

Discovery of the Antibiotic Phosacetamycin via a New Mass Spectrometry-Based Method for Phosphonic Acid Detection

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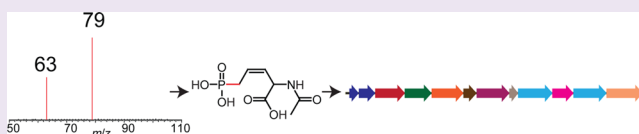
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S Supporting Information

ABSTRACT: Naturally occurring phosphonates such as phosphinothricin (Glufosinate, a commercially used herbicide) and fosfomycin (Monuroil, a clinically used antibiotic) have proved to be potent and useful biocides. Yet this class of natural products is still an under explored family of secondary metabolites. Discovery of the biosynthetic pathways responsible for the production of these compounds has been simplified by using gene based screening approaches, but detection and identification of the natural products the genes produce have been hampered by a lack of high-throughput methods for screening potential producers under various culture conditions. Here, we present an efficient mass-spectrometric method for the selective detection of natural products containing phosphonate and phosphinate functional groups. We have used this method to identify a new phosphonate metabolite, phosacetamycin, whose structure, biological activity, and biosynthetic gene cluster are reported.



Phosphonates and phosphinates are compounds that have a C–P or C–P–C bond motif, respectively. These molecules are extensively used as inhibitors of enzymes that recognize substrates with phosphoryl or carboxyl substituents.¹ The chemical stability of phosphonates and their nearly identical geometry to phosphate ester and carboxylic acid transition states^{1,2} make this class of compounds ideal as mimics of intracellular metabolites. The higher pK_a values for phosphonates (2–3 for pK_{a1} and 8–9 for pK_{a2})³ relative to phosphate esters (<1–2 for pK_{a1} and 6–7 for pK_{a2})⁴ favors divalent metal ion binding for phosphonic acids and has also been proposed to play a role in enzyme inhibitory mechanisms.^{5,6} Examples of these potent and widely used compounds include both synthetic molecules^{7,8} and products of natural origin.^{9–12} Common to all characterized phosphonate and phosphinate biosynthetic pathways except the angiotensin converting enzyme inhibitor, K-26¹³ is the phosphoenolpyruvate phosphonomutase (PepM) enzyme encoded by the *ppm* gene.¹ The commonality of *ppm* to many phosphonate biosynthetic pathways offers a way to prescreen organisms for the genetic capacity to produce phosphonates, but so far a robust and sensitive method for screening cultures for the presence and identity of phosphonate compounds has not been developed. Phosphorus nuclear magnetic resonance spectroscopy (³¹P NMR) is a robust and extremely selective technique that allows for detection of molecules with a C–P bond due to the characteristic chemical shift range¹⁴ in complex matrixes; however, the relatively low sensitivity, throughput,¹⁵ and limited structural information ³¹P NMR provides prompted development of a complementary method that would address

these limitations. The flexibility of liquid chromatography (LC) and the level of detailed information that can be obtained from complex samples using mass spectrometry and tandem mass spectrometry (MS and MS/MS) make LC–MS and LC–MS/MS attractive tools for the screening of microbial extracts for the presence of high value or high interest compounds; however, detection and identification of small hydrophilic organic acids such as phosphinates, phosphonates, phosphorylated compounds, and carboxylic acids present an analytical challenge when employing this approach. The reason analysis of these types of compounds can be problematic using LC–MS is that they are found in matrixes that have a high concentration of nonvolatile salts, which are a major source of interference. Selective removal of these nonvolatile salts is required to make the sample suitable for mass spectrometric analysis and presents a challenge due to their high concentration in biological samples and comigration with small organic acids through most chromatographic media. An additional challenge that arises when conducting the analysis of secondary metabolites such as phosphonates is that they are present in much lower concentrations than phosphorylated metabolites and phosphate salts. For example, phosphate and phosphorylated metabolites can be in the range of 1–10 mM,¹⁶ whereas phosphonate metabolites such as fosfomycin are typically present in much lower concentrations.¹⁷ To solve this problem, we developed a

