Supporting Information

In vitro Phosphinate Methylation by PhpK from *Kitasatospora Phosalacinea*

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Materials. All reagents were obtained from EMD Millipore/Merck KGaA, Thermo Fisher Scientific, or Research Products International Corporation unless otherwise indicated. SAM was from Safeway and was purified via reverse-phase HPLC using a C4 column (Grace Vydac) and a linear gradient of 100% 0.1% aqueous formate to 100% acetonitrile, concentrated by rotary evaporation, and stored in 0.1 M H_2SO_4. MTAN was overexpressed and purified as a hexahistidine-tagged recombinant protein according to a literature procedure.[1] NAcDMPT was synthesized by Asischem by modifying literature procedures.[2,3] NAcPT was synthesized by reacting 6 equivalents of acetic anhydride with a slurry of PT (Sigma-Aldrich) in acetic acid.

Cloning of phpK from Kitasatospora phosalacinea. *K. phosalacinea* DSM 43860 was obtained from the United States Department of Agriculture Agricultural Research Service Culture Collection as a lyophilized powder. The strain was reconstituted in liquid media and grown at 30 °C. Subsequently, *K. phosalacinea* was streaked onto agar plates to obtain single colonies. Genomic DNA from single colonies was isolated using the Wizard Genomic DNA purification kit (Promega). The *phpK* gene was amplified from *K. phosalacinea* genomic DNA using the polymerase chain reaction, ligated into the pETBlue-1 vector (EMD Millipore/Merck KGaA) according to the manufacturer's directions, and transformed into NovaBlue Gigasingles competent cells (EMD Millipore/Merck KGaA). Plasmids were isolated with the Spin Miniprep Kit (Qiagen). The resulting construct was sequenced and compared to the published *phpK* sequence from *S. viridochromogenes*. Due to poor expression, the *phpK* gene was re-amplified from the pETBlue-1 construct and ligated into the pET-30a (+) vector (EMD Millipore/Merck KGaA). The primers used were 5'-GAG AAG **CAT ATG** AAG CAC TGC ATA GTC-3' and 5'-TTT **GCG GCC GC**T TAT TAT CGA AAC TCA CTC CCT-3' (reverse complement; Sigma-Aldrich and Operon). The restriction sites (*Nde*I and *Not*I) are in bold type. After

transformation into NovaBlue GigaSingles competent cells, colonies were isolated for plasmid preparation as described above.

Overexpression and purification of PhpK. After sequencing, the plasmid (denoted KPPhpK-pET-30a G4) was transformed by electroporation into *Escherichia coli* Rosetta 2 (DE3) pLysS cells (EMD/Merck KGaA) for protein overexpression. Cells (3 mL) were transferred from an overnight culture into 2 L flasks containing 500 mL of Luria-Bertani broth supplemented with kanamycin (50 μg/mL) and $FeCl_3$ (50 μM). Cultures were grown at 37° C to an OD_{600} of 0.6-0.8, and protein overexpression was induced by the addition of isopropyl β-D-thiogalactoside (0.5 mM). Cells were incubated for 4 additional hours prior to harvesting by centrifugation. 1 L of culture typically yields ~2.5 g of cell paste. Cells were frozen at -20 or -80 °C until needed.

All purification steps were performed in an anaerobic chamber (Coy Laboratory Products). We modified a literature procedure for the solubilization and refolding of an iron-sulfur/corrinoid protein.[4] Cells were thawed and resuspended in 20 mM HEPES or EPPS, pH 8 (Buffer A) supplemented with 1 mM $MgSO_4$, 1 mM DTT, Benzonase (EMD Millipore/Merck KGaA) and 0.5 mM PMSF or AEBSF. After sonication, the lysate was centrifuged at high speed. The pellet was solubilized in buffer A with 1 mM $MgSO_4$, 1 mM DTT, and 6 M urea and centrifuged again. The resulting supernatant was transferred to a clean container, and SAM, ferrous ammonium sulfate, sodium sulfide, DTT, and PMSF or AEBSF were added. This mixture was diluted with buffer lacking urea and allowed to refold anaerobically overnight. The refolded mixture was centrifuged at high speed to remove precipitants and concentrated in an anaerobic chamber using an Amicon stirred ultrafiltration cell equipped with a YM-30 filter (EMD Millipore/Merck KGaA). The protein was centrifuged again and filtered before being

