 1.99 (t, 1H, *J* = 2.6), 1.96 (s, 3H)

6-Heptynoyl-*S*-*N*-acetylcysteamine (**7**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (bs, 1H), 3.43 (q, 2H, *J* = 6.1), 3.03 (t, 2H, *J* = 6.6), 2.61 (t, 2H, *J* = 7.3), 2.21 (dt, 2H, *J* = 7.1, 2.7), 1.97 (s, 3H), 1.96 (t, 1H, *J* = 2.7), 1.79 (m, 2H, *J* = 7.6), 1.60-1.53 (m, 2H, *J* = 7.1)

4-Pentynoyl-*S*-ethanethiol (**8**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.90 (q, 2H, *J* = 7.4), 2.77 (t, 2H, *J* = 7.2), 2.53 (dt, 2H, *J* = 6.8 and 2.6), 1.98 (t, 1H, *J* = 2.6), 1.25 (t, 3H, *J* = 7.4)

5-Hexynoyl-*S*-ethanethiol (**9**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.88 (q, 2H, *J* = 7.4), 2.68 (t, 2H, *J* = 7.2), 2.25 (dt, 2H, *J* = 6.9 and 2.8), 1.98 (t, 1H, *J* = 2.7), 1.88 (m, 2H, *J* = 7.8), 1.25 (t, 3H, *J* = 7.4)

6-Heptynoyl-*S*-ethanethiol (**10**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.87 (q, 2H, *J* = 7.4), 2.57 (t, 2H, *J* = 7.3), 2.21 (dt, 2H, *J* = 7.0, 2.6), 1.95 (t, 1H, *J* = 2.6), 1.79 (m, 2H, *J* = 7.8), 1.60-1.53 (m, 2H, *J* = 7.5), 1.25 (t, 3H, *J* = 7.4)

Synthesis of 15

To a round-bottom flask charged with a stir bar and **2** (50 μL, 0.37 mmol, 2.0 eq) under an atmosphere of argon was added anhydrous tetrahydrofuran (THF; 1 mL) and magnesium ethoxide (21.4 mg, 0.19 mmol, 1.0 eq). To a separate round-bottom flask charged with a stir bar and 5-hexynoic acid (63 μL, 0.56 mmol, 3.0 eq) under an atmosphere of argon was added anhydrous THF (3 mL) and carbonyldiimidazole (CDI; 90.8 mg, 0.56 mmol, 3.0 eq). Reactions were stirred at room temperature. After 1 hr, the reaction generating the magnesium salt of methylmalonyl ethanethioester was concentrated *in vacuo*. To the residue, the solution of the acyl imidazole was added, and the reaction was stirred under argon for an additional 16 hrs. The reaction was concentrated *in vacuo* and resuspended in 0.1 M HCl (10 mL) and ethyl acetate (50 mL). The organic layer was washed with 0.1 M HCl (1 x 30 mL) and then with saturated sodium chloride (1 x 10 mL). The organic layer was dried over anhydrous sodium sulfate, and product was isolated *in vacuo*. The product was purified by dry flash column chromatography (silica, eluting with 5:95 ethyl acetate:hexanes) to afford compound **15**.

(2*RS*)-Methyl-3-oxo-hept-6-ynoyl-*S*-ethanethiol (**15**) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74 (q, 2H, *J* = 7.1), 2.92 (q, 2H, *J* = 7.4), 2.79 (m, 2H, *J* = 27.6), 2.22 (m, 2H), 1.94 (t, 1H, *J* = 2.6), 1.38 (d, 3H, *J* = 7.1), 1.26 (t, 3H, *J* = 7.4)

Synthesis of sulforhodamine B azide

Synthesis of 2-azidoethanol followed previously described method (2). A 25 mL Schlenk flask was charged with 2-azidoethanol (17 mg, 0.19 mmol, 1.1 eq), TEA (2.6 μL, 0.19 mmol, 1.1 eq) and DCM (5 mL). The resulting solution was cooled to 0 °C and allowed to stir for 10 min. A sulforhodamine B acid chloride solution in 5 mL DCM (100 mg, 0.17 mmol, 1.0 eq) was subsequently added dropwise via syringe. The resulting solution was stirred at 0 °C for 30 min and then allowed to stir at room temperature. Upon reacting for 12 hrs, the product was isolated *in vacuo* to afford a metallic green residue. The product was first to elute upon purification by dry flash column chromatography (silica, eluting with 90:10 chloroform:methanol).

Engineering EryMod6TE mutant plasmids

The formation of EryMod6TE plasmid was described previously (3). Site-directed mutagenesis of EryMod6TE plasmid was performed to engineer mutant plasmids using single-step mutagenesis protocol outlined by Liu and coworkers with the following primers (italics depicts overlapping region and alanine residue is underlined) (4): ΔKS forward 5'-*ACCTGT GCCGTCGGAGAAGGTCGG*ACCACCGGAATGTGAGGCGAAACCG-3'; ΔKS reverse 5'-GGC TTAGAAGGTCCGGCGGTAACCG*TGGACACGGCAGCCTCTTCCAGCC*-3'; ΔAT forward 5'-*CGGCAATAGCCAGTACGAGTCCCG*CTTTAACGCCGCCGGCAGCAC-3'; ΔAT reverse 5'-CGT GTGGGGTGAGCCCGTCA*GCCGTTATCGGTCATGCTCAGGGC*-3'; ΔTE forward 5'-*CAG*

*GCCACGCCGCCGGCG*CACTCATGGCCTATGCACTCGCGAC-3'; ΔTE reverse 5'-CGTG GGAACCACTGTTTGGAAAGCACCATC*GTCCGGTGCGGCGGCCGC*-3'.

PCR reaction conditions to engineer **Δ**KS, **Δ**AT and **Δ**TE plasmids was performed using the following conditions (50 mL total volume reactions): 1X Phusion GC buffer, 5 ng EryMod6TE plasmid, 1 mM each corresponding forward and reverse primers, 200 mM dNTPs, 3% v/v DMSO and 3 U Phusion DNA polymerase.

PCR reaction conditions to engineer **Δ**KS+AT plasmid was performed using the following conditions (50 mL total volume reactions): 1X Phusion GC buffer, 5 ng **Δ**AT plasmid, 1 mM each **Δ**KS forward and reverse primers, 200 mM dNTPs, 3% v/v DMSO and 3 U Phusion DNA polymerase.

PCR reaction conditions to engineer **Δ**KS+TE plasmid was performed using the following conditions (50 mL total volume reactions): 1X Phusion GC buffer, 5 ng **Δ**TE plasmid, 1uM each **Δ**KS forward and reverse primers, 200 mM dNTPs, 3% v/v DMSO and 3 U Phusion DNA polymerase.

PCR reaction conditions to engineer **Δ**AT+TE plasmid was performed using the following conditions (50 mL total volume reactions): 1X Phusion GC buffer, 5 ng **Δ**TE plasmid, 1 mM each **Δ**AT forward and reverse primers, 200 mM dNTPs, 3% v/v DMSO and 3 U Phusion DNA polymerase.

Protein expression, dialysis, and purification

EryMod6TE expression plasmids were transformed into *E. coli* K207-3 cells (5), while the glucose dehydrogenase (GDH) and the EryTE expression plasmids were each transformed into *E. coli* BL21(DE3) cells (6). Starter cultures (60 mL) were grown overnight and used to inoculate Luria broth supplemented with 50 mg/L kanamycin (6 L, 37 °C). Cultures were cooled to 15 °C after cells reached OD_{600} = 0.4, and protein expression was induced (0.5 mM IPTG). After 16 hrs, cells were harvested by centrifugation (4,500 x g, 10 min) and resuspended in 40 mL lysis buffer (0.5 M NaCl, 30 mM HEPES pH 7.5). Cells were lysed via sonication, and cellular debris was removed (centrifugation at 30,000 x g, 60 min). EryMod6TE and GDH lysates were dialyzed against 1 L lysis buffer at 4 °C. After 4 hrs, the buffer was exchanged with 1 L fresh lysis buffer, and allowed to dialyze for an additional 16 hrs. Lysates were centrifuged (4,000 x g, 10 min), flash-frozen, and stored at -80 °C until further use. EryTE was further purified for TE-mediated hydrolysis experiments by passing the cell lysate over a nickel-NTA column equilibrated with lysis buffer. The column was washed with lysis buffer containing 15 mM imidazole (25 mL) and protein was eluted with lysis buffer containing 150 mM imidazole (5 mL).

Conditions for EryMod6TE reactions

The following stock solutions were prepared: 1.5 M Tris-HCl pH 7.5, 5 M NaCl, 3 M glucose, 100 mM $NADP^+$, 0.5 M **1** and **2** in DMSO, and 0.1 M **3, 6-10** in DMSO.