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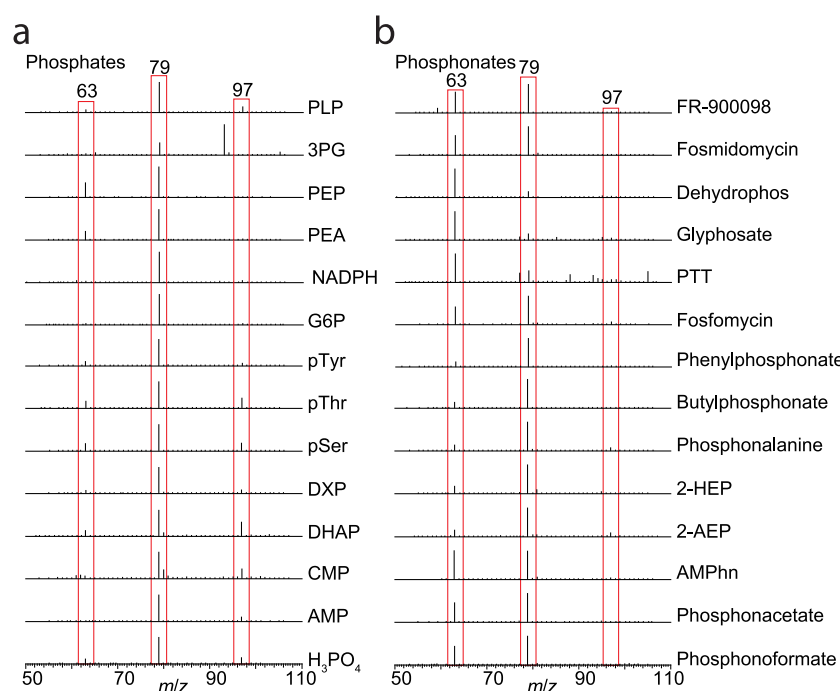


Figure 1. Differential fragmentation patterns of phosphonates and phosphorylated compounds. (a) Phosphate standards subjected to direct infusion mass spectrometry with source induced fragmentation. (b) Phosphonate standards subjected to direct infusion mass spectrometry with source induced fragmentation. PLP, pyridoxal 5' phosphate; 3PG, 3 phosphoglycerate; PEP, phosphoenoyl pyruvate; PEA, phosphoethanolamine; NADPH, reduced nicotinamide adenine dinucleotide phosphate; G6P, glucose 6-phosphate; pTyr, phosphotyrosine; pThr, phosphothreonine; pSer, phosphoserine; DXP, 1-deoxy D-xylulose 5-phosphate; DHAP, dihydroxyacetone phosphate; CMP, cytidine monophosphate; AMP, adenosine monophosphate; PTT, phosphinothricin tripeptide; 2-HEP, 2-hydroxyethyl phosphonate; 2-AEP, 2-aminoethyl phosphonate; AMPhn, aminomethyl phosphonate.

phosphonate enrichment protocol based on iron-immobilized metal affinity chromatography (IMAC), patterned after phosphopeptide enrichment strategies¹⁸ that include steps to reduce the background of contaminating phosphorylated compounds and phosphate salts. We couple this enrichment to hydrophilic interaction chromatography (HILIC)¹⁹ for the separation and precursor ion scanning mass spectrometry for the selective detection of phosphonate metabolites. The detection method can be scaled up for preparative scale purification for full structure elucidation and biological activity determination. Application of the method enabled discovery of a novel phosphonate antibiotic that we designated phosacetamycin, whose structure, bioactivity, and biosynthetic gene cluster is reported here. We also propose the biosynthetic pathway of phosacetamycin based upon sequencing of the biosynthetic gene cluster.

The current lack of methods to quickly detect and identify phosphonate and phosphinate metabolites first prompted the development of a high-throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) based screening platform that would allow rapid evaluation of microbes whose genomes contain *ppm* and therefore the genetic capacity for the production of phosphonate compounds (*ppm+* strains). Phosphonates, like phosphorylated compounds, fragment to give characteristic negative ions at m/z 79 and 63 corresponding to the elimination of PO_3^- and PO_2^- , respectively.²⁰ Unlike phosphorylated compounds, however, we found phosphonates preferentially fragment to give the m/z 63 ion (Figure 1). The differential fragmentation patterns of phosphonates relative to phosphates provides a potential way to discriminate between highly abundant phosphorylated

compounds and phosphate from the cell extracts and culture media of microorganisms and phosphonate metabolites of interest. Unfortunately not all phosphate derivatives were observed to obey this trend; phosphoenoyl pyruvate (PEP), phosphoethanolamine (PEA), phosphotyrosine (pTyr), and phosphoric acid all gave intense peaks at m/z 63 that were of similar or greater intensity than the peak at m/z 97 (Figure 1a).

