In vitro Characterization of a Heterologously Expression Non-Ribosomal Peptide Synthetase Involved in Phosphinothricin Tripeptide Biosynthesis

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SUPPORTING INFORMATION

1. Chemical Synthesis of Substrates

Substrates. AcDMPT was synthesized using the radical addition of hypophosphorous acid to vinyl glycine as the key step as described previously (1) and similar to a procedure reported while this work was in progress (2).

General
All NMR spectra were recorded on Varian U500 spectrometers. ^1H NMR spectra are referenced to TMS at 0 ppm or CHCl₃ at 7.26 ppm, and ^13C NMR spectra are referenced to CDCl₃ at 77.7 ppm. Mass spectrometry (MS) experiments were carried out by the Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign. Fractions collected during silica gel column chromatography were analyzed by TLC. Unless otherwise specified, all compounds and solvents were obtained from Fisher or Aldrich. THF was distilled from sodium/benzophenone, and CH₂Cl₂ was distilled form CaH₂ prior to use.

1.1. Synthesis of AcDMpt.
(S)-2-Aminobutyrolactone hydrobromide (1). To a mixture of distilled water (20 mL) and isopropanol (20 mL) was added L-methionine (5 g, 33.5 mmol), acetic acid (8 mL) and bromoacetic acid (5.4 g, 38.9 mmol). The mixture was stirred at 50 °C for 30 min until all solid was dissolved. The solution was heated at reflux for 2.5 h. After cooling to room temperature, the solvent was removed using a rotary evaporator to afford a sticky oil. The oil was dissolved in dioxane (10 mL) and stirred for 1 h, and then filtered to give a pink solid (4.2 g) in a yield of 83%. $^1$H NMR (400 MHz, D$_2$O) δ 4.44-4.39 (m, 1H), 4.27-4.21 (m, 2H), 2.62-2.58 (m, 1H), 2.27-2.23 (m, 1H).

![Chemical Structure](image-url)

(S)-Aminobromobutyrate hydrobromide (2). To a high pressure seal tube were added 33% HBr in acetic acid (25 mL) and the lactone 1 (18 mmol). The tube was sealed and heated at 75 °C for 6 h. The resulting suspension was cooled to room temperature and filtered. The residue was washed with diethyl ether (20 mL × 3) to afford a white solid in a yield of 75%. $^1$H NMR (400 MHz, D$_2$O) δ 4.06 (td, $J = 6.4, 1.2$ Hz, 1H), 3.50-3.39 (m, 2H), 2.39 (dt, $J = 22.0, 6.4$ Hz, 1H), 2.22 (dt, $J = 22.0, 6.4$ Hz, 1H).

$^1$H NMR

![Chemical Structure](image-url)

(S)-Methyl aminobromobutanoate hydrobromide (3). To methanol (20 mL) was added AcCl (5 mL) at 0 °C. After addition the solution was stirred at 0 °C for 10 min. Compound 2 (10 mmol) was added in one portion. The solution was slowly heated to reflux and was kept at reflux for 2 h. After cooling to RT the solvent was removed by rotary evaporation to give a white solid in quantitative yield.
(S)-N-Acetyl-2-amino-4-bromobutanoate methyl ester (4). The ester 3 (10 mmol) was dissolved in THF (200 mL), followed by addition of Et₃N (6 mL) and AcCl (0.9 mL). The resulting mixture was stirred at RT overnight. The reaction was quenched by the addition of water (20 mL), and was extracted with ethyl acetate (60 mL × 3). The combined organic layers were washed with water (30 mL) and brine (30 mL), and dried over sodium sulfate. After filtration and removal of the solvent from the filtrate using a rotary evaporator, the resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:2) to afford a 93% yield of desired product.