Tris-HCl pH 7.5 (150 mM), NaCl (100 mM), glucose (500 mM), $NADP^+$ (10 mM), extender unit **1** or **2** (5 mM), priming unit **3** or **6-10** (2.5 mM), pure DMSO (5% v/v), water, dialyzed GDH lysate (10% v/v), and dialyzed EryMod6TE lysate (25% v/v) were combined in a microcentrifuge tube (200 mL reaction volume). After addition of each dialyzed enzyme lysate, reactions were gently mixed by pipetting and then centrifuged (3000 x g, 5 sec). Reactions were incubated at 23 °C for 16 hrs before being subjected to CuAAC with sulforhodamine B azide. Negative control reactions contained all substrates except for EryMod6TE dialyzed lysate. Reactions were performed in duplicate.

Preparative *in vitro* synthesis of reduced diketide 5

Tris-HCl pH 7.5 (150 mM), NaCl (100 mM), glucose (500 mM), $NADP^+$ (10 mM), extender unit **2** (5 mM), priming unit **3** (2.5 mM), pure DMSO (total 5% v/v), water, dialyzed GDH lysate (10% v/v), and dialyzed EryMod6TE lysate (25% v/v) were combined in a conical tube in a total volume of 25 mL. After the addition of each dialyzed lysate, reactions were gently mixed by pipetting and then centrifuged (3000 x g, 5 sec). After 16 hrs, the reaction was washed using diethyl ether (1 x 50 mL). The aqueous layer was then acidified using 12 N HCl (2 mL) and products were extracted with ethyl acetate (3 x 150 mL). The organic layer was dried over sodium sulfate and concentrated *in vacuo*. Diketide **5** was purified by dry

flash column chromatography (silica, eluting with 10:89:1 ethyl acetate:hexanes:acetic acid) and characterized by ^{1}H NMR.

Phosphate-containing reaction conditions

Tris-HCl pH 7.5 (150 mM), NaCl (100 mM), extender unit **2** (5 mM), priming unit **3** (2.5 mM), pure DMSO (total 5% v/v), Na_2HPO_4 pH 7 (0, 10, 50 or 100 mM), water, dialyzed GDH lysate (10% v/v) and dialyzed EryMod6TE lysate (25% v/v) were combined in a microcentrifuge tube (200 mL total volume). After addition of each dialyzed lysate, reactions were gently mixed by pipetting and then centrifuged (3000 x g, 5 sec). Reactions were incubated at 23 °C for 16 hrs before being subjected to CuAAC with sulforhodamine B azide. Negative control reactions contained all substrates except for EryMod6TE dialyzed lysate. Reactions were performed in duplicate.

Glycerol-containing reaction condition

Reactions were prepared as described in "Conditions for EryMod6TE reactions" except for the use of 10% v/v glycerol and EryMod6TE knockouts (25% v/v; ΔKS, ΔAT, ΔTE, ΔKS+AT, ΔKS+TE, and ΔAT+TE). In the experiment with purified EryTE, Tris-HCl pH 7.5 (150 mM), NaCl (100 mM), priming unit **3** (2.5 mM), glycerol (10% v/v), water, and nickel-purified EryTE (25% v/v) were added to a microcentrifuge tube (200 mL total volume). Reactions were incubated at 23 °C for 16 hrs before being subjected to CuAAC with sulforhodamine B azide.

CuAAC reaction conditions

The following stock solutions were prepared: 10 mM sulforhodamine B azide in DMSO, 1 M sodium ascorbate, and 0.5 M copper(II) sulfate.

DMSO (50% v/v), sulforhodamine B azide (1 mM), EryMod6TE reaction (0.75 mM), and sodium ascorbate (40 mM) were combined (48 mL total volume) in a microcentrifuge tube. Reactions were vortexed and centrifuged (3000 x g, 5 sec). Copper(II) sulfate (20 mM) was then added and immediately vortexed for 3 seconds to initiate the click reaction. Samples were incubated at 23°C for 1 hr and stored at -80 °C until further use.

HPLC and fluorescence detection

Reactions subjected to CuAAC were centrifuged to remove debris (20,000 x g, 2 min). Samples (20 mL) were analyzed using a Waters Symmetry® C18 3.5 μm 4.6 x 75 mm column on a Beckman Coulter System Gold® 126 Solvent Module equipped with a Jasco FP-2020 Plus Intelligent Fluorescence Detector (λ_{ex} = 565 nm, λ_{em} = 586 nm). The mobile phases consisted of degassed, deionized water + 0.1% TFA (solvent A) and degassed acetonitrile + 0.1% TFA (solvent B). A linear gradient (flow rate = 1 mL/min) of 75% to 55% B over 40 min followed by 10 min at 100% B was used to analyze reactions. Data was analyzed using 32 Karat Software.

LC/MS analysis

Whole reactions or compounds collected from HPLC runs were subjected to positive-ESI LC/MS (Agilent Technologies 1200 Series HPLC with a Gemini C18 column coupled to an Agilent Technologies 6130 quadrupole mass spectrometer). Mobile phases consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). A linear gradient (flow rate = 0.7 mL/min) of 5%–95% B over 12 min was used.

High-Resolution Mass Spectrometry

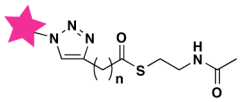
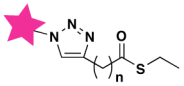
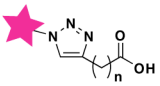
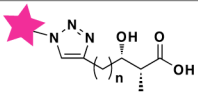
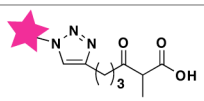
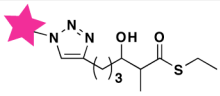
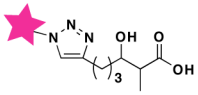
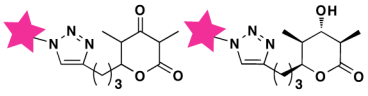
All samples were subject to positive electrospray ionization and masses were characterized on an Agilent 630 Accurate Mass Q-Tof LC/MS.

References:

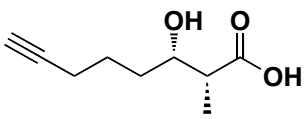
1. Pohl, N. L., Gokhale, R. S., Cane, D. E., and Khosla, C. (1998) Synthesis and incorporation of an *N*-acetylcysteamine analogue of methylmalonyl-CoA by a modular polyketide synthase. *J. Am. Chem. Soc.* **120**, 11206-11207.
2. Lu, X. and Bittman, R. (2005) Synthesis of a photoactivatable (2*S*,3*R*)-sphingosylphosphorylcholine analogue. *J. Org. Chem.* **70**, 4746-4750.
3. Hughes, A. J. and Keatinge-Clay, A. T. (2011) Enzymatic extender unit generation for *in vitro* polyketide synthase reactions: structural and functional showcasing of *Streptomyces coelicolor* MatB. *Chem. Biol.* **18**, 165-176.
4. Liu, H. and Naismith, J. (2008) An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotech.* **8***,* 91-100.
5. Murli, S., Kennedy, J. Dayem, L. C., Carney, J. R., and Kealey, J. T. (2003) Metabolic engineering of Escherichia coli for improved 6-deoxyerythronolide B production. *J. Ind. Microbiol. Biotech.* **30**, 500-509.
6. Piasecki, S. K. and Keatinge-Clay, A. T. (2012) Monitoring biocatalytic transformations mediated by polyketide synthase enzymes in cell lysate via fluorine NMR. *SynLett* **23***,* 1840-1842.

High-Resolution Mass Spec Analysis – Positive ESI

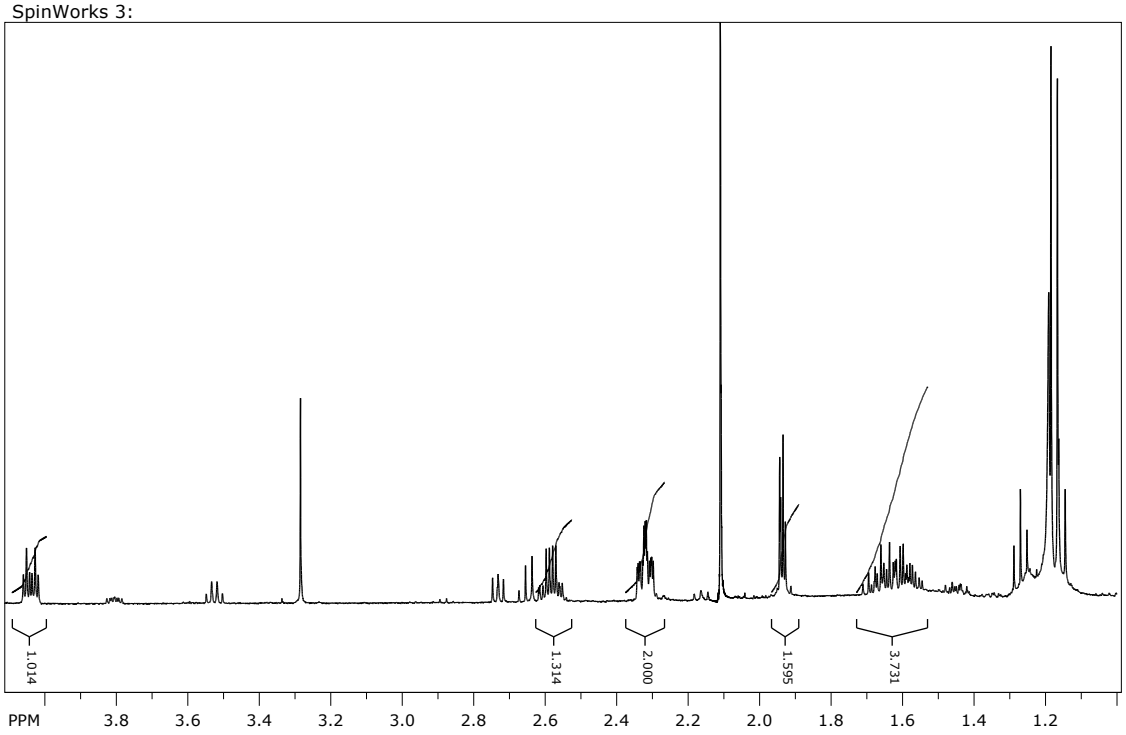
Table S1. High Resolution Mass Spec Analysis of Compounds

