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Unusual transformations in the biosynthesis of the antibiotic phosphinothricin tripeptide

Joshua A.V. Blodgett[1], **Paul M. Thomas**[2], **Gongyong Li**[2,#], **Juan E. Velasquez**[2], **Wilfred A. van der Donk**[2], **Neil L. Kelleher**[2], and **William W. Metcalf**[1,*]

[1]Department of Microbiology, University of Illinois at Urbana-Champaign, B103 CLSL, 601 S. Goodwin, Urbana, IL 61801

[2]Department of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Matthews Ave., Urbana, IL 61801

Abstract

Phosphinothricin-tripeptide (PTT, phosphinothricyl-alanyl-alanine) is a natural product antibiotic and potent herbicide that is produced by *Streptomyces hygroscopicus* ATCC 21705[1] and *Streptomyces viridochromogenes* DSM 40736[2]. PTT has attracted widespread interest due to its commercial applications and unique phosphinic acid functional group. Despite intensive study since its discovery in 1972 (see[3] for a comprehensive review), a number of steps early in the PTT biosynthetic pathway remain uncharacterized. Here we report a series of interdisciplinary experiments involving the construction of defined *S. viridochromogenes* mutants, chemical characterization of accumulated intermediates, and *in vitro* assay of selected enzymes to examine these critical steps in PTT biosynthesis. Our results indicate that early PTT biosynthesis involves a series of heretofore undescribed catalyses, including a highly unusual reaction for carbon bond cleavage. In sum, we define a more complex pathway for early PTT biosynthesis that includes biochemically unprecedented and chemically interesting steps.

Keywords

Streptomyces viridochromogenes; phosphinothricin; biosynthesis; bialaphos; phosphonate metabolism

[*]Corresponding Author: Department of Microbiology, University of Illinois at Urbana-Champaign, 601 South Goodwin Avenue, Urbana, IL 61801, Phone: 217-244-1943, Fax: 217-244-6697, metcalf@uiuc.edu.
[#]Present Address: Shanghai Chemspec Corp., No. 3 Lan 1273, Tong Pu Road, Shanghai, China 200333.

Accession codes

Sequence data from the PTT biosynthetic gene cluster that was used in designing the experiments reported here was derived solely from previously available sequence (GenBank accession number AY632421). Sequence data generated in this study that corrects apparent sequencing errors in *S. hygroscopicus* PTT biosynthetic genes (found in GenBank accessions AB029917.1 and D37878.1) were deposited in GenBank under accession numbers EF486265 and EF486266 respectively.

Author Contributions

W.W.M. and J.A.V.B. designed most experiments and wrote the manuscript. J.A.V.B. conducted all microbiological, genetic and molecular biological experiments. NMR analyses were conducted by J.A.V.B. and G.L.. G.L., J.E.V. and W.A.V. designed and carried out chemical syntheses and analyses. P.M.T. and N.L.K. designed and conducted the mass spectrometry experiments.

The bioactive portion of the PTT (also known as bialaphos, **1**) tripeptide is phosphinothricin (PT, **2**). PT is the only known naturally occurring compound to incorporate a direct carbon to phosphorus to carbon (C-P-C) bond motif. The phosphinic acid functional group comprised by this bonding arrangement allows PT to act as a glutamine synthetase inhibitor, where it mimics the tetrahedral transition state of the catalytic intermediate γ-glutamyl phosphate[4]. PTT is a member of a growing family of secondary metabolites containing phosphonate or phosphinate functional groups. This family includes fosfomycin (**3**), a clinically utilized antibacterial[5], and fosmidomycin (**4**), a potent antimalarial agent currently undergoing field trials[6].

PTT has become a model for the biosynthesis of phosphonic acid antibiotics based on numerous studies[3], leading to a pathway that is regarded as mostly solved. It should be noted, however, that many of the PTT non-producing mutants analyzed to define the pathway were generated by random chemical mutagenesis and some mutations were not rigorously mapped to individual genes. Accordingly, the resulting biosynthetic model is based upon a patchwork of chemical, enzymatic, and genetic data. Further, though many PTT biosynthetic genes were previously cloned and sequenced, the full gene cluster was not completely sequenced from either producer until very recently, when the cluster from *S. viridochromogenes* was isolated and characterized[7, 8]. Sequence analysis of the cluster revealed that only about half of the genes identified had rigorous genetic or biochemical data establishing their roles in PTT biosynthesis. Some of the remaining genes were assigned tentative biosynthetic roles based upon sequence homology, but others could not be confidently assigned despite their location in the core of the PTT gene cluster (Fig. 1a).

The first two steps in PTT biosynthesis are well established[9–11], but the chemical logic of the previously proposed pathway (Fig. 1b) suggested that a number of other reactions found early in the pathway could be biochemically interesting. These include the unusual cleavage of phosphonoacetaldehyde (**5**) into hydroxymethylphosphonate (HMP, **6**) (step III) and the subsequent oxidation of hydroxymethylphosphonate directly into phosphonoformate (**7**) (step IV). In addition, the utilization of phosphonoformate to directly replace the phosphate of phosphoenolpyruvate (**8**), thereby producing the intermediate carboxyphosphonoenolpyruvate (CPEP, **9**) (step V)[12], is also unexpected. This is because the enzyme that is reported to be involved, CPEP synthase, shares significant identity with enolase and related proteins[13]. Because these enzymes usually carry out dehydration reactions, the proposed reaction is biochemically inconsistent with the expected activity the protein[14]. Based upon these observations, the early steps in PTT biosynthesis clearly warranted further investigation.