(S)-N-Acetyl-2-amino-4-(phenylselenyl)butanoate naphthalenylmethyl ester (6). To a solution of NaOH (0.41 g) in H₂O/EtOH (40 mL, 1:2) was added the ester 4 (3.14 g). The solution was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was dried at low pressure overnight. The resulting solid was dissolved in DMF (20 mL), followed by (2-bromomethyl) naphthalene (3.3 g). The mixture was stirred at room temperature overnight. To the resulting solution was added water (100 mL) to give a white suspension. The suspension was filtered and the product was purified by silica gel column chromatography (EtOAc:hexane = 1:2) to give the desired compound in 90% yield. ¹H NMR (500 MHz, CDCl₃) δ 7.86-7.79 (m, 4H), 7.52-7.50 (m, 2H), 7.42-7.38 (m, 3H), 7.20-7.15 (m, 3H), 6.15 (d, J = 8.0 Hz, 1H), 5.33 (d, J = 12.5 Hz, 1H), 5.29 (d, J = 12.5 Hz, 1H), 4.81-4.79 (m, 1H), 2.85-2.80 (m, 2H), 2.26-2.24 (m, 1H), 2.07-2.04 (m, 1H), 2.00 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 171.4, 170.2, 133.4, 133.3, 133.2, 129.6, 129.4, 128.8, 128.3, 128.0, 128.9, 127.4, 126.7, 126.0, 67.8, 52.6, 33.3, 23.4.

¹H NMR
(S)-N-Acetyl-2-amino-3-butenoate-2-naphthalenylmethyl ester (7). The starting material was dissolved in dichloromethane (150 mL). To the solution was added H₂O₂ (30%, 20 mL). The mixture was heated at reflux for 5 h (40-45 °C). After cooling to room temperature, the organic layer was separated and washed with water (960 mL × 6), until the KI test paper gave a negative result. The solution was dried over sodium sulfate, filtered, and concentrated using a rotary evaporator. The crude product was purified by silica gel column chromatography (EtOAc:hexane = 1:2) to afford 1.2 g of the alkene (47%). ^1H NMR (500 MHz, CDCl₃) δ 7.86-7.82 (m, 5H), 7.52-7.48 (m, 2H), 7.44-7.42 (m, 1H), 6.22 (d, J =  5.5 Hz, 1H), 5.92 (ddd, J = 17.5, 10.5, 5.5 Hz, 1H), 5.38-5.23 (m, 5H), 2.06 (s, 3H); ^13C NMR (125 MHz, CDCl₃) δ 170.8, 169.8, 133.4, 133.3, 132.7, 132.2, 128.8, 128.2, 128.0, 127.7, 126.7, 125.9, 118.1, 67.9, 54.8.

^1H NMR
(S)-N-Acetyldemethylphosphinothricin-2-naphthalenylmethyl ester (8). The starting material (1.2 g) was dissolved in MeOH (4 mL), followed by addition of ammonium hypophosphite (0.5 g). To the mixture was added a solution of Et₃B in hexane (1 M, 4.2 mL). The mixture was vigorously stirred at air atmosphere for 2 h at which TLC showed consumption of the starting material. The reaction was quenched with 5% KHSO₄ (945 mL), and extracted with EtOAc (30 mL × 2). The organic layer was washed with water and brine (10 mL), dried over sodium sulfate, and filtered. The solvent was removed using a rotary evaporator and after verification of the presence of the desired product by $^{31}$P NMR spectroscopy, the crude product was carried forward to the next step without further purification. $^{31}$P NMR (202 MHz, CDCl₃) δ 33.82.

$^{31}$P NMR, $^1$H-decoupled
(S)-N-Acetyldemethylphosphinotricin sodium salt (9). To a solution of sodium hydroxide (43 mg) in H₂O/EtOH (2 mL/2 mL) was added the ester 8. The reaction was stirred at room temperature for 1 h. TLC showed consumption of the starting material. The ethanol was removed under reduced pressure. The aqueous layer was extracted with EtOAc (5 mL × 2). The aqueous solution was dried under reduced pressure to afford the desired product, 148 mg (78%). ¹H NMR (500 MHz, D₂O) δ 6.73 (dd, J = 508.5, 1.5 Hz, 1H), 3.97-3.95 (m, 1H), 1.81-1.72 (m, 4H), 1.64-1.61 (m, 1H), 1.39-1.32 (m, 2H); ³¹P NMR (202 MHz, CDCl₃) δ 30.39.

¹H NMR
$^{31}$P NMR, $^1$H decoupled

$^{31}$P NMR, H-P coupled
1.2. Synthesis of D,L-N-AcPt, L-N-AcGlu, L-N-AcAsp, and L-N-AcAP4.

