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Investigation of enzymatic C–P bond formation using multiple quantum HCP nuclear magnetic resonance spectroscopy

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Abstract

The biochemical mechanism for the formation of the C–P–C bond sequence found in L-phosphinothricin, a natural product with antibiotic and herbicidal activity, remains unclear. To obtain further insight into the catalytic mechanism of PhpK, the *P*-methyltransferase responsible for the formation of the second C–P bond in L-phosphinothricin, we utilized a combination of stable isotopes and two-dimensional nuclear magnetic resonance spectroscopy. Exploiting the newly emerged Bruker QCI probe (Bruker Corp.), we specifically designed and ran a ¹³C-³¹P multiple quantum ¹H-¹³C-³¹P (HCP) experiment in ¹H-³¹P two-dimensional mode directly on a PhpK-catalyzed reaction mixture using ¹³CH₃-labeled methylcobalamin as the methyl group donor. This method is particularly advantageous because minimal sample purification is needed to maximize product visualization. The observed 3:1:1:3 multiplet specifically and unequivocally illustrates direct bond formation between ¹³CH₃ and ³¹P. Related nuclear magnetic resonance experiments based upon these principles may be designed for the study of enzymatic and/or synthetic chemical reaction mechanisms.

Keywords

NMR; ¹H; ¹³C; ³¹P; phosphinates; methylation; enzymatic reaction mechanisms; cobalamin; radical S-adenosyl-L-methionine

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Introduction

L-Phosphinothricin (PT; L-2-amino-4-hydroxymethylphosphinylbutanoate) and its derivatives, bialaphos and phosalacine, contain the only stable, naturally occurring carbon–phosphorus–carbon (C–P–C) bonding sequences.^[1] PT, a methyl phosphinate, has been commercially used as an herbicide for over 20 years and is a relatively potent treatment for tuberculosis infections.^[1–4] Other phosphonates and phosphinates are interesting drug candidates because they are extremely stable and mimic biological carboxylates and/or phosphoester-containing compounds.

L-Phosphinothricin is naturally produced by *Streptomyces hygroscopicus*, *Streptomyces viridochromogenes*, and *Kitasatospora phosalacinea*.^[5–7] In one of the final steps of PT biosynthesis, the phosphinate substrate 2-acetylamino-4-hydroxyphosphinylbutanoate (*N*-acetyldemethylphosphinothricin, NAcDMPT) is methylated to produce *N*-acetylphosphinothricin (NAcPT) (Fig. 1), completing the C–P–C bond sequence. This reaction is catalyzed by the *P*-methyltransferase enzyme, PhpK. Its mechanism has been proposed to require the use of radical *S*-adenosyl-L-methionine (SAM) chemistry (Fig. 1A).^[8,9] Proteins of the radical SAM super-family typically bind a reducible four-iron, four-sulfur [(4Fe-4S)] cluster with a highly conserved CxxxCxxC cysteine sequence. An electron is donated from this cluster to homolytically cleave SAM, generating a highly reactive 5'-deoxyadenosyl radical (5'-dA•) that participates in catalysis.^[10]

We previously used a two-dimensional (2D) ¹H–³¹P gradient heteronuclear single quantum correlation (gHSQC) nuclear magnetic resonance (NMR) experiment to illustrate the unusual *P*-methyltransferase activity of PhpK from *K. phosalacinea*.^[11] Alternative analytical methods have proven nearly intractable for the study of PhpK because of separation difficulties and interference from other reaction components. 2D ¹H–³¹P NMR experiments benefit from the nearly 100% natural abundance of ³¹P. This contrasts with ¹H–¹³C and ¹H–¹⁵N correlation spectra, which usually require isotopic enrichment and/or high sample concentrations considering the low natural abundances of ¹³C and ¹⁵N. This issue is partially offset by the high sensitivity afforded by large and relatively uniform ¹J_{HC} and ¹J_{HN} coupling constants. In contrast, typical ³¹P-containing compounds such as nucleotides and other organophosphates instead often possess multiple bond ⁿJ_{HP} couplings via H–C–O–P linkages. These couplings are significantly smaller and are highly variable depending upon local geometry, thus limiting the usage of ¹H–³¹P gHSQC for small molecule characterization.