Previous analyses indicated that location of the group of genes comprised of *phpC*, *phpD*, and *phpE* in the PTT gene cluster suggested that they could have roles in the conversion of phosphonoacetaldehyde to phosphonoformate (Fig 1b, steps III and IV) [7, 15]. The role of *phpC* was unknown and its homology to type III (iron-dependent) alcohol dehydrogenases did not suggest an obvious function for the gene in PTT biosynthesis. To determine its role, we constructed and characterized a Δ*phpC* mutant of *S. viridochromogenes* (WM6547). Initial bioassay results indicated that the Δ*phpC* mutant retained the ability to produce PTT when grown in liquid culture or on ISP2 (a glucose-yeast-malt medium) plates. However,

the amount of PTT produced appeared to be less than that produced by the parent strain (Supplementary Fig. 1). When concentrated culture supernatants were examined by ^{31}P NMR, the characteristic peak of PTT at 42 ppm was not observed. Instead, a strong resonance (δ 17.2) that was identified as 2-aminoethylphosphonate (AEP, **10**) (by spiking the sample with authentic standard) was observed. AEP was also identified in the sample by LC/MS (Table 1, Supplementary Fig. 2).

Although AEP was not known or expected to be an early PTT intermediate, we reasoned that it could represent a derivative of the biosynthetic intermediate phosphonoacetaldehyde because the recovery of amino derivatives of carbonyl-containing PTT biosynthetic intermediates from blocked mutants is not unprecedented. Phosphinoalanine (**11**) and aminoethylphosphinic (**12**) acid, likely degradation products of the established intermediate phosphinopyruvate (**13**), have been isolated from a *S. hygroscopicus* non-producing mutant[16]. Further, previous groups working on phosphonate biosynthesis have utilized AEP as a physiological equivalent of phosphonoacetaldehyde[1718]. The conversions of carbonyls to amino groups, as well as the reverse reactions, are likely carried out by aminotransferases. This is supported by the observation that many of these enzymes often display relaxed substrate specificity[19].

If AEP is a phosphonoacetaldehyde derivative, then phosphonoacetaldehyde should be the substrate for the alcohol dehydrogenase encoded by *phpC*. To test if PhpC acts as a phosphonoacetaldehyde reductase, we purified a histidine-tagged PhpC fusion after over-expression in *Streptomyces lividans.* His-PhpC catalyzed the *in vitro* reduction of phosphonoacetaldehyde to hydroxyethylphosphonate (HEP, **14**) with either NADH (**15**) or NADPH (**16**) as a cofactor using ^{31}P NMR spectroscopy (Fig. 2) and LC/MS (Table 1, Supplementary Fig. 3) to monitor the reaction. The reaction proceeds to apparent completion in the presence of NADH, but to a lesser extent with NADPH, suggesting that NADH is the preferred cofactor. These data support the hypothesis that the *in vivo* accumulation of aminoethylphosphonate results from a side reaction, and further suggests that the reduction of phosphonoacetaldehyde to hydroxyethylphosphonate is step III (Fig. 3) of the PTT biosynthetic pathway.

The discovery of hydroxyethylphosphonate as a biosynthetic intermediate is interesting because the compound has previously been isolated from the culture broth of a PTT non-producing *S. hygroscopicus* mutant[20], but the compound has largely been regarded as the product of a side reaction[3]. We found data to directly establish hydroxyethylphosphonate as a true biosynthetic intermediate after we characterized a Δ*phpD* mutant (WM6601). The mutant failed to produce PTT under any conditions tested and ^{31}P NMR analysis of culture supernatants from this mutant revealed the presence of hydroxyethylphosphonate (δ 19.1). The accumulation of this compound was additionally confirmed by LC/MS (Table 1, Supplementary Fig. 4). The secretion of hydroxyethylphosphonate by the Δ*phpD* mutant suggested that this intermediate might be the substrate for PhpD. To test this, we over-expressed PhpD in *E. coli* and examined its ability to convert hydroxyethylphosphonate to another phosphonate *via* LC/MS and ^{31}P NMR spectroscopy. Our NMR results (Fig. 2b) demonstrated that extracts from cells carrying a PhpD expression plasmid, but not from the plasmid-free host, convert hydroxyethylphosphonate (δ 19.6) into

hydroxymethylphosphonate (δ 17.1). These results were confirmed by LC/MS (Table 1, Supplementary Fig. 5).

The direct conversion of 2-hydroxyethylphosphonate into hydroxymethylphosphonate (Fig. 3, step IV) by PhpD is worthy of special attention because the reaction has few, if any, biochemical precedents. PhpD is not homologous to any protein of known function in the current Genbank release[7] and therefore we cannot propose a mechanism based upon bioinformatic analysis. It is difficult to envision a simple chemical mechanism for the reaction, but our initial biochemical results firmly establish hydroxyethylphosphonate-cleavage activity to PhpD.

Hydroxymethylphosphonate, the product of the PhpD reaction, is a recognized PTT biosynthetic intermediate that was first isolated during the analysis of a genetically uncharacterized non-producing mutant of *S. hygroscopicus*[20]. During our characterization of a Δ*phpE* mutant (WM6602), we found that hydroxymethylphosphonate (HMP, δ 16.4) accumulated in culture broth after ^{31}P NMR analysis, with LC/MS independently verifying the compound (Table 1, Supplementary Fig. 6) in the sample. Bioassays of liquid cultures indicated that the Δ*phpE* mutant does not produce PTT; however, weak bioactivity was noted when the strain was assayed after growth on ISP2 plates (Supplementary Fig. 1) The *phpE* gene encodes a protein with similarity to members of the 2-hydroxyacid alcohol dehydrogenase family of enzymes, which, taken together with our data, suggests that PhpE is an alcohol dehydrogenase whose substrate is hydroxymethylphosphonate (Fig. 3, step V). Initial attempts to demonstrate this activity after over-expression of the protein in both *E. coli* and *S. lividans* failed (data not shown).