L-2-Amino-4-phosphonobutyric acid (L-AP4) was purchased from Tocris Bioscience. Both L-Glu and L-Asp were purchased from Sigma-Aldrich, and PT was obtained from Matrix Scientific. Acetylation of the commercially available amino acids was achieved by first neutralization to pH 7.0 with potassium carbonate followed by addition of acetic anhydride. After acidification of the product using hydrochloric acid, the obtained acetylated product was purified using HPLC.

2. General Molecular Biology Methods. All molecular biology techniques, such as polymerase chain reaction (PCR), transformation and plasmid preparation, were performed using standard procedures (Sambrook and Maniatis) (3). Restriction enzymes, T4 DNA ligase, and polymerases were purchased from either New England Biolabs or Invitrogen. Oligonucleotides used as primers in PCR were purchased from Operon. 3H-Acetyl coenzyme A (AcCoA) was purchased from MP Biomedicals, and radioactive tetrasodium ³²P-pyrophosphate was purchased from Perkin Elmer. All DNA constructs prepared in this work were verified by sequencing at the Carver Biotechnology Center at UIUC.

Construction of Plasmids.

**pET15b-phsA.** The phsA gene was amplified from genomic DNA of *S. viridochromogenes* DSM 40736 by PCR using a forward primer 5’-GGGAATTCCATATGACCGCAGCGACCCGGACA–3’ containing a *Nde*I restriction site and a reverse primer 5’-TATAAACGCGGATCCCTACGTCCCCTTCAGTTTC–3’ containing a *Bam*HI restriction site. Successful PCR products were digested with *Nde*I and *Bam*HI and subsequently ligated into the double-digested pET15b vector (Novagen) with T4 DNA ligase.

**pMAL-c2X-phsA.** The phsA gene was amplified by PCR as described above using a forward primer 5’-GGAAGGATTTCAGAATTCATGACCGCAG-3’ containing an *Eco*RI site and a reverse primer 5’-TATAAACGCGGATCCCTACGTCCCCTTCAGTTTC-3’ containing a *Bam*HI site. The PCR product and pMAL-c2X vector were treated with these restriction enzymes and then ligated to produce a vector encoding PhsA fused at its *N*-terminus to maltose binding protein.

**pET28b-malE-phsA.** The malE-phsA gene was amplified by PCR from pMAL-c2X-phsA using as forward primer 5’-GCCGCTCGCCCATGGCTATGAAAATCGAAGAAGGTAAACTGG-3’ and as reverse primer 5’-AGAGCGAAGAGCCTCGAGCGTCCCCTTCAGTGTCGCCG-3’ to introduce *Nco*I and *Xho*I restriction sites, respectively. The PCR product and pET28b vector were treated with these enzymes and then ligated to produce a vector encoding MBP-PhsA with a hexahistidine tag (His₆) at its *C*-terminus.

**pET28b-malE-phsA(S16A).** The first PCR was performed to amplify a 1.5 kb fragment of *phsA* containing the S16A coding region using a forward primer 5’-CCCGCTCGCCCATGGCTATGAAAATCGAAGAAGGTAAACTG-3’ and as reverse primer 5’-AGAGCGAAGAGCGTCCCCTTCAGTGTCGCCG-3’ and a reverse primer 5’-TGAAGCGACCGCCGATTACGTCG-3’ containing a *Bam*HI site. The PCR product and pET28b vector were treated with these enzymes and then ligated to produce a vector encoding MBP-PhsA with a hexahistidine tag (His₆) at its *C*-terminus.

3. Protein Purification

**Overexpression of His-tagged PhsA.** *E. coli* Rosetta (DE3) carrying pET15b-phsA was grown in 3 L of Luria-Bertani (LB) medium containing 100 µg/mL ampicillin and 20 µg/mL of chloramphenicol. The strain with pET28b-malE-phsA was grown in 3 L of LB with 50 µg/mL kanamycin and 20 µg/mL of chloramphenicol as resistant markers. Both strains were grown at 37 °C and induced with isopropyl-
\[\text{β-D-thiogalactopyranoside (IPTG)} \text{ (final concentration 0.1 mM)} \text{ at } \text{OD}_{600} = 0.6. \text{ The cultures were grown at } 18 \, ^\circ\text{C overnight following induction.}