loaded onto an anionic exchange column (DEAE-5PW, Tosoh Biosciences) connected to an HPLC (Shimadzu) and eluted with a gradient of from 0-1 M KCl in buffer A. Fractions containing PhpK were pooled and concentrated as described above. Subsequently, the hypothesized iron-sulfur clusters in PhpK were anaerobically reconstituted with an excess of sodium sulfide, ferrous ammonium sulfate, and DTT overnight at ~20 °C. The reconstituted protein was centrifuged to remove precipitants and dialyzed into buffer A containing 1 mM $MgSO_4$. Aliquots of PhpK were frozen in the anaerobic chamber and stored in liquid nitrogen for future use. PhpK can be stored at concentrations of 20 mg/mL or more as assessed by the method of Waddell.[5] The typical yield of PhpK using this procedure is ~10 mg from 1 g of cell paste. In our hands, the protein is ~80-90% pure as assessed by SDS-PAGE (Figure S1).

EPR sample preparation and spectral collection. All manipulations for sample preparation were performed anaerobically. PhpK was thawed in the anaerobic chamber prior to sample set-up. After mixing protein with other components in concentrations similar to those used for assays (*vide infra*), samples were transferred to 4 mm EPR tubes (Norell), frozen in cold isopentane, and stored in liquid nitrogen. Low-temperature X-band EPR spectra were acquired on a Varian Line spectrometer equipped with a Varian E102 microwave bridge, an Oxford Instruments ESR-900 continuous flow helium flow cryostat, an Oxford Instruments 3120 temperature controller, and interfaced with a Linux system. Spectra were obtained and manipulated using the Xemr program (Jussi Eloranta, http://www.csun.edu/~jeloranta/xemr). A Varian gaussmeter and a Hewlett-Packard 5255A frequency converter and 5245L electronic counter were employed to measure the magnetic field strength and the frequency, respectively. A Hewlett-Packard 432A power meter was used to calibrate the microwave power. Spectra were acquired as the average of four 2 min scans. EPR spectra were recorded under the following

conditions: 9.252 GHz, 16 G modulation amplitude, 3400 G center field, 1000 G sweep width, 1 mW power, 8000 gain, 0.3 s time constant, 10 K.

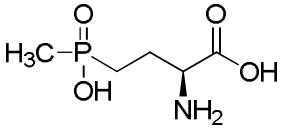
Enzymatic assays and NMR spectral collection. For assays, PhpK [100-200 μM] was thawed in the anaerobic chamber and incubated ~10 min with an excess of SAM [2-5 mM]. Sodium dithionite [4-5 mM] was added, and the mixture was allowed to incubate for ~4 hr. NAcDMPT [1 mM], $CH_3Cbl(III)$ [1-2 mM], and MTAN were added, and the reaction was allowed to incubate anaerobically for 18 hours or more. A control reaction was performed containing all components except sodium dithionite (Figure S4). The reaction mixtures were removed from the glove box, treated with NH_4OH, and centrifuged to remove precipitants. To approximate a zero time point (Figure S5), the sample was immediately removed from the glove box, exposed to air, and treated with NH_4OH in a span of approximately 30 seconds. The supernatant was then filtered through polyethersulfone centrifugal filters (molecular weight cutoff 10,000 or 30,000 Daltons; VWR) to remove protein. The resulting solution was loaded onto a Kromasil C-18 reverse-phase column (4.6 x 150 mm; Sigma-Aldrich) attached to a Beckman-Coulter System Gold HPLC. NAcDMPT and NAcPT were separated from some reaction components using a linear gradient from 100% buffer B (0.1% formic acid in water) to 100% acetonitrile. Appropriate fractions were collected, pooled, and concentrated by rotary evaporation. The resulting syrup was dissolved in D_2O (Cambridge Isotope Labs) to a final volume of ~500-550 μL for NMR spectroscopy. Two-dimensional ^{1}H-^{31}P gHSQC NMR spectra were collected using a Varian 600 MHz spectrometer or a Bruker 700 MHz spectrometer using a coupling constant of 15 Hz at 22 °C. Phosphoric acid was used as the standard. The positions of the peaks varied slightly between samples due to differences in final pH, and peak positions also

varied slightly between spectrometers. NMR spectra were processed with NMRPipe software (http://spin.niddk.nih.gov/NMRPipe/).