The growing need for detection of ³¹P-containing compounds at low concentrations has revived interest in ¹H-detected, multidimensional ³¹P NMR. These studies have been facilitated by the ever-increasing sensitivity of NMR probes for ¹H detection, such as the newly emerged Bruker QCI cryogenic probe. For instance, ¹H–³¹P–³¹P correlated spectroscopy (COSY) experiments have been developed to characterize crude methylerythritol phosphate pathway products and polyphosphorylated small molecules.^[12,13] These studies demonstrated that enzymatic reactions could be studied *in situ* at low substrate concentrations without isotopic enrichment or purification. Other recent

studies have employed 2D heteronuclear multiple bond correlation for the identification of C–P compounds and the elucidation of enzymatic activities.^[14–17]

Because CH₃Cbl is typically a cofactor *in vivo*,^[18] we wished to more closely examine the role of this molecule in *P*-methylation. PhpK requires reducing equivalents, SAM, and methylcobalamin (CH₃Cbl) for activity and is apparently only capable of single turnover when reduced by sodium dithionite.^[11] Here, we exploit the high sensitivity of the new Bruker QCI probe with a new triple resonance, multiple quantum (MQ) version HCP experiment in ¹³C-³¹P to unequivocally illustrate PhpK-catalyzed bond formation between ¹³CH₃-methyl and the ³¹P-phosphinate. This method is advantageous because NMR data can be collected with minimal sample purification. Triple resonance single quantum HCP was first used for sequential backbone assignment of ¹³C-labeled RNA. H–C–P correlations were built through one bond *J*-coupling of ¹J_{CH} (–145 Hz) and multiple bond *J*-couplings of ^{*n*}J_{CP} where *n* = 2 or 3.^[19,20]

Later, to obtain RNA phosphodiester backbone conformation restraints, HCP spectral resolution along ¹³C was doubled using combined evolution technique for measurement of ³J_{C2'P} and ³J_{C4'P} couplings and ³¹P anisotropic chemical shift change.^[21] Ultimately, related NMR experiments may be designed for quadruple and/or broadband triple resonance probes and applied to the study of enzymatic and/or synthetic organic reaction mechanisms to confirm bond formation between specific magnetic nuclei.

Results and discussion

In Fig. 2, we present our ¹³C-³¹P MQ HCP experiment. ¹H, ¹³C, and ³¹P radio-frequency pulses are applied at 4.77, 23.0, and 56.0 ppm, respectively, and ¹³C- and ³¹P-globally optimized alternating-phase rectangular pulses decouplings are applied with field strengths γB₂ of 4.54 kHz and 2.5 kHz, respectively. All pulsed field gradients are applied along the *z*-axis with a 1 ms duration followed by a gradient recovery period of 200 μs. Because ^{*n*}J_{HP} is usually small and variable, we did not use it for direct coherence transfer from ¹H to ³¹P as is done in gHSQC. Instead, 2D ¹H-³¹P correlations are built through tandem insensitive nuclei enhanced by polarization transfer (INEPT) steps for coherence transfer from ¹H to ¹³C and then from ¹³C to ³¹P, making use of one bond *J*-couplings of ¹J_{CH} and ¹J_{CP}. This contrasts with reported RNA HCP experiments in which multiple bond *J*-couplings of ^{*n*}J_{CP}, which are usually variable and relatively small, are used for coherence transfer.^[19,21]

The relevant coherence transfer pathway can be described as shown in Fig. 3. Initial ¹H polarization of the methyl group is excited by a 90° pulse on ¹H followed by an INEPT period for coherence transfer to ¹³C (point *b*), which is again followed by another INEPT period (point *b* to point *c*) for coherence transfer from ¹³C to ³¹P. ¹³C-³¹P MQ coherence is generated by the pulse on ³¹P with phase φ₃ (point *d*). During time period *t*₁, the chemical shift of ³¹P is encoded together with the *J*_{HC} coupling through simultaneous application of 180° pulses on ¹³C and ¹H, but the chemical shift of ¹³C is not encoded. After the time period *t*₁, 180° pulses are applied on ¹H (blank with phase ‘y’) and ³¹P followed by a short compensation delay δ to refocus both the *J*_{CH} coupling effect and the chemical shift of ³¹P during the *t*₁ first data point interval to eliminate the phasing issue. Application of the 90°

pulses on ^{31}P (point *e*) transfers the coherence back to ^{13}C (point *f*), coherence is then transferred back to ^1H and refocused for detection during the reverse INEPT period.