**Purification of MBP-PhsA-His}_6. \text{ The cells were harvested by centrifugation (15 min, 5000g), resuspended in buffer A (50 mM Tris, 300 mM NaCl, 15% glycerol (v/v), 10 mM } \text{β-mercaptoethanol, pH 7.8 at 4 \, ^\circ\text{C}} \text{ with protease inhibitor cocktail tablets (Complete Mini, EDTA-free, Roche), and disrupted by incubation with 0.2 mg/mL of lysozyme followed by sonication (pulse sequence of 5 s on and 9.9 s off, 15 min). The lysate was cleared by centrifugation (30 min, 30,000xg), and purified using Ni}^{2+} \text{ affinity resin (Qiagen) equilibrated with buffer A containing 10 mM imidazole. The cell lysate was passed through a 45 \, \mu\text{m filter before application to the resin. The column (1 x 10 cm) was washed with 20 column volumes of buffer A containing 25 mM imidazole to remove any proteins bound nonspecifically. Elution of the protein was achieved with buffer A with increasing imidazole concentrations in a step gradient from 25 mM to 500 mM with 25 mM increments (total volume = 200 mL), and the fractions containing the desired protein as determined by SDS-PAGE and Coomassie Blue staining were concentrated using Amicon Ultra with a 30 kDa membrane (Millipore). Imidazole and NaCl were removed using a PD-10 desalting column (GE Healthcare) pre-packed with Sephadex G-25 medium. The protein was eluted with buffer B (20 mM Tris, 25 mM NaCl, pH 7.5), and was further purified on a HiTrap Q HP anion exchange column (GE Healthcare) pre-equilibrated with buffer B. Elution of the enzyme was carried out with buffer B in a step gradient of NaCl, from 25 mM to 500 mM with 25 mM increments (total volume = 220 mL). The purified protein MBP-PhsA-His}_6 \text{ was concentrated using Amicon Ultra (30 kDa), mixed with glycerol to a final concentration of 20\% (w/v), flash frozen in liquid nitrogen, and stored at -80 \, ^\circ\text{C. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.}

**Conversion of Apo-MBP-PhsA-His}_6 to Holo-MBP-PhsA-His}_6 by Sfp. \text{ For } \text{in vitro} \text{ priming of MBP-PhsA-His}_6 \text{ to its holo form, 9.6 } \mu\text{M MBP-PhsA-His}_6 \text{ was incubated with 25 } \mu\text{M CoA and 1 nmol Sfp (4) for 1 h at 25 \, ^\circ\text{C in assay buffer (50 mM HEPES pH 7.5, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM MgCl}_2) in a final volume of 150 } \mu\text{L.} \]
Detection of Posttranslational Modification Activity by Sfp.

**BODIPY-CoA in-gel Fluorescence Labeling.** MBP-PhsA-His<sub>6</sub> was incubated with Sfp (4 µM) in a buffer containing 10 mM MgCl<sub>2</sub>, 2.4 mM DTT and 0.16 mM BODIPY-CoA (5) for 1-2 h at 30 °C. The reaction was quenched by adding equal volume of SDS-PAGE loading buffer. The reaction mixture was analyzed by running a 10% SDS-PAGE, exciting the fluorophore at 460 nm, and detecting at 520 nm (Figure S1). Subsequently, the gel was stained with Coomassie blue.

![Figure S1. SDS-PAGE of MBP-PhsA-His<sub>6</sub> treated with BODIPY-CoA and Sfp and visualized at 520 nm. Lane 1, MBP-PhsA-His<sub>6</sub>; lane 2, MBP-PhsA-His<sub>6</sub> treated with BODIPY-CoA; lane 3, MBP-PhsA-His<sub>6</sub> treated with BODIPY-CoA and Sfp. Lanes 4-6 and 7-9 have the same composition as lanes 1-3 but more material was loaded into the wells of the gel.](image).