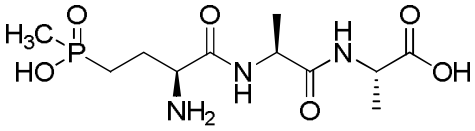
Abbreviations: SAM, *S*-adenosyl-L-methionine; HPLC, high performance liquid chromatography; NAcDMPT, 2-acetylamino-4-hydroxyphosphinylbutanoate or *N*-acetyldemethylphosphinothricin; NAcPT, 2-acetylamino-4-hydroxymethylphosphinylbutanoate or *N*-acetylphosphinothricin; PT, 2-amino-4-hydroxymethylphosphinylbutanoate or phosphinothricin; OD, optical density; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EPPS, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; PMSF, phenylmethylsulfonyl fluoride; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; CH_3Cbl(III), methylcobalamin; gHSQC, gradient heteronuclear single quantum correlation; E.COSY, exclusive correlation spectroscopy.

1. Cornell, K.A., Swarts, W.E., Barry, R.D., and Riscoe, M.K. (1996) *Biochem. Biophys. Res. Commun., 228*, 724–732.
2. Xiao, Y., Lee, K., and Liu, P. (2008) *Org Lett., 10*, 5521-5524.
3. Lee, J.H., Evans, B.S., Li, G., Kelleher, N.L., and van der Donk, W.A. (2009) *Biochemistry, 48*, 5054-5056.
4. Lu, W.P., Schiau, I., Cunningham, J.R., and Ragsdale, S.W. (1993) *J. Biol. Chem., 268,* 5605-5614.
5. Waddell, W.J. (1956) *J. Lab. Clin. Med., 48*, 311-314.

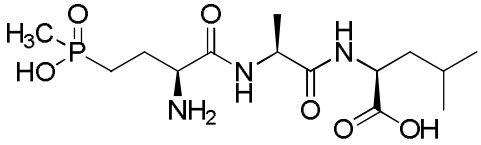
Chart S1. Natural Products Containing a C-P-C Linkage



Phosphinothricin



Bialaphos (L-PT-L-Ala-L-Ala)



Phosalacine (L-PT-L-Ala-L-Leu)

Figure S1. SDS-PAGE of PhpK. Lanes, left to right: 1, Molecular weight marker (top to bottom: 175, 83, 62, 47.5, 32.5, 25 kD); 2, refolded PhpK; 3, pooled, concentrated PhpK after anionic exchange purification; 4, reconstituted PhpK; 5, dialyzed PhpK.

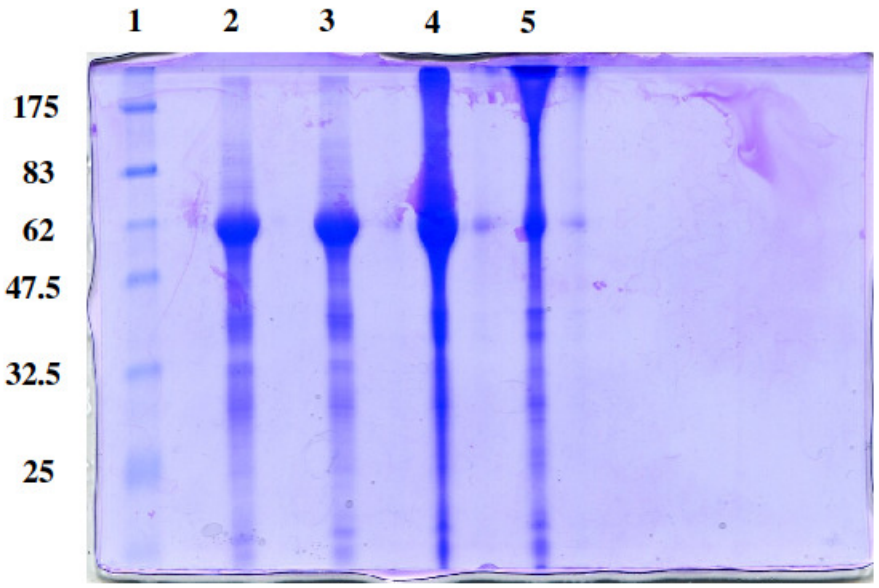


Figure S2. Ultraviolet-visible spectrum of PhpK. This spectrum corresponds to a sample containing approximately 6.3 mg/mL purified PhpK.