The ^{13}C - ^{31}P MQ HCP experiment is performed in ^1H - ^{31}P 2D mode. The final detection term $H_x(\omega_p, J_{\text{HC}})$ indicates that it is encoded by both the chemical shift of ^{31}P and the J_{HC} coupling. The term $(\omega_p, J_{\text{HC}}, \gamma_{\text{C}}g_1)$ indicates coherence is timely encoded by the chemical shift of ^{31}P (ω_p) and J -coupling of J_{HC} , and it is spatially encoded by the gradient g_1 when coherence is on ^{13}C ($\gamma_{\text{C}}g_1$). Gradient selection is achieved by application of the refocusing gradient g_2 with $\gamma_{\text{C}}g_1 = \gamma_{\text{H}}g_2$ (Fig. 3, last line) during the reverse INEPT period $2\tau_1$, provided the pulse width of field gradients g_1 and g_2 is the same. Thus, the 2D ^1H - ^{31}P spectrum is ^{13}C -edited and filtered, rendering a much clearer spectrum through direct J_{HP} coherence transfer compared with a conventional 2D ^1H - ^{31}P spectrum. The resulting clarity allows this specially designed HCP experiment to clearly identify and differentiate the functional group of interest, in this case, $^{13}\text{CH}_3$ - ^{31}P , from the background.

Evolution of the ^{13}C - ^{31}P MQ coherence is encoded by both the ^{31}P chemical shift Hamiltonian and the J_{CH} coupling Hamiltonian. Temporarily ignoring the chemical shift effect of ^{31}P , the MQ coherence term $4H_zC_yP_y$ will behave similarly to coherence $2H_zC_y$ under the J_{CH} coupling Hamiltonian for a CH_3 -group, which will give a typical 3:1:1:3 peak splitting pattern.^[22] Splitting occurs along the ^{31}P dimension because the simultaneous evolution of the J_{CH} coupling Hamiltonian with the ^{31}P chemical shift Hamiltonian during t_1 .

Figure 4 shows the 2D ^1H - ^{31}P spectrum of the resulting MQ HCP of the reaction mixture. The region including the signal of the target product is outlined by the dashed line and expanded to the right. The cross-peak of interest is observed at 1.35 ppm and 56.5 ppm along the ^1H and ^{31}P dimensions, respectively. Slices taken along the ^{31}P dimension at the chemical shifts indicated by broken lines are shown at bottom right. The informative 3:1:1:3 peak splitting pattern evident at 1.35 ppm ^1H and 56.5 ppm ^{31}P unequivocally illustrates that $^{13}\text{CH}_3$ is directly attached to ^{31}P .^[22] The additional 1:0:1 peak splitting pattern observed along the ^{31}P dimension at 1.70 ppm of ^1H indicates a ^{31}P - $^{13}\text{CH}_2$ group, which is a contaminant that may result from cyclization of the ^{13}C -labeled methyl phosphinate. Our control spectra show no evidence of this peak.

As illustrated in Fig. 5, the doublet-quadruplet splitting pattern observed at 13.4 ppm in the ^{13}C spectrum of the reaction mixture provides additional evidence for direct attachment of the ^{13}C -labeled methyl to the ^{31}P atom. The splitting pattern of the ^{31}P - $^{13}\text{CH}_3$ group of the NAcPT product is highlighted in the zoomed-in panel. Despite the fact that the ^{31}P - $^{13}\text{CH}_3$ -containing product is enriched by isotopic labeling, its concentration is so low that its signal intensity is easily masked. Thus, the observed ^{13}C signals are very weak compared with those of the NAcDMPT reactant. Thus, without prior knowledge, it would be extremely difficult to identify the product on the basis of only the ^{13}C spectrum of the sample. Therefore, the data collected from our newly designed HCP experiment are clearly more specific and informative than the ^{13}C spectrum alone. Indeed, our method is advantageous for the analysis and/or identification of unknown compounds that can be produced with ^{13}C labeling.