**Radioassay of the Phosphopantetheinylation Process.** The activity of Sfp on MBP-PhsA-His<sub>6</sub> was also determined by measuring the incorporation of ³H-labeled acetyl CoA into apo-MBP-PhsA-His<sub>6</sub> (13 µM) under the phosphopantetheinylation conditions described above except for the use of ³H-acetyl CoA (20 mCi/mmol, 10 µCi/mL, 52 µM) in place of CoA and the use of lower Sfp concentration (0.1 µM). Reactions were initiated by addition of Sfp, and at each time-point, a 30 µL aliquot was taken from the reaction mixture, and quenched with 240 µL of 10% trichloroacetic acid (TCA). BSA (100 µg) was added as a carrier protein, and the precipitated protein was pelleted by centrifugation. After the supernatant was discarded, the pellet was washed three times with 240 µL of 10% TCA before resuspension in 100 µL formic acid. Liquid scintillation cocktail (ScientiSafe Econo 1, Fisher Scientific) was added to the protein suspension, and the radioactivity of tritium bound to the protein was quantified by liquid scintillation counting.
Figure S2. Time-dependent progress of the transfer of radiolabeled phosphopantetheinyl group from $^3$H-acetyl CoA to MBP-PhsA-His$_6$ by the PPTase Sfp.

4. Activity Assays

**Kinetic Analysis of the Pyrophosphate Exchange Reaction.** Reactions were carried out in 90 µL containing 50 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, 2 mM Na$_4$PP$_i$, 1 µCi ($^{32}$P)-PP$_i$, 0.005 - 4 mM of substrate and 0.52 µM of holo MBP-PhsA-His$_6$. Substrate concentrations in stock solutions were determined by $^{31}$P NMR spectroscopy. The reaction mixture was equilibrated at 30 °C before initiation of the reaction with 2 mM ATP. Aliquots were taken at each time-point, and analyzed by thin layer chromatography (TLC) (6, 7) using a polyethyleneimine plate (Scientific Adsorbents Inc., Atlanta, GA) pre-run in water. ATP and pyrophosphate were resolved using a developing solvent of 750 mM KH$_2$PO$_4$, pH 3.5, and 4 M urea. Separated radiolabeled products were visualized using a phosphorimaging plate (Fujifilm, BAS-MS2040) and the scanned image (Storm$^{TM}$) was analyzed using ImageQuant software. In addition to the expected exchange of $^{32}$P from pyrophosphate into ATP, small amounts of radiolabeled ADP and trace amounts of P$_i$ were also observed, as has been seen in other studies of adenylation domains (8, 9). ADP is typically not identified using the charcoal adsorption method as both ATP and ADP are adsorbed to the charcoal. The formation of these additional products was dependent on both PhsA and substrate. Exclusion of these radiolabeled products resulted in the same trends (larger apparent $k_{cat}$ for AcDMPT, smaller apparent $K_m$ for AcPT) as including them in the analysis. For apo-MBP-PhsA-His$_6$, the protein was treated under the same conditions as for holo protein.
Figure S3. Schematic representation of the PhsA assay. ATP is utilized for activation of the amino acid substrate as an adenylate with a release of pyrophosphate. The reverse reaction results in incorporation of phosphorus from pyrophosphate into ATP and can be monitored when using radiolabeled PPi (the pyrophosphate exchange assay). Adenylated substrate is loaded onto the phosphopantetheine arm of the T domain of PhsA by attack of the thiol onto the carboxyl carbon of the adenylate to have the amino acid covalently loaded.