If PhpE is a hydroxymethylphosphonate dehydrogenase, then its expected product would be phosphonoformaldehyde (**17**). Therefore, to produce the known intermediate phosphonoformate, the activity of an aldehyde dehydrogenase would likely be required. Sequence homology suggests that *phpJ* encodes an aldehyde dehydrogenase that could have this function[8]. We found that a Δ*phpJ* mutant (WM6548) produced PTT in both liquid and plate-grown cultures. Nevertheless, ^{31}P NMR analysis of culture supernatants from this mutant indicated the accumulation of a compound with a strong resonance (δ 9.8) corresponding to aminomethylphosphonate (AMPn, **18**). Two additional small peaks were also noted in the spectrum; one corresponding to hydroxymethylphosphonate (δ 16.4), the other consistent with PTT (δ 43.3). LC/MS analysis independently confirmed the presence of aminomethylphosphonate in the sample (Table 1, Supplementary Fig. 7). Given the proposed activity of PhpJ, we expected to find phosphonoformaldehyde in the culture broth of the *phpJ* mutant, not aminomethylphosphonate. These results, however, are reminiscent of the accumulation of aminoethylphosphonate by the *phpC* mutant. Thus, we surmise that non-specific aminotransferases similar to those that convert phosphonoacetaldehyde into aminoethylphosphonate perform the analogous reaction on phosphonoformaldehyde, giving aminomethylphosphonate. Our genetic results therefore suggest that step VI (Fig. 3) of PTT biosynthesis is the oxidation of phosphonoformaldehyde to phosphonoformate.

Our characterization of a *phpF* mutant suggested that it is involved in the next step of PTT biosynthesis. The gene encodes a nucleotidyltransferase homolog, and although *phpF* has

been sequenced in both *S. hygroscopicus* and *S. viridochromogenes*, a proposed role for it in PTT biosynthesis has never been suggested. We found that a Δ*phpF* mutant (WM6546) fails to produce PTT after growth in MYG broth, though low level PTT biosynthesis was noted when the strain was grown on ISP2 plates (Supplementary Fig. 1). ^{31}P NMR analysis of concentrated culture supernatants from this mutant revealed 2 unique peaks (δ 1.1, 4.4), which correspond to phosphonoformate and phosphite (**19**), respectively. In addition, a minor peak corresponding to hydroxymethylphosphonate (S/N~9, δ 16.7) was also detected. Phosphite is a known product of the spontaneous decarboxylation of phosphonoformate[3], thus its presence in a sample containing phosphonoformate is not surprising. These data suggested that PhpF plays an important role in PTT biosynthesis, probably acting after the formation of phosphonoformate.

To verify that PhpF acts after the synthesis of phosphoformate, we performed a chemical feeding experiment. The Δ*phpF* mutant was unable to produce PTT when grown in MYG broth supplemented with 0.5 mM phosphonoformate, using a bioassay to score for antibiotic production. Conversely, both the Δ*phpD* and Δ*phpE* mutants were able to convert this compound to PTT, indicating that it can be taken up and metabolized by strains used in our study. Therefore *phpD* and *phpE* act before phosphonoformate is produced in the PTT pathway, whereas *phpF* acts after phosphonoformate production.

To address the role of *phpF* biochemically, we purified a histidine-tagged PhpF fusion by nickel-affinity chromatography after over-expression in *E. coli.* Nucleotidyltransferase activity was examined by ^{31}P NMR spectroscopy in an assay that included His-PhpF, CTP (**20**), phosphonoformate and inorganic pyrophosphatase (Fig. 2c). In these reactions, a small portion of unreacted phosphonoformate (δ 1.7) remains and a significant amount of phosphate (δ 2.6) has accumulated. Further, two new peaks are observed [δ −8.6 (d, *J*=29.9 Hz), −10.1 (d, *J*=29.6 Hz)]. The spin-spin coupling (*J)* observed for this new product is much broader than that seen for CTP (Fig. 2c), suggesting that the compound is unlikely to be another phosphoanhydride (such as CDP, **21**). Similar, but lower-level activity was also observed when UTP (**22**) and dCTP (**23**) were used as co-substrates with phosphonoformate (Supplemental Fig. 8); however, because these reactions never approached completion we assume that CTP is the *in vivo* substrate.

In the preceding assay we included inorganic pyrophosphatase to drive the reaction to completion, because we assumed that pyrophosphate (PP_i, **24**) would be displaced from CTP during catalysis. To verify this, we performed an assay in the absence of pyrophosphatase, which resulted in the partial conversion of the substrates to product with the concomitant accumulation of pyrophosphate (δ −4.7) (Fig. 2c). To show that the reactions described above are dependent upon His-PhpF, a control assay using heat inactivated His-PhpF was performed. The ^{31}P NMR assay results indicated that activity of the protein was abolished and peaks corresponding to the unreacted substrates phosphonoformate (δ 1.7) and the phosphoanhydride of CTP [δ −5.1 (d, *J*= 14.1 Hz), −9.97 (d, *J*=15.3 Hz), −18.6] were observed.

Our *in vitro* experiments with His-PhpF strongly suggested that the enzyme catalyzes the displacement of the β and γ phosphate of CTP (as pyrophosphate) by phosphonoformate to

produce a novel intermediate: cytidine monophosphate-5'-phosphonoformate (CMP-5'-PF, **25**). LC/MS analysis of the PhpF-catalyzed reaction detected CMP-5'-PF and a mass corresponding to an ammonium adduct (**26**) of the product (Table 1, Supplementary Fig. 9). To further confirm the structure of CMP-5'-PF, tandem mass spectrometry (MS^2) was performed, providing additional supporting data (see Supplementary Data).