Compound		Formula	High Resolution Mass: (exp) obs
		$C_{29}H_{34}N_5O_7S_2$	(628.1899) 628.1897
	**3***	$C_{39}H_{48}N_6NaO_9S_3$	(863.2543) 863.2526
	6*	$C_{38}H_{46}N_6NaO_9S_3$	(849.2386) 849.2374
	7*	$C_{40}H_{50}N_6NaO_9S_3$	(877.2699) 877.2681
	**8***	$C_{36}H_{43}N_5NaO_8S_3$	(792.2172) 792.2195
	9*	$C_{37}H_{45}N_5NaO_8S_3$	(806.2328) 206.2328
	10*	$C_{38}H_{47}N_5NaO_8S_3$	(820.2485) 820.2451
	**4***	$C_{35}H_{42}N_5O_9S_2$	(740.2424) 740.2427
	11*	$C_{34}H_{39}N_5NaO_9S_2$	(748.2087) 748.2082
	12*	$C_{36}H_{43}N_5NaO_9S_2$	(776.2400) 776.2379
	**5***	$C_{38}H_{47}N_5NaO_{10}S_2$	(820.2662) 820.2635
	13*	$C_{37}H_{45}N_5NaO_{10}S_2$	(806.2506) 806.2499
	14*	$C_{39}H_{50}N_5O_{10}S_2$	(812.2999) 812.2984
	**16***	$C_{38}H_{45}N_5NaO_{10}S_2$	(818.2506) 818.2482
	**17***	$C_{40}H_{51}N_5NaO_9S_3$	(864.2747) 864.2734
	**18a* (*syn*)**	$C_{38}H_{47}N_5NaO_{10}S_2$	(820.2662) 820.2635
	18b* (*anti*)	$C_{38}H_{47}N_5NaO_{10}S_2$	(820.2662) 820.2639
	**19***	$C_{41}H_{49}N_5NaO_{10}S_2$	(858.2819) 858.2790
	20*	$C_{41}H_{51}N_5NaO_{10}S_2$	(860.2975) 860.2968

^{1}H NMR of **5**



SpinWorks 3:



file: ...\ajh0419_64frac5t_s2pul_H1.fid\fid block# 1 expt: "s2pul"
transmitter freq.: 399.807110 MHz
time domain size: 51282 points
width: 6410.26 Hz = 16.0334 ppm = 0.125000 Hz/pt
number of scans: 64

freq. of 0 ppm: 399.804738 MHz
processed size: 65536 complex points
LB: 0.100 GF: 0.0000
Hz/cm: 49.904 ppm/cm: 0.12482

Phosphate increases substrate turnover

Increased turnover numbers for triketide lactone formation by the bimodular DEBS1+TE (the first polypeptide of the erythromycin PKS fused to EryTE) were observed with increases in phosphate concentration (Pieper 1996). To determine whether phosphate has a similar effect on single-module constructs, EryMod6TE was incubated with 0–100 mM phosphate, priming unit **3**, extender unit **2**, and the NADPH regeneration system (Figure S1A). After 2 and 16 hrs, reactions were subjected to CuAAC with sulforhodamine B azide and analyzed by HPLC (Figure S1B-C).

After 2 hrs, 20% of **3** was converted to **5** in the absence of phosphate, while 31% had been converted in the presence of 100 mM phosphate (Figure S1B). After 2 hrs, 43% of **3** remained unreacted in the absence of phosphate, whereas only 20% remained unreacted in the presence of 100 mM phosphate. After 16 hrs, no **3** remained and no precipitate was observed in reactions containing at least 50 mM phosphate (Figure S1).

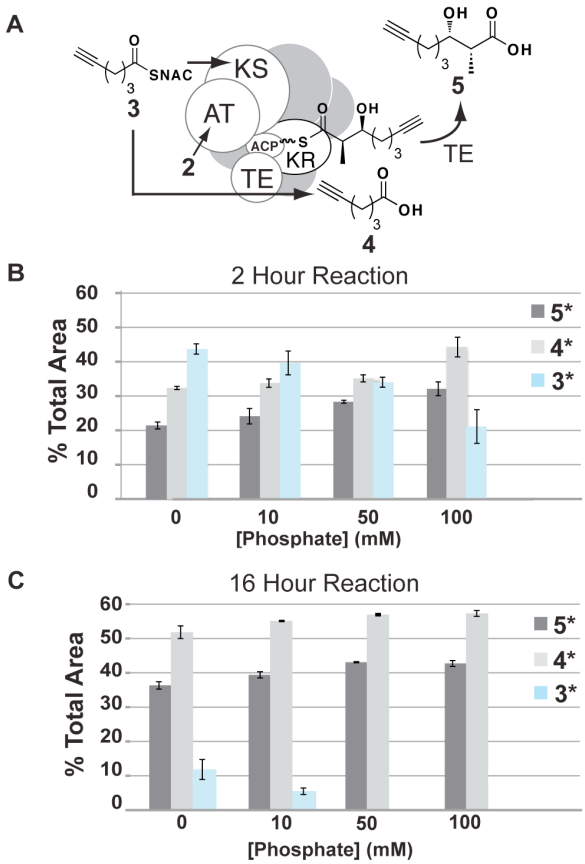


Figure S1. Phosphate enhances turnover. **A**) EryMod6TE operating as a reduced diketide synthase. **B**) Comparison of peak areas for 2 hr reactions shows increased substrate turnover with phosphate concentration. **C**) Comparison of peak areas for 16 hr reactions shows complete substrate turnover when the phosphate concentration is at least 50 mM.

Pieper, R., Ebert-Khosla, S., Cane, D. E., and Khosla, C. (1996) Erythromycin biosynthesis: kinetic studies on a fully active modular polyketide synthase using natural and unnatural substrates. *Biochemistry* **35**, 2054-2060.

TE-catalyzed glycerolysis

Previously, glycerol has been used to prevent precipitation of EryMod6TE in dialyzed lysate (Hughes 2012 and Harper 2012); however, HPLC analysis of EryMod6TE-catalyzed reactions in the presence of glycerol yielded a large, unanticipated peak (**21***, Figure S2B). ^{1}H NMR analysis confirmed that this peak resulted from priming unit glycerolysis. A panel of single and double knockout mutants of EryMod6TE was assayed for the ability to generate **21** (ΔKS, ΔAT, ΔTE, ΔKS+AT, ΔKS+TE, and ΔAT+TE; catalytic nucleophiles replaced by alanines). Through these experiments, TE activity was linked to the generation of **21** (Figure S2C). TE-catalyzed glycerolysis was confirmed when **21*** was observed from the incubation of purified EryTE with **3** in a glycerol-containing buffer (Figure S2D). ^{1}H NMR characterization of **21** yielded the following spectra: (400 MHz, $CDCl_3$) δ 4.18-4.07 (m, 2H), 3.91-3.86 (m, 1H), 3.67-3.52 (m, 2H), 2.45 (t, 2H, J = 7.4), 2.22 (m, 2H), 1.92 (t, 1H, J = 2.7), 1.80 (m, 2H, J = 7.3).