Our NMR results clearly support a reaction mechanism in which CH₃Cbl is the direct methyl group donor. Two potential mechanisms are diagrammed in Fig. 1. Radical SAM chemistry (Fig. 1A) leads to the highly reactive 5'-dA•, which could abstract the H atom from the NAcDMPT phosphinate to form 5'-deoxyadenosine (5'-dA) and a substrate phosphonyl radical. The resulting phosphonyl radical reacts with CH₃Cbl to give NAcPT and cobalamin (Cbl) in the +2 oxidation state (Cbl(II)).^[8,9] Alternatively, nucleophilic substitution (Fig. 1B) requires deprotonation of the phosphinate. The resulting nucleophile attacks CH₃Cbl via S_N2 chemistry to form the NAcPT product and leaves Cbl in the +1 oxidation state (Cbl(I)).

Although the observed requirement of reducing equivalents for methylation *in vitro* favors the first mechanism, we cannot rule out the second mechanism, in part because of our NMR findings reported here. Interestingly, we observed complete exchange of the phosphinyl proton with deuterium, as shown by the characteristic 1:1:1 triplet at 1.70 ppm ¹H and 53.5 ppm ³¹P in Fig. 6. This result indicates that deprotonation of the phosphinyl proton occurs nonenzymatically despite an unfavorable predicted pK_a. In this particular sample, we believe that deprotonation and subsequent exchange was facilitated by the low resulting sample pH after cationic exchange to remove buffer salts that would have affected the cryoprobe. We are currently investigating the rate of phosphinyl proton exchange in the presence of PhpK to determine its potential physiological and catalytic relevance.

Our previous results suggested that PhpK was only capable of single turnover *in vitro*, because the observed amount of NAcPT product formed was approximately stoichiometric with enzyme.^[11] To reduce spectral collection times for the NMR experiments described herein, we combined several reaction mixtures to maximize ¹³C-labeled product. We unexpectedly observed ¹²CH₃-NAcPT product, which is associated with the small cross-peak centered at 1.36 ppm of ¹H and 56.5 ppm of ³¹P, in addition to the expected ¹³C-labeled product (Fig. 6). We had not observed this cross-peak in our previous experiments with PhpK.^[11] Integration of cross-peak volumes indicates that 5–10% ¹²CH₃-NAcPT was formed. Mass spectral analysis of the original ¹³CH₃Cbl suggests that it is 93.91% ¹³C-labeled and thus contains approximately 6.09% ¹²CH₃. Thus, our NMR results remain consistent with single turnover and indicate that PhpK is apparently unable to remethylate Cbl under our current reaction conditions.

Concluding remarks

In summary, we specifically designed a triple resonance ¹³C-³¹P MQ HCP experiment to illustrate that *P*-methylation catalyzed by PhpK from *K. phosalacinea* uses ¹³CH₃Cbl and not another source as the direct methyl group donor. The definitive 3:1:1:3 multiplet pattern in the 2D ¹H-³¹P spectrum of the ¹³C-³¹P MQ HCP experiment (Fig. 6) unequivocally illustrates direct ³¹P-¹³CH₃ bond formation. These spectroscopic results agree with previous experiments using cell-free extracts, which demonstrated that ¹⁴CH₃-Cbl was the only source of the methyl group in the PhpK reaction.^[23] Other recently characterized cobalamin-dependent radical SAM methyltransferases such as TsrM and GenK have recently been shown to use SAM as a methyl group donor *in vitro*, even in the presence of excess CH₃Cbl.^[24,25] In particular, our results contrast with those reported for TsrM for which no

incorporation of ^{13}C -label was observed even in the presence of 25-fold excess $^{13}\text{CH}_3\text{Cbl}$.^[24] Under our reaction conditions, we clearly observed transfer of ^{13}C -label to the product, indicating that the primary methyl donor is CH_3Cbl . Further experiments using isotopically labeled methyl donors, such as $^{13}\text{CH}_3\text{-SAM}$, and/or alternative reducing agents are needed to determine conditions for potential remethylation of Cbl by PhpK *in vitro*.

The essentially 100% natural abundance of ^{31}P ensures that ^{13}C -edited ^1H - ^{31}P 2D spectra have high sensitivity. These spectra are significantly more specific and informative than the ^{13}C spectrum alone. Another major advantage of our method is the minimal sample clean-up needed prior to spectral collection. For PhpK, this is particularly useful because separating the compounds of interest is difficult, and the level of turnover is low for this apparently suicidal enzyme. Therefore, we are able to visualize maximal product formation using this MQ HCP experiment. For these reasons, we anticipate that similar, specially designed NMR experiments can confirm specific bond formation between NMR-active nuclei. Quadruple or broadband triple resonance probes may be used to apply these approaches at either room and/or cryogenic temperatures. Such methods may thus be widely applicable to the study of a variety of reaction mechanisms. These types of experiments may become especially important as natural products containing C–P bonds continue to be explored because of their potential significance in biological, ecological, and medical contexts.^[8,16,26,27] In addition, our results set the stage for future mechanistic investigations of the PhpK *P*-methyltransferase using NMR spectroscopy to delineate the roles of the players in this complex methylation reaction.