Our identification of the CMP-5'-PF intermediate is important for two reasons. First, it establishes that CPEP biosynthesis probably doesn't occur as shown in Fig. 1b. Second, CMP-5'-phosphonoformate can be viewed as an activated intermediate primed for transfer of the phosphonoformate group to an appropriate acceptor molecule. This fits well with our recent suggestion that the PhpG and PhpH proteins could work in concert to produce CPEP *in vivo* via a reaction series analogous to the phosphoglycerate mutase and enolase reactions of glycolysis[7]. PhpG is a close homolog of an autophosphorylating phosphoglycerate mutase from the archaeon *Sulfolobus solfatericus*[21]. The *Sulfolobus* enzyme has been shown to catalyze the transfer of the γ-phosphate of ATP (**27**) directly to an active site serine, creating a phosphoprotein intermediate. This phosphate group is subsequently transferred to 3-phosphoglycerate (**28**), yielding 2-phosphoglycerate (**29**) as a product. We suggest that PhpG catalyzes an analogous reaction using the activated CMP-5'-phosphonoformate intermediate to donate phosphonoformate to the active site of the enzyme. The enzyme bound phosphonoformate could then be donated to a compound such as 3-phosphoglycerate, yielding a phosphonoformylated intermediate that would then serve as the substrate for the PhpH enolase, creating CPEP (Fig. 3).

We tested the above hypotheses regarding the functions of *phpG* and *phpH* by mutational analysis. Bioassays of a Δ*phpG* mutant (WM6549) indicated that PTT was not produced by liquid-grown cultures, although the strain did exhibit some bioactivity when grown on solid ISP2 medium. The Δ*phpH* mutant (WM6604) failed to produce PTT under all conditions tested. Unfortunately, no significant accumulation of phosphonates was observed in either strain based upon ^{31}P NMR analysis. Nevertheless, because PTT production is eliminated in the *phpH* mutant and strongly reduced in the *phpG* mutant, it seems likely that both genes are involved in the biosynthesis of this antibiotic. Neither mutant was able to convert phosphonoformate to PTT in chemical feeding experiments; thus, both *phpG* and *phpH* clearly act after the formation of phosphonoformate in the PTT pathway. Because we failed to obtain additional data to support our proposed roles for *phpG* and *phpH*, this portion of our model remains speculative. However, based upon the evidence presented above, we believe this model to be more credible than that previously proposed.

During our analysis of PTT biosynthesis, we expected our mutants to be deficient for biosynthesis of the antibiotic. We were surprised to find that many of our mutants can produce PTT to some extent, despite using strains that were rigorously validated by both PCR and DNA hybridization techniques. We suggest that the apparent bypass of some of our mutations occurs when other cellular enzymes can substitute at some low level for PTT pathway-specific enzymes. The observation that most bypassed genes deleted in this study encode proteins that are similar to common metabolic enzymes supports this idea. Further, we surmise that the bypass phenotype could be directly responsible for the failure of previous efforts to identify mutants in the new steps that we described here because similar

phenomena have not been documented in any other reports on PTT biosynthesis. This observation demonstrates the importance of using a directed approach for pathway analyses because, despite the biosynthetic bypasses, our mutants still secreted sufficient quantity of biosynthetic intermediates to allow identification. Although not fully blocked for PTT biosynthesis, the mutants clearly possess strong physiological bottlenecks for intermediate conversions.

Finally, our data have implications for the biosynthesis of other phosphonic acids. For example, the biosynthetic pathways of fosfomycin and PTT were long thought to share only the phosphoenolpyruvate phosphomutase and phosphonopyruvate decarboxylase catalyzed reactions, implying that phosphonoacetaldehyde would be the last common intermediate between the pathways[22]. It was recently shown that a PhpC homolog is required for fosfomycin biosynthesis, and hydroxyethylphosphonate is now believed to be a biosynthetic intermediate in this pathway[23, 24]. These results suggest that the reduction of phosphonoacetaldehyde by enzymes similar to PhpC may represent a common step in other phosphonate biosynthetic pathways as well.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Details on gene cloning can be found in Supplementary Methods and the oligonucleotides used in the cloning experiments are listed in Supplementary Table 2. *Escherichia coli* strains were grown on TYE solid medium or Luria-Bertani (LB) liquid medium supplemented with antibiotics where appropriate[25]. All *E. coli* strains were grown at 37°C for DNA cloning and manipulation; conditions for protein over expression experiments are specifically noted in the Supplementary Methods. Antibiotic concentrations used for cloning and plasmid maintenance in *E. coli* were chloramphenicol (Cm) 12 μg/mL, apramycin (Apr) 30 or 50 μg/mL, ampicillin (Amp) 100 μg/mL. *Streptomyces viridochromogenes* strains were grown on ISP2 (Difco, Sparks, MD) solid medium or in MYG liquid medium[17] with antibiotics added where appropriate. *S. viridochromogenes* strains were incubated at 30 °C, unless specifically noted. Antibiotics used in ISP2 medium were thiostrepton (Thio) 50 μg/mL; streptomycin (Str) 20 or 100 μg/mL; nalidixic acid (Nal) 25 μg/mL; and Apr 30 μg/mL. Antibiotic concentrations used in *Streptomyces* liquid cultures are noted where appropriate. Intergenic conjugations between *E. coli* donor strains and *S. viridochromogenes* recipients were carried out essentially as described in[26], except that 2 μl of the donor/recipient mix was spotted to the conjugation plates with a micropipettor instead of using a pin replicator. *S. viridochromogenes* spores were obtained by spreading MYG grown cultures to SMMS medium[27] for confluence. After green spores developed, they were harvested by rolling sterile 3 mm glass beads over the plates. Beads were gathered and washed with TX buffer[28] to create spore suspensions. Bioassays for PTT production were performed against both sensitive and resistant strains of *Bacillus subtilis* as previously described[7]. Production in liquid cultures was measured by disk diffusion and production on solid medium was assayed by applying pieces of inoculated agar directly to the assay plate.

Generation of *Streptomyces viridochromogenes* mutants

The mutants used in this study were constructed using a selection/counterselection scheme that resulted in the generation of unmarked deletions of genes from the chromosome (Supplementary Fig. 10). All putative mutants were initially screened by PCR and were later confirmed by Southern blot. A detailed description of the procedures used for mutagenesis is located in Supplementary Methods.