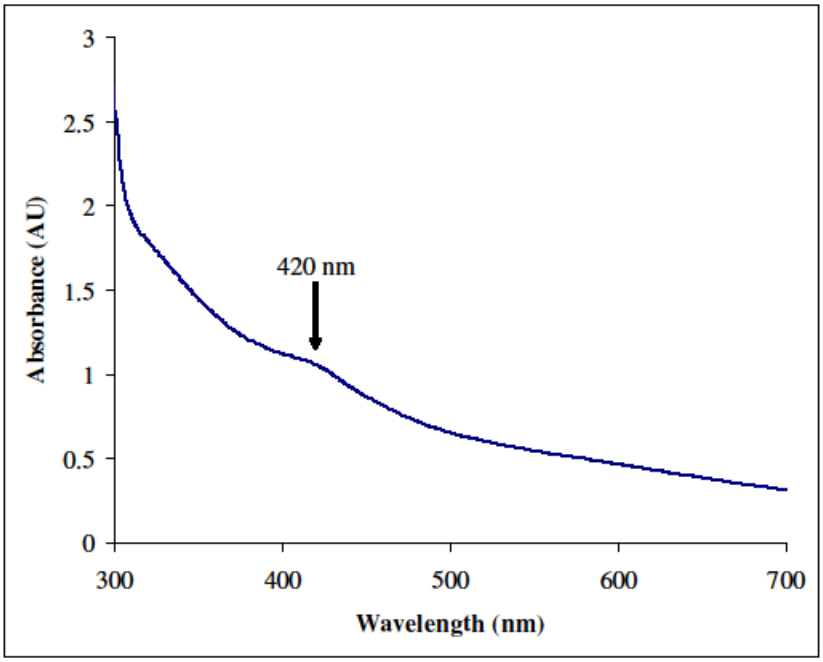


Figure S3. EPR data for PhpK. Spectra have been normalized to equivalent gain. Bottom (black), PhpK only; middle (blue), PhpK + dithionite; top (red), PhpK + dithionite + SAM + $CH_3Cbl(III)$ + NAcDMPT.

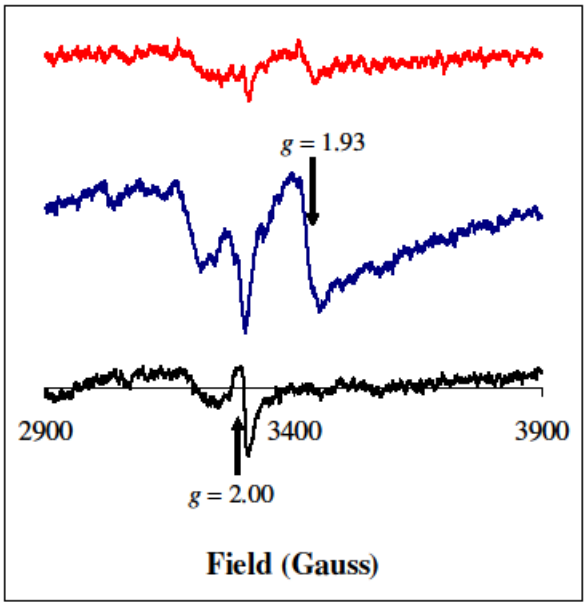


Figure S4. H-P gHSQC spectrum of the partially purified PhpK control reaction containing all components except sodium dithionite. The major crosspeak at (1.36, 29.7) ppm, surrounded by artifacts along the ^{31}P axis, corresponds to the C-4 methylene group of the substrate, NAcDMPT. Additional crosspeaks are observed at (1.60, 29.7) ppm and (1.80, 29.7) ppm corresponding to the C-3 methylene group of NAcDMPT and (6.77, 29.7) ppm corresponding to the phosphinyl proton of NAcDMPT.