Methods

Sample preparation

NACDMPT was synthesized by AsisChem Inc. (Watertown, MA, USA) by modifying literature procedures.^[28,29] $^{13}\text{CH}_3\text{Cbl}$ was synthesized according to a literature procedure using 99% ^{13}C $^{13}\text{CH}_3\text{I}$ (Sigma-Aldrich, St. Louis, MO, USA) as the starting material.^[30] Mass spectrometric analysis of the resulting $^{13}\text{CH}_3\text{Cbl}$ indicated that it contained ~6.09% $^{12}\text{CH}_3$ -methyl.

PhpK from *K. phosalacinea* was overexpressed, refolded, and purified as described previously.^[11] Iron–sulfur cluster reconstitution and set-up of PhpK-catalyzed reactions were carried out as described previously^[11] with the following modifications. Twenty-seven 1ml reactions were incubated in the anaerobic chamber overnight. After incubation, the reactions were removed from the anaerobic chamber, quenched with NH_4OH (~10% final concentration), and allowed to partially evaporate in a fume hood overnight. PhpK was removed from the pooled reactions using polyethersulfone centrifugal filters (VWR, Radnor, PA, USA) or Pierce Protein Concentrators (Thermo Fisher Scientific Inc., Rockford, IL, USA). The resulting filtrate was partially purified using cation exchange resin (AG-50, Acros Organics, Geel, Belgium) equilibrated in and eluted with deionized water to remove buffer salts. The eluent was concentrated via rotary evaporation to dryness and resuspended in 500ul D_2O (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA) for NMR analysis. The final concentration of NACDMPT substrate was ~50 mM, and the final concentration of NAcPT product was ~0.5mM or ~1% relative to substrate. NMR

experiments. Initial NMR spectra were collected at 22°C using a 600 MHz Varian spectrometer. MQ HCP data were collected at 25°C on a 700 MHz Bruker Avance spectrometer equipped with a 5mm z-gradient quadrupole resonance cryogenic QCI probe. The HCP experiment was run in 2D mode without ^{13}C chemical shift evolution. Complex data points of 2048×150 were collected along the ^1H and ^{31}P dimensions with spectral widths of 16 and 9ppm, respectively. Spectral folding shifted the NAcDMPT-associated cross-peaks along the ^{31}P axis compared with our previous work.^[11] In addition, the observed peaks are downshifted compared with previous studies^[11] because of the high acidity of the sample after cationic exchange to remove buffer salts. Scans of 16 per FID and an inter-scan delay of 2 s resulted in a total data acquisition time of ~3h. The ^1H - ^{31}P gHSQC spectrum (Fig. 6) was acquired with similar parameters, that is, 2048×150 complex data, 16 scans per FID, and an inter-scan delay of 2 s, resulting in a total acquisition time of ~3h. The ^{13}C spectrum (Fig. 5) was acquired using zg30 sequence with 8192 scans and an inter-scan delay of 1.5 s, resulting in a total data acquisition time of ~4h. NMR data were processed using NMRPipe software.^[31]