Identification of biosynthetic intermediates accumulated by *Streptomyces viridochromogenes* mutants

Starter cultures (15 mL MYG in 125 mL Erlenmeyer flask w/sterile glass beads to provide mechanical disruption of mycelial clumps) of *S. viridochromogenes* mutants were inoculated from ISP2 plates and grown for 2 days with agitation. Cultures were homogenized to break up mycelial clumps and 200 μL of each was used to inoculate 100 mL MYG medium in 500 mL baffled flasks. These cultures were incubated for 4 days with continuous shaking, after which mycelia were removed from the culture supernatant *via* centrifugation. Culture supernatants were concentrated *in vacuo* and subsequently analyzed by ^{31}P NMR as outlined above. In general, due to the complex mixture of compounds in the exhausted culture media, peaks in the range of phosphate and phosphate esters (δ 5 to δ −20) are not reported. In addition, shifts in the phosphonate/phosphinate range (~δ 8 to 45) are only reported if they have a signal-to-noise (S/N) ratio >7:1. Phosphonate intermediates were identified by the addition of authentic standards to the samples and not by resonance peak value alone because ^{31}P NMR phosphonate signals are strongly pH dependent[29]. Coupled liquid chromatography/mass spectrometry (LC/MS) was used as an independent line of evidence to verify the identity of the accumulated intermediates. Samples (50 μL concentrated supernatant) were directly injected onto a 2.1 mm × 100 mm Atlantis Hydrophilic Interaction Chromatography (HILIC) silica column (Waters, Milford, MA), operated at 200 μL/min on a Thermo Surveyor HPLC (ThermoFisher Scientific, San Jose, CA). Samples were separated during a 30 minute gradient from 20:80 A:B to 60:40 A:B where A was 100 mM ammonium acetate + 0.1 % acetic acid and B was acetonitrile. The eluent was directly infused onto a linear ion trap/Fourier-Transfrom hybrid mass spectrometer (LTQ-FT, ThermoFisher Scientific, San Jose, CA) where Fourier-Transform mass spectrometry (FT-MS) data were acquired in negative ion mode at 50,000 resolving power. Phosphonate intermediates were identified by accurate mass analysis, retention time comparison and comparison of tandem MS fragmentation patterns against authentic standards.

^{31}P NMR methods

All NMR experiments were performed at the Varian Oxford Center for Excellence in NMR laboratory at the University of Illinois, Urbana-Champaign. Biological and enzymatic samples were routinely assayed for the presence of phosphonates using ^{1}H-decoupled ^{31}P NMR. Samples were suspended in 20% D_2O and resonance peak values are reported against an 85% phosphoric acid standard (δ 0). Spectra were acquired on either a Varian Unity 500 spectrometer equipped with a 5 mm Nalorac Quad probe manually tuned for phosphorus at 202.28 Mhz or on a Varian Inova 600 spectrometer equipped with a 5 mm Varian 600DB AutoX probe with ProTune accessory tuned for phosphorus at 242.79 Mhz.