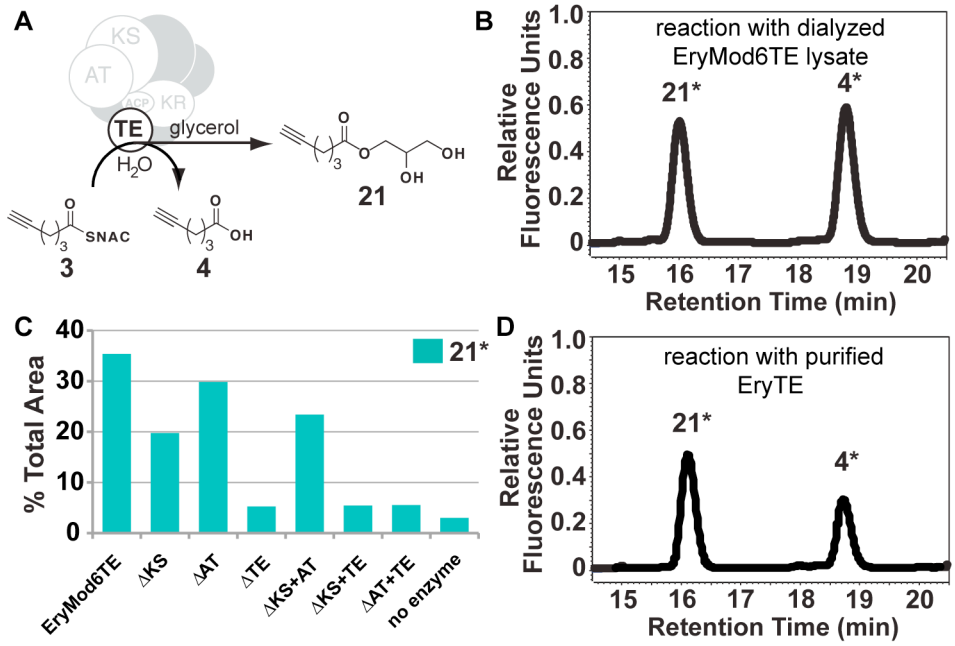
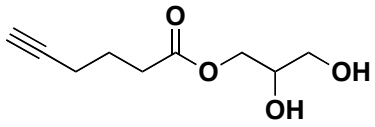


Figure S2. EryTE-mediated glycerolysis of priming units. **A**) TE-mediated hydrolysis has been implicated in low biocatalytic product yields. Here EryTE is also shown to catalyze glycerolysis in reaction buffers containing glycerol. **B**) HPLC trace (λ_{ex} = 565 nm, λ_{em} = 586 nm) reveals the generation of both the hydrolysis product **4** and the glycerolysis product **21** from priming unit **3** from reactions employing dialyzed EryMod6TE lysate. **C**) EryMod6TE mutants containing an active EryTE produce significant quantities of glycerolysis product **21**. **D**) HPLC trace (λ_{ex} = 565 nm, λ_{em} = 586 nm) reveals the generation of both the hydrolysis product **4** and the glycerolysis product **21** from priming unit **3** from a reaction employing purified EryTE.

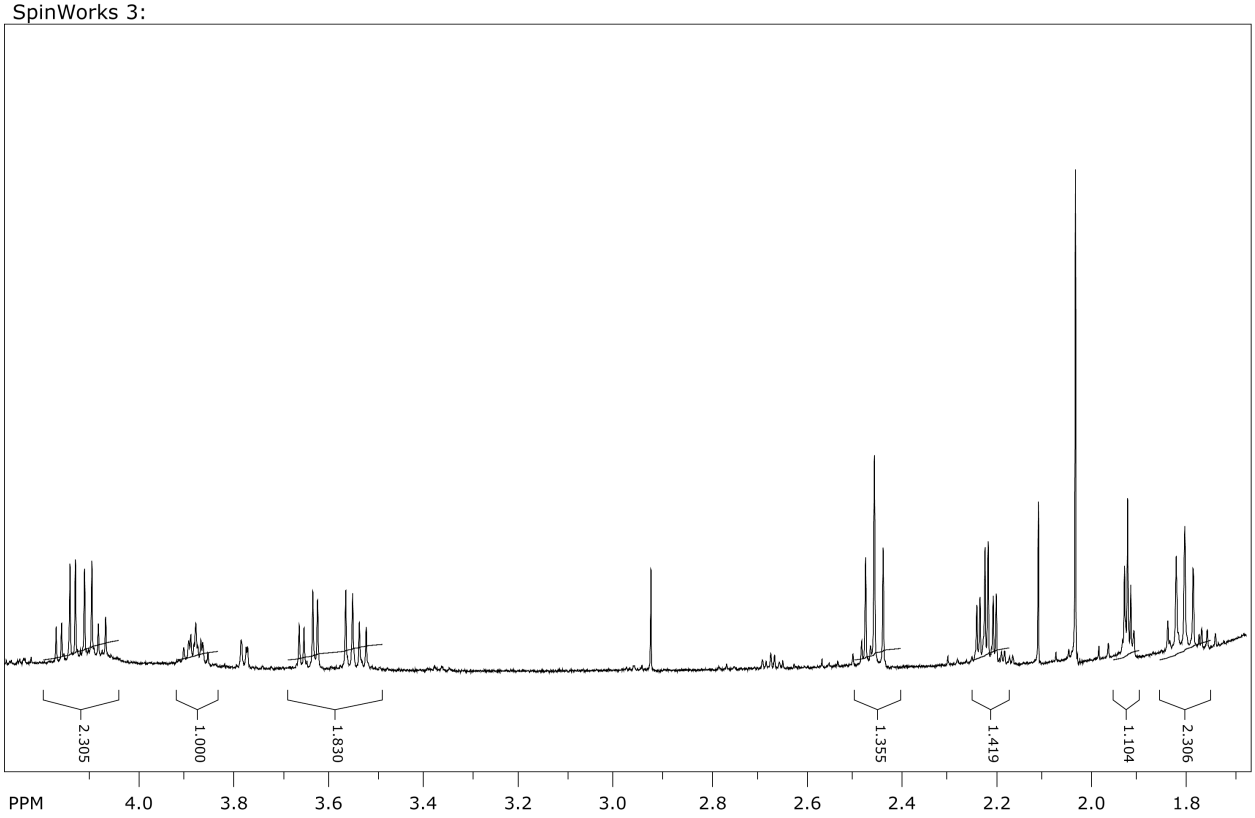
Hughes, A. J., Detelich, J. F., and Keatinge-Clay, A. T. (2012) Employing a polyketide synthase module and thioesterase in the semipreparative biocatalysis of diverse triketide pyrones. *MedChemComm* **3**, 956-959.

Harper, A. D., Bailey, C. B., Edwards, A. D., Detelich, J. F., and Keatinge-Clay, A. T. (2012) Preparative, *in vitro* biocatalysis of triketide lactone chiral building blocks. *ChemBioChem* **13**, 2200-2203.

^{1}H NMR of **21**

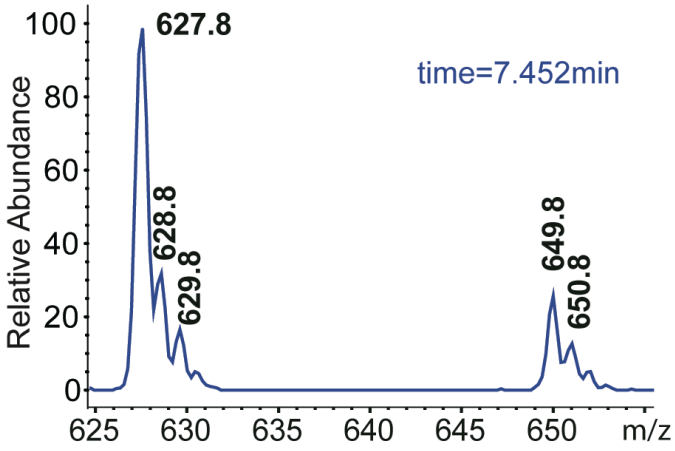


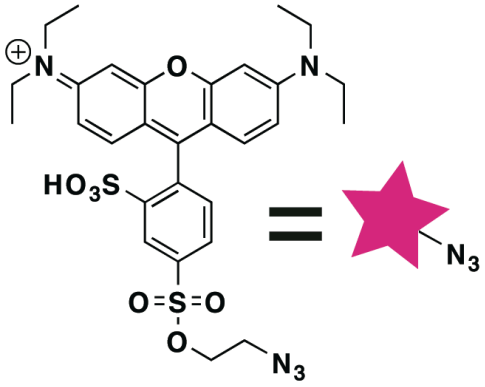
SpinWorks 3:



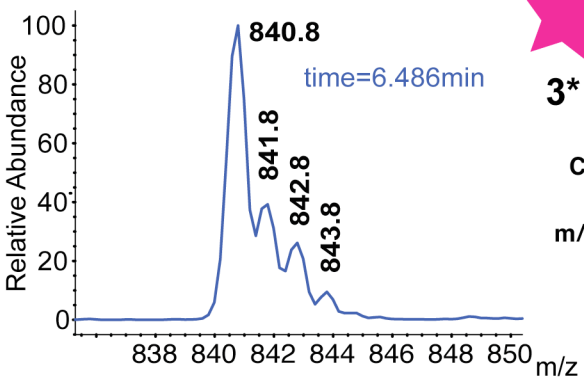
file: ...\AJH101712_frac5a_s2pul_H1.fid\fid block# 1 expt: "s2pul"
transmitter freq.: 399.807110 MHz
time domain size: 51282 points
width: 6410.26 Hz = 16.0334 ppm = 0.125000 Hz/pt
number of scans: 8