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References

1. Thompson, C.J.; Seto, H. *Genetics and Biochemistry of Antibiotic Production*. Butterworth Heinemann Bio/Technology; Oxford: 1995. p. 197-222.
2. Hoerlein G. *Rev Environ Contam Toxicol*. 1994; 138:73–145. [PubMed: 7938785]
3. Harth G, Horwitz MA. *J Exp Med*. 1999; 189:1425–1436. [PubMed: 10224282]
4. Harth G, Horwitz MA. *Infect Immun*. 2003; 71:456–464. [PubMed: 12496196]
5. Bayer E, Gugel KH, Hägele K, Hagenmaier H, Jessipow S, König WA, Zähler H. *Helv Chim Acta*. 1972; 55:224–239. [PubMed: 5010035]
6. Seto H, Sasaki T, Imai S, Tsuruoka T, Ogawa H, Satoh A, Inouye S, Niida T, Otake N. *J Antibiot*. 1982; 36:96–98. [PubMed: 6678916]
7. Omura S, Murata M, Hanaki H, Hinotozawa K, Oiwa R, Tanaka H. *J Antibiot*. 1984; 37:829–835. [PubMed: 6480502]
8. Metcalf WW, van der Donk WA. *Annu Rev Biochem*. 2009; 78:65–94. [PubMed: 19489722]
9. Booker SJ. *Curr Opin Chem Biol*. 2009; 13:58–73. [PubMed: 19297239]
10. Sofia HJ, Chen G, Hetzler BG, Reyes-Sindola JF, Miller NE. *Nucleic Acids Res*. 2001; 29:1097–1106. [PubMed: 11222759]
11. Werner WJ, Allen KD, Hu K, Helms GL, Chen BS, Wang SC. *Biochemistry*. 2011; 50:8986–8988. [PubMed: 21950770]
12. Majumdar A, Shah MH, Bitok JK, Hassis-LeBeau ME, Meyers CLF. *Mol BioSys*. 2009; 5:935–944.

13. Majumdar A, Sun Y, Shah M, Meyers CLF. J Org Chem. 2010; 75:3214–3223. [PubMed: 20408590]
14. Borisova SA, Circello BT, Zhang JK, van der Donk WA, Metcalf WW. Chem Biol. 2010; 17:28–37. [PubMed: 20142038]
15. Peck SC, Gao J, van der Donk WA. Methods Enz. 2012; 516:101–123.
16. Yu X, Price NPJ, Evans BS, Metcalf WW. J Bact. 2014; 196:1768–1779. [PubMed: 24584498]
17. Cioni JP, Doroghazi JR, Ju KS, Yu X, Evans BS, Lee J, Metcalf WW. J Nat Prod. 2014; 77:243–249. [PubMed: 24437999]
18. Frey, PA. Comprehensive Natural Products II: Chemistry and Biology. Vol. 7. Elsevier; Philadelphia, PA: 2010. p. 501-546.
19. Heus HA, Wijmenga SS, Vandeven FJM, Hilbers CW. J Am Chem Soc. 1994; 116:4983–4984.
20. Marino JP, Schwalbe H, Anklin C, Bermel W, Crothers DM, Griesinger C. J Am Chem Soc. 1994; 116:6472–6473.
21. O’Neil-Cabello E, Wu ZR, Bryce DI, Nikonowicz EP, Bax A. J Biomol NMR. 2004; 30:61–70. [PubMed: 15452435]
22. Tugarinov V, Hwang PM, Ollerenshaw JE, Kay LE. J Am Chem Soc. 2003; 125:10420–10428. [PubMed: 12926967]
23. Kamigiri K, Hidaka T, Imai S, Murakami T, Seto H. J Antibiot. 1992; 45:781–787. [PubMed: 1624380]
24. Pierre S, Guillot A, Benjdia A, Sandström C, Langella P, Berteau O. Nat Chem Biol. 2012; 8:957–959. [PubMed: 23064318]
25. Kim HJ, McCarty RM, Ogasawara Y, Liu YN, Mansoorabadi SO, Levieux J, Liu HW. J Am Chem Soc. 2013; 135:8093–8096. [PubMed: 23679096]
26. Watanabe Y, Nakajima M, Hoshino T, Jayasimhulu K, Brooks EE, Kaneshiro ES. Lipids. 2001; 36:513–519. [PubMed: 11432465]
27. Yu X, Doroghazi JR, Janga SC, Zhang JK, Circello B, Griffin BM, Labeda DP, Metcalf WW. Proc Natl Acad Sci U S A. 2013; 110:20759–20764. [PubMed: 24297932]
28. Xiao Y, Lee K, Liu P. Org Lett. 2008; 10:5521–5524. [PubMed: 19053721]
29. Lee JH, Evans BS, Li G, Kelleher NL, van der Donk WA. Biochemistry. 2009; 48:5054–5056. [PubMed: 19432442]
30. Tollinger M, Derer T, Konrat R, Krautler B. J Mol Catal A: Chem. 1997; 116:147–155.
31. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. J Biomol NMR. 1995; 6:277–293. [PubMed: 8520220]

Figure 1.