Other methods

See Supplementary Methods for details on strain construction, protein overexpression, enzyme assay conditions, and the sources and synthesis of phosphonic acids used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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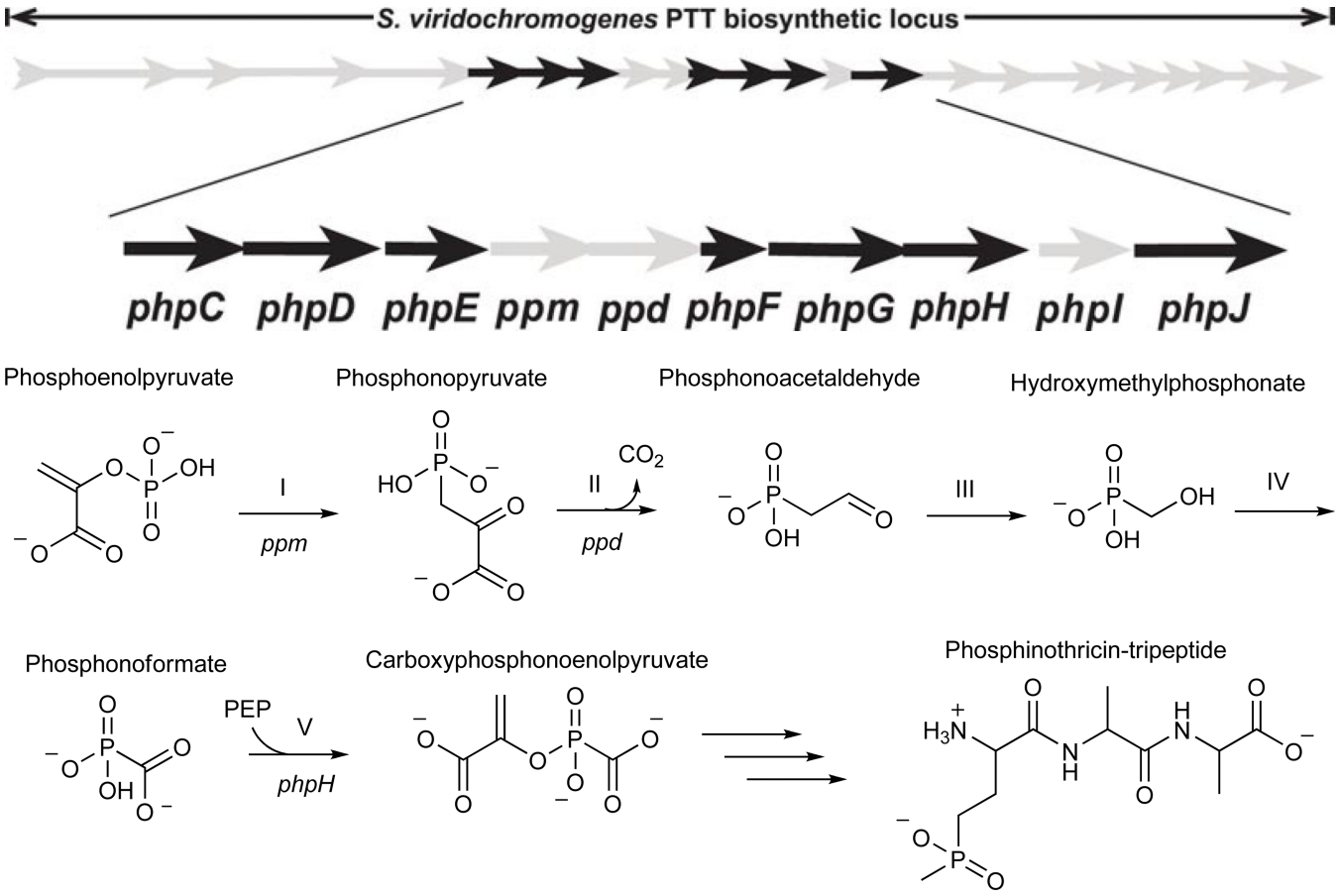
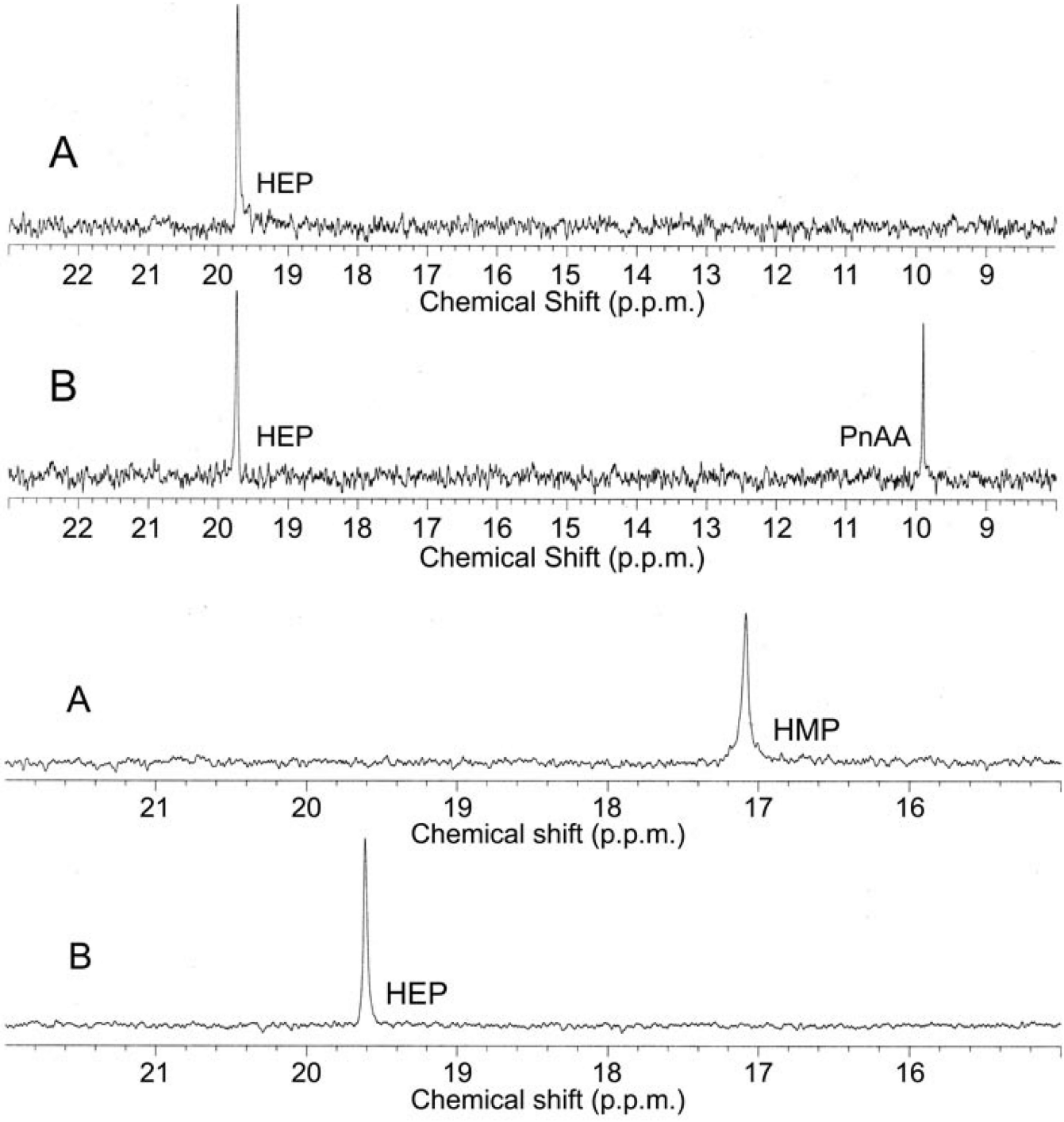


Figure 1.

Map of PTT biosynthetic genes and model for early steps in PTT biosynthesis. (**a**) Detail of genes in the core of the PTT biosynthetic gene cluster. Genes shown in grey were not studied in this work because they have experimentally established roles in PTT biosynthesis or because homology has allowed roles to be assigned by inference. The open reading frames in black did not have experimentally assigned roles, except for *phpH*, where this gene is expected to function in CPEP synthesis. (**b**) Model for carboxyphosphonoenolpyruvate biosynthesis from phosphonoacetaldehyde *via* the intermediates hydroxymethylphosphonate and phosphonoformate (adapted from[3]). Steps referred to in the text are denoted by roman numerals, and *S. viridochromogenes* genes thought to be involved in each step[7] are indicated where possible.