For this reason, steps must be taken to reduce the background of phosphate and phosphate esters in the samples (Supplementary Figures S1–S3). The following protocol has proven effective in removing phosphate derivatives. Extracts from *ppm+* strains are first pretreated with alkaline phosphatase and phosphodiesterase to minimize contamination with phosphorylated metabolites. Phosphate released from digested metabolites and from the culture medium is precipitated by the addition of calcium acetate. Excess Ca^{2+} is then removed by precipitation with NH_4HCO_3 . The pretreated samples are desalted and enriched by iron IMAC. The phosphonate enriched samples are then subjected to HILIC chromatography and analyzed by a precursor ion screening method specific for phosphorus containing compounds²⁰ using a hybrid quadrupole linear-ion trap mass spectrometer to efficiently screen extracts for the presence of phosphonates. Next, the putative phosphonates are reanalyzed using a high-resolution accurate mass instrument in order to determine the molecular formula, dereplicate, and begin structural elucidation. The rigorous pretreatment steps were found to be essential for the detection of phosphonate compounds present at low concentrations in samples with a large excess of phosphorylated species and phosphate.

In order to validate our approach, we applied this sample preparation and analysis on a *ppm*⁺ actinomycete, *Streptomyces aureus* NRRL B-2808. Application of our sample preparation and analysis methods readily identified an unknown phosphonate metabolite with ion formula C₇H₁₁O₆NP⁻ (Figures 2 and

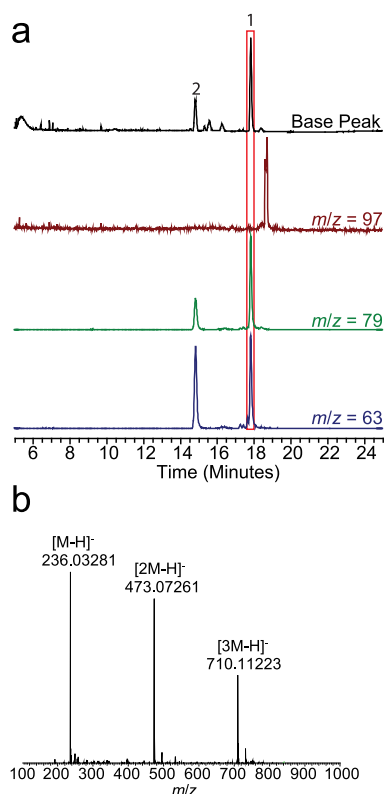


Figure 2. Identification of a new phosphonate from the culture of a *ppm*⁺ actinomycete. (a) LC-FTMS base peak chromatogram (top) and ITMS extracted ion chromatograms (*m/z* 97, 79, and 63) from source fragmentation (lower). (b) FTMS spectrum of LC peak 1 corresponding to unknown phosphonate with an ion formula of C₇H₁₁O₆NP⁻. LC peak 2 is a contaminant peak from the glass wash; see Supplementary Figure S5.

S4). LC peak 2 in Figure 2 was identified as a contaminant from the glass wash (Supplementary Figure S5). The presence of the phosphonate moiety was further supported by ³¹P NMR analysis; the chemical shift (17.90 ppm) and coupling constant (*J* = 21.2 Hz) are consistent with a C–P bond (Supplementary Figure S6). Further investigation into the structure of this new compound using MS², MS³, and MS⁴ analyses (Supplementary Figures S7–S9) indicated the presence of carboxylic acid, acetyl, and phosphonate groups by virtue of the fragment ions *m/z* 192.04278, 150.03248, and 79.1, respectively. This metabolite's molecular formula was not present in any chemical databases, so we scaled up the culture volume in order to obtain material sufficient for full structural characterization. During initial screening of *ppm*⁺ strains, cultures were grown on solid medium; however, after initial detection of phosacetamycin, we determined that soy flour mannitol liquid medium was optimal for production. Phosacetamycin was purified using essentially the same scheme as the screening method with the addition of three stages of HPLC to purify the compound to homogeneity; HILIC, aqueous normal phase and reversed phase chromatographies all guided by mass-based fractionation.