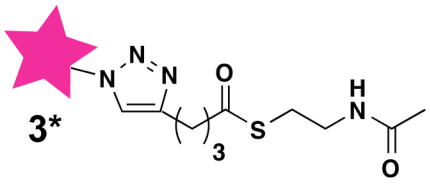
freq. of 0 ppm: 399.804737 MHz
processed size: 65536 complex points
LB: 0.100 GF: 0.0000
Hz/cm: 41.854 ppm/cm: 0.10469



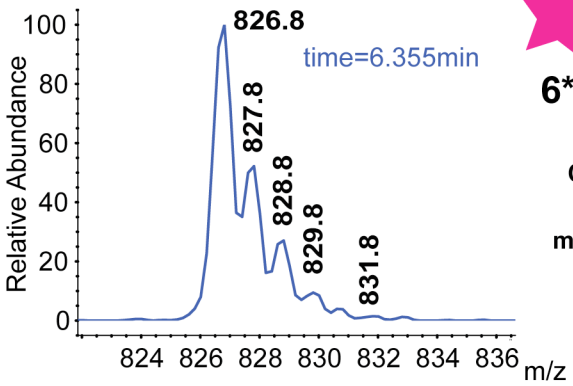


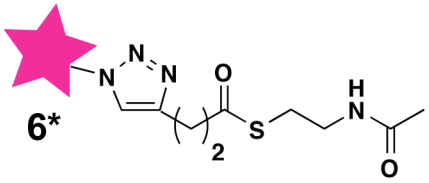
Chemical Formula: $C_{29}H_{34}N_5O_7S_2^+$
Exact Mass: 628.19
m/z: 628.19 (100.0%), 629.19 (35.1%), 630.19 (11.6%), 630.20 (5.0%), 631.19 (3.1%), 631.20 (1.0%)



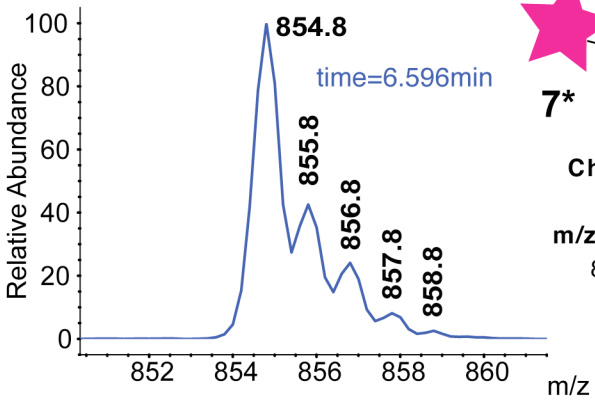


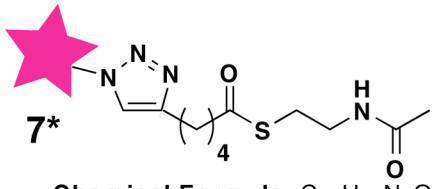
Chemical Formula: $C_{39}H_{49}N_6O_9S_3^+$
Exact Mass: 841.27
m/z: 841.27 (100.0%), 842.28 (43.1%), 843.27 (14.6%), 843.28 (11.9%), 844.27 (6.4%), 842.27 (4.6%), 844.28 (2.4%), 845.27 (1.7%)



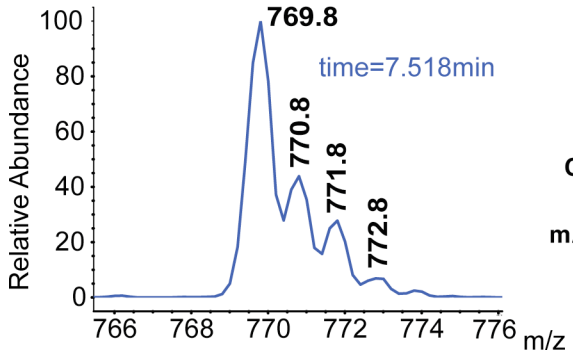


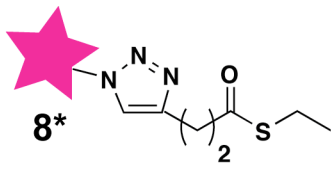
Chemical Formula: $C_{38}H_{47}N_6O_9S_3^+$
Exact Mass: 827.26
m/z: 827.26 (100.0%), 828.26 (44.4%), 829.25 (13.6%), 829.26 (12.2%), 830.26 (7.0%), 828.25 (2.2%), 831.26 (1.6%), 830.27 (1.2%)



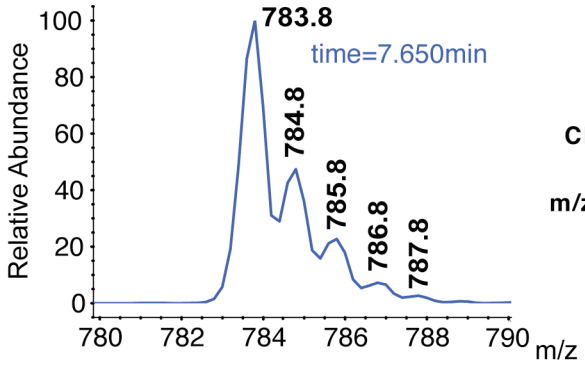


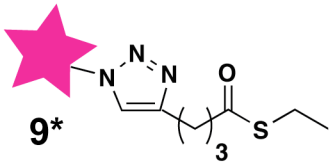
Chemical Formula: $C_{40}H_{51}N_6O_9S_3^+$
Exact Mass: 855.29
m/z: 855.29 (100.0%), 856.29 (46.6%), 857.28 (13.6%), 857.29 (13.0%), 858.29 (6.6%), 856.28 (2.2%), 858.30 (2.2%), 859.29 (1.7%)



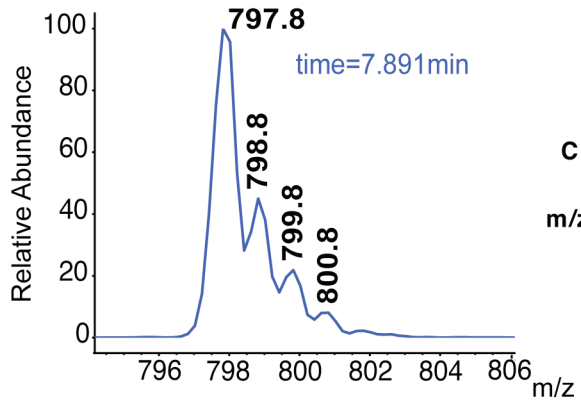


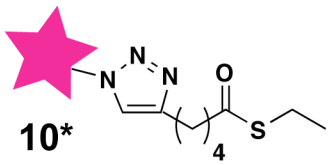
Chemical Formula: $C_{36}H_{44}N_5O_8S_3^+$
Exact Mass: 770.23
m/z: 770.24 (100.0%), 771.24 (39.7%), 772.23 (13.6%), 772.24 (11.0%), 773.23 (5.8%), 771.23 (4.2%), 774.24 (1.4%), 773.24 (1.2%)



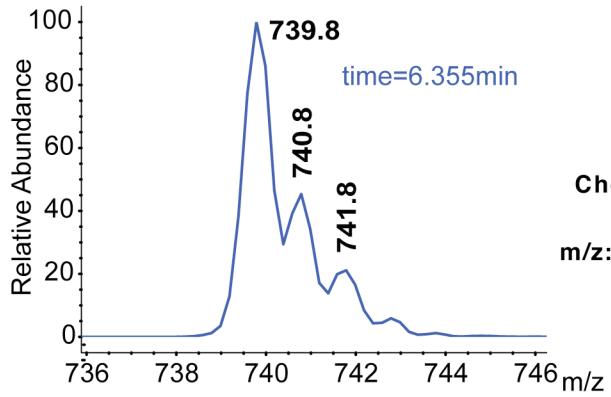


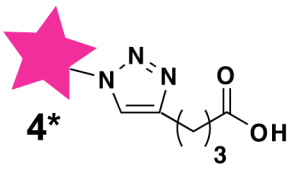
Chemical Formula: $C_{37}H_{46}N_5O_8S_3^+$
Exact Mass: 784.25
m/z: 784.25 (100.0%), 785.25 (44.3%), 786.25 (15.3%), 786.26 (9.8%), 787.25 (6.0%), 787.26 (1.9%), 788.25 (1.6%)