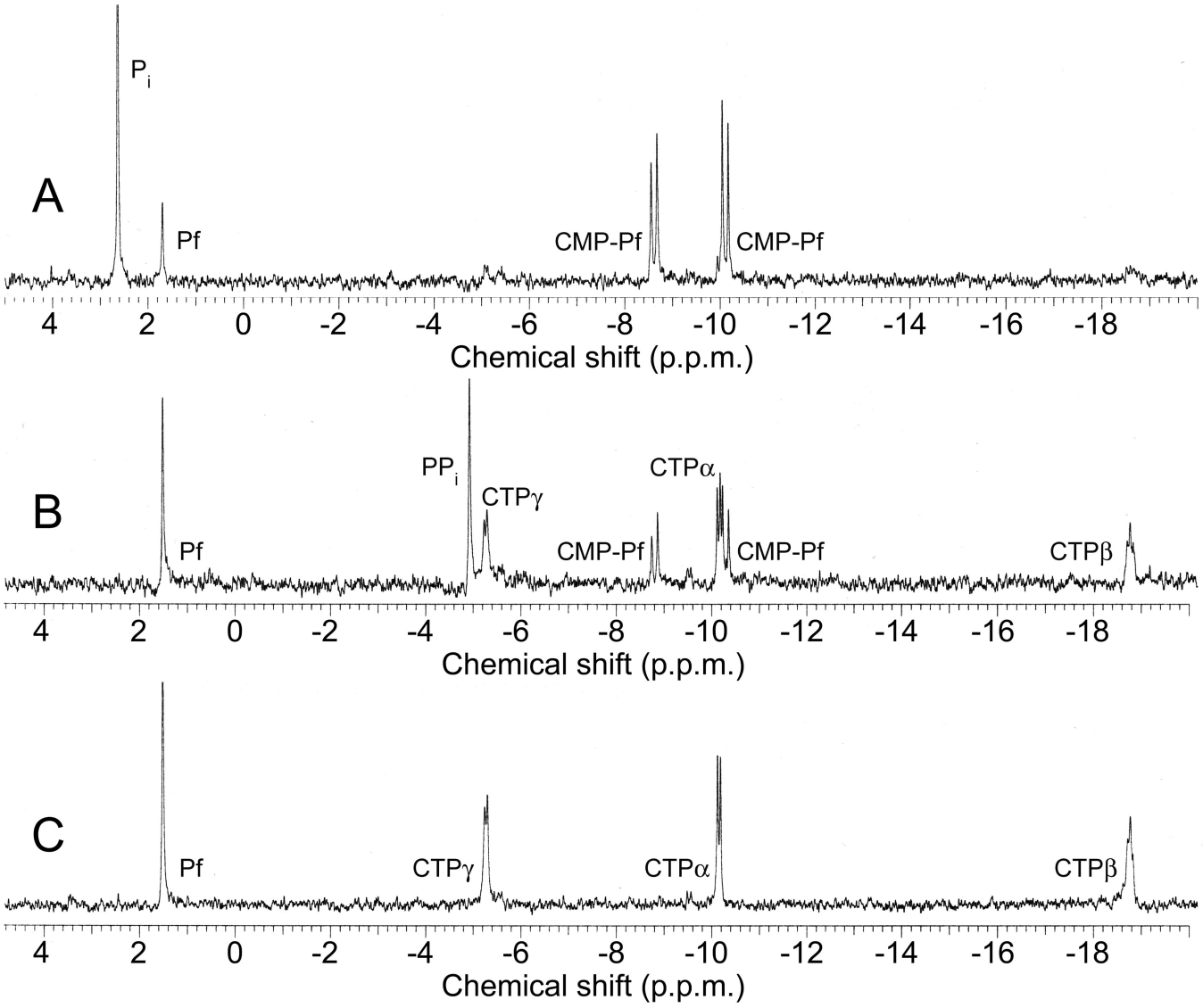


Figure 2.

In vitro reconstitution of early PTT biosynthetic reactions monitored by ^{31}P NMR. **(a)** ^{31}P NMR results of the His-PhpC catalyzed reduction of phosphonoacetaldehyde (PnAA, δ 9.9) to hydroxyethylphosphonate (HEP, δ 9.7) in the presence of (A) NADH and (B), the same reaction performed in the presence of NADPH. (**b**) ^{31}P NMR results confirming the direct *in vitro* conversion of hydroxyethylphosphonate to hydroxymethylphosphonate by PhpD. (A), an assay performed in the presence of Rosetta(DE3)pLysS/pJVD26 extract containing PhpD, showing the conversion of hydroxyethylphosphonate into hydroxymethylphosphonate (HMP, δ 17.1). (B) a control assay containing hydroxyethylphosphonate (HEP, δ 19.6) and Rosetta(DE3)pLysS extract. (**c**) ^{31}P NMR results of assays establishing the CTP-phosphonoformate nucleotidyltransferase activity of His-PhpF. (A) A reaction containing His-PhpF, inorganic pyrophosphatase, CTP and phosphonoformate showing the accumulation of phosphate (δ 2.6), and the conversion of substrates into CMP-5'-phosphonoformate (δ −8.6, −10.1). The top of the phosphate peak is

not shown to fit the spectrum to the figure. (B), the same reaction as in A in the absence of pyrophosphatase showing the partial conversion of phosphonoformate and CTP into CMP-5'-phosphonoformate (δ −8.6, −10.1) and pyrophosphate (δ −4.7) and (C) a control assay containing heat-inactivated His-PhpF, CTP (δ −5.1, −9.97, −18.6) and phosphonoformate (δ 1.7), The putative α, β, and γ designations of the CTP phosphorus nuclei have been assigned by comparison to those analogously found in ATP [30].