Phosacetamycin was obtained as a white, amorphous powder with a yield of 100 μg per liter. The ¹H NMR spectrum (Supplementary Table S1) displayed signals corresponding to one tertiary methyl group at δ 1.89 ppm, one methylene group at δH 2.46, one methane group at δ 4.76 ppm, and two olefinic protons at δ 5.66 and 5.45 ppm, which indicated that the other four protons in the molecular formula were exchangeable. The ¹H decoupled ³¹P NMR spectrum of compound 1 revealed a signal at 17.90 ppm. A direct P–CH₂ bond was evidenced by triplet splitting (*J* = 21.2 Hz) in the ¹H-coupled ³¹P NMR spectrum and by the large coupling constant of *J* = 128.3 Hz in the ¹³C NMR spectrum for a CH₂ signal at 28.5 ppm. The structure of phosacetamycin was found to be analogous to 2-amino-5-phosphono-3-pentenoic acid (APPA), a constituent of the rhizocticins (rhizocticin A, Gly-APPA; rhizocticin B, Val-Gly-APPA; rhizocticin C, Ile-Gly-APPA; rhizocticin D, Leu-Gly-APPA)²¹ and plumbemycins (plumbemycin A, Ala-Asp-APPA; plumbemycin B Ala-Asn-APPA),²² by ¹H–¹H COSY correlations of H-1 with H-2, H-3 with H-2 and H-4, ¹H–¹H TOCSY correlations of H-1, H-2, H-3, and H-4, and ¹H–¹³C HMBC correlations of H-4 with C-5. APPA and phosacetamycin differ in that phosacetamycin possesses an acetamide group, which has additional signals corresponding to C-1' (δc 173.1) and C-2' (δc 21.8) and ¹H–¹³C HMBC correlations of C-1' with H-4 and H-2'. NMR spectra are displayed in Supplementary Figures S10–S18.

The absolute configuration of phosacetamycin was determined to be L as in the rhizocticins and plumbemycins.²³ Free APPA was obtained by digestion of phosacetamycin in 6 M HCl at 95 °C for 24 h. A standard of L-APPA was prepared by digestion of rhizocticin in 6 M HCl as described previously.²³ The APPA liberated from phosacetamycin, and the standard was analyzed using a chiral column, CrownPak CR+ (Supplementary Figure S19). On this column, the D-form amino acids always elute before their L-form counterparts.²³ As expected, some epimerization to the D-form was observed²³ and served as an internal standard.

The antimicrobial properties of phosacetamycin were investigated using a disk diffusion assay. Zones of growth inhibition were measured for phosacetamycin as well as standard antimicrobial agents; the data are summarized in Table 1. In contrast to the rhizocticins and the plumbemycins, which exhibit exclusively antifungal and antibacterial activity,

Table 1. Biological Activity Data for Phosacetamycin and Standard Antibiotics Against Representative Microorganisms

	zone of inhibition (diameter, mm)		
	phosacetamycin	kanamycin	nystatin
<i>Escherichia coli</i> imp ASR	10.5	15.0	NT ^a
<i>Escherichia coli</i> MG1655	10.0	15.0	NT ^a
<i>Escherichia coli</i> WM6242 – IPTG	11.0	15.5	NT ^a
<i>Escherichia coli</i> WM6242 + IPTG	10.5	15.5	NT ^a
<i>Serratia marcescens</i> ATCC 274	0	17.0	NT ^a
<i>Salmonella typhimurium</i> LT2 ATCC 700720	0	13.5	NT ^a
<i>Staphylococcus aureus</i> ATCC 25923	10.0	18.0	NT ^a
<i>Bacillus subtilis</i> 168	9.5	17.0	NT ^a
<i>Paecilomyces variotii</i>	12	NT ^a	31
<i>Saccharomyces cerevisiae</i>	0	NT ^a	26

^aNot tested.