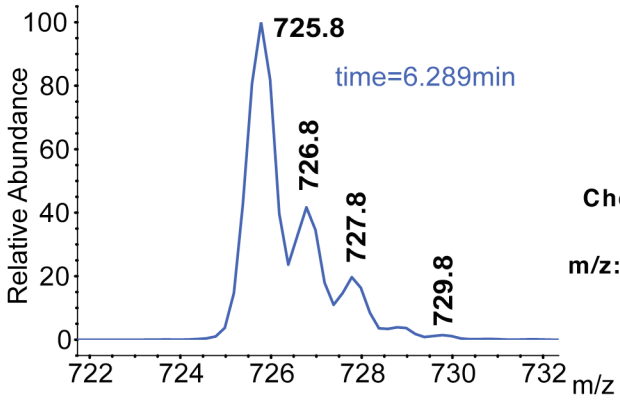


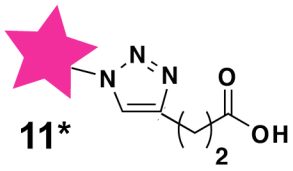
Chemical Formula: $C_{38}H_{48}N_5O_8S_3^+$
Exact Mass: 798.27
m/z: 798.27 (100.0%), 799.27 (44.4%), 800.26 (13.6%), 800.27 (11.8%), 801.27 (6.8%), 799.26 (1.8%), 802.27 (1.5%), 801.28 (1.2%)



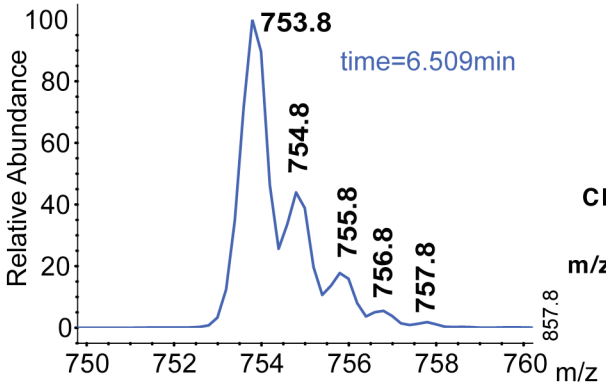


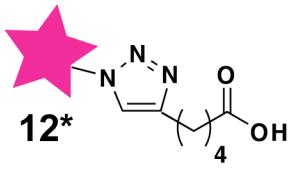
Chemical Formula: $C_{35}H_{42}N_5O_9S_2^+$
Exact Mass: 740.24
m/z: 740.24 (100.0%), 741.25 (38.7%), 742.24 (9.8%), 742.25 (9.8%), 743.24 (3.8%), 741.24 (3.4%), 743.25 (1.8%)



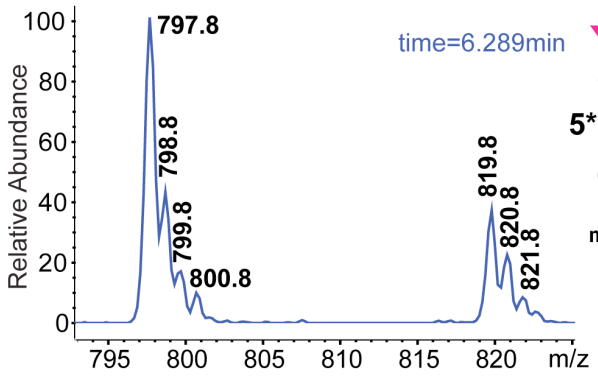


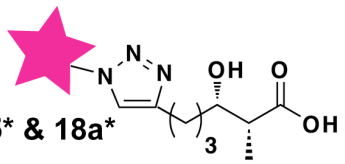
Chemical Formula: $C_{34}H_{40}N_5O_9S_2^+$
Exact Mass: 726.23
m/z: 726.23 (100.0%), 727.23 (39.2%), 728.23 (9.8%), 728.22 (9.1%), 729.23 (4.4%), 727.22 (1.8%)



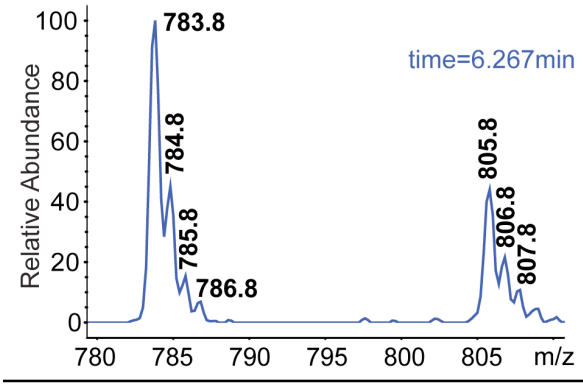


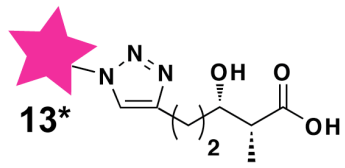
Chemical Formula: $C_{36}H_{44}N_5O_9S_2^+$
Exact Mass: 754.26
m/z: 754.26 (100.0%), 755.26 (43.2%), 756.26 (10.6%), 756.25 (9.1%), 757.26 (3.9%), 757.27 (1.7%)



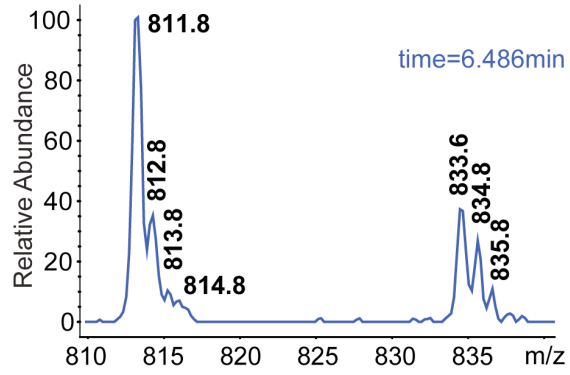


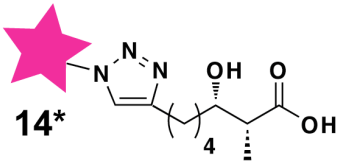
Chemical Formula: $C_{38}H_{48}N_5O_{10}S_2^+$
Exact Mass: 798.28
m/z: 798.28 (100.0%), 799.29 (42.0%), 800.29 (11.4%), 800.28 (9.8%), 801.28 (4.0%), 799.28 (3.4%), 801.29 (2.4%)



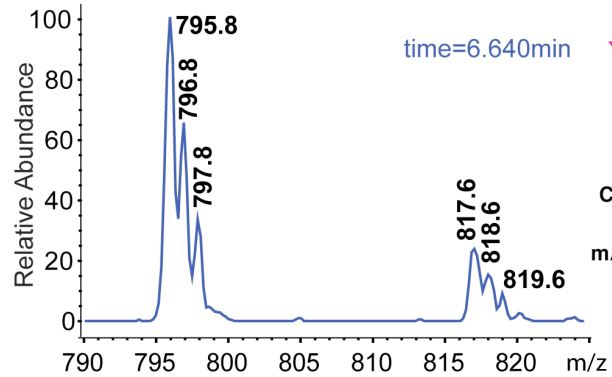


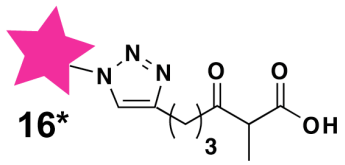
Chemical Formula: $C_{37}H_{46}N_5O_{10}S_2^+$
Exact Mass: 784.27
m/z: 784.27 (100.0%), 785.27 (44.4%), 786.26 (9.1%), 786.28 (8.2%), 787.27 (4.1%), 786.27 (3.5%), 787.28 (1.9%)



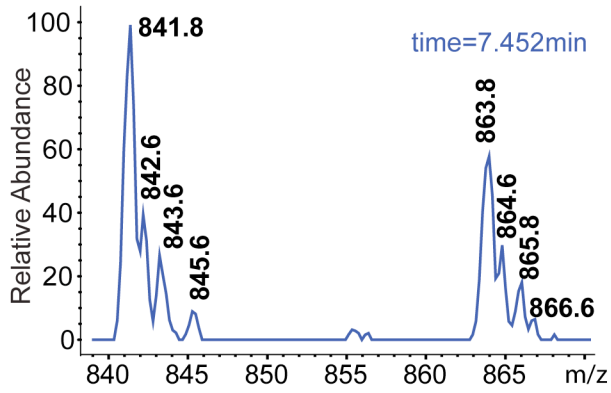


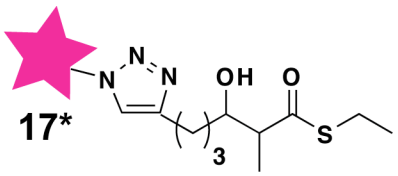
Chemical Formula: $C_{39}H_{50}N_5O_{10}S_2^+$
Exact Mass: 812.30
m/z: 812.30 (100.0%), 813.30 (46.0%), 814.30 (12.6%), 814.31 (9.1%), 815.30 (4.2%), 815.31 (2.3%), 816.30 (1.1%)