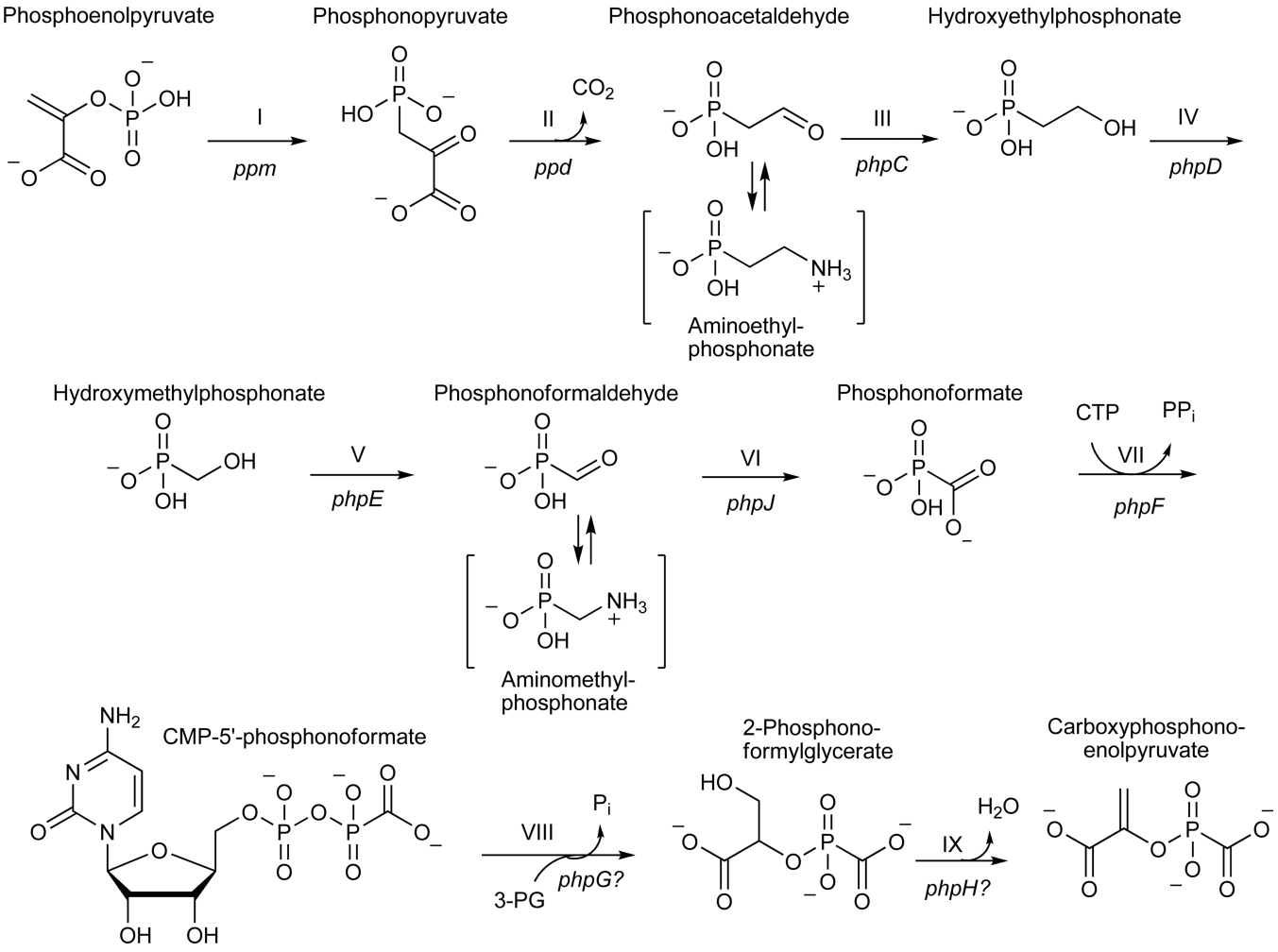


Figure 3.

An alternative pathway for carboxyphosphonoenolpyruvate biosynthesis from phosphonoacetaldehyde supported by data presented in this work. Steps referred to in the text are indicated by roman numerals and are annotated with their respective genetic determinants. Bracketed intermediates were identified from blocked mutants and likely represent modifications of the corresponding aldehyde intermediates.

Table 1

LC/MS data acquired for early PTT biosynthetic intermediates

Sample	Compound	Formula	Retention time (min)[a]	Ion detected	Calculated (*m/z*)	Measured (*m/z*)[b]
WM6492 (Δ*phpC*)	AEP	$C_2H_8NO_3P$	22.5/ 22.5	$[M-H]^-$	124.0169	124.0168/ 124.0169
PhpC *in vitro*	HEP	$C_2H_7O_4P$	15.8/ 16.4	$[M-H]^-$	125.0009	125.0010/ 125.0009
WM6601 (Δ*phpD*)	HEP	$C_2H_7O_4P$	16.2/ 16.4	$[M-H]^-$	125.0009	125.0009/ 125.0009
PhpD *in vitro*	HMP	CH_5O_4P	15.7/ 16.9	$[M-H]^-$	110.9853	110.9852/ 110.9853
WM6602 (Δ*phpE*)	HMP	CH_5O_4P	17.6/ 16.9	$[M-H]^-$	110.9853	110.9852/ 110.9853
WM6488 (Δ*phpJ*)	AMPn	CH_6NO_3P	22.1/ 22.8	$[M-H]^-$	110.0013	110.0011/ 110.0013
PhpF *in vitro*	CMP-5'-PF	$C_{10}H_{15}N_3O_{12}P_2$	9.6	$[M+H]^+$	432.0204	432.0203
PhpF *in vitro*	CMP-5'-PF, NH_4^+	$C_{10}H_{15}N_3O_{12}P_2$	9.6	$[M+NH_4]^+$	449.0469	449.0468

[a,b]Retention times and measured mass to charge ratio (*m/z*) values are listed such that the first value represents our experimental results and the second value is derived from the analysis of a corresponding authentic standard (if available).