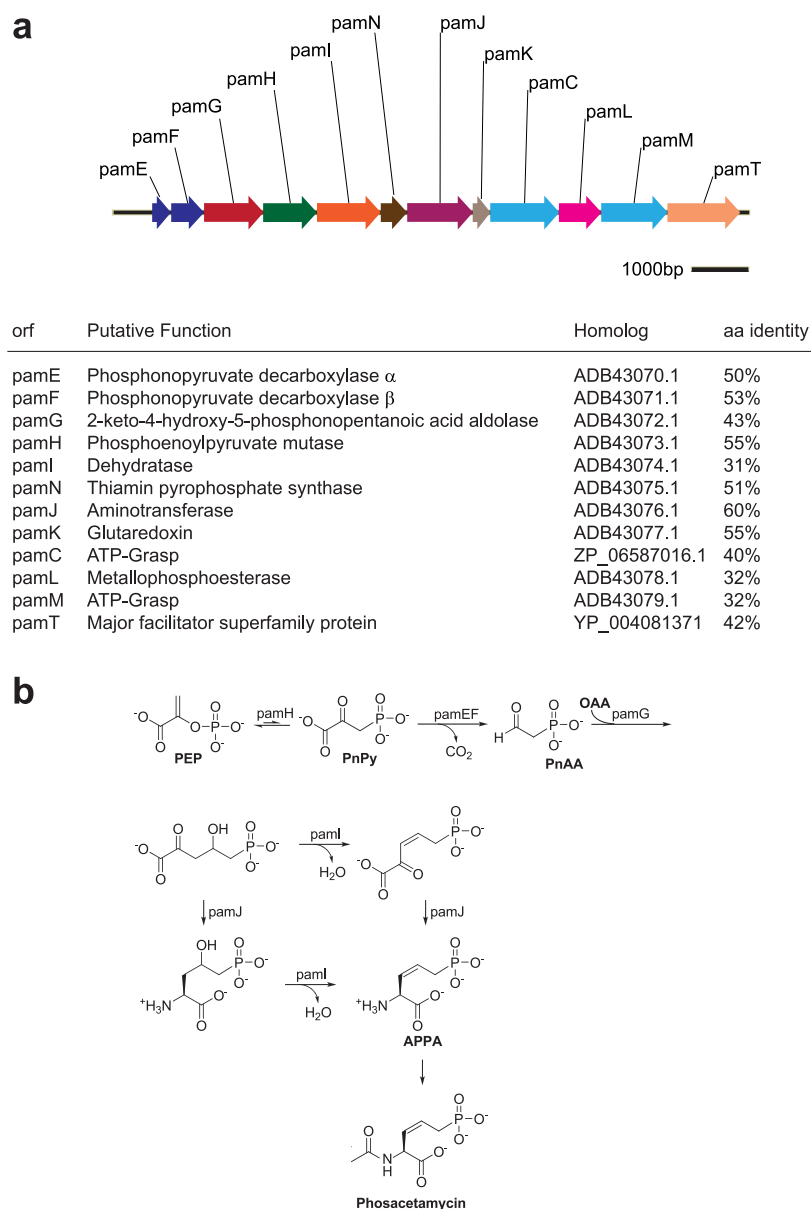


Figure 3. Phosacetamycin biosynthetic gene cluster. (a) ORF map and annotation of the phosacetamycin biosynthetic genes. (b) Proposed biosynthetic pathway for phosacetamycin. To date, experimental evidence is lacking that would establish the order of the *pamI* and *pamJ* reactions. OAA, oxaloacetic acid.

respectively,^{21,24} phosacetamycin was found to inhibit growth of both bacteria and fungi. The fungal and bacterial selectivity of the rhizotocins and plumbemycins is dependent on the amino acids appended on the APPA moiety of these compounds,²⁵ presumably due to differential peptide transport. Phosacetamycin was tested against three strains of *Escherichia coli*, MG1655 (wild-type K12), an increased membrane permeability (*imp*) mutant²⁶ and an engineered strain, WM6242, which has a phosphonate transport system, *phnCDE*, under the control of an IPTG inducible promoter (P_{tac}).²⁷ The sensitivity of *E. coli* strains MG1655, *imp*, and WM6242 (with or without the phosphonate transport system induced) were quite similar for phosacetamycin suggesting that, at least for *E. coli*, the ability of phosacetamycin to inhibit growth is not limited by transport.