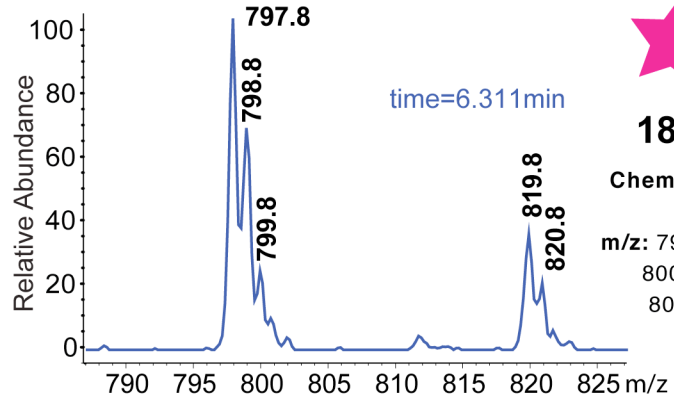


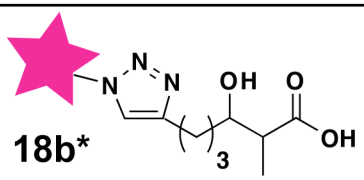
Chemical Formula: $C_{38}H_{46}N_5O_{10}S_2^+$
Exact Mass: 796.27
m/z: 796.27 (100.0%), 797.27 (45.5%), 798.26 (9.1%), 798.28 (8.6%), 799.27 (4.2%), 798.27 (3.5%), 799.28 (2.0%), 800.27 (1.0%)



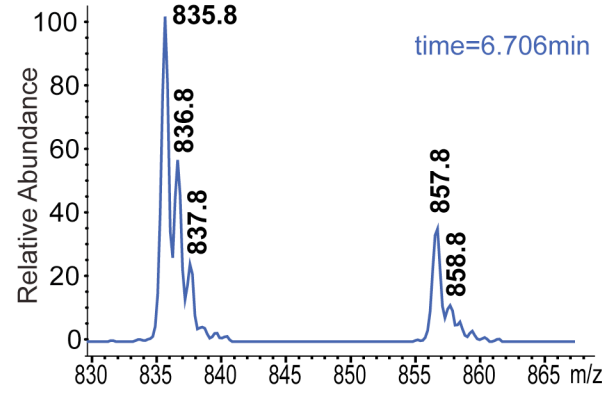


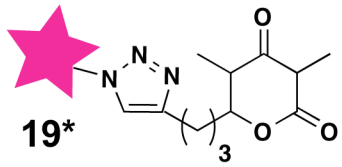
Chemical Formula: $C_{40}H_{52}N_5O_9S_3^+$
Exact Mass: 842.29
m/z: 842.29 (100.0%), 843.30 (44.2%), 844.29 (14.4%), 844.30 (12.5%), 845.29 (6.5%), 843.29 (4.2%), 845.30 (2.5%), 846.30 (1.6%)



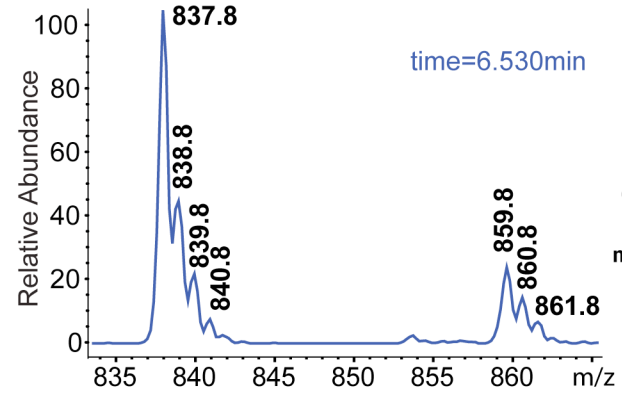


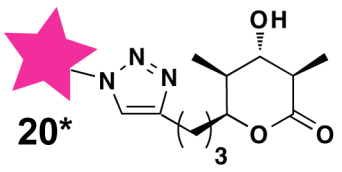
Chemical Formula: $C_{38}H_{48}N_5O_{10}S_2^+$
Exact Mass: 798.28
m/z: 798.28 (100.0%), 799.29 (42.0%), 800.29 (11.4%), 800.28 (9.8%), 801.28 (4.0%), 799.28 (3.4%), 801.29 (2.4%)





Chemical Formula: $C_{41}H_{50}N_5O_{10}S_2^+$
Exact Mass: 836.30
m/z: 836.30 (100.0%), 837.30 (48.2%), 838.30 (12.7%), 838.31 (10.0%), 839.30 (4.4%), 839.31 (2.5%), 840.30 (1.2%)





Chemical Formula: $C_{41}H_{52}N_5O_{10}S_2^+$
Exact Mass: 838.32
m/z: 838.32 (100.0%), 839.32 (45.3%), 840.32 (13.4%), 840.31 (9.1%), 841.31 (4.3%), 839.31 (3.4%), 841.33 (1.5%), 841.32 (1.4%), 842.32 (1.2%)

Compound **1** Reaction Analysis

Trial 1

	RET Time	AREA	% Total AREA
5*	18.727	15787002	40.5
4*	19.294	19825587	50.9
3*	20.825	3343245	8.6

Trial 2

	RET Time	AREA	% Total AREA
5*	18.679	16962289	40.8
4*	19.249	21039755	50.6
3*	20.800	3606768	8.7

Compound **1**		
	AVERAGE	**STDEV**
5*	40.6	0.2
4*	50.7	0.2
3*	8.6	0.1

Compound **2** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.447	11912678	33.1
4*	19.030	17413347	48.5
3*	20.539	6613850	18.4

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.699	15345004	34.5
4*	19.270	19658811	44.1
3*	20.787	9527100	21.4

Compound **2**		
	AVERAGE	**STDEV**
5*	33.8	0.9
4*	46.3	3.0
3*	19.9	2.1

Compound **1** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.444	8176075	20.6
3*	20.910	31563023	79.4

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.490	6260615	17.5
3*	21.007	29561878	82.5

Compound **1** CONTROL		
	AVERAGE	**STDEV**
4*	19.0	2.2
3*	81.0	2.2

Compound **2** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.444	5960798	15.8
3*	20.977	31720433	84.2

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.485	5514672	15.0
3*	21.087	31295599	85.0

Compound **2** CONTROL		
	AVERAGE	**STDEV**
4*	15.4	0.6
3*	84.6	0.6

Compound **6** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
13*	17.809	9013751	20.7
11*	18.680	22850133	52.6
6*	20.194	11593317	26.7

Trial 2

	Ret. Time (min)	AREA	% Total AREA
13*	17.592	8347381	16.6
11*	18.484	25467750	50.8
6*	19.930	16329898	32.6

Compound **6**		
	AVERAGE	**STDEV**
13*	18.7	2.9
11*	51.7	1.3
6*	29.6	4.2

Compound **8** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
13*	17.867	7061148	20.6
11*	18.714	23420844	68.3
8*	35.014	3821288	11.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
13*	17.842	10990448	26.6
11*	18.752	25630547	62.1
8*	35.048	4675599	11.3

Compound **8**		
	AVERAGE	**STDEV**
13*	23.6	4.3
11*	65.2	4.4
8*	11.2	0.1

Compound **6** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	**AREA**	**% Total AREA**
11*	18.737	172204	0.5
6*	20.024	37911066	99.5

Trial 2

	Ret. Time (min)	**AREA**	**% Total AREA**
11*	18.465	2424183	6.1
6*	19.745	37329938	93.9

Compound **6** CONTROL		
	AVERAGE	**STDEV**
11*	3.3	4.0
6*	96.7	4.0

Compound **8** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	**AREA**	**% Total AREA**
11*	18.755	212419	0.6
8*	33.681	36043103	99.4

Trial 2

	Ret. Time (min)	**AREA**	**% Total AREA**
11*	18.827	2710007	6.1
8*	33.460	41899558	93.9

Compound **8** CONTROL		
	AVERAGE	**STDEV**
11*	3.3	3.9
8*	96.7	3.9

Compound **3** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.620	15828462	28.3
4*	19.092	33896564	60.6
3*	20.719	6183995	11.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.464	11380788	27.1
4*	19.105	23650841	56.3
3*	20.509	6981340	16.6

Compound **3**		
	AVERAGE	**STDEV**
5*	27.7	0.9
4*	58.5	3.1
3*	13.8	3.9

Compound **9** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.692	14020189	31.5
4*	19.374	26711265	60.0
9*	36.719	3814999	8.6

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.697	14966330	32.9
4*	19.342	26192166	57.6
9*	36.746	4290108	9.4

Compound **9**		
	AVERAGE	**STDEV**
5*	32.2	1.0
4*	58.8	1.6
9*	9.0	0.6

Compound **3** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.359	6049863	15.9
3*	20.760	31884840	84.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.330	6555358	16.5
3*	20.849	33124632	83.5