Figure S4. Concentration dependence of the observed initial rates of amino acid-stimulated ATP-pyrophosphate exchange. (A) Apo-MBP-PhsA-His6 (open circles) and holo-MBP-PhsA-His6 (closed circles) with D,L-AcPT (B) Apo-MBP-PhsA-His6 with L-AcDMPT (open squares) or L-N-AcGlu (open diamonds), and holo-MBP-PhsA-His6 with L-AcDMPT (closed squares) or L-N-AcGlu (closed diamonds). The lines were obtained by fitting the data to the Michaelis-Menten equation.
FTMS Analysis of MBP-PhsA-His₆ after loading with AcPT and AcDMPT. Samples for FTMS were prepared in the same manner as above except that higher concentrations of protein were utilized. A solution of 12.4 µM MBP-PhsA-His₆ or its S16A mutant in 70 mM Tris-HCl, 10 mM MgCl₂, 2 mM DTT, 4 µM Sfp, and 200 µM CoA was incubated at 30 °C for 30 min for phosphopantetheinylation. To the solution was then added ATP 10 mM and 25, 250 or 450 µM AcDMPT or AcPT (final concentrations) for 1 h at 30 °C. Samples were quenched at 5, 10 30 and 60 minutes after substrate removal. All reactions were quenched with 5% formic acid, and centrifuged to remove any particulates before analysis by LC-MS. All mass spectral data were collected on a 7 Tesla LTQ-FT (Thermo Fisher Scientific) equipped with a Surveyor MS pump and autosampler. Reverse phase liquid chromatography (RPLC) was carried out on a Jupiter 2.0 X 50 mm C4 column (Phenomenex) using water (solvent A) and acetonitrile (solvent B) with 0.1% formic acid. LC-MS assays used a gradient program starting at 30% B increasing to 65% B over 15 min followed by a 20 min re-equilibration to initial conditions. The mass spectrometric method consisted of a low resolution ion trap full MS scan followed by a pseudo-MS² scan (NS at 75 V) for the phosphopantetheine (PPant) ejection assay (10, 11). Note that all species entering the mass spectrometer are fragmented simultaneously giving rise to highly complicated MS/MS data; however, the <5 part-per-million mass accuracy is the key to monitoring the specific m/z channels corresponding to the ions from PPant ejection. LC-MS data was analyzed using QualBrowser software (Thermo Fisher). For quantitation of PPant ejection products, Nozzle-Skimmer dissociation (NS) scans were summed over the period of MBP-PhsA-His₆ elution for each LC-MS run to obtain the total normalized levels of the PPant ejection ions. The predominant product was set to 100% and the substrate with lower activity was normalized to that value. Results from five replicate assays were used for comparison, with an observed precision of 9.7, 7.3 and 4.1 % at 1 sigma for the 25, 250 and 450 µM assays, respectively. The data with the wild-type MBP-PhsA-His₆ are shown in Figure S5.

![Figure S5](image-url)  
*Figure S5.* Peak intensities of PPant ejection ions resulting from loaded AcPT and AcDMPT with wild type MBP-PhsA-His₆. Data are the same as in Figure 3 in the main text.
Evaluation of the importance of the thioesterase motif in PhsA for loading selectivity. If the thioesterase motif in PhsA were to selectively remove AcDMPT (but not AcPT) after loading onto the Ppant arm of the PCP domain, the higher ATP-Pi exchange activity of AcDMPT at high substrate concentration would not be at odds with the lower amounts of loaded AcDMPT seen in Figure S5. Two experiments were conducted to investigate the possible involvement of this thioesterase motif. First, the putative catalytic Ser was mutated to Ala (MBP-PhsA-His₆ S16A). The experiment described above was repeated and the relative loading onto the T-domain was determined. As shown in Figure S6, the AcPT remained preferentially loaded at all substrate concentrations, suggesting the thioesterase motif is not involved in any post-loading editing.

In a second complementary experiment, potential time dependent hydrolysis of thioester bound intermediates after loading was investigated with wild type MBP-PhsA-His₆. First, 12.4 µM holo-MBP-PhsA-His₆ or its S16A mutant in 70 mM Tris-HCl, 10 mM MgCl₂, 10 mM ATP, 2 mM DTT was incubated with 1 mM AcDMPT or AcPT. Then, the proteins were passed through a Microbiospin 6 column (Bio-rad) previously equilibrated with 70 mM Tris-HCl, 10 mM MgCl₂, 2 mM DTT. This selectively removed small molecule substrates and allowed for monitoring the potential hydrolysis of thioester bound intermediates. As shown in Figure S7, the ratio of AcDMPT to AcPT did not change much during a 1 h time course suggesting that post-loading editing also does not occur in the wt protein.
Figure S6. Competition loading results determined by the LC-FTMS PPant ejection assay at the indicated substrate concentrations with the S16A mutant of MBP-PhsA-His6. A. Proposed structures of PPant ejection ions colored to match corresponding peaks. B. Peak intensities of PPant ejection ions resulting from loaded AcPT and AcDMPT.
Figure S7. Relative peak intensities of AcDMPT to AcPT at 5, 10, 30 and 60 min after passage through a gel filtration spin column to remove small molecule substrates. Diamonds, wt MBP-PhsA-His$_6$; squares MBP-PhsA-His$_6$ S16A.
Figure S8. Expanded view of Figure 2 in the main text including the pantetheine ejection ion at m/z 261.126 highlighted in red.
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