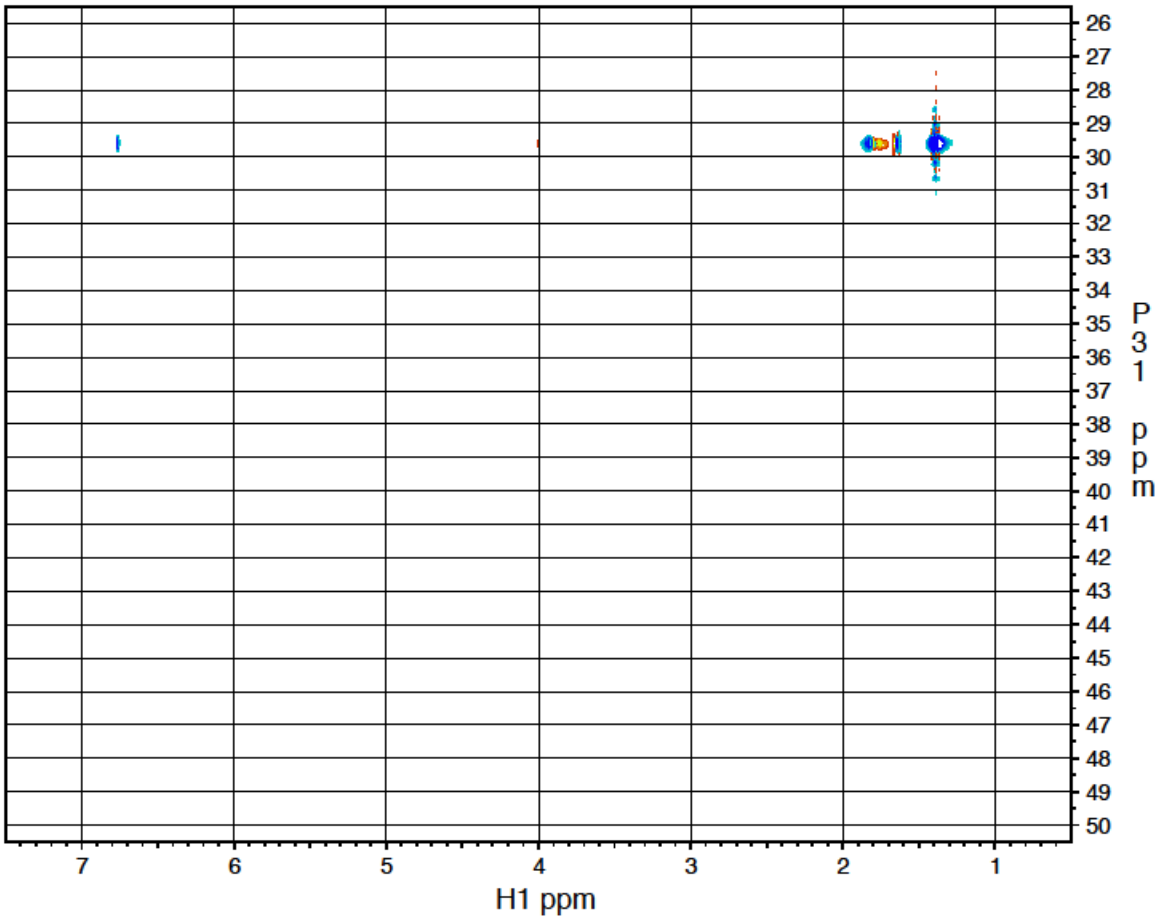


Figure S5. H-P gHSQC spectrum of the partially purified, immediately quenched PhpK reaction. The major crosspeak at (1.36, 29.7) ppm, surrounded by artifacts along the ^{31}P axis, corresponds to the C-4 methylene group of the substrate, NAcDMPT. Additional substrate-associated crosspeaks are observed at (1.60, 29.7) ppm and (1.80, 29.7) ppm corresponding to the C-3 methylene group of NAcDMPT and (6.77, 29.7) ppm corresponding to the phosphinyl proton of NAcDMPT. The crosspeak at (1.08, 43.5) ppm corresponds to the methyl group added by PhpK in NAcPT.