Compound **3** CONTROL		
	AVERAGE	**STDEV**
4*	16.2	0.4
3*	83.8	0.4

Compound **9** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.300	594660	4.0
9*	36.803	14175016	96.0

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.314	500041	2.8
9*	36.754	17355117	97.2

Compound **9** CONTROL		
	AVERAGE	**STDEV**
4*	3.4	0.9
9*	96.6	0.9

Compound **7** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
14*	20.08	11581283	25.46
12*	20.68	32761857	72.03
7*	22.69	1138304	2.50

Trial 2

	Ret. Time (min)	AREA	% Total AREA
14*	20.16	10643821	23.85
12*	20.71	33422377	74.89
7*	22.70	564438	1.26

Compound **7**		
	AVERAGE	**STDEV**
14*	24.7	1.1
12*	73.5	2.0
7*	1.9	0.9

Compound **10** Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
14*	20.16	10535136	22.95
12*	20.66	34727449	75.66
10*	39.71	637867	1.39

Trial 2

	Ret. Time (min)	AREA	% Total Area
14*	20.09	8001594	19.65
12*	20.62	30425013	74.71
10*	38.95	2299424	5.65

Compound **10**		
	AVERAGE	**STDEV**
14*	21.3	2.3
12*	75.2	0.7
10*	3.5	3.0

Compound **7** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
12*	20.814	23100604	41.1
7*	22.619	33168434	58.9

Trial 2

	Ret. Time (min)	AREA	%total AREA
12*	20.655	25015046	43.3
7*	22.619	32755287	56.7

Compound **7** CONTROL		
	AVERAGE	**STDEV**
12*	42.2	1.6
7*	57.8	1.6

Compound **10** CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
12*	20.822	9162547	51.7
10*	39.851	8562374	48.3

Trial 2

	Ret. Time (min)	AREA	% Total AREA
12*	20.795	12754036	62.3
10*	39.769	7724401	37.7

Compound **10** CONTROL		
	AVERAGE	**STDEV**
12*	57.0	7.5
10*	43.0	7.5

Compound **3** – 0 mM Phosphate – 2 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.665	14806840	21.3
4*	19.232	22840606	32.8
3*	20.822	31899164	45.9

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.867	13023723	22.8
4*	19.407	19204279	33.5
3*	20.987	25014238	43.7

0 mM Phosphate- 2 hrs		
	AVERAGE	**STDEV**
5*	22.0	1.0
4*	33.2	0.5
3*	44.8	1.5

Compound **3** – 10 mM Phosphate – 2 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.580	15802446	23.1
4*	19.244	23049643	33.7
3*	20.774	29464589	43.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.890	13553917	26.4
4*	19.424	18227682	35.5
3*	21.020	19587930	38.1

10 mM Phosphate- 2 hrs		
	AVERAGE	**STDEV**
5*	24.8	2.3
4*	34.6	1.2
3*	40.6	3.5

Compound **3** – 50 mM Phosphate – 2 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.464	16814392	29.4
4*	19.019	21047074	36.8
3*	20.539	19366060	33.8

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.777	15811837	28.8
4*	19.325	19405246	35.3
3*	20.905	19771894	36.0

50 mM Phosphate- 2 hrs		
	AVERAGE	**STDEV**
5*	29.1	0.4
4*	36.0	1.1
3*	34.9	1.5

Compound **3** – 100 mM Phosphate – 2 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.492	20585641	31.5
4*	19.125	28318670	43.3
3*	20.640	16505850	25.2

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.862	19556396	34.4
4*	19.404	26989181	47.5
3*	21.020	10325964	18.2

100 mM Phosphate - 2 hrs		
	AVERAGE	**STDEV**
5*	32.9	2.1
4*	45.4	2.9
3*	21.7	5.0

Compound **3** – 0 mM Phosphate – 16 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.669	16536904	37.1
4*	19.250	23683668	53.1
3*	20.779	4360206	9.8

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.740	15832878	35.6
4*	19.330	22474980	50.5
3*	20.864	6184094	13.9

0 mM Phosphate - 16 hrs		
	AVERAGE	**STDEV**
5*	36.3	1.1
4*	51.8	1.8
3*	11.8	2.9

Compound **3** – 10 mM Phosphate – 16 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.712	19279548	40.0
4*	19.317	26531272	55.1
3*	20.825	2328798	4.8

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.382	19807554	38.8
4*	19.004	28157616	55.1
3*	20.495	3145642	6.2

10 mM Phosphate - 16 hrs		
	AVERAGE	**STDEV**
5*	39.4	0.9
4*	55.1	0.0
3*	5.5	0.9

Compound **3** – 50 mM Phosphate – 16 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.617	24264845	43,2
4*	19.374	31903020	56.8

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.642	20931892	43.0
4*	19.279	27803314	57.0

50 mM Phosphate – 16 hrs		
	AVERAGE	**STDEV**
5*	**43.1**	**0.2**
4*	**56.9**	**0.2**

Compound **3** – 100 mM Phosphate – 16 hrs - Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
5*	18.745	22205349	42.1
4*	19.300	30532660	57.9

Trial 2

	Ret. Time (min)	AREA	% Total AREA
5*	18.657	19918332	43.3
4*	19.212	26066248	56.7

100 mM Phosphate – 16 hrs		
	AVERAGE	**STDEV**
5*	42.7	0.9
4*	57.3	0.9

Compound **3** – 0 mM Phosphate – 16 hrs CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.317	6028704	14.9
3*	19.945	34549471	85.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.295	6749666	16.2
3*	20.769	34841101	83.8

0 mM Phosphate CONTROL		
	AVERAGE	**STDEV**
4*	15.5	1.0
3*	84.5	1.0

Compound **3** – 10 mM Phosphate – 16 hrs CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.164	6257779	15.1
3*	20.692	35150517	84.9

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.279	6576249	15.4
3*	20.592	36009401	84.6

10 mM Phosphate CONTROL		
	AVERAGE	**STDEV**
4*	15.3	0.2
3*	84.7	0.2

Compound **3** – 50 mM Phosphate – 16 hrs CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	AREA	% Total AREA
4*	19.275	8543020	19.9
3*	20.612	34470898	80.1

Trial 2

	Ret. Time (min)	AREA	% Total AREA
4*	19.310	7864313	18.3
3*	20.894	35083973	81.7

50 mM Phosphate CONTROL		
	AVERAGE	**STDEV**
4*	19.1	1.1
3*	80.9	1.1

Compound **3** – 100 mM Phosphate – 16 hrs CONTROL Reaction Analysis

Trial 1

	Ret. Time (min)	**AREA**	**% Total AREA**
4*	19.294	7648977	18.5
3*	20.710	33648585	81.5

Trial 2

	Ret. Time (min)	**AREA**	**% Total AREA**
4*	19.274	9948370	23.0
3*	20.677	33388648	77.0

100 mM Phosphate CONTROL		
	AVERAGE	**STDEV**
4*	20.7	3.1
3*	79.3	3.1

Compound **15** Reaction Analysis

Trial 1

	Ret. Time (min)	**AREA**	**% Total AREA**
18a*	18.590	13305217	23.8
18b*	19.122	7131187	12.8
20*	21.575	18279563	32.7
16*	22.539	5606334	10.0
19*	23.498	11526588	20.6

Trial 2

	Ret. Time (min)	**AREA**	**% Total AREA**
18a*	18.845	12387653	24.4
18b*	19.372	7118427	14.0
20*	21.860	16615907	32.7
16*	22.774	4820335	9.5
19*	23.727	9887625	19.5

Compound **15**		
	AVERAGE	**STDEV**
18a*	24.1	0.4
18b*	13.4	0.9
20*	32.7	0.0
16*	9.8	0.4
19*	20.0	0.8

Compound **3** EryMod6TE Mutant Reaction Analysis

AREA

	EryMod6TE	**ΔKS**	**ΔAT**	**ΔTE**
21*	2614798	1554339	2198173	372883
4*	826717	1509532	1235584	859962
3*	3951371	4797879	3931098	5868967
Total AREA	7392886	7861750	7364855	7101812

	ΔKS+AT	**ΔKS+TE**	**ΔAT+TE**	**no EryMod6TE**
21*	1708062	479870	409617	166666
4*	1138868	1127082	993593	0
3*	4447477	7212606	6012292	6333238
Total AREA	7294407	8819558	7415502	6499904

	% Total Area		
	21*	**4***	**3***
EryMod6TE	35.4	11.2	53.4
ΔKS	19.8	19.2	61.0
ΔAT	29.8	16.8	53.4
ΔTE	5.2	12.1	82.6
ΔKS+AT	23.4	15.6	61.0
ΔKS+TE	5.4	12.8	81.8
ΔAT+TE	5.5	13.4	81.1
no EryMod6TE	3.0	0.0	97.4