Potential mechanisms for PhpK catalysis. (A) Radical *S*-adenosyl-L-methionine (SAM) and (B) S_N2 nucleophilic substitution.

Figure 2.

Pulse sequence of ^{13}C - ^{31}P MQ HCP experiment confirming connectivity of $^{13}\text{CH}_3$ to ^{31}P . Narrow and wide bars indicate non-selective 90° and 180° pulses. The delays are $\tau_1 = 1.92$ ms (taken $J_{\text{CH}} = 130\text{ Hz}$); $\tau_2 = 2.78$ ms (taken $J_{\text{CP}} = 90\text{ Hz}$); The short delay δ together with the blank 180° pulse applied on ^1H with phase 'y' refocuses the chemical shift of ^{31}P and the J -coupling effect between ^1H and ^{13}C (J_{CH}) during the t_1 first data point interval. Phase cycling is as follows: $\phi_1 = 2[y], 2[-y]$; $\phi_2 = y, -y$; $\phi_3 = 4[x], 4[-x]$; $\phi_{\text{rec}} = x, -x, -x, x, -x, x, x, -x$. All other radio-frequency pulses are applied with phase x unless indicated otherwise. Quadrature detection in the P (t_1) dimension is achieved using States-TPPI applied to the phase ϕ_3 . Quadrature detection in the ^{13}C (t_2) dimension is achieved by using echo-antiecho-TPPI applied to phase ϕ_2 and by flipping the polarity of gradient g_1 every another FID (as indicated by the filled and open sine bells). Gradient strengths (percentage of highest gradient current, 10 A) are: g_3 : 21.2 G/cm (40%); g_4 : 21.2 G/cm (40%). The selective gradient pairs are: g_1 : 42.4 G/cm (80%); g_2 : 10.653 G/cm (20.1%).

$$\begin{aligned}
 H_z \xrightarrow{90_x^0} -H_y(a) \xrightarrow{INEPT} 2H_xC_z \xrightarrow{90_{H,y}^0, 90_{C,\emptyset_1}^0} \xrightarrow{INEPT} -4H_zC_yP_z(c) \xrightarrow{90_{P,\emptyset_3}^0} 4H_zC_yP_y(d) \xrightarrow{t_1, \delta} 4H_zC_yP_y(\omega_P, J_{HC})(e) \\
 \xrightarrow{90_{P,x}^0} 4H_zC_yP_z(\omega_P, J_{HC})(f) \xrightarrow{2\tau_2, g_1} 2H_zC_x(\omega_P, J_{HC}, \gamma_C g_1)(g) \xrightarrow{90_{C,\emptyset_2}^0, 90_{H,x}^0, 2\tau_1, g_2} H_x(\omega_P, J_{HC})
 \end{aligned}$$

Figure 3.

Coherence transfer pathway of ^{13}C - ^{31}P MQ HCP experiment.

Figure 4.

^1H - ^{31}P correlation spectrum of the ^{13}C - ^{31}P MQ HCP experiment. The region including the signal of the target NAcPT product is expanded to the right. The cross-peak for NAcPT is observed at 1.35 and 56.5 ppm along the ^1H and ^{31}P dimensions, respectively. Slices taken along the ^{31}P dimension indicated by the broken lines at the chemical shift of ^1H 1.35 ppm are shown at bottom right.

Figure 5.

One-dimensional ^{13}C spectrum of the PhpK reaction sample. The spectrum of the targeted reaction product is emphasized in the zoomed-in panel. The ^{13}C peak of the product shows a doublet-quadruplet pattern due to the J_{CP} (doublet) and J_{CH} (quadruplet, $-\text{CH}_3$ group) couplings. Multiplets from a minor impurity are indicated by red arrows.

Figure 6.

^1H - ^{31}P gHSQC spectrum illustrating H-D exchange of NAcDMPT. The 1:1:1 cross-peak splitting pattern centered at 1.70 ppm of ^1H and 53.5 ppm of ^{31}P is a characteristic of J_{PD} coupling. The cross-peak centered at 1.36 ppm of ^1H and 56.5 ppm of ^{31}P corresponds to $^{13}\text{CH}_3\text{-NAcPT}$. The 1:1 cross-peak splitting pattern centered at 1.70 ppm ^1H and 56.5 ppm ^{31}P corresponds to the methylene at C-4 of the NAcPT product.