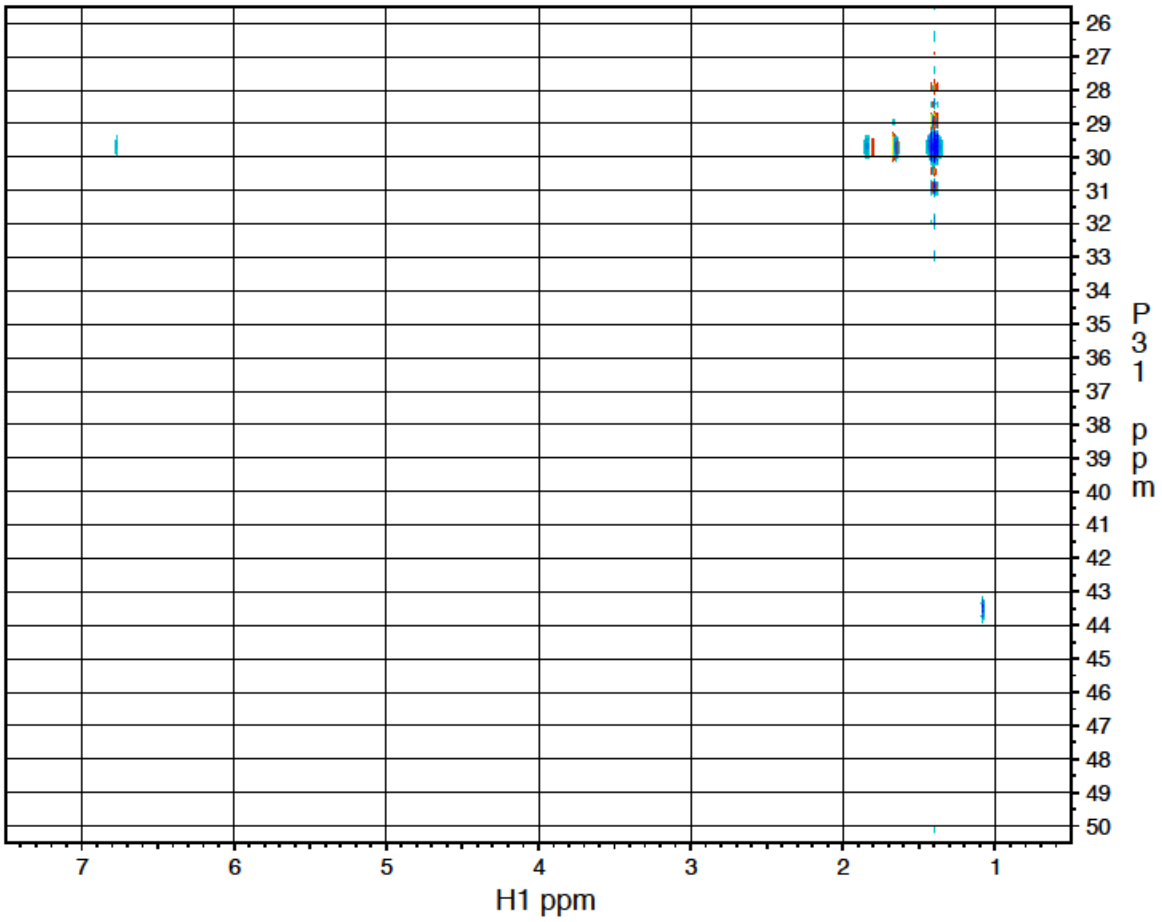


Figure S6. H-P gHSQC spectrum of the partially purified $^{12}CH_3$-Cbl(III) PhpK reaction allowed to incubate for ~18 h. The major crosspeak at (1.36, 30.0) ppm {surrounded by the artifacts at (1.36, 27.5) ppm, (1.36, 32.5) ppm, and (1.40, 30.0) ppm} corresponds to the C-4 methylene group of the substrate, NAcDMPT. The crosspeak at (1.07, 44.0) ppm corresponds to the methyl group added by PhpK in NAcPT.

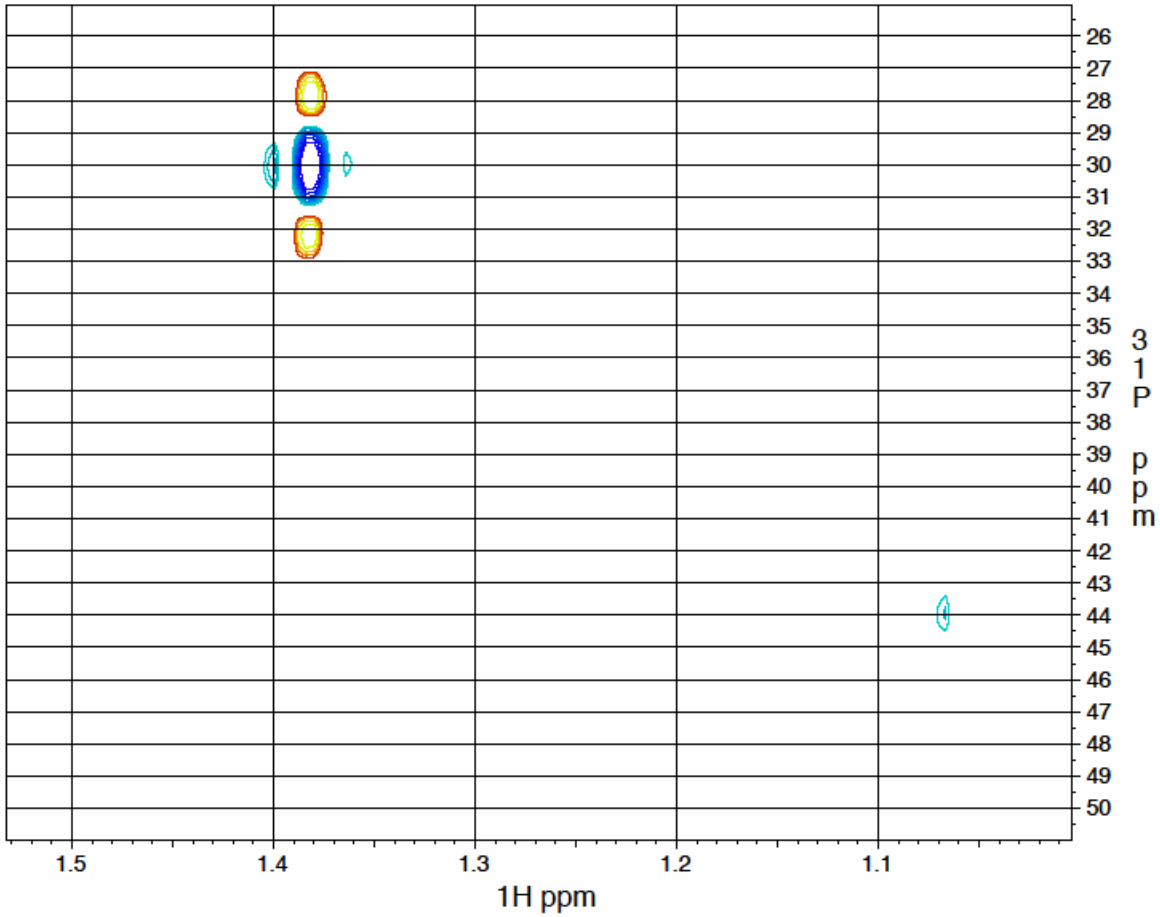


Figure S7. H-P gHSQC spectrum of the partially purified $^{12}CH_3$-Cbl(III) and $^{13}CH_3$-Cbl(III) PhpK reaction. The E.COSY pattern (indicated by the asterisks) of the H-P crosspeaks (centered at (1.06, 43.8) ppm and indicated by the "x") is observed due to the passive couplings of ^{1}H and ^{31}P to the attached ^{13}C nuclei of NAcPT derived using $^{13}CH_3$-Cbl(III) as the methyl group donor; please compare Figure 1 from the text. The center crosspeak at (1.06, 43.8) ppm indicated by the "x" arises from NAcPT derived using $^{12}CH_3$-Cbl(III) as the methyl group donor.