The 11074 bp biosynthetic gene cluster of phosacetamycin was determined by whole genome sequencing of the producing

organism, *Sm. aureus* NRRL B-2808, and is depicted in Figure 3. The contig containing the coding sequence for the *ppm* gene was found to be flanked by a set of genes homologous to the rhizotocin gene cluster from *B. subtilis* ATCC 6633.²⁵ Two ORFs (open reading frames), *pamC* (a predicted ATP-Grasp enzyme) and *pamT* (a predicted major facilitator superfamily transport protein), do not have homologues in the rhizotocin gene cluster (Figure 3a). On the basis of the shared similarity between the phosacetamycin and rhizotocin gene clusters, phosacetamycin biosynthesis is proposed to follow rhizotocin biosynthesis for the APPA moiety (Figure 3b). The mechanism for the installation of the acetyl group onto APPA is unknown at this time and is the subject of future work.

Here, we have demonstrated a set of methods for the selective enrichment, detection, and identification of phosphonate compounds present in low concentrations in complex mixtures. Efficient sample preparation and data collection are

important in high-throughput screening applications of LC–MS/MS. The quantity and depth of information that is returned by metabolomic screening efforts necessitates the rapid identification of samples of interest for further analysis and distillation of features of interest from background signals and known compounds (dereplication). We have successfully used these methods to identify a previously unknown phosphonate metabolite, phosacetamycin. The methods described above can be extended to screen a larger culture collection using an expanded set of culture conditions in order to detect or elicit phosphonate production, optimize yield, or detect engineered, non-natural phosphonates in enzyme and pathway engineering studies.

METHODS

Culture Media, Chemicals, and Supplies. Culture medium was purchased from Becton Dickson. IMAC Hypercel Resin and Acroprep filter plates were purchased from Pall Life Sciences. A Sequant Zic-pHILIC HPLC column (2.1 × 150 mm) and PolyCatA column (9.4 × 150 mm) were purchased from the Nest Group. A Cogent Diamond Hydride column (7.8 × 150 mm) was purchased from Microsolv. Synergi RP Fusion columns (9.4 × 150 mm) were purchased from Phenomenex. All other chemicals were from Sigma-Aldrich unless otherwise specified (St. Louis, MO).

Mass Spectrometry. Mass spectrometry was performed using a Q-Trap 5500 (AB SCIEX) at the Roy J. Carver Biotechnology Facility at the University of Illinois, a custom 11T LTQ-FT Ultra (Thermo Scientific Inc.) previously described,²⁸ or an Agilent single quadrupole MSD/SL coupled to Agilent 1200 HPLCs. Detailed experimental procedures are described in the Supporting Information.

NMR Spectroscopy. NMR data were acquired using an Agilent 600 MHz spectrometer equipped with a 5 mm HCN pulsed field gradient triple resonance probe, OneNMR probe, or ICG probe (Agilent) and controlled from a workstation running Vnmj 3.0. Detailed experimental procedures and NMR data are presented in the Supporting Information.

Bioassays. Biological activity was determined using a disk diffusion assay. Fifty micrograms of phosacetamycin or reference compounds were spotted onto a 6 mm paper disk on a seeded agar plate. Bioassay plates were incubated at 37 or 30 °C as appropriate for 12–24 h. Zones of inhibition were measured using calipers and recorded as the diameter of the clear zone around the disk.

DNA Sequencing and Analysis. Strain *Sm. aureus* NRRL B-2808 was sequenced using the Illumina HiSeq platform at the University of Illinois at Urbana–Champaign Roy J. Carver Biotechnology Center. Assembly and gene prediction was performed as in ref 29. Detailed genome analysis procedures are described in the Supporting Information. The biosynthetic gene cluster for phosacetamycin is deposited under Genbank accession no. KC733813.

ASSOCIATED CONTENT

Supporting Information

Additional experimental procedures